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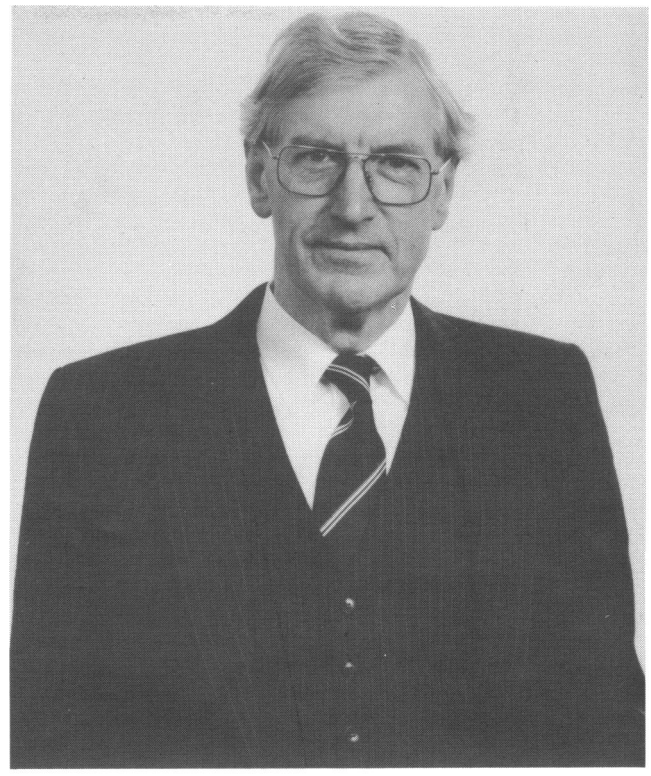
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Dr S J Coker
Department of Pharmacology
University of Liverpool
P.O. Box 147
LIVERPOOL L69 3BX

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OBITUARY

A.M. Barrett, B Pharm, PhD, FIBiol



A.M. Barrett

Michael Barrett who, for many years, had been actively involved in the affairs of the British Pharmacological Society, died on 21st July 1994. Three years previously, ill health had compelled him to take early retirement from the University of Buckingham where he was Vice-Chancellor. Since then he and I had met regularly and our lunch-time conversations at a delightful pub in the lovely Cotswold countryside had become a routine part of life in retirement for both of us. When I learned that his death had occurred very soon after our last meeting I was deeply shocked.

He was educated at Cheltenham Grammar School and at the School of Pharmacy, University of London, where members of the academic staff were impressed not only by his bright intellect but also by his charm and engaging personality. In 1953 he obtained the B Pharm degree with first class honours in pharmacology and chemistry and then, at Gladwyn Buttle's suggestion, became my first PhD student. I look back on our association at that time with great pleasure and still remember our long discussions on topics ranging from the design of experiments, the need for proper controls and the importance of replication, to conservation and the ethics of animal experiments, interspersed with cricket, the theatre and current affairs. He was a very confident and articulate young man who possessed a flair for argument in a clear, concise and convincing manner. He was never aggressive and always polite. We had worked together for a couple of years on the pharmacological actions of corticosteroids on the secretion of adrenocorticotrophic hormone by the anterior pituitary gland when Michael seized the opportunity to work in Cleveland, Ohio, with George Sayers, who was then the world leader in this field of endocrinology. He returned a year later even more confident! His thesis, written in a typically elegant manner, was completed and he was successful in obtaining the PhD degree. He was appointed to a lectureship in pharmacology at the School of Pharmacy and developed an enviable reputation as an enthusiastic and stimulating teacher. Our close association ceased then until nearly forty years later but I continued to follow his career with great interest and a certain amount of pride. I began to sense that Michael was beginning to find the ambience of the laboratory tedious and boring. Although he enjoyed discussing pharmacological data, his enthusiasm for laboratory work was beginning to make way for aspirations in a different direction.

However, after a few years at the School of Pharmacy, he accepted an appointment as a research pharmacologist at ICI where he worked on factors affecting the mobilisation of free fatty acids and, subsequently, on β-adrenoceptor blocking drugs. His colleagues were particularly impressed by the careful consideration which he gave to the design and control of his experiments and by the meticulous manner in which he performed them. The work of the group was published in several papers and review articles and, of course, its theoretical and practical importance is well known.

His appointment, in 1970, to the Chair of Pharmacology at Leeds opened the door to a different career in which he could fulful his real ambition. He began to indulge his interest in University organisation and to develop his remarkable administrative skills. He actively sought to be elected Pro-Vice-Chancellor and was eventually successful. In the absence of the Vice-Chancellor, Lord Boyle, who was terminally ill with cancer, Michael used his administrative skill to guide the University through the difficult period when numerous premature retirements of members of the academic staff were being negotiated and financial cuts were being made. He also exhibited his organisational ability outside the University as administrator of the Leeds International Piano Competition and Chairman of the Leeds Eastern Area Health Authority.

In 1985 he became Vice-Chancellor of the University of Buckingham where he demonstrated further his flair for academic leadership. He encouraged the expansion of post-graduate studies, the further development of science departments and the evolution of links between the University and other institutions which led to the establishment of new degree courses in, for example, agriculture and land management. He was responsible for the attraction of considerable funding from sources outside the University. Many academic innovations were introduced and new halls of residence were built during his term of office but the period was marred for him by personal tragedies. His wife, Pat, died suddenly leaving a son and daughter both of whom were still at school. A year later he had to undergo major heart surgery and his subsequent ill health forced his early retirement. He was a devoted parent who spared no effort in trying to compensate his children for the tragic loss of their mother. At the same time he became involved in local politics particularly those concerned with health and education.

He became a member of the British Pharmacological Society in 1963 since when he has always been active in its affairs. He was a member of the editorial board, he served as Meetings Secretary and as General Secretary and was a member of the Committee. For several years he was also Secretary-General of the International Union of Pharmacology. This year he was elected to Honorary Membership of the Society.

He seemed to know everybody but he had few close friends. I knew him for forty years, always liked him and admired him for his charm and gracious manner. He was an excellent student, a delightful colleague and a good friend. He will be sadly missed.

J.R. Hodges

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Respiratory effects of baclofen and 3-aminopropylphosphinic acid in guinea-pigs

[1]John A. Hey, Garfield Mingo, Donald C. Bolser, William Kreutner, Dawn Krobatsch & Richard W. Chapman

Allergy, Schering-Plough Research Institute, Kenilworth, New Jersey, U.S.A.

1 The effects of the $GABA_B$ receptor agonists, baclofen and 3-aminopropylphosphinic acid (3-APPi) given by the subcutaneous or intracerebroventricular (i.c.v.) route were examined on minute ventilation ($\dot{V}$), tidal volume (V_T) and respiratory rate (f) due to room air and carbon dioxide (CO_2)-enriched gas hyperventilation in conscious guinea-pigs.

2 Baclofen (0.3–10 mg kg^{-1}, s.c.) produced a dose-dependent inhibition of $\dot{V}$ and f due to room air and CO_2 inhalation. The maximum inhibition of room air breathing $\dot{V}$ was 85% ± 3 and f was 74% ± 3 at 10 mg kg^{-1}, s.c. The maximum effects on CO_2-induced hyperventilation were 68% ± 9 and 51% ± 6, for $\dot{V}$ and f respectively. Only the highest dose of baclofen studied (10 mg kg^{-1}) produced a significant inhibition of V_T due to room air breathing (46% ± 6) and CO_2 breathing (38% ± 11).

3 3-APPi (0.3–100 mg kg^{-1}, s.c.) did not affect $\dot{V}$, V_T or f due to room air breathing or CO_2 inhalation at any dose tested. Also, i.c.v. administration of 3-APPi (100 μg) did not affect ventilatory responses due to room air breathing or CO_2 inhalation.

4 Pretreatment with the $GABA_B$ antagonist, CGP 35348 3-aminopropyl-(diethoxymethyl) phosphinic acid (3–30 mg kg^{-1}, s.c.) blocked the respiratory depressant effects of baclofen (3 mg kg^{-1}, s.c.) in a dose-related fashion.

5 Intracerebroventricular (i.c.v.) administration of CGP 35348 (50 μg) blocked the respiratory depressant effects of baclofen. CGP 35348 given alone either i.c.v. or s.c. had no effects on respiration due to room air or CO_2 inhalation.

6 Pretreatment with either the $GABA_A$ antagonist bicuculline (30 mg kg^{-1}, s.c.) or the opioid antagonist, naloxone (1 mg kg^{-1}, s.c.) had no effect on the respiratory depressant action of baclofen (3 mg kg^{-1}, s.c.).

7 These results show that baclofen inhibits ventilation due to room air breathing, and attenuates the hyperventilation response to CO_2 inhalation. The peripherally acting $GABA_B$ agonist, 3-APPi had no effect on ventilation. These findings demonstrate that the respiratory depressant effects of baclofen are due to activation of CNS $GABA_B$ receptors and indicates that only $GABA_B$ receptor agonists that penetrate into the CNS may cause respiratory depression.

Keywords: 3-Aminopropylphosphinic acid; baclofen; CNS; $GABA_B$ receptors; minute volume; respiration

Introduction

Recent studies have found that the $GABA_B$ receptor agonists, baclofen and 3-aminopropylphosphinic acid (3-APPi) inhibit a variety of neurally-evoked responses in the airways including tachykinin-mediated and cholinergic bronchospasm, airway microvascular leakage and cough (Belvisi *et al.*, 1989; Chapman *et al.*, 1991; Danko *et al.*, 1992; Bolser *et al.*, 1993; Chapman *et al.*, 1993b). These effects are produced by inhibition of the release of neurotransmitters and/or neuromodulators from pulmonary nerves. On the other hand, baclofen inhibits cough primarily by activation of $GABA_B$ receptors in the central nervous system (CNS), while 3-APPi exerts predominantly a peripheral antitussive effect (Bolser *et al.*, 1994).

$GABA_B$ receptor agonists also produce respiratory depression by inhibiting activity of respiratory neurones in the CNS (Lalley, 1983; DeFeudis, 1984; Da Silva *et al.*, 1987; Schmid *et al.*, 1989; Wagner & Dekin, 1993). Therefore, highly lipophilic $GABA_B$ agonists, such as baclofen, that rapidly penetrate into the CNS produce respiratory depression when given parenterally in large doses (Lipscomb & Meredith, 1980; Lalley, 1983; DaSilva *et al.*, 1987). On the other hand, 3-APPi is a $GABA_B$ agonist that does not penetrate into the CNS (Hills & Howson, 1992) and would not be expected to depress respiratory function, although this has not been studied.

[1] Author for correspondence at: Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ, U.S.A. 07033-0539.

The purpose of the present study was to determine the respiratory effects of baclofen and 3-APPi in guinea-pigs. We also studied the effect of a selective $GABA_B$ receptor antagonist, CGP 35348 3-aminopropyl-(diethoxymethyl) phosphinic acid (Olpe *et al.*, 1990) on the respiratory depressant effects of baclofen to verify the selectivity and site of action of this $GABA_B$ agonist.

Methods

Male Hartley guinea-pigs (Charles River Labs, Wilmington, MA, U.S.A.) ranging in weight from 350 to 450 g were used in these studies. The animals were given food and water *ad libitum*.

Pulmonary measurements

The guinea-pigs were placed in a head-out, whole body pressure plethysmograph (Penn-Century, Philadelphia, PA, U.S.A.). Copper gauze was placed inside the plethysmograph to keep the interior temperature of the plethysmograph constant. Latex collars were placed over the animals head to provide an airtight seal between the guinea-pig and the plethysmograph. A pressure transducer (Statham-Gould, San Juan, PR; Model P23XL) was connected to an outlet port in the plethysmograph wall and measured the pressure changes inside the plethysmograph. This pressure signal was visually

displayed on a chart recorder (Grass Instruments, Quincy, MA, U.S.A.; Model RPS 7C8) and used to measure tidal volume (V_T) and respiratory frequency (f). Minute ventilation ($\dot{V}$) was derived from these two measurements.

A transparent chamber was placed over the head of the guinea-pig to facilitate the inhalation of a hypercapnic gas mixture (10% CO_2, 21% O_2, 69% N_2), which was delivered from a compressed gas source (SOS Gases, Inc., Kearney, NJ, U.S.A.) at a constant flow of 40 ml s^{-1}. The guinea-pigs were exposed to the hypercapnic gas mixture for 10 min to achieve steady-state conditions and for ventilation to stabilize at an elevated level (Danko & Chapman, 1988).

Experimental protocol

The guinea-pigs were allowed to equilibrate in the plethysmograph for 15 min after which time measurements of V_T, f and $\dot{V}$ were obtained during room air breathing. The guinea-pigs were then exposed to the CO_2-enriched gas mixture and V_T, f and $\dot{V}$ were measured. Following exposure to the CO_2-enriched gas the guinea-pigs were taken out of the plethysmograph and injected subcutaneously with saline, baclofen or 3-APPi. Thirty minutes after the administration of these drugs, the guinea-pigs were placed into the plethysmograph and measurements of V_T, f and $\dot{V}$ were obtained during room air breathing and after exposure to the CO_2-enriched gas mixture. For studies involving antagonist, these compounds were given subcutaneously 10 min before baclofen.

In other experiments, CGP 35348 or 3-APPi was given into the CNS through chronically implanted intracerebroventricular cannulae (Mauser *et al.*, 1988; McLeod *et al.*, 1991). For implantation of the intracerebroventricular (i.c.v.) cannulae, guinea-pigs were anaesthetized with ketamine (30 mg kg^{-1}, i.m.) and placed in a rodent stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.). A mid-line incision was made through the scalp to expose the skull and a small hole was drilled through the skull 0.5 mm anterior, and 2.0 mm lateral to the bregma. Through this hole a stainless steel cannula was inserted 4.5 mm below the skull surface to penetrate the lateral cerebral ventricle (McLeod *et al.*, 1991). Three anchoring screws were placed into the skull and the i.c.v. cannula was secured to the screws with dental acrylic cement. The scalp was sutured and the animals were left to recover for 1 week before study. All injections through the i.c.v. cannula were made with a microlitre syringe (Hamilton, Reno, NV, U.S.A.) and administered in artificial CSF in a volume of 10 µl.

Compounds

Compounds used in this study included (±)-baclofen, naloxone HCl (Research Biochemicals Inc., Natick MA, U.S.A.), bicuculline methiodide (Sigma Chemical Co., St. Louis, MO, U.S.A.), 3-APPi (Schering-Plough Research Institute, Kenilworth, NJ, U.S.A.) and CGP 35348 (Ciba-Geigy Corp., Basel, Switzerland).

Statistics

All data are represented as mean ± s.e.mean. Statistical differences between means were evaluated with Student's *t* test or one-way analysis of variance. Differences were considered significant if $P<0.05$.

Results

Table 1 shows the effect of baclofen and 3-APPi on V_T, f and $\dot{V}$ during room air breathing and after exposure to a carbon dioxide-enriched gas mixture. Baclofen (0.3–10 mg kg^{-1}, s.c.) produced a dose-dependent inhibition of f and $\dot{V}$ due to room air breathing and during CO_2 inhalation (Table 1). On the other hand, the inhibition of V_T by baclofen was seen only at 10 mg kg^{-1}. 3-APPi (0.3–100 mg kg^{-1}, s.c.) had no significant effect on V_T, f and $\dot{V}$ during room air breathing or during CO_2 inhalation (Table 1). Similarly, i.c.v 3-APPi did not alter normal ventilation responses to room air breathing or to CO_2-stimulated ventilation. In these studies, the changes of $\dot{V}$ after 3-APPi (100 µg, i.c.v.) were $-7 \pm 9\%$ for room air and $1 \pm 12\%$ for CO_2 hyperventilation ($n = 9$). In contrast, i.c.v. baclofen (3 µg; $n = 6$) significantly inhibited room air $\dot{V}$ ($-68 \pm 6\%$) and CO_2-stimulated $\dot{V}$ ($-60 \pm 8\%$).

Pretreatment with CGP 35348 (3–30 mg kg^{-1}, s.c.) reduced the respiratory depressant effects of baclofen (3 mg kg^{-1}, s.c.) during CO_2 inhalation (Figure 1). The dose of CGP 35348 that produced a 50% blockade of baclofen's inhibitory effect was 5.9 mg kg^{-1}. Intracerebroventricular administration of CGP 35348 (50 µg, i.c.v.) also reduced the respiratory depressant effects of baclofen (Figure 2). On the other hand, CGP 35348 (30 mg kg^{-1}, s.c.) had no significant effect on the respiratory depression caused by subcutaneous administration of pentobarbitone. In these studies, pentobarbitone (30 mg kg^{-1}, s.c.) caused a $64 \pm 2\%$ reduction in $\dot{V}$ during CO_2 inhalation. In the presence of CGP 35348 (30 mg kg^{-1}, s.c.), pentobarbitone reduced $\dot{V}$ by $71 \pm 6\%$.

Neither pretreatment with the $GABA_A$ antagonist, bicuculline (30 mg kg^{-1}, s.c.) nor with the opioid antagonist, naloxone (1 mg kg^{-1}, s.c.) inhibited the respiratory depressant effects of baclofen (3 mg kg^{-1}, s.c.) (Table 2). This dose of naloxone reverses the respiratory depressant effect of morphine (30 mg kg^{-1}, s.c.; data not shown).

Discussion

Previous studies in animals (Schmid *et al.*, 1989) and man (Lipscomb & Meredith, 1980) have demonstrated that baclofen is a powerful respiratory depressant. In conscious guinea-pigs, we found that subcutaneous administration of baclofen inhibited ventilation during room air breathing and attenuated the hyperventilatory response to carbon dioxide inhalation. The effects of baclofen were blocked by administration of the centrally acting $GABA_B$ antagonist, CGP 35348, but not by the $GABA_A$ antagonist, bicuculline, nor by naloxone, an opioid antagonist. On the other hand, 3-APPi, which is a peripherally acting $GABA_B$ agonist that does not penetrate into the CNS (Hills & Howson, 1992) had no effect on ventilation. These results demonstrate that the respiratory depressant effects of baclofen are specific to $GABA_B$ receptor activation and identify that only $GABA_B$ receptor agonists that penetrate into the CNS cause respiratory depression.

The respiratory depressant effects of baclofen were seen on both room air ventilation and on the ventilatory responses to carbon dioxide. Inhalation of carbon dioxide was used in these studies because it is a potent respiratory stimulant that causes consistently stable increases in f and $\dot{V}$ that are sensitive to drugs affecting the respiratory pattern (Danko & Chapman, 1988). Previous studies in anaesthetized cats reported that intravenous baclofen produces an apneustic breathing pattern with a large reduction in respiratory rate and an increase in tidal volume (Lalley, 1983; DaSilva *et al.*, 1987). In our study, baclofen produced the greatest reductions in respiratory rate and smaller reductions in tidal volume, but there was no evidence of apneustic breathing with doses as high as 10 mg kg^{-1}, s.c. This may reflect the fact that our studies were performed in the absence of general anaesthesia or possibly to a species difference. Involvement of CNS $GABA_B$ receptor activation was confirmed by blocking the respiratory depressant effects of baclofen with i.c.v. administration of the $GABA_B$ receptor antagonist CGP 35348 (Olpe *et al.*, 1990). These findings are in agreement with previously published results that show a central site of action of baclofen that inhibits respiration (Lalley, 1983; DaSilva *et al.*, 1987; Schmid *et al.*, 1989).

Table 1 Effect of baclofen and 3-aminopropylphosphinic acid (3-APPi) on ventilation during breathing of room air and carbon dioxide

Compound[a]	Dose (mg kg^{-1}, s.c.)	n	% change[b] Room air breathing V_T	f	$\dot{V}$	Carbon dioxide breathing[c] V_T	f	$\dot{V}$
Saline	–	18	−4 ± 6	−5 ± 3	−9 ± 6	−1 ± 6	−5 ± 3	−8 ± 4
Baclofen	0.3	12	−15 ± 9	−6 ± 4	−18 ± 6	23 ± 17	−9 ± 4	−8 ± 7
	1	12	0 ± 8	−36 ± 5*	−38 ± 8*	−13 ± 11	−27 ± 4*	−26 ± 9*
	3	19	3 ± 8	−52 ± 5*	−54 ± 4*	−4 ± 7	−34 ± 5*	−56 ± 6*
	10	6	−46 ± 6*	−74 ± 3*	−85 ± 3*	−38 ± 11*	−51 ± 6*	−68 ± 9*
3-APPi	0.3	10	−8 ± 13	−8 ± 7	−4 ± 8	7 ± 7	−6 ± 3	1 ± 8
	3	8	0 ± 13	8 ± 5	−12 ± 3	−6 ± 10	0 ± 4	3 ± 10
	30	9	−8 ± 4	−3 ± 4	−13 ± 3	−20 ± 9	−4 ± 5	−5 ± 20
	100	9	−15 ± 3	1 ± 6	−14 ± 6	−3 ± 6	5 ± 7	−5 ± 14

[a]Compounds given 30 min before measurement. [b]Mean ± s.e.mean % change of room air and CO_2 enriched gas breathing ventilation due to compound, compared with respective baseline values. Baseline values for room air breathing are: $V_T = 2.5 \pm 0.2$, $f = 122 \pm 5$ and $\dot{V} = 309 \pm 20$. Baseline ventilation values due to carbon dioxide breathing are: $V_T = 7.5 \pm 0.4$, $f = 114 \pm 3$ and $\dot{V}$ 862 ± 55. [c]Exposure to 10% CO_2, 21% O_2, 69% N_2 for 10 min.
*$P < 0.05$ compared to values before administration of compound.

Table 2 Effect of selective antagonists on the respiratory depressant effects of baclofen during carbon dioxide breathing

First[a]	Treatment	Second[b]	n	Percent change[c] of minute volume
Saline		Baclofen	17	−46 ± 4*
Bicuculline	(30 mg kg^{-1})	Baclofen	6	−40 ± 13*
Bicuculline	(30 mg kg^{-1})	Saline	6	+1 ± 8
Naloxone	(1 mg kg^{-1})	Baclofen	12	−69 ± 7*
Naloxone	(1 mg kg^{-1})	Saline	6	−18 ± 10

[a]Compounds given 10 min before baclofen.
[b]Baclofen (3 mg kg^{-1}, s.c.) or saline given 30 min before measurement of minute volume during CO_2 inhalation.
[c]Mean ± s.e.mean percent change of minute volume due to baclofen or saline.
*$P < 0.05$ compared to values before administration of compounds.

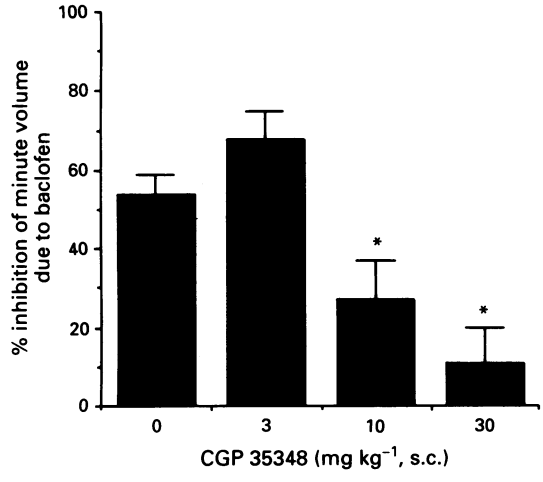


Figure 1 Blockade of baclofen-induced depression of CO_2 breathing with the $GABA_B$ antagonst, CGP 35348. Columns represent mean ± s.e.mean percentage inhibition of minute volume due to baclofen (3 mg kg^{-1}, s.c.) ($n = 6-12$); *$P < 0.05$, represents statistically significant differences from ventilation responses in animals that received baclofen alone.

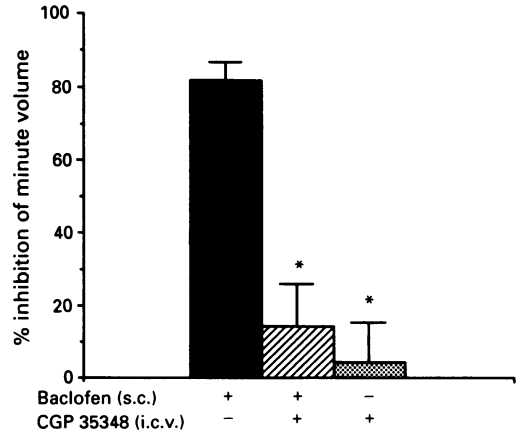


Figure 2 Effect of intracerebroventricular (i.c.v.) CGP 35348 on baclofen-induced depression of CO_2 breathing in the guinea-pig. Solid column is baclofen (3 mg kg^{-1}, s.c.) + i.c.v. artificial CSF; cross-hatched column is baclofen (3 mg kg^{-1}, s.c.) + i.c.v. CGP 35348 (50 μg). Stippled column is saline (s.c.) + i.c.v. CGP 35348 (50 μg). Values represent mean ± s.e.mean. *$P < 0.05$, compared to animals that received baclofen alone. Note that CGP 35348 given alone did not affect ventilation responses to CO_2 breathing.

We previously found that baclofen and 3-APPi are potent antitussive agents that inhibit the cough reflex by acting at central and peripheral $GABA_B$ receptors respectively. In the present study, baclofen inhibited ventilation at doses of 1 mg kg^{-1}, s.c. and higher. This is greater than the dose of baclofen ($ED_{30} = 0.04$ mg kg^{-1}, s.c.) required to inhibit cough in guinea-pigs (Bolser *et al.*, 1993). Therefore, the antitussive activity of baclofen can be dissociated from its ability to exert central respiratory depressant effects. This may reflect either a peripheral site of action of baclofen that inhibits cough or an effect on components of the central cough generation system that may be more sensitive and/or automatically separate from neurones controlling ventilation and ventilatory responses to CO_2.

3-APPi is a potent $GABA_B$ receptor agonist that inhibits neurally-evoked tachykinin and cholinergic mediated bronchospasm (Chapman *et al.*, 1993a) and cough (Bolser *et al.*, 1994) in guinea-pigs. This compound demonstrates antitussive activity in guinea-pigs with an ED_{30} of 0.36 mg kg^{-1}, subcutaneously (Bolser *et al.*, 1993). In our study, 3-APPi had no effect on ventilation or on the ventilatory responses to CO_2 inhalation at doses as high as 100 mg kg^{-1}, s.c.; this dose is 278 fold greater than its antitussive ED_{30} (Bolser *et al.*, 1993). Although a possible explanation for this result is that 3-APPi does not cross the blood-brain barrier (Hills & Howson, 1992), the present finding that centrally administered 3-APPi does not inhibit ventilation argues against this mechanism playing a significant role in the lack of respiratory depressant effects of 3-APPi. Taken together, the most likely explanation for these results is that baclofen inhibits ventilation by activation of 3-APPi-insensitive $GABA_B$ receptors in the CNS.

We have previously reported that $GABA_B$ receptor agonists inhibit a variety of neural responses in the airways that may be important in the pathogenesis of airway obstructive diseases, like asthma (Chapman *et al.*, 1993b). Therefore, $GABA_B$ agonists may be therapeutically useful for the treatment of these airway disorders. The results from this study indicate that CNS penetration could limit the therapeutic usefulness of a $GABA_B$ receptor agonist due to respiratory depression. However, $GABA_B$ agonists that preferentially activate peripheral $GABA_B$ receptors and do not penetrate the blood-brain barrier may be devoid of the liability to this potential side effect.

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References

BELVISI, M.G., ICHINOSE, M. & BARNES, P.J. (1989). Modulation of non-adrenergic, non-cholinergic neural bronchoconstriction in guinea-pig airways via GABA-B receptors. *Br. J. Pharmacol.*, **97**, 1225–1231.

BOLSER, D.C., AZIZ, S.M., DEGENNARO, F.C., KREUTNER, W., EGAN, R.W., SIEGEL, M.I. & CHAPMAN, R.W. (1993). Antitussive effects of GABA-B agonists in the cat and guinea pig. *Br. J. Pharmacol.*, **110**, 491–495.

BOLSER, D.C., DEGENNARO, F.C., O'REILLY, S., CHAPMAN, R.W., KREUTNER, W. & HEY, J.A. (1994). Peripheral and central sites of action of GABA-B agonists to inhibit the cough reflex in the cat and guinea pig. *Br. J. Pharmacol.*, **113**, 1344–1348.

CHAPMAN, R.W., DANKO, G., DEL PRADO, M, EGAN, R.W., KREUTNER, W., RIZZO, C.A. & HEY, J.A. (1993a). Further evidence for prejunctional GABA-B inhibition of cholinergic and peptidergic bronchoconstriction in guinea pigs: Studies with new agonists and antagonists. *Pharmacology*, **46**, 315–323.

CHAPMAN, R.W., DANKO, G., RIZZO, C., EGAN, R.W., MAUSER, P.J. & KREUTNER, W. (1991). Prejunctional GABA-B inhibition of cholinergic, neurally-mediated airway contractions in guinea pigs. *Pulmon. Pharmacol.*, **4**, 218–224.

CHAPMAN, R.W., HEY, J.A., RIZZO, C.A. & BOLSER, D.C. (1993b). GABA-B receptors in the lung. *Trends Pharmacol. Sci.*, **14**, 26–29.

DANKO, G. & CHAPMAN, R.W. (1988). Simple, noninvasive method to measure the antibronchoconstriction activity of drugs in conscious guinea pigs. *J. Pharmacol. Methods*, **19**, 165–173.

DANKO, G., HEY, J.A., DEL PRADO, M., KREUTNER, W. & CHAPMAN, R.W. (1992). GABA-B inhibition of peptidergic airway microvascular leakage in guinea pigs. *Pharmacol. Commun.*, **1**, 203–209.

DA SILVA, A.M.T., HARTLEY, B., HAMOSH, P., QUEST, J.A. & GILLIS, R.A. (1987). Respiratory depressant effects of GABA-A and B-receptor agonists in the cat. *J. Appl. Physiol.*, **62**, 2264–2272.

DEFEUDIS, F.V. (1984). GABA and respiratory function. *Gen. Pharmacol.*, **15**, 441–443.

HILLS, J.M. & HOWSON, W. (1992). The GABA-B receptor profile of a series of phosphinic acids-agonist and antagonist activity in a range of peripheral tissues. In *GABA outside the CNS*, ed. Erdö, S.L. pp. 249–260. Berlin, Heidelberg, New York: Springer-Verlag.

LALLEY, P.M. (1983). Biphasic effects of baclofen on phrenic motoneurons: possible involvement of two types of γ-aminobutyric acid (GABA) receptors. *J. Pharmacol. Exp. Ther.*, **226**, 616–645.

LIPSCOMB, D.J. & MEREDITH, T.J. (1980). Baclofen overdose. *Postgrad. Med. J.*, **56**, 108–109.

MAUSER, P.J., EDELMAN, N.H & CHAPMAN, R.W. (1988). Central nervous system control of airway tone in guinea pigs: the role of histamine. *J. Appl. Physiol.*, **65**, 2024–2029.

MCLEOD, R.L., GERTNER, S.B. & HEY, J.A. (1991). Modulation of cardiovascular function by central histamine H_3 receptors in conscious guinea pigs. *Eur. J. Pharmacol.*, **209**, 141–142.

OLPE, H.R., KARLSSON, G., POZZA, M.F., BRUGGER, F., STEINMAN, M., RIEZEN, H.V., FAGG, G., HALL, R.G., FROESTL, W. & BITTIGER, H. (1990). CGP 35348: A centrally active blocker of GABA-B receptors. *Eur. J. Pharmacol.*, **187**, 27–38.

SCHMID, K., BÖHMER, G. & GEBAUER, K. (1989). GABA-B receptor mediated effects on central respiratory system and their antagonism by phaclofen. *Neurosci. Lett.*, **99**, 305–310.

WAGNER, P.G. & DEKIN, M.S. (1993). GABA-B receptors are coupled to a barium-insensitive outward rectifying potassium conductance in promoter respiratory neurons. *J. Neurophysiol.*, **69**, 286–289.

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Unexpected prosecretory action component of loperamide at μ–opioid receptors in the guinea-pig colonic mucosa *in vitro*

Wolfgang Kromer

Byk Gulden, Department of Pharmacology, Byk-Gulden-Str. 2, D-78467 Konstanz, Germany

1 In a voltage clamp setting (Ussing chamber), the antidiarrhoeal drug, loperamide (Lop) slightly augmented prostaglandin E_1 (PGE_1) plus theophylline-stimulated net chloride secretion above control values at low concentrations (10^{-10} and 10^{-9} M) but inhibited it at higher concentrations (10^{-6} and 10^{-5} M). The apparently weak prosecretory action component of Lop was turned into a clear cut antisecretory effect by pretreatment with 2×10^{-7} M naloxonazine plus 10^{-7} M CTOP-NH_2 (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2), two selective μ opioid receptor antagonists. This indicates a prosecretory effect of loperamide at μ opioid receptors. The antisecretory effect of low Lop concentrations, uncovered by μ opioid receptor blockade, was prevented by additional blockade of κ opioid receptors by 5×10^{-9} M nor-binaltorphimine (nor-BNI).

2 The nonselective opioid antagonist, naloxone, at 10^{-6} M did not significantly reduce either PGE_1 plus theophylline-stimulated net chloride secretion in Lop-free controls or the antisecretory action of Lop. By contrast, the partial agonist ethylketocyclazocine (EKC), which activates κ but blocks μ opioid receptors, concentration-dependently inhibited PGE_1 plus theophylline-stimulated net chloride secretion without any consistent prosecretory action component. Nor-BNI at 5×10^{-8} M significantly blocked the antisecretory action of EKC.

3 It is concluded that, in the guinea-pig colonic mucosa under the present conditions, μ opioid receptors mediate enhancement and κ opioid receptors inhibition of PGE_1-stimulated net chloride secretion by low Lop concentrations. The two opposite actions are largely masked by superimposition. An opioid receptor-independent mechanism of action contributes to the antisecretory effect of Lop at high concentrations.

Keywords: Opioid receptor types; colonic chloride secretion; loperamide; guinea-pig colonic mucosa

Introduction

As a basis of its antidiarrhoeal action, loperamide displays net antisecretory effects in the human jejunum *in vivo* (Hughes *et al.*, 1984). A similar effect in the rat *in vivo* is blocked by the competitive opioid antagonist, naloxone, suggesting its mediation by opioid receptors (Piercey & Ruwart, 1979). Apart from this opioid-specific antisecretory action, non-opioid action components of loperamide have been suggested, i.e., calcium channel blocking-like properties (Burleigh, 1988) and inhibition of calmodulin at micromolar concentrations (Stoll *et al.*, 1988). However, irrespective of the particular mechanisms of the antisecretory/antidiarrhoeal action of loperamide, its predominant peripheral action has been attributed to its unique pharmacokinetic disposition (Miyazaki *et al.*, 1982). This may be due to enterohepatic shunting (see Awouters *et al.*, 1983; Ooms *et al.*, 1984) and accumulation of loperamide within the intestinal wall possibly caused by its surface tension-lowering properties (Wüster & Herz, 1978).

The opioid-specific action component of loperamide has been characterized by its binding to and activation of opioid receptors in 'much the same way as do classical opiate agonists' (Awouters *et al.*, 1983). Little if anything is known about the receptor-subtype preference of loperamide and its relation to the antisecretory/antidiarrhoeal effect. Interestingly, the μ-selective opioid agonist DAMGOL has recently been shown to enhance PGE_1 plus theophylline-stimulated net chloride secretion in the guinea-pig colonic mucosa *in vitro*, while the μ-selective opioid antagonist, CTOP-NH_2 (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) and the κ-selective agonist, U69593, inhibited it (Kromer, 1991; 1993). Therefore, the present experiments have been performed in order to search for a prosecretory action component of loperamide and, if present, identify the opioid receptor types responsible for the prosecretory and antisecretory action components of this antidiarrhoeal drug.

Methods

Experimental system and conditions

Mucosal preparations with adherent submucosal plexus, free of circular and longitudinal muscle layers, were obtained from the distal colon of male guinea-pigs (350–450 g body wt) and mounted in Ussing-type chambers (see Kromer, 1991). Short circuit current (I_{SC}) was measured as an indicator of net anion secretion in the presence of 100 μM amiloride after stimulation with 1 μM prostaglandin E_1 (PGE_1) plus 100 μM theophylline. Amiloride was used to block Na^+ fluxes. Under these conditions, the attribution of changes in I_{SC} to changes in Cl^- secretion is based on control experiments performed in the absence of Cl^- (substitution by gluconate) and has been discussed elsewhere (Kromer, 1991). The composition of the buffer was (mM): NaCl 113.6, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 1.2, Na_2HPO_4 2.4, NaH_2PO_4 0.6, $NaHCO_3$ 21, glucose 10, pH 7.4, 37°C, gassed with 95% O_2 plus 5% CO_2.

Experimental protocol

After at least 45 min of tissue equilibration, drugs were administered in a single experiment in the order, and according to the time schedule, shown in Figure 1. Drug concentra-

tions are also indicated there. In contrast to the opioid agonists, loperamide and EKC, opioid antagonists (if used) were always present both in test preparations and in agonist-free controls.

Amiloride was applied to the mucosal side and PGE_1 to the serosal side. All of the other compounds were applied to either side. I_{SC} recordings were made every 2.5 min. The difference between the mean value of four recordings after PGE_1 and the basal value immediately before stimulation was taken as ΔI_{SC} (see Figure 1). Note that, in Figures 2–4, ΔI_{SC} in controls (i.e., either loperamide- or EKC-free preparations otherwise pretreated as indicated in the respective horizontal line in Figure 1 and stimulated by PGE_1 plus theophylline) has been set at zero. Accordingly, the absolute value of ΔI_{SC} measured in the control preparation of one intestine has been subtracted from the ΔI_{SC} values of all the other preparations taken from the same intestine. $1\ \mu A = 0.037\ \mu Eq \times h^{-1} \times cm^{-2}$. Seven concentrations of loperamide (10^{-11} to 10^{-5} M) have been used in adjacent preparations, one concentration each, plus one control preparation, all taken from the same animal. Thus, eight possible locations along the distal colon were randomly allocated to the various drug concentrations. The distal 5 cm proximal to the anus were discarded. For a repeat experiment shown in Figure 6, only one loperamide concentration (10^{-9} M) was used.

Drugs

Amiloride HCl, loperamide HCl, naltrindole HCl, PGE_1 (all Sigma, Germany); CTOP-NH_2 (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (Peninsula, U.S.A.); ethylketocyclazocine methane sulphonate (Sterling-Winthrop Res. Inst., U.S.A.); naloxonazine, nor-binaltorphimine HCl (Research Biochemicals Inc., U.S.A.); naloxone HCl (Endo Labs. Inc, U.S.A.); theophylline (Byk Gulden, Germany). Aqueous solutions were prepared except for PGE_1 and loperamide. A stock solution of PGE_1 was made in absolute ethanol, and 10 µl were added to 10 ml bathing solution. Loperamide was dissolved in methanol and diluted with water to a stock solution of 10% (vol/vol) methanol. After a further dilution with water, 100 µl were added to 10 ml bathing solution.

Statistical analysis

The statistical significance of ΔI_{SC} values (see above) was evaluated in the Student's t test for unpaired (Figure 2–4) or paired samples (Figure 6), two sided reading.

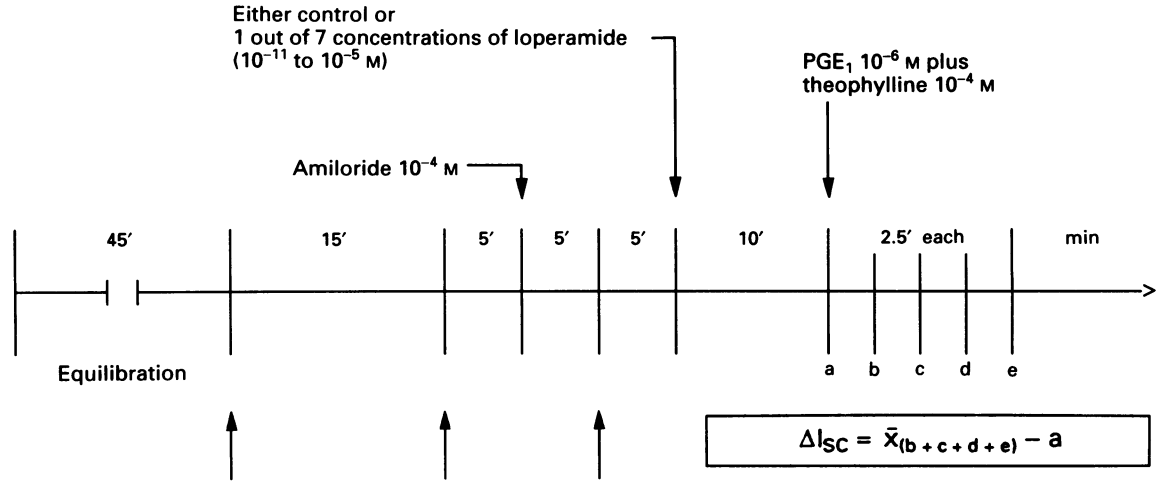


Figure 2:	Naloxonazine	Naloxonazine	CTOP-NH_2	each	10^{-7} M (▽)
	Naloxonazine	Naloxonazine	CTOP-NH_2	each	10^{-7} M
			plus nor-BNI		5×10^{-9} M (□)
	——	——	nor-BNI		5×10^{-9} M (○)
	——	——	Control		(▼)
Figure 3:	——	——	Naloxone		10^{-6} M (◇)
	——	——	Naltrindole		10^{-9} M (●)
	——	——	Control		(▼)
Figure 4:	——	——	Control with loperamide (Lop)		(▼)
	——	——	Control with EKC instead of Lop		(△)
	——	——	Prior to EKC: nor-BNI 5×10^{-8} M		(▲)
Treatment above time axis: uniform for all tissues, except for EKC					(△; ▲)

Figure 1 Time schedule of a single experiment, the basis for calculation of ΔI_{SC} within a single experiment, and the different treatments used. Note that, in Figures 2–4, ΔI_{SC} in controls (i.e., either loperamide- or ethylketocyclazocine (EKC)-free preparations otherwise pretreated as indicated in the respective horizontal line and stimulated by prostaglandin E_1 (PGE_1) plus theophylline: see above) has been set at zero. Accordingly, the absolute value of ΔI_{SC} measured in the control preparation of one intestine has been subtracted from the ΔI_{SC} values of all the other preparations taken from the same intestine. For absolute control values, see legends to Figures 2–4. Dashes (-) indicate that no naloxonazine was administered at this time point.

Results

At relatively low concentrations of 10^{-10} and 10^{-9} M, loperamide tended to enhance (not statistically significant) net chloride secretion stimulated by PGE_1 plus theophylline (Figure 2). The higher loperamide concentrations of 10^{-6} and 10^{-5} M produced a clear cut inhibition. Selective blockade of μ opioid receptors by pretreatment of the preparations with both naloxonazine and CTOP-NH_2 largely prevented the prosecretory action component of loperamide thereby unmasking its antisecretory action in the same low concentration range (Figure 2). Additional blockade of κ opioid receptors by nor-BNI prevented this antisecretory action component of loperamide, shifting the concentration-response curve back to that obtained with loperamide alone. Thus, the latter data represent a superimposition of prosecretory and antisecretory action components (Figure 2). Nor-BNI alone tended to augment slightly the prosecretory effect of loperamide on PGE_1 plus theophylline-stimulated net chloride secretion at low concentrations and to impair slightly the antisecretory action of high loperamide concentrations (Figure 2).

No effect on the prosecretory or antisecretory action component of loperamide was found with 10^{-6} M naloxone (Figure 3). The δ-selective antagonist, naltrindole (Portoghese *et al.*, 1988) at 10^{-9} M affected only the action of 10^{-9} M loperamide (Figure 3). The biological significance of this finding remains unclear.

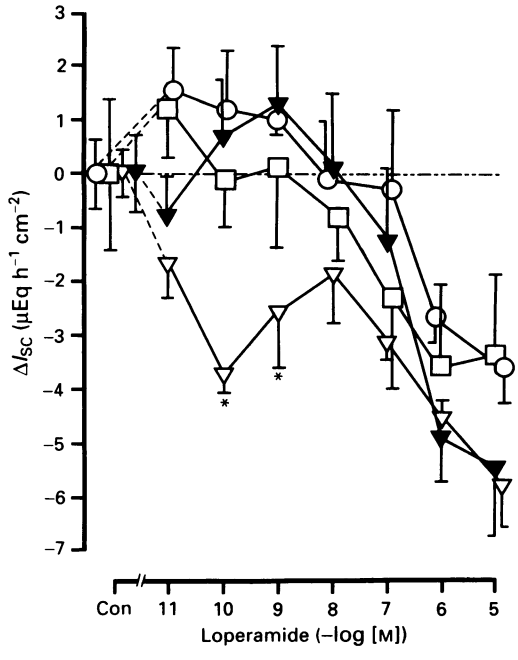


Figure 2 ΔI_{SC} (ordinate scale) normalized by subtraction of ΔI_{SC} values observed in control preparations from the same animals (see Figure 1) and plotted against loperamide concentration (abscissa scale). The mean ΔI_{SC} from controls of any one treatment group has been subtracted from each single ΔI_{SC} control value in order to maintain variation in normalized controls. For symbols and general explanation, see Figure 1. Absolute ΔI_{SC} values in loperamide-free controls were: 7.4 ± 0.6 (▼); 7.1 ± 0.3 (▽); 6.1 ± 1.3 (□); 5.2 ± 0.5 (○) (μEq h^{-1} cm^{-2}). Note that loperamide (▼) tended to enhance ΔI_{SC} above control values in a non-significant fashion at low (see also Figure 5), and significantly inhibited ΔI_{SC} at high concentrations. Enhancement was turned into inhibition by μ opioid receptor blockade by naloxonazine plus CTOP-NH_2 (▽), and was shifted back to control level by additional blockade of κ opioid receptors by nor-binaltorphimine (nor-BNI) (□). The κ antagonist nor-BNI alone (without μ opioid receptor blockade) caused a slight and insignificant elevation of ΔI_{SC} at very low and high loperamide concentrations (○). $n = 15$ (▼) and 4 (▽, □, ○). Values are mean ± s.e.mean P values are *<0.05 (▽ *vs* ▼).

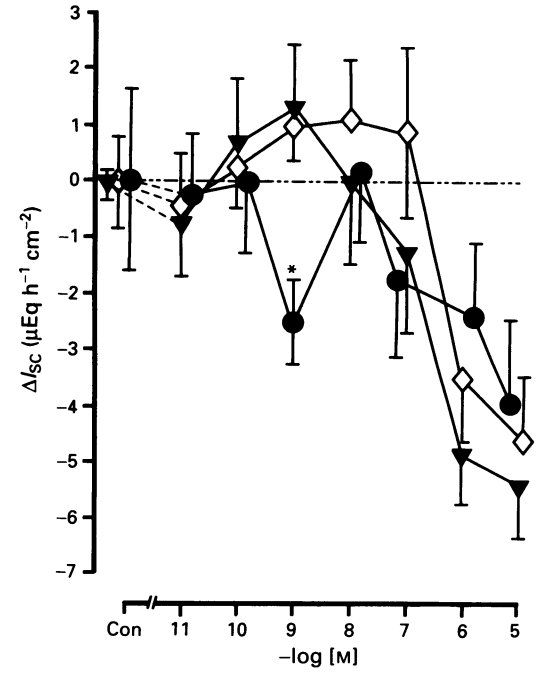


Figure 3 Concentration-response curves for loperamide in the presence of either naloxone or naltrindole. For symbols and general explanation, See Figure 1 and legend to Figure 2. Data of loperamide without pretreatment (▼) have been taken from Figure 2 for comparison. Absolute ΔI_{SC} values in controls were: 5.6 ± 0.7 (◇; naloxone 10^{-6} M; $n = 4$) and 6.9 ± 1.5 (●; naltrindole 10^{-9} M; $n = 4$) (μEq h^{-1} cm^{-2}). Values are mean ± s.e.mean. P value is *<0.05 (● *vs* ▼).

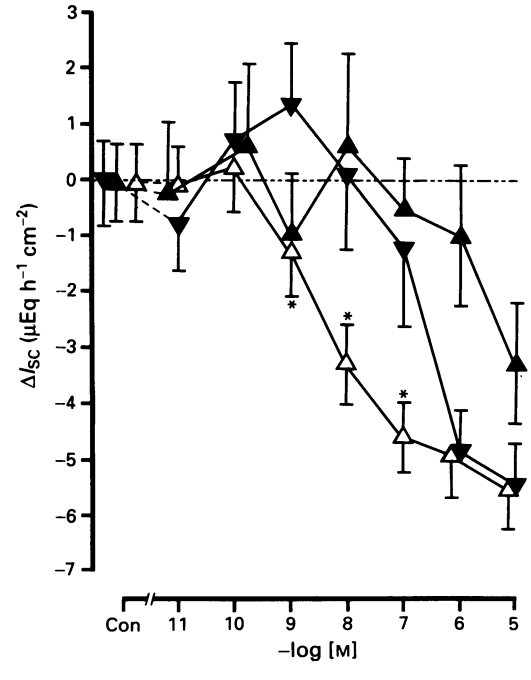


Figure 4 Concentration-response curves for ethylketocyclazocine (EKC) in the absence and presence of the κ-antagonist nor-binaltorphimine (nor-BNI). For symbols and general explanation, see Figure 1 and legend to Figure 2. Data of loperamide (▼) have been taken from Figure 2 for comparison. Absolute ΔI_{SC} values in controls were: 7.2 ± 0.6 (△); 7.3 ± 0.4 (▲) (μEq h^{-1} cm^{-2}). Note that EKC was used instead of loperamide either without (△; $n = 15$) or after pretreatment of the preparations with 5×10^{-8} M nor-BNI (▲; $n = 4$). Values are mean ± s.e.mean. P values are *<0.05 (△ *vs* ▼).

The partial agonist, ethylketocyclazocine (EKC), which activates κ but blocks μ opioid receptors, did not display any overall prosecretory effect on PGE_1 plus theophylline-stimulated net chloride secretion, but concentration-dependently inhibited it to the same maximum degree as observed with loperamide (Figure 4). However, the IC_{50} value of EKC was about two orders of magnitude lower (ca. 4×10^{-9} M) compared to that of loperamide. The κ-selective antagonist, nor-BNI at 5×10^{-8}M inhibited the antisecretory action of EKC (Figure 4).

Maximum net chloride secretion in the presence of loperamide (minus control value) was taken as an indicator of the stimulatory action component of loperamide, irrespective of the particular concentration at which it was found. The majority of the intestines used disclosed a prosecretory action component of loperamide (Figure 5a). Most of the stimulatory effects of loperamide have been observed at low concentrations (10^{-10} and 10^{-9} M; Figure 5b). Although a relatively large variation in the values of loperamide-induced elevation of net chloride secretion prevented the data from becoming statistically significant (Figure 2), Figure 5a clearly shows that most of the intestines displayed enhanced net chloride secretion in response to one of the low loperamide concentrations.

The experiments shown in Figure 2 were performed in late March/early April 1993. Repeat experiments done from October to December 1993 failed to confirm the antisecretory action of low loperamide concentrations in the presence of μ opioid receptor blockade by both naloxonazine and CTOP-NH_2 (data not shown). No obvious reasons for this failure could be found. However, another repeat experiment (Figure 6) done in May 1994 clearly confirmed the above result of the experiment shown in Figure 2. Thus, a seasonal variation may explain the discrepancies but this is still hypothetical.

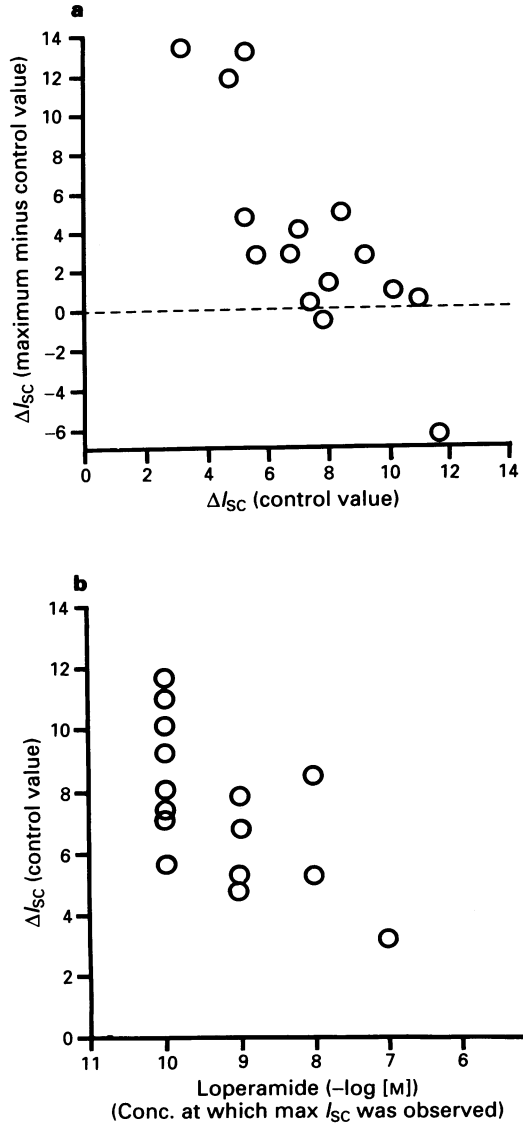


Figure 5 (a) Maximum ΔI_{SC} values (minus ΔI_{SC} in the corresponding loperamide-free control) obtained in the presence of any one concentration of loperamide (ordinate scale) plotted against the ΔI_{SC} value in the loperamide-free control preparation taken from the same animal (abscissa scale). One symbol represents one animal, $n = 15$. Same data as shown in Figures 2–4 (▼). (b) ΔI_{SC} values obtained in loperamide-free control preparations (ordinate scale) plotted against the loperamide concentration which produced the maximum ΔI_{SC} in test preparations taken from the same animals (abscissa scale). Same data as shown in (a).

Discussion

In contrast to the traditional view (for review, see Kromer, 1988), the present data clearly demonstrate that loperamide displays a prosecretory action component mediated, at least in the guinea-pig colon under the present *in vitro* conditions, by μ opioid receptors. An antisecretory action of loperamide at low concentrations, under the same experimental condi-

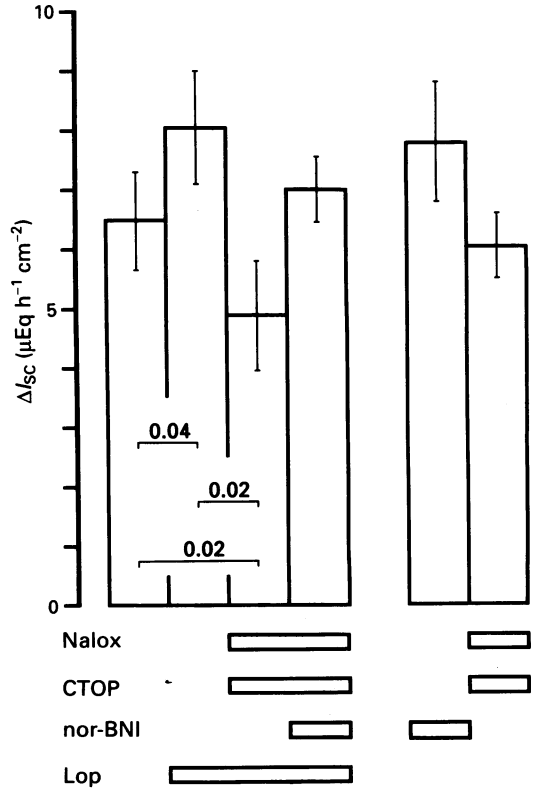


Figure 6 The influence of loperamide in the absence or presence of μ opioid receptor blockade by naloxonazine plus CTOP-NH_2 on net chloride secretion (ΔI_{SC}) stimulated by prostaglandin E_1 (PGE_1) plus theophylline. Repeat experiment to that shown in Figure 2. The horizontal bars indicate the presence of either naloxonazine 2×10^{-7} M (Nalox), CTOP-NH_2 10^{-7} M (CTOP), nor-binaltorphimine 5×10^{-9} M (nor-BNI), or loperamide 10^{-9} M (Lop). See Figure 1 for the time course of a single experiment. Values are mean ± s.e.mean. *P* values are indicated within the columns. $n = 4$. Note that loperamide alone increases net chloride secretion, an effect converted to a clear-cut decrease by concomitant μ opioid receptor blockade. This decrease in ΔI_{SC} relative to controls is prevented by κ opioid receptor blockade by nor-binaltorphimine.

tions, is due to activation of κ opioid receptors. These conclusions are based on selective blockade of the two opposite action components by either μ-selective (naloxonazine: Ling *et al.*, 1986; Paul & Pasternak, 1988; CTOP-NH_2: Gulya *et al.*, 1986) or κ-selective opioid antagonists (norbinaltorphimine: Portoghese *et al.*, 1987; for a review on opioid receptor type-selective ligands, see Kosterlitz, 1991). In the absence of any selective opioid receptor blockade, the two opposite actions are masked by superimposition but, under μ opioid receptor blockade, the antisecretory action component at κ opioid receptors becomes evident (Figure 2). Concomitant blockade of both μ and κ opioid receptors by either selective antagonists in combination or by a nonselectively high concentration of naloxone prevented both of the two action components thus producing the same overall result as their superimposition in the absence of antagonists. δ Opioid receptors seem to be of no major functional significance in the control of net chloride secretion in the guinea-pig colon *in vitro*, at least when stimulated by PGE_1 plus theophylline (Kromer, 1991; Kromer & Beubler, 1993). It has been shown in a previous publication by substitution of gluconate for chloride (Kromer, 1991) that changes in I_{SC} caused by alterations in opioid receptor activation relate to changes in net chloride secretion.

Naloxonazine (2×10^{-7} M) plus CTOP-NH_2 (10^{-7} M) did not completely prevent a rudimentary 'prosecretory' action component of loperamide but strongly impaired and shifted it to higher loperamide concentrations (i.e., 10^{-8} M, Figure 2). This failure of low antagonist concentrations to block completely the prosecretory action at high loperamide concentrations might be explained by an at least partial failure of naloxonazine to inhibit irreversibly μ opioid receptors (Cruciani *et al.*, 1987), in conjunction with accumulation of loperamide in the intestinal tissue (Wüster & Herz, 1978). Since multiple μ opioid receptor subtypes may exist (Pasternak & Wood, 1986), a combination of naloxonazine and CTOP-NH_2 was used in the present study to try to block more or less all of them. It may be noted in this context that loperamide produced different K_D values, in binding studies on guinea-pig myenteric plexus membranes, of about 10^{-9} and 10^{-7} M (Mackerer *et al.*, 1976; Clay *et al.*, 1977). Nevertheless, except for naloxone, low antagonist concentrations have been chosen in the present study in order to keep antagonist effects selective for the respective opioid receptor types, accepting potential incomplete blockade at high loperamide concentrations.

Ethylketocyclazocine (EKC) was used as a partial agonist activating κ (Pfeiffer *et al.*, 1982; Cheng *et al.*, 1992) but blocking μ opioid receptors in the nanomolar range, in just the same way as ketazocine-like compounds (Magnan *et al.*, 1982; Gillan & Kosterlitz, 1982; Sheldon *et al.*, 1987). As expected on grounds of this partial agonist activity, EKC did not produce any consistent prosecretory effect but inhibited net chloride secretion, by a factor of almost 100, more potently than loperamide. A concentration of 5×10^{-8} M of the κ-selective antagonist nor-BNI was used to demonstrate opioid receptor mediation of the net antisecretory action of EKC. This concentration of nor-BNI may block μ and δ opioid receptors to some extent, but this should be insignificant when used against a κ-selective agonist like EKC.

Although the failure of EKC to enhance net chloride secretion above control values is reasonably explained by its μ-antagonist properties, the greater antisecretory potency of EKC compared to loperamide is less easily explained. It is difficult to assess, on the basis of available binding data, to what degree a lower binding affinity of loperamide, compared with EKC, to functional κ opioid receptors may contribute to this difference (see Clay *et al.*, 1977). Because of the unique physicochemical properties of loperamide, its tissue concentration and, along with it, its pharmacodynamic effect increases over time (Wüster & Herz, 1978). Since it was the purpose of the present study to unravel opioid receptor-mediated actions, and not to investigate the pharmacokinetic properties, preincubation time with loperamide was the same as with EKC. Longer preincubation times might further increase the antisecretory effect of loperamide and, thereby, obscure its prosecretory action component.

Naloxone at a high concentration of 10^{-6} M, which blocks more or less all opioid receptor types, did not significantly affect the action of loperamide (Figure 3). This may reflect blockade of both prosecretory and antisecretory action components. The antisecretory activity of high concentrations of loperamide in the presence of naloxone is probably not related to activation of any opioid receptors and may be due, instead, to blockade of calcium channels (Reynolds *et al.*, 1984) or inactivation of calmodulin (Zavecz *et al.*, 1982).

The highest frequency (as opposed to magnitude) of the prosecretory action component of lopermide in the absence of μ opioid receptor blockade was found at low loperamide concentrations of 10^{-10} and 10^{-9} M (Figure 5b). It should be noted that the antisecretory action of these low concentrations of loperamide, in the presence of μ opioid receptor blockade, was observed in intestines that displayed absolute ΔI_{SC} values (see legend to Figure 2) in the sole presence of naloxonazine plus CTOP-NH_2 that were not different from those seen in drug-free control preparations. This is consistent with the view that κ opioid receptors were not endogenously activated and still available for activation by loperamide.

An antisecretory action of the μ-selective opioid antagonist, CTOP-NH_2, was also observed with regard to inhibition of net water secretion stimulated by PGE_2 in the jejunal loop of the anaesthetized rat (Kromer & Beubler, 1993). Hence, net prosecretory/antiabsorptive action components of loperamide may relate to a general μ opioid receptor mechanism not restricted to the guinea-pig colon *in vitro*.

In conclusion, this is the first time that a prosecretory action component of loperamide at low concentrations has been detected and related to activation of μ opioid receptors. It is superimposed on an antisecretory action component mediated by activation of κ opioid receptors, when secretion was stimulated by PGE_1 plus theophylline. At micromolar concentrations, loperamide displays an additional antisecretory action component that is largely independent of opioid receptors.

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References

AWOUTERS, F., NIEMEGEERS, C.J.E. & JANSSEN, P.A.J. (1983). Pharmacology of antidiarrhoeal drugs. *Annu. Rev. Pharmacol. Toxicol.*, **23**, 279–301.

BURLEIGH, D.E. (1988). Opioid and non-opioid actions of loperamide on cholinergic nerve function in human isolated colon. *Eur. J. Pharmacol.*, **152**, 39–46.

CHENG, J., ROQUES, B.P., GACEL, G.A., HUANG, E. & PASTERNAK, G.W. (1992). κ_3 opiate receptor binding in the mouse and rat. *Eur. J. Pharmacol.*, **226**, 15–20.

CLAY, G.A., MACKERER, C.R. & LIN, T.K. (1977). Interaction of loperamide with [^{3}H]naloxone binding sites in guinea-pig brain and myenteric plexus. *Mol. Pharmacol.*, **13**, 533–540.

CRUCIANI, R.A., LUTZ, R.A., MUNSON, P.J. & RODBARD, D. (1987). Naloxonazine effects on the interaction of enkephalin analogs with mu-1, mu and delta opioid binding sites in rat brain membranes. *J. Pharmacol. Exp. Ther.*, **242**, 15–20.

GILLAN, M.G.C. & KOSTERLITZ, H.W. (1982). Spectrum of the μ-, δ- and κ-binding sites in homogenates of rat brain. *Br. J. Pharmacol.*, **77**, 461–469.

GULYA, K., PELTON, J.T., HRUBY, V.J. & YAMAMURA, H.I. (1986). Cyclic somatostatin octapeptide analogues with high affinity and selectivity toward mu opioid receptors. *Life Sci.*, **38**, 2221–2229.

HUGHES, S., HIGGS, N.B. & TURNBERG, L.A. (1984). Loperamide has antisecretory activity in the human jejunum *in vivo*. *Gut*, **25**, 931–935.

KOSTERLITZ, H.W. (1991). Opioid receptor subtypes: past, present and future. In *Neurobiology of Opioids*. ed. Almeida, O.F.X. & Shippenberg, T.S. pp. 3–9, Berlin: Springer.

KROMER, W. (1988). Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacol. Rev.*, **40**, 121–162.

KROMER, W. (1991). Voltage-clamp experiments reveal receptor type-dependent modulation of chloride secretion in the guinea-pig colonic mucosa by intestinal opioids. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 360–367.

KROMER, W. (1993). Synergistic opioid inhibition of colonic Cl^- secretion by κ-opioid receptor agonism plus μ-opioid receptor antagonism. *Eur. J. Pharmacol.*, **230**, 235–237.

KROMER, W. & BEUBLER, E. (1993). Dual opioid modulation of chloride/water secretion in the guinea-pig colon *in vitro* and the rat jejunum *in vivo*. *J. Auton. Pharmacol.*, **13**, 315–321.

LING, G.S.F., SIMANTOV, R., CLARK, J.A. & PASTERNAK, G.W. (1986). Naloxonazine actions *in vivo*. *Eur. J. Pharmacol.*, **129**, 33–38.

MACKERER, C.R., CLAY, G.A. & DAJANI, E.Z. (1976). Loperamide binding to opiate receptor sites of brain and myenteric plexus. *J. Pharmacol. Exp. Ther.*, **199**, 131–140.

MAGNAN, J., PATERSON, S.J., TAVANI, A. & KOSTERLITZ, H.W. (1982). The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn-Schmied. Arch. Pharmacol.*, **319**, 197–205.

MIYAZAKI, H., NAMBU, K. & HASHIMOTO, M. (1982). Loperamide in rat intestines: a unique disposition. *Life Sci.*, **30**, 2203–2206.

OOMS, L.A.A., DEGREYSE, A.D. & JANSSEN, P.A.J. (1984). Mechanisms of action of loperamide. *Scand. J. Gastroenterol.*, **19** (suppl. 96), 145–155.

PASTERNAK, G.W. & WOOD, P.J. (1986). Minireview: multiple mu opiate receptors. *Life Sci.*, **38**, 1889–1898.

PAUL, D. & PASTERNAK, G.W. (1988). Differential blockade by naloxonazine of two μ opiate actions: analgesia and inhibition of gastrointestinal transit. *Eur. J. Pharmacol.*, **149**, 403–404.

PFEIFFER, A., SADEE, W. & HERZ, A. (1982). Differential regulation of the μ-, δ-, and κ-opiate receptor subtypes by guanyl nucleotides and metal ions. *J. Neurosci.*, **2**, 912–917.

PIERCEY, M.F. & RUWART, M.J. (1979). Naloxone inhibits the antidiarrhoeal activity of loperamide. *Br. J. Pharmacol.*, **66**, 373–375.

PORTOGHESE, P.S., LIPKOWSKI, A.W. & TAKEMORI, A.E. (1987). Binaltorphimine and nor-binaltorphimine, potent and selective κ-opioid receptor antagonists. *Life Sci.*, **40**, 1287–1292.

PORTOGHESE, P.S., SULTANA, M. & TAKEMORI, A.E. (1988). Naltrindole, a highly selective and potent non-peptide δ opioid receptor antagonist. *Eur. J. Pharmacol.*, **146**, 185–186.

REYNOLDS, I.J., GOULD, R.J. & SNYDER, S.H. (1984). Loperamide: blockade of calcium channels as a mechanism for antidiarrhoeal effects. *J. Pharmacol. Exp. Ther.*, **231**, 628–632.

SHELDON, R.J., NUNAN, L. & PORRECA, F. (1987). Mu antagonist properties of kappa agonists in a model of rat urinary bladder motility *in vivo*. *J. Pharmacol. Exp. Ther.*, **243**, 234–240.

STOLL, R., RUPPIN, H. & DOMSCHKE, W. (1988). Calmodulin-mediated effects of loperamide on chloride transport by brush border membrane vesicles from human ileum. *Gastroenterol.*, **95**, 69–76.

WÜSTER, M. & HERZ, A. (1978). Opiate agonist action of antidiarrheal agents *in vitro* and *in vivo* – findings in support of selective action. *Naunyn-Schmied. Arch. Pharmacol.*, **301**, 187–194.

ZAVECZ, J.H., JACKSON, T.E., LIMP, G.L. & YELLIN, T.O. (1982). Relationship between anti-diarrheal activity and binding to calmodulin. *Eur. J. Pharmacol.*, **78**, 375–377.

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Mediation of noradrenaline-induced contractions of rat aorta by the α_{1B}-adrenoceptor subtype

[1]R. Testa, L. Guarneri, E. Poggesi, I. Simonazzi, C. Taddei & A. Leonardi

Research and Development Division, Recordati S.p.A., 20148 Milan, Italy

1 The subtypes of α_1-adrenoceptor mediating contractions to exogenous noradrenaline (NA) in rat aorta have been examined in both biochemical and functional studies.

2 Incubation of rat aortic membranes with the irreversible α_{1B}-adrenoceptor antagonist, chloroethylclonidine (CEC: 10 μM) did not change the K_D of [^{3}H]-prazosin binding in comparison to untreated membranes, but reduced by 88% the total number of binding sites (B_{max}).

3 Contractions of rat aortic strips to NA after CEC (50 μM for 30 min) incubation followed by repetitive washing, showed a marked shift in the potency of NA and a partial reduction in the maximum response. The residual contractions to NA after CEC incubation were not affected by prazosin (10 nM).

4 The competitive antagonists prazosin, terazosin, (R)-YM-12617, phentolamine, 5-methylurapidil and spiperone inhibited contractions to NA with estimated pA_2 values of 9.85, 8.54, 9.34, 7.71, 7.64 and 8.41, respectively.

5 The affinity of the same antagonists for the α_{1A}- and α_{1B}- adrenoceptors was evaluated by utilizing membranes from rat hippocampus pretreated with CEC, and rat liver, respectively. 5-Methylurapidil and phentolamine were confirmed as selective for the α_{1A}-adrenoceptors, whereas spiperone was α_{1B}-selective.

6 A significant correlation was found between the pA_2 values of the α_1-adrenoceptor antagonists tested and their affinity for the α_{1B}-adrenoceptor subtype, but not for the α_{1A}-subtype.

7 In conclusion, these findings indicate that in rat aorta most of the contraction is mediated by α_{1B}-adrenoceptors, and that the potency (pA_2) of an antagonist in this tissue should be related to its antagonistic effect on this subtype of the α_1-adrenoceptor population.

Keywords: Rat aorta; α_{1A}-adrenoceptors; α_{1B}-adrenoceptors; prazosin; terazosin; (R)-YM-12617; phentolamine; 5-methylurapidil; spiperone

Introduction

Since the subdivision of α_1-adrenoceptors into α_{1A}- and α_{1B}-subtypes (Morrow & Creese, 1986; Han *et al.*, 1987a,b), the subtype(s) of α_1-adrenoceptor present in rat aorta have been variously classified as α_{1B} (Han *et al.*, 1990; Eltze & Boer, 1992) both α_{1A} and α_{1B} (Tian *et al.*, 1990) or atypical (Muramatsu *et al.*, 1991; Oriowo *et al.*, 1992).

Recently, Aboud *et al.* (1993) stated that contractions of rat aorta induced by noradrenaline (NA) are mediated by non-α_{1A}, non α_{1B}-adrenoceptors, due both to the high potency of the α_{1A}-selective antagonists and the sensitivity to the selective α_{1B}-adrenoceptor alkylating agent, chloroethylclonidine (CEC) (Han *et al.*, 1987a).

This paper describes our attempts to characterize the α-adrenoceptor(s) in rat aorta by use of CEC both in biochemical and functional studies. In addition, since no absolutely subtype-selective antagonists are available, the potency of several α_1-adrenoceptor antagonists on rat aorta was also evaluated and compared to their affinity for the α_{1A}- and α_{1B}-adrenoceptor subtypes determined in rat hippocampus pretreated with CEC and rat liver, respectively. Some of the affinities of the antagonists for the α_1-adrenoceptor subtypes were previously published by our group (Testa *et al.*, 1993; Taddei *et al.*, 1993).

Methods

Male Sprague Dawley rats (300–450 g) were obtained from Charles River Italia.

[1] Author for correspondence.

Radioligand binding in rat aortic membranes

Preparation of membranes Rats were killed by cervical dislocation and the thoracic aorta dissected, washed in saline and homogenized in 50 vols of 50 mM Tris-HCl buffer (pH 7.4) in a Polytron (speed 7, 2 × 20 s). The homogenate obtained was filtered through four layers of cheesecloth, divided in two parts and centrifuged at 49000 *g* for 10 min. The pellets were resuspended in 50 vol of the ice-cold buffer, incubated for 30 min at 37°C and recentrifuged at 49000 *g* for 10 min. When the effects of irreversible receptor inactivation were studied, the buffer contained CEC (10 μM). The pellets were washed once more in drug-free ice-cold buffer and finally suspended in 160 vol of Tris-HCl.

[^{3}H]-prazosin binding studies Aliquots (2 ml) of aortic membranes were incubated for 30 min at 25°C with different concentrations of [^{3}H]-prazosin ranging from 0.05 to 8 nM. Non-specific binding was determined in the presence of 10 μM phentolamine. Incubations were terminated by vacuum filtration over 0.2% polyethyleneimine pretreated Whatman GF/B fibre filters using a Brandel cell harvester. The filters were then washed with 3 × 3 ml of ice-cold buffer and the radioactivity retained on the filters was counted in 10 ml of Filter Count (Packard) in a liquid scintillation spectrometer (counting efficiency of 40%).

Functional studies using rat aortic strips

Spiral strips were prepared from each artery (1 strip per aorta, fragments about 3 cm long were used) and suspended in 20 ml organ baths containing Krebs bicarbonate buffer of

the following composition (mM): NaCl 112.0, KCl 5.0, $CaCl_2$ 2.5, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 12.0 and glucose 11.1, equilibrated at 37°C with 95% O_2: 5% CO_2 and containing desmethylimipramine (0.1 μM) and corticosterone (1 μM) to block neuronal and extraneuronal uptake of NA, (±)-propranolol (1 μM) to block β-adrenoceptors and yohimbine (0.1 μM) to block α_2-adrenoceptors.

Tissues were subject to a passive load of 1.5 g and change in length measured with isotonic transducers (Basile 7006).

Tissues were allowed to equilibrate for 60 min and primed twice with 10 μM NA. After washing, the preparations were equilibrated for a further 60 min and a cumulative concentration-response curve to NA generated (generally from 1 nM to 100 μM). Following washout of NA and re-equilibration of the tissue (45 min), the drug to be tested was added and after 30 min, a second NA cumulative concentration-response curve constructed.

When CEC was the test-drug, following the 30 min incubation with the alkylating agent, the tissues were washed extensively three times (in 0.5 h) before constructing the second NA concentration-response curve. In order to evaluate the effects of prazosin in tissues previously treated with CEC, vascular preparations, subjected to the same protocol as above and after developing the second NA concentration-response curve, were washed with drug-free Krebs solution. After re-equilibration (45 min) the tissues were further incubated for 30 min with the antagonist before generating a third NA concentration-response curve. Proper control preparations treated with CEC and following vehicle incubation were prepared to verify any time-dependent change of NA response.

Vascular endothelium was not specifically removed; however, no particular care was taken to avoid stretching or damaging the luminal surface of the vessels, or to preserve its integrity.

Affinity for the α_{1A}- and α_{1B}-adrenoceptor subtypes

The affinity of different antagonists for the α_{1A}- and α_{1B}-adrenoceptor subtypes was evaluated by studying their ability to displace specific [^{3}H]-prazosin binding from membranes of rat hippocampus pretreated with CEC (α_{1A}) and rat liver (α_{1B}) according to the method of Morrow & Creese (1986) and Taddei *et al.* (1993), respectively.

Data analysis

Values are arithmetic means ± s.e.mean. Saturation binding data were obtained from pools of rat thoracic aortae, and the experiments were conducted on four different pools of tissue.

The saturation curves (Scatchard analysis) were analyzed according to the method of Munson & Rodbard (1980), using the non-linear curve-fitting programme LIGAND (from N.I.H.) to determine the apparent dissociation constant (K_D) and maximum number of binding sites (B_{max}) for [^{3}H]-prazosin.

The displacement curves of the antagonists on the α_{1A}- and α_{1B}- adrenoceptor subtypes were analysed by non-linear curve fitting of the logistic equation according to the method reported by De Lean *et al.* (1978), utilizing the ALLFIT programme (from N.I.H.). The IC_{50} values and pseudo-Hill slope coefficients were estimated by the programme. The value for the inhibition constant, K_i, was calculated by use of the Cheng & Prusoff (1973) equation:

$$K_i = IC_{50}/(1 + L/K_D),$$

where L is the concentration of [^{3}H]-prazosin used.

In the isolated vascular preparations, 5–10 preparations were used when the effects of CEC were studied, and at least 2 preparations were taken from different animals for each concentration of the other antagonists tested. Only one antagonist concentration was used for each preparation.

The concentration-response curves (before and after antagonist incubation) were analysed by non-linear curve fitting of the logistic equation as reported for the binding studies. The statistical significance of the differences between the parameters of the control concentration-response curve and the curve obtained after drug incubation (EC_{50}, slope, E_{max}), was examined by simultaneous fitting with the considered parameter shared as reported by De Lean *et al.* (1978).

The dissociation constant of the agonist and the fraction of receptors inactivated by incubation with CEC were evaluated according to Furchgott & Bursztyn (1967).

Schild-plot parameters (pA_2 and slope) were evaluated by linear regression analysis according to Tallarida & Murray (1987). The dose-ratios (DRs) (EC_{50} ratios of NA) observed after incubation with the different concentrations of the antagonists were always corrected for time-dependent changes in first and second curve locations, determined in vehicle-treated time-matched control curves. Identity of the experimentally derived DRs with a Schild plot slope of unity was assessed by the *F* test for goodness of fit, according to Munson & Rodbard (1980).

Correlation analysis between the functional activity of the antagonists in rat aortic strips and their binding affinities at α_1-adrenoceptor subtypes was assessed by least-squares linear regression.

Drugs

The following drugs were used: [^{3}H]-prazosin (7-methoxy-^{3}H: specific activity 76.2 Ci mmol^{-1}, batch 2825-078, NEN Research Product, Dupont de Nemours Italiana); chloroethylclonidine 2HCl, spiperone HCl, desmethylimipramine HCl, 5-methylurapidil, (Research Biochemical Incorporated, Natick, U.S.A.); pargyline HCl, (−)-noradrenaline $C_4H_6O_6$, prazosin HCl, phentolamine-HCl, corticosterone, (±)-propranolol HCl, yohimbine HCl, (Sigma-Aldrich, Italy); nicardipine HCl, and (R)-YM-12617 HCl (tamsulosin) were synthesized in Recordati laboratories.

In binding studies, the compounds were dissolved in absolute ethanol. For the isolated vascular preparations, CEC, prazosin, terazosin, and phentolamine were dissolved in distilled water; nicardipine, 5-methylurapidil, and spiperone were dissolved in aqueous N,N-dimethylformamide and Tween 80 (both 1% v/v final concentration) as 1 mM stock solutions and further diluted as necessary with distilled water; (R)-YM-12617 was dissolved in dimethylsulphoxide and water (1:1) at 1 mM, and further diluted as necessary with distilled water.

Results

Effects of CEC on [^{3}H]-prazosin binding in rat aortic membranes

The incubation of rat aortic membranes with 10 μM CEC did not change the K_D of [^{3}H]-prazosin binding in comparison to untreated membranes (K_D values were 1.68 ± 0.53 and 1.28 ± 0.33 nM, respectively). In contrast, the total number of binding sites labelled by [^{3}H]-prazosin were significantly reduced (by about 88%) from 1.80 ± 0.22 pmol g^{-1} fresh tissue (untreated), to 0.22 ± 0.08 pmol g^{-1} (after CEC). A representative Scatchard plot of saturation assay in rat aorta membranes is shown in Figure 1.

The nonlinear regression analysis revealed that the data were best described by a model for one binding site, both before and after CEC incubation.

Antagonism of noradrenaline-induced contractions in rat aortic strips

The cumulative addition of NA to spiral strips of rat thoracic aorta produced concentration-dependent contractions with a

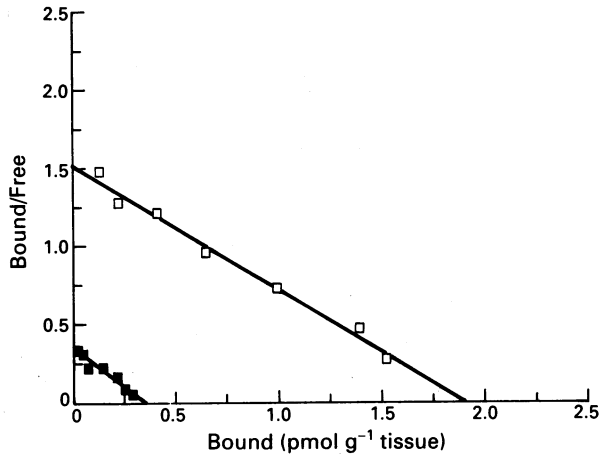


Figure 1 Scatchard plot representative from a single experiment of [^{3}H]-prazosin saturation binding in rat aortic membranes pretreated (■) or not pretreated (□) with 10 μM chloroethylclonidine. In both cases, the data were consistent with binding to a single site with B_{max} = 0.22 pmol g^{-1} (K_D = 1.68 nM) and B_{max} = 1.80 pmol g^{-1} (K_D = 1.28 nM), respectively.

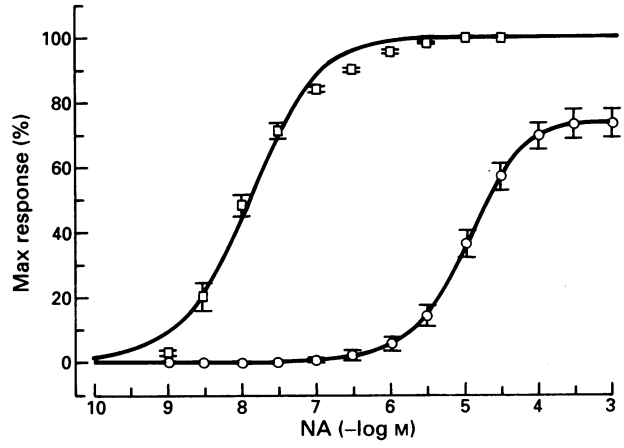


Figure 2 Effects of chloroethylclonidine (50 μM, 30 min incubation followed by washout) on contractions to noradrenaline (NA) in rat aorta. Responses, expressed as a percentage of the maximum contraction obtained in the control (□) cumulative concentration-response curve, are presented as mean ± s.e. from at least 5 experiments.

mean pEC_{50} value ($-\log EC_{50}$ = concentration that produced 50% of the maximal tension attainable) of 7.35 (range 6.82–7.96), in agreement with that (7.77) reported by Ruffolo & Waddel (1982).

The second of the paired control concentration-response curves to NA was, in general, slightly shifted to the right of the first curve, yielding a DR in the range 1.3–2.5.

The slopes and maxima of these curves were not statistically different.

The NA concentration-response curve was partially but significantly depressed (by about 25%) and markedly shifted to the right after 30 min incubation (followed by repetitive washout) with 50 μM CEC (Figure 2).

The plot of reciprocals of equieffective NA concentrations before and after CEC incubation (not shown), according to the method of Furchgott & Bursztyn (1967), enabled calculation of K_A and the fraction (q) of the initial receptor pool remaining after receptor inactivation. The pK_A value (4.15; $-\log K_A$, M) estimated for the agonist was clearly different from that previously reported (6.58) by other authors utilizing dibenamine as an irreversible antagonist (Ruffolo & Waddel, 1982). The number of remaining receptors (q = 0.0008; <0.1%) suggests that almost all the adrenoceptors were inactivated by CEC.

To verify this hypothesis, the effect of prazosin (10 nM, 30 min incubation) on NA-induced contraction of aorta strips was evaluated in tissues pretreated, or not, with CEC. As shown in Figure 3, prazosin induced a marked shift to the right (by about 85 fold) of the NA concentration-response curve (corrected DR = 34; apparent pK_B = 9.5) in untreated strips, but was practically inactive on strips previously treated for 30 min (followed by repetitive washout) with 50 μM CEC.

The addition of the calcium entry blocker, nicardipine, (up to 10 μM) caused only a non-significant (in comparison to the matched control preparations), non-concentration-dependent rightward shift and a 8–15% depression of the maximum of the concentration-response curve for the NA-induced contractions (Figure 4). This suggests that dihydropyridine-sensitive Ca^{2+} channels are unlikely to be involved in receptor-effector coupling in this tissue.

In order to confirm the results described above, the potency of different α_1-adrenoceptor antagonists in inhibiting the noradrenaline-induced contractions of rat aorta was assessed.

The antagonists caused parallel, concentration-related shifts to the right of the NA concentration-response curves,

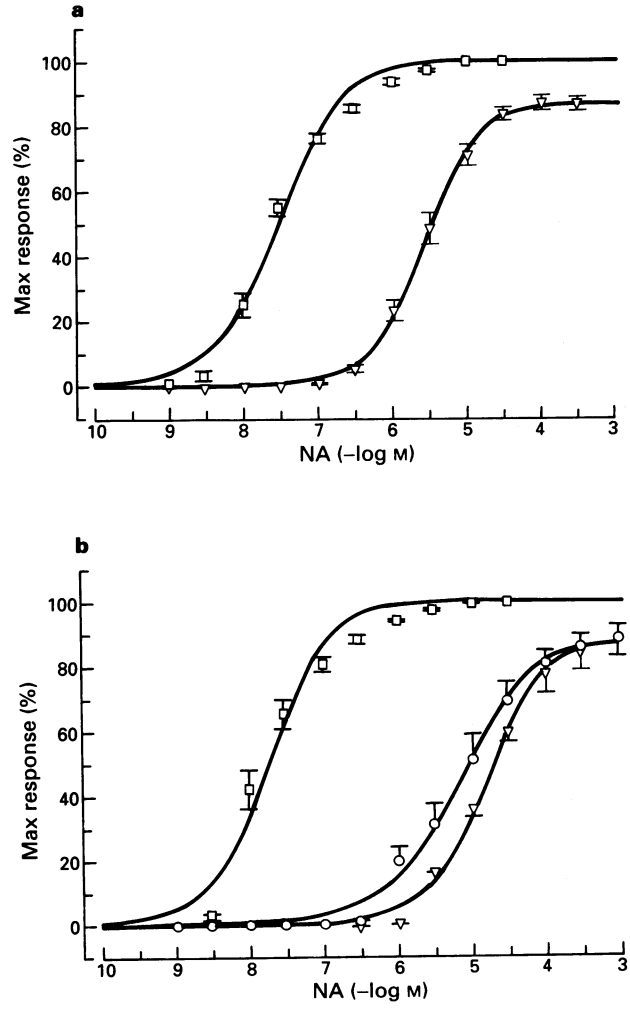


Figure 3 Effects of prazosin (10 nM, 30 min incubation) on contractions to noradrenaline (NA) in rat aorta untreated and previously treated with chloroethylclonidine (CEC). In both cases the responses are expressed as a percentage of the maximum contraction obtained in the control (□) cumulative concentration-response curve. (a) NA-response curve before (□) and after prazosin (▽) in untreated tissues. (b) Responses to NA in tissues before (□), following 30 min CEC incubation and washout (○), and after further addition of prazosin (▽). Data are presented as mean ± s.e. from at least 3 experiments in both cases.

without affecting significantly the maximum contraction caused by the agonist in comparison to that observed in the matched control preparations. The slopes of the Schild plots obtained with these compounds were found to be close to, and not significantly different from, unity, suggesting that simple competitive antagonism occurred.

The calculated pA_2 values (slope constrained to unity) given in Table 1 show that prazosin and (R)-YM-12617 were the most potent antagonists. It is noticeable that, in our experimental conditions, spiperone proved more potent than 5-methylurapidil as an antagonist of NA-induced contractions.

Affinity for the α_{1A}- and α_{1B}-adrenoceptor subtypes

The potency of the compounds in displacing the [^{3}H]-prazosin specific binding in the different tissues tested is summarized in Table 2. Prazosin and terazosin showed the same affinity for the two α_1-adrenoceptor subtypes, whereas 5-methylurapidil (Figure 5a) and (R)-YM-12617 were selective for the α_{1A}-subtype, and spiperone (Figure 5b) was selective for the α_{1B}-subtype.

The pseudo-Hill coefficients for the inhibition of [^{3}H]-prazosin binding were found to be close to and not significantly different from unity (not shown), indicating a simple competitive antagonism.

The investigated antagonists generated a significant linear correlation between their functional potency in terms of pA_2 values evaluated on rat aorta and their binding affinity for the α_{1B}-adrenoceptor subtype, but not with the affinity for α_{1A}-subtype (Figure 6). The obtained equations are the following (Y = X for identity; in parentheses the s.e. mean of the slope; r^2 = correlation coefficient):

(α_{1A}-adrenoceptor) $Y = 0.978\ (\pm 0.031)X$; $r^2 = 0.38$, NS
(α_{1B}-adrenoceptor) $Y = 0.946\ (\pm 0.024)X$; $r^2 = 0.77$, $P < 0.01$

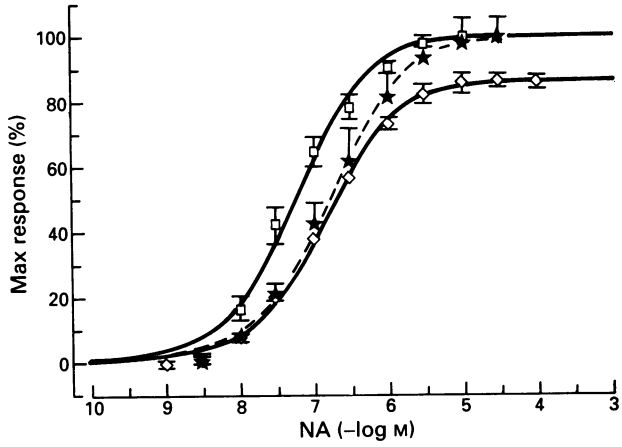


Figure 4 Effects of vehicle (★) and 10 μM nicardipine (◇) on contractions to noradrenaline (NA) in rat aorta. In both cases the responses are expressed as percentage of the maximum contraction obtained in the control (□) cumulative concentration-response curve. The points are the mean ± s.e. from at least 3 experiments.

Table 1 Estimated potency of α_1-antagonists, expressed as pA_2 values from constrained Schild plots (slope = 1.00), for competitive antagonism against noradrenaline-induced contractions of rat aortic strips

Drugs	*pA_2*
Prazosin	9.85 ± 0.10
Terazosin	8.54 ± 0.05
(R)-YM-12617	9.34 ± 0.07
Phentolamine	7.71 ± 0.13
5-Methylurapidil	7.64 ± 0.04
Spiperone	8.41 ± 0.11

Results are presented as mean ± s.e. of $n = 6-10$ preparations for each pA_2 determination.

Table 2 Affinities of antagonists (expressed as K_i, nM) for the α_{1A}- and α_{1B}-adrenoceptor subtypes

Drugs	*α_{1A}*	*α_{1B}*
Prazosin	0.93 ± 0.19	0.36 ± 0.11
Terazosin	5.81 ± 1.57	3.55 ± 0.46
(R)-YM-12617	0.20 ± 0.03	4.06 ± 0.95
Phentolamine	16.37 ± 4.75	89.33 ± 2.51
5-Methylurapidil	4.66 ± 1.82	220.24 ± 30.29
Spiperone	37.81 ± 5.82	2.03 ± 0.09

Rat hippocampus membranes pretreated with CEC (α_{1A}) and rat liver membranes (α_{1B}) were utilized as the source of receptors. The slope of all the displacement curves were not statistically different from unity. Data are the mean ± s.e.mean of 2–4 experiments, each in triplicate.

Discussion

To characterize the population of α_1-adrenoceptors present in rat aorta, we studied the effects of CEC incubation on [^{3}H]-prazosin binding in membrane preparations from this tissue, and the effects of this selective α_{1B} irreversible antagonist on

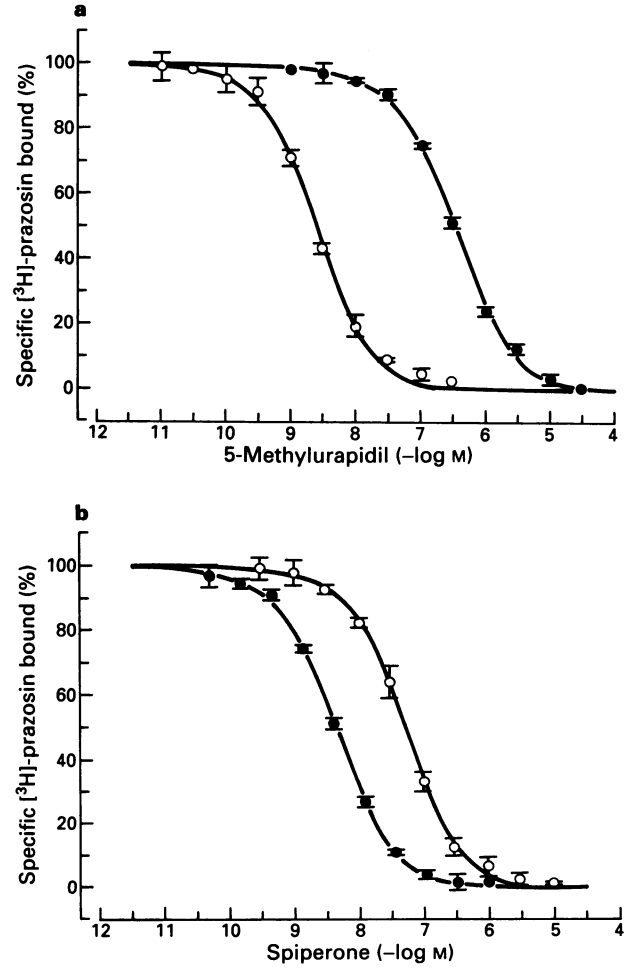


Figure 5 (a) 5-Methylurapidil and (b) spiperone/[^{3}H]-prazosin competition in rat hippocampal (α_{1A}, ○) and liver (α_{1B}, ●) membranes. The points represent the mean ± s.e. percentage reduction of the specific binding from 3 experiments each in triplicate. Lines through the data were fitted by computer, assuming displacement from a single recognition site.

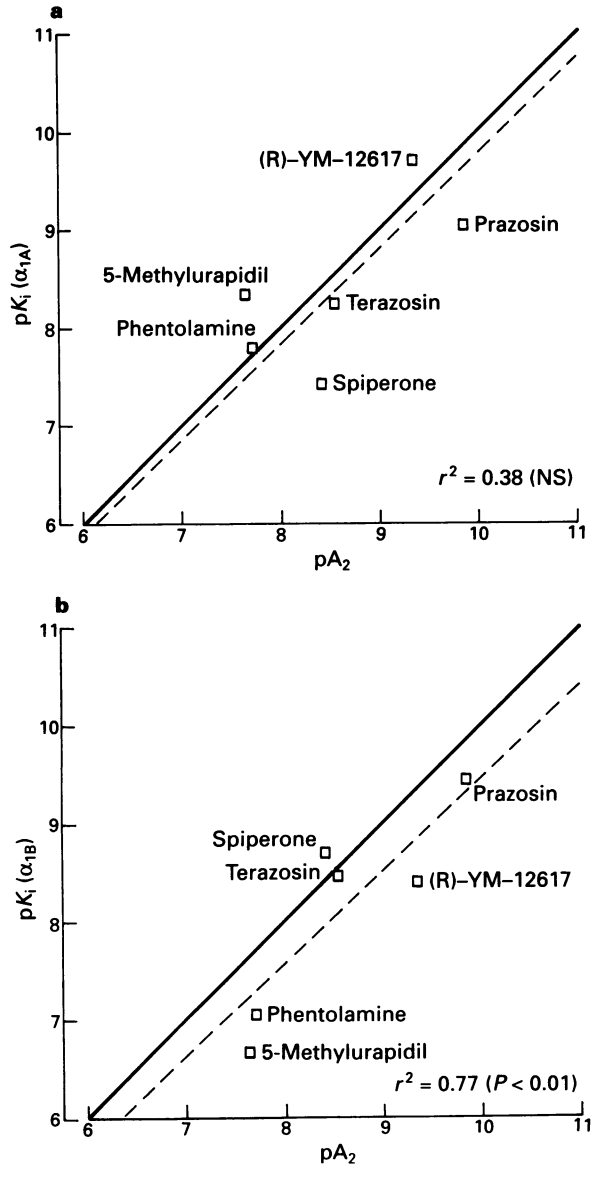


Figure 6 Correlation between the pA_2 values of the investigated α-antagonists on noradrenaline-induced contractions of rat aorta, and their affinity expressed as pK_i values for α_{1A} (a) and α_{1B}-adrenoceptor subtypes (b). Solid lines represent the 'equivalence' lines (slope = 1). Dashed lines are the evaluated regression lines, Y = mX (in parentheses the s.e. of the slope): α_{1A}-adrenoceptor Y = 0.978 (±0.031)X; α_{1B}-adrenoceptor Y = 0.946 (±0.024)X.

NA-induced contractions, as well as the potency of several competitive antagonists in inhibiting contractions to NA.

Incubation of rat aorta membranes with CEC induced a significant decrease (88%) in the total number of binding sites without affecting the K_D for [^{3}H]-prazosin.

Nonlinear regression analysis of the saturation experiments revealed that the data best fit to a model for one binding site, indicating that a single population of receptors is labelled by [^{3}H]-prazosin.

In the functional studies with rat aorta, 50 μM CEC induced a marked parallel shift to the right of the NA concentration-response curve and a depression in the maximum tension attainable. The EC_{50} of the agonist increased from 12 nM to 10 μM. The results are in agreement with previously published data reported by other authors (Han *et al.*, 1990; Aboud *et al.*, 1993). Moreover, we found that the receptors remaining after incubation of rat aortic strips with CEC were insensitive to prazosin. In these experiments, while prazosin (10 nM) in absence of CEC treatment showed a 34 fold shift to the right of the NA concentration-response curve, after CEC incubation a non-significant shift of the NA curve was observed with the drug. These findings are in agreement with those reported by Oriowo & Bevan (1990), who demonstrated that CEC unmasks a non-α-adrenoceptor site in the rat aorta, insensitive to phenoxybenzamine, prazosin, WB4101 and yohimbine. The K_A value of the agonist calculated after CEC incubation (4.15) is in agreement with these observations.

In fact, it has been reported that the K_A of NA in this tissue utilizing dibenamine as irreversible antagonist (Ruffolo & Waddel, 1982) is 6.58 and we confirmed this result utilizing phenoxybenzamine as irreversible antagonist (K_A = 6.46; data not shown). These findings indicate, on the whole, that the receptors remaining after CEC incubation cannot be considered α_1-adrenoceptors.

In addition, it is well known that the smooth muscle contractions elicited by α_{1A}-subtype receptor activation are dependent on Ca^{2+} influx through the dihydropyridine-sensitive channels, while contractions elicited by the activation of α_{1B}-adrenoceptors are independent of extracellular Ca^{2+} influx (Minneman, 1988; Han *et al.*, 1990). In our experimental conditions, the calcium entry blocker, nicardipine caused only a non-significant, non-dose-dependent slight rightward shift and depression of the maximum of the concentration-response curve to NA, as reported by Han *et al.* (1990) after nifedipine incubation.

The competitive antagonists prazosin, terazosin, (R)-YM-12617, phentolamine, 5-methylurapidil and spiperone inhibited contractions to NA with pA_2 values of 9.85, 8.54, 9.34, 7.71, 7.64 and 8.41, respectively. Though the present functional evaluations did not provide the most precise estimates of the antagonists affinity, owing to the limited series of experiments and at least in some cases the narrow range of concentrations tested, the pA_2 value of prazosin (or the apparent pK_B = 9.5) derived is in agreement with previously reported data showing affinity values (pA_2) of 10.42 (Hamed *et al.*, 1983), 10.30 (Martinotti *et al.*, 1991), 9.89 (Muramatsu *et al.*, 1990), 9.45 (Aboud *et al.*, 1993) and 10.6 (Oriowo *et al.*, 1990). Also our pA_2 values for phentolamine and 5-methylurapidil are similar to those previously obtained by other authors (Muramatsu *et al.*, 1990; Hamed *et al.*, 1983; Aboud *et al.*, 1993).

Recently, Aboud *et al.* (1993) stated that contractions of rat aorta to NA are mediated by non-α_{1A}, non-α_{1B}-adrenoceptors, due to the simultaneous presence of sensitivity to CEC (α_{1B}) and to the high potency of selective α_{1A}-antagonists. Therefore, we evaluated the affinity of the tested antagonists for the α_{1A}-adrenoceptor subtype in rat hippocampal membranes after CEC inactivation (Morrow & Creese, 1986), and the affinity for the α_{1B}-adrenoceptor subtype in rat liver membranes since this tissue contains mainly this α_1-subtype (Garcia-Sainz *et al.*, 1992; Taddei *et al.*, 1993). Rat aortic membranes were not utilized to evaluate the affinity for the α_{1B}-adrenoceptors because of the low number of specific binding sites for [^{3}H]-prazosin. We confirmed that prazosin and terazosin do not discriminate between the α_1-adrenoceptor subtypes, whereas 5-methylurapidil and (R)-YM-12617 were confirmed as clearly the most active on α_{1A}-adrenoceptors, and spiperone on α_{1B}-adrenoceptor subtypes. The selectivity ratios (pK_i hippocampus/pK_i liver) obtained by us with phentolamine and spiperone (5.5 and 0.04, respectively) were fully in agreement with those previously reported by Michel *et al.* (1989), who used rat submaxillary gland and liver as sources of α_{1A}- and α_{1B}-adrenoceptor subtypes, respectively.

The pA_2 values obtained with the antagonists in the course of our experiments gave significant correlation with their affinity for the α_{1B}-adrenoceptor, and showed a lower degree of correlation with their affinity for the α_{1A}-adrenoceptor subtype.

When some of these antagonists were tested on rabbit urethra, a tissue containing mainly the α_{1A} subtype, their

functional potency correlated better with their binding affinity for the α_{1A}-adrenoceptor than for the α_{1B}-subtype (Testa *et al.*, 1993), supporting the soundness of this approach.

In our opinion, all the findings reported in the present work clearly indicate that in rat aorta most of the contraction to NA is mediated by α_{1B}-adrenoceptors, and that the potency (pA_2) of an antagonist in this tissue parallels its receptor binding affinity for this subtype of the α_1-adrenoceptor population.

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Note added during revision of the manuscript

In a recent paper, Eltze (1994) reported an excellent correlation between the affinities (pA_2 values) of several α_1-adrenoceptor antagonists (including those used in our study) evaluated on guinea-pig spleen, and their pK_i values for the rat liver α_{1B}-adrenoceptors (reported by Taddei *et al.*, 1993), concluding that the α_1-adrenoceptor mediating smooth muscle contraction of this tissue can be best characterized as being of the B subtype.

Furthermore, this author showed a significant correlation between the affinities (pA_2 values) of these antagonists on guinea-pig spleen, and the affinities (pA_2 values) of the same compounds evaluated on rat aorta, but not with the affinities (pA_2 values) evaluated on rat vas deferens (a tissue expressing the α_{1A}-adrenoceptors).

References

ABOUD, R., SHAFII, M. & DOCHERTY, J.R. (1993). Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.*, **109**, 80–87.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzyme reaction. *Biochem. Pharmacol.*, **22**, 3099.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

ELTZE, M. & BOER, R. (1992). SDZ NVI 085 discriminates between rat vas deferens alpha$_{1a}$- and rat aorta alpha$_{1b}$-adrenoceptors. *Arch. Pharmacol.*, **345**, R108.

ELTZE, M. (1994). Characterization of the α_1-adrenoceptor subtype mediating contraction of guinea-pig spleen. *Eur. J. Pharmacol.*, **260**, 211–220.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. NY Acad. Sci.*, **144**, 882–898.

HAMED, A.T., JOHNSON, T.D., CHARLTON, K.G. & CLARKE, D.E. (1983). Pharmacological characterization of α-adrenoceptor subtypes in rat isolated thoracic aorta. *J. Auton. Pharmacol.*, **3**, 265–273.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987a). Heterogeneity of α_1-adrenergic receptors revealed by chloroethylclonidine. *Mol. Pharmacol.*, **32**, 505–510.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987b). α_1-adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature*, **329**, 333–335.

HAN, C., LI, J. & MINNEMAN, K.P. (1990). Subtypes of α_1-adrenoceptors in rat blood vessels. *Eur. J. Pharmacol.*, **190**, 97–104.

GARCIÁ-SÁINZ, J.A., ROMERO-AVILA, M.T., ALCÁNTARA-HERNÁNDEZ, R., MACÍAS-SILVA, M., OLIVARES-REYES, J.A. & GONZÁLES-ESPINOSA, C. (1992). Species heterogeneity of hepatic α_1-adrenoceptors: α_{1A}, α_{1B} and α_{1C} subtypes. *Biochem. Biophys. Res. Commun.*, **186**, 760–767.

MARTINOTTI, E., BRESCHI, M.C., OMINI, C., PANI, M., CIUCCI, M.A. & NIERI, P. (1991). Comparative study of postsynaptic α-adrenoceptors in aorta obtained from human and other mammalian species. *Pharmacol. Res.*, **23**, 57–69.

MICHEL, A.D., LOURY, D.N. & WHITING, R.L. (1989). Identification of a single α_1-adrenoceptor corresponding to the α_{1_a}-subtype in rat submaxillary gland. *Br. J. Pharmacol.*, **98**, 883–887.

MINNEMAN, K.P. (1988). α_1-Adrenoceptor subtypes, inositol phosphates, and sources of cell Ca^{++}. *Pharmacol Rev.*, **40**, 87–119.

MORROW, A.L. & CREESE, I. (1986). Characterization of α_1-adrenoceptor subtypes in rat brain: a reevaluation of [^{3}H]WB4101 and [^{3}H] prazosin binding. *Mol. Pharmacol.*, **29**, 321–330.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

MURAMATSU, I., OHMURA, T., KIGOSHI, S., HASHIMOTO, S. & OSHITA, M. (1990). Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle. *Br. J. Pharmacol.*, **99**, 197–201.

MURAMATSU, I., KIGOSHI, S. & OHMURA, T. (1991). Subtypes of α_1-adrenoceptors involved in noradrenaline induced contractions of rat thoracic aorta and dog carotid artery. *Jpn. J. Pharmacol.*, **57**, 535–544.

ORIOWO, M.A. & BEVAN, J.A. (1990). Chloroethylclonidine unmask a non-$_\alpha$-adrenoceptor noradrenaline binding site in the rat aorta. *Eur. J. Pharmacol.*, **178**, 243–246.

ORIOWO, M.A., NICHOLS, A.J. & RUFFOLO, R.R. JR. (1992). Receptor protection studies with phenoxybenzamine indicate that a single α_1-adrenoceptor may be coupled to two signal transduction processes in vascular smooth muscle. *Pharmacology*, **45**, 17–26.

RUFFOLO, R.R. JR. & WADDELL, J.E. (1982). Receptor interactions of imidazolines: α-adrenoceptors of rat and rabbit aortae differentiated by relative potencies, affinities and efficacies of imidazoline agonists. *Br. J. Pharmacol.*, **77**, 169–176.

TADDEI, C., POGGESI, E., LEONARDI, A. & TESTA, R. (1993). Affinity of different α_1-agonists and antagonists for the α_1-adrenoceptors of rabbit and rat liver membranes. *Life Sci.*, **53**, PL177–181.

TALLARIDA, R.J. & MURRAY, R.B. (1987). *Manual of Pharmacologic Calculations with Computer Programs*, 2nd edition. Berlin: Springer-Verlag.

TESTA, R., GUARNERI, L., IBBA, M., STRADA, G., POGGESI, E., TADDEI, C., SIMONAZZI, I. & LEONARDI, A. (1993). Characterization of α_1-adrenoceptor subtypes in prostate and prostatic urethra of rat, rabbit, dog and man. *Eur. J. Pharmacol.*, **249**, 307–315.

TIAN, W.N., GUPTA, S. & DETH, R.C. (1990). Species differences in chloroethylclonidine antagonism at vascular α_1 adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **253**, 877–883.

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Effects of the selective I_1 imidazoline receptor agonist, moxonidine, on gastric secretion and gastric mucosal injury in rats

[1]*†Gary B. Glavin & *,**Donald D. Smyth

Departments of *Pharmacology and Therapeutics, †Surgery and **Internal Medicine, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada, R3E 0W3

1 Previous reports of the effects of α_2-adrenoceptor stimulation on gastric secretion are inconsistent because it was not clear whether the compounds were activating α_2-adrenoceptors and/or newly described imidazoline receptors. In the present experiments, the effects of moxonidine, an I_1-imidazoline receptor agonist and antihypertensive agent, on gastric secretion and on experimental gastric mucosal injury were examined.

2 Moxonidine (0.01, 0.1 and 1.0 mg kg^{-1}, i.p.) potently inhibited basal (non-stimulated) gastric acid secretion in conscious rats with an ED_{50} of 0.04 mg kg^{-1}. Two hours following administration of the highest dose of moxonidine (1.0 mg kg^{-1}), gastric acid output was completely suppressed. Moxonidine also significantly increased intragastric pH, at the two highest doses.

3 The α_2-adrenoceptor agonist, clonidine (0.01, 0.1 and 1.0 mg kg^{-1}, i.p.) decreased basal acid secretion at the lowest dose (37%) and at the highest dose (46%), while the intermediate dose did not affect gastric acid output.

4 In an ethanol-induced model of gastric mucosal injury, moxonidine decreased the length of lesions at the lowest and highest doses (0.01 and 1.0 mg kg^{-1}) as well as the number of the lesions, at the highest dose (1.0 mg kg^{-1}).

5 In pylorus-ligated rats, moxonidine significantly decreased acid secretion (all doses), total secretory volume (1.0 mg kg^{-1}) as well as pepsin output (1.0 mg kg^{-1}).

6 In comparison to clonidine, moxonidine appears to be a more potent anti-secretory and gastric-protective compound. These data indicate a potential role for imidazoline receptor agonists in the management of gastroduodenal diseases associated with hypertension. The relative contribution of the central and peripheral effects of moxonidine to these gastrointestinal actions remains to be determined.

Keywords: Moxonidine; imidazoline receptor; clonidine; gastric acid secretion; pepsin; gastric ulcer; ethanol; pylorus ligation

Introduction

Although not a consistent finding, many studies have indicated an association between hypertension and gastroduodenal ulcer disease. Epidemiological data indicate that hypertension and gastric ulcer disease significantly overlap in man (Mitana *et al.*, 1990). However, laboratory investigations reveal some inconsistencies with the clinical reports. Both Paré & Schimmel (1986) and Shichijo *et al.* (1991) reported that spontaneously hypertensive rats develop less gastric mucosal damage in response to either chemical or stress challenge. Consequently, several groups examined the direct effects of known antihypertensive agents upon gastric secretion and experimental gastric mucosal damage. The non-selective β-adrenoceptor antagonist, propranolol, was generally associated with gastroprotective and anti-secretory effects (Sankary *et al.*, 1986; Bhandare *et al.*, 1990; Ray *et al.*, 1993). Conversely, the α_2-adrenoceptor agonist, clonidine, has been associated with both gastroprotection as well as with exacerbation of gastric pathology. Kunchandy *et al.* (1985) reported that clonidine decreased gastric lesions in rats, while Del Soldato (1986) indicated that, depending upon the dose of clonidine used, gastric mucosal injury was either lessened or worsened. Recently, Al-Bekairi *et al.* (1993) demonstrated that clonidine exacerbated ethanol-induced gastric mucosal damage, but also decreased gastric acid secretion and enhanced gastric adherent mucus. Because the acid and pepsin-inhibitory effects of clonidine were blocked by idazoxan in control rats but not in cholinoceptor stimulated rats, Tazi-Saad *et al.* (1992) suggested that the effects of clonidine were most likely mediated centrally, rather than locally on secretory cells in the gut.

Recent studies have indicated that many effects previously attributed to α_2-adrenoceptor stimulation may in fact involve a novel class of receptor, the imidazoline receptor. It is now appreciated that the antihypertensive action of clonidine is primarily mediated by stimulation of central I_1-imidazoline receptors in the nucleus reticularis lateralis rather than by α_2-adrenoceptors (Tibirica *et al.*, 1991; Ernsberger *et al.*, 1993). Thus, it is not clear whether the previously reported effects of clonidine on gastric secretion were mediated by α_2-adrenoceptors and/or imidazoline receptors. Due to the inconsistency in the literature regarding the gastrointestinal actions of traditional centrally-acting antihypertensive agents (α_2-adrenoceptor agonists), we have now tested the effects of moxonidine in two models of gastric secretion and in a model of experimental gastric mucosal injury.

Methods

Gastric acid secretion

Male Sprague-Dawley rats (180 ± 10 g at the start of the study) were implanted with chronic indwelling gastric cannulae as described previously (Paré *et al.*, 1977). After a 14-day recovery period, all cannulae remained firmly attach-

[1] Author for correspondence.

ed and produced no untoward effects. Secretory testing in sessions of 3 h occurred in the following sequence: vehicle (saline 1.0 ml kg^{-1}), moxonidine 0.01 mg kg^{-1}, 0.1 mg kg^{-1}. 1.0 mg kg^{-1} i.p. and vehicle again. Each of these sessions was separated by a 96 h period. Within each secretory testing session, a baseline collection was followed by the injection (i.p.) of the vehicle or moxonidine and two subsequent post treatment collections. Thus, each animal served as its own control and all drug treatments were preceded by a 1 h baseline collection period in which no injection occurred. All drug treatments were both preceded and followed by vehicle injections. The volume of secretion was recorded and aliquots of each sample were titrated to pH 7.0 with 0.01 M NaOH in a Mettler DL-21 autotitrator. The results are expressed as μmol h^{-1}. For purposes of comparison, other animals were prepared as described above, but given vehicle and clonidine at doses of 0.01, 0.1 and 1.0 mg kg^{-1} i.p. as described as above.

Ethanol-induced gastric mucosal injury

Rats ($n = 5$ per group) were randomly assigned to treatment conditions. They were deprived of food, but not water, for 24 h prior to being given 1.0 ml of 75% (v/v) ethyl alcohol (Canadian Industrial Alcohols and Chemicals Ltd., Corbyville, Ontario, Canada) p.o. by gavage (Robert *et al.*, 1979). Vehicle or moxonidine at doses of 0.01, 0.1, or 1.0 mg kg^{-1} was given i.p. 5 min prior to ethanol. Following ethanol and drug treatment, rats were returned to individual cages without food or water for 2 h, after which time they were killed by cervical dislocation. The stomachs were removed, everted, washed and fixed in 10% (v/v) buffered formalin and the number and cumulative length (in millimeters) of gastric glandular mucosal injury determined under a dissecting microscope with an ocular micrometer by a treatment-'blinded' observer.

Pylorus ligation

Rats ($n = 6$ per group) were randomly assigned to treatment conditions. They were deprived of food, but not water, for 24 h, then, immediately before and throughout the experiment, no access to water was allowed. Under ether anesthesia, a midline laparotomy was used to expose the pylorus which was then ligated with 2-0 silk. Care was taken to avoid trauma to the surrounding vasculature. The incision was closed with silk sutures, and vehicle (saline) or moxonidine (0.01, 0.1 or 1.0 mg kg^{-1}, i.p.) administered. Four hours later, all rats were killed by cervical dislocation and the stomachs were removed and the total volume of secretion was recorded. The samples were then centrifuged at 3000 *g* to remove residual debris and aliquots taken for determination of acid content as described above. The results were expressed as μmol h^{-1}. Other aliquots were taken for pepsin determination according to the method of Dupuy & Szabo (1986) and the results expressed as pepsin output (mg h^{-1}). Gastric mucosal injury was assessed as described above.

Drugs

Moxonidine was a gift from Beiersdorf AG, Hamburg, Germany and was dissolved in 0.9% (v/v) saline and injected i.p. in a volume of 1.0 ml kg^{-1}. Clonidine HCl was obtained from Sigma, St. Louis, MO, U.S.A., dissolved in distilled water and injected i.p. in a volume of 1.0 ml kg^{-1}.

Data analysis

All data are expressed as mean ± s.e.mean. Conscious gastric acid secretion data were analysed by repeated measures ANOVA followed by the least significant difference test for *post-hoc* comparisons. Data from the other experiments were analysed by factorial ANOVA followed by the Tukey HSD (honestly significant difference) test for *post-hoc* comparisons (Kirk, 1982).

Results

Gastric acid secretion

Moxonidine significantly reduced basal (non-stimulated) gastric acid secretion when given doses of 0.1 (63% inhibition) and 1.0 (65% inhibition) mg kg^{-1}, i.p. (Figure 1; ED_{50} = 0.04 mg kg^{-1}). The suppression of gastric secretion persisted into the second hour following moxonidine administration and, in the case of the 1.0 mg kg^{-1} dose, completely suppressed acid output in this second hour (Figure 1). Moxonidine also increased gastric pH ($P < 0.05$) at the two higher antisecretory doses (Table 1).

Clonidine at a dose of 0.01 mg kg^{-1} inhibited gastric secretion by 37% in the hour immediately following its injection. At the highest dose of clonidine (1.0 mg kg^{-1}), acid output decreased by 46% in both the first and second hours after its administration. The intermediate dose of clonidine (0.10 mg kg^{-1}) did not affect gastric acid secretion (Figure 2).

Ethanol-induced gastric injury

Ethanol-induced gastric mucosal injury was significantly reduced by the lowest (0.01 mg kg^{-1}) and highest (1.0 mg kg^{-1}) doses of moxonidine (Table 2). At the highest dose, moxonidine significantly reduced both the number and the severity of ethanol-induced gastric lesions.

Pylorus ligation

In pylorus-ligated rats, moxonidine significantly reduced acid secretory volume at the highest dose tested (Table 3). Gastric acid output was significantly suppressed by all doses of moxonidine (58%, 66% and 68% inhibition at doses of 0.01, 0.1 and 1.0 mg kg^{-1}, respectively), while pepsin secretion was significantly reduced by the highest dose of moxonidine. Gastric mucosal injury consequent to pylorus ligation determined by the length of the lesions was significantly reduced only by the 0.1 mg kg^{-1} dose of moxonidine.

Discussion

In light of numerous differences that exist in response to α_2-adrenoceptor agonists which contain either a phenethylamine or imidazoline type moiety, a novel class of receptor (imidazoline receptors) has been postulated to account for the actions of the latter group of agents (Hamilton, 1992; Michel & Ernsberger, 1992). Early studies had documented that compounds such as idazoxan, rauwolscine and yohimbine were specific antagonists of α_2-adrenoceptors relative to α_1-adrenoceptors (Doxey *et al.*, 1984). Subsequent studies, however, demonstrated that [^{3}H]-idazoxan labelled sites were not adrenoceptors and not labelled with specific tritiated α_2-adrenoceptor antagonists such as rauwolscine and yohimbine (Michel *et al.*, 1989; 1990). These non-adrenoceptor sites have been recently classified into I_1- and I_2-imidazoline receptor subtypes based on their selectivity for [^{3}H]-clonidine and [^{3}H]-idazoxan, respectively (Ernsberger *et al.*, 1992; Reis *et al.*, 1992). Compounds acting as agonists of I_1-imidazoline receptors have been found to be potently antihypertensive but exhibit less sedation and anti-salivary effects compared to other centrally acting antihypertensive agents, including clonidine (Chrisp & Faulds, 1992). This decreased incidence of side effects of I_1-specific agents, which previously limited the use of these centrally-acting agents, may conceivably result in an increased interest in this class of antihypertensive compound. Thus, description of additional actions of these agents that may have therapeutic benefits will be of interest.

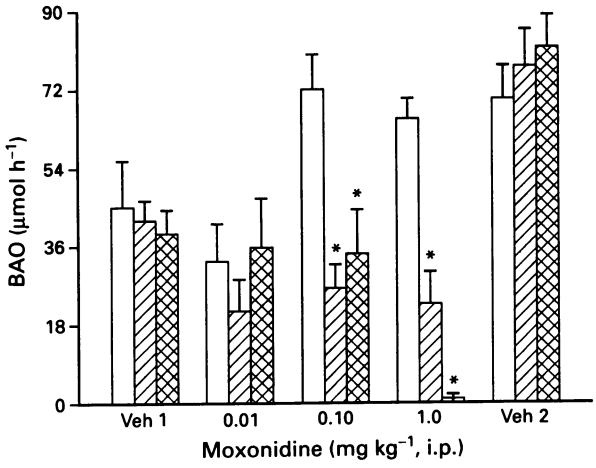


Figure 1 Effects of moxonidine on basal (non-stimulated) gastric acid output (BAO) in conscious rats. Data are presented as mean ± s.e.mean, ($n = 8$). Injection of vehicle or moxonidine occurred at the beginning of the second hour of collection. Significant difference in BAO in the second (hatched columns) or the third (cross-hatched columns) hour of collection is shown as $*P<0.05$ compared with the preinjection baseline hour (open columns).

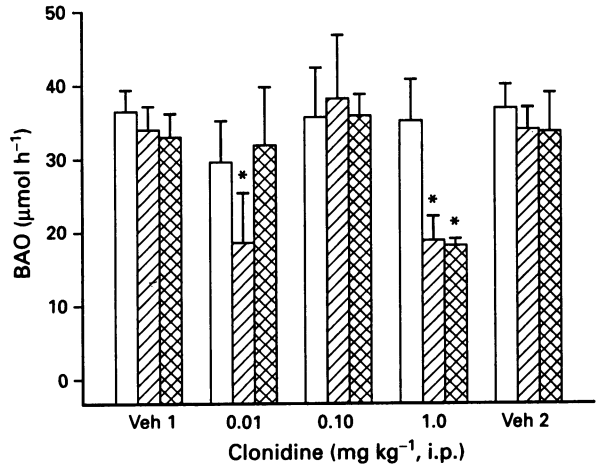


Figure 2 Effects of clonidine on basal (non-stimulated) gastric acid output (BAO) in conscious rats. Data are presented as mean ± s.e.mean ($n = 8$). Injections of vehicle as clonidine occurred at the beginning of the second hour of collection. Significant difference in BAO in the second (hatched columns) and the third (cross-hatched columns) hour is shown as $*P<0.05$ compared with pre-injection baseline hour (open columns).

Table 1 Effect of moxonidine on the pH of basal secretion in conscious rats (mean ± s.e.mean, $n = 8$)

	Moxonidine (mg kg^{-1})				
	Vehicle 1	*0.01*	*0.1*	*1.0*	*Vehicle 2*
Hour 1	2.2 (0.2)	4.0 (0.3)	1.8 (0.1)	1.9 (0.1)	2.3 (0.1)
Hour 2	2.4 (0.3)	5.3 (0.3)	3.9 (0.3)*	5.5 (0.2)*	1.9 (0.1)
Hour 3	2.4 (0.2)	2.6 (0.2)	3.4 (0.2)*	8.0 (0.2)*	1.7 (0.1)

*Significantly different from hour 1 ($P<0.05$).

Table 2 Effects of moxonidine on ethanol-induced gastric mucosal injury (mean ± s.e.mean, $n = 5$)

Moxonidine (mg kg^{-1})	*Number of gastric lesions*	*Length of gastric lesions* (mm)
0 (Saline)	19.2 ± 2.2	62.6 ± 5.8
0.01	14.2 ± 2.8	37.2 ± 9.3*
0.1	15.8 ± 2.3	56.2 ± 13.4
1.0	6.8 ± 0.9*#	16.4 ± 4.2*#

*Significantly different from saline vehicle ($P<0.05$); #Significantly different from moxonidine, 0.01 mg kg^{-1} ($P<0.05$).

Table 3 Effect of moxonidine on gastric acid output, secretion volume, pepsin output and mucosal injury in pylorus-ligated rats (mean ± s.e.mean, $n = 6$)

Moxonidine (mg kg^{-1})	*Acid output* (μmol h^{-1})	*Secretion vol.* (ml)	*Pepsin output* (mg h^{-1})	*Number of gastric lesions*	*Length of gastric lesions* (mm)
0 (Saline)	33.0 (2.1)	6.4 ± 0.5	15.6 ± 1.9	2.3 ± 1.8	7.5 ± 2.1
0.01	13.7 (0.8)*	8.3 ± 0.7	19.3 ± 2.3	2.2 ± 0.9	12.0 ± 4.2
0.1	11.3 (1.1)*	6.6 ± 1.0	13.3 ± 2.7#	2.2 ± 0.7	4.8 ± 2.3#
1.0	10.5 (1.2)*	5.2 ± 0.6*#	11.5 ± 2.5*#	2.3 ± 0.9	5.3 ± 2.6

Pylorus ligation 4 h; moxonidine i.p. at time 0 (immediately after surgery). *Significantly different from vehicle ($P<0.05$); #Significantly different from moxonidine, 0.01 mg kg^{-1} ($P<0.05$).

A role for imidazoline receptors and for compounds that are active at these sites, in gastrointestinal function and pathology has not been indicated until now. Moxonidine, an I_1-imidazoline receptor agonist, has been found to exhibit a 70 to 600 fold greater selectivity for the I_1-imidazoline receptor relative to the α_2-adrenoceptor, depending on the tissue studied and the assay conditions (Ernsberger *et al.*, 1992; 1993). In the present study, moxonidine beneficially altered gastric function at doses which were less than those previously shown to be required to decrease blood pressure in spontaneously hypertensive rats (Chrisp & Faulds, 1992; Irzyniec *et al.*, 1992). Gastric acid secretion in conscious rats was inhibited with an ED_{50} of 0.04 mg kg^{-1}, i.p. Moxonidine also reduced both acid and pepsin secretion in pylorus-ligated rats and reduced ethanol-induced gastric mucosal injury. Interestingly, moxonidine reduced not only aggressive, pro-ulcerogenic factors such as gastric acid and pepsin, but also appeared to enhance defensive, anti-ulcerogenic mechanisms that have become the target of recent attention in gastrointestinal drug development (Glavin & Hall, 1992). In particular, intragastric pH was significantly elevated by moxonidine, suggesting a possible action on gastric bicarbonate secretion that remains to be investigated.

The present data with moxonidine are in contrast to comparable experiments with clonidine. For example, Al-Bekairi *et al.* (1993) observed that clonidine exacerbated ethanol-induced gastric damage. In these studies, clonidine also decreased gastric acid secretion to a maximum of 63% at a dose of 0.3 mg kg^{-1}. In the present experiments, the lowest and highest doses of clonidine resulted in 37% and 46%, inhibition of basal gastric acid secretion, respectively, while the dose of 0.10 mg kg^{-1} did not influence acid output. In contrast, moxonidine, at doses of 0.1 and 1.0 mg kg^{-1}, initially resulted in 63% and 65% inhibition of basal acid

output, respectively, but in the case of the 1.0 mg kg^{-1} dose, this inhibition persisted into the subsequent hour of testing during which 100% suppression of gastric secretion was observed. The contrasting gastrointestinal actions of clonidine and moxonidine thus lend further support to the hypothesis that these compounds act at different sites, α_2-adrenoceptors and imidazoline receptors, respectively. The relative selectivity of these agonists for their respective receptors as reported by others would support this contention. Clonidine displayed a 5 fold selectivity for the α_{2B}-adrenoceptor over the I_1-imidazoline receptor. Conversely, moxonidine displayed up to a 600 fold selectivity for the I_1-imidazoline receptor (Ernsberger *et al.*, 1992).

Similarly, previous reports of α_2-adrenoceptor-mediated changes on acid secretion with other purported selective agonists must be re-examined. SKF 35886, a purported α_2-adrenoceptor agonist, was found to decrease secretion and increase absorption of fluid in the rabbit intestine (Fondacaro *et al.*, 1989). However, studies in our laboratory have indicated that SKF 35886 (2,6 dimethyl clonidine) has a 5 fold greater selectivity for the I_1-imidazoline receptor over the α_2-adrenoceptor (Smyth *et al.*, unpublished findings). In fact, we have documented a number of differences with regard to the renal actions of clonidine (α_2-adrenoceptor agonist) and 2,6 dimethyl clonidine (Smyth *et al.*, 1992; Li & Smith, 1993) consistent with these two agonists functioning at two unique receptors.

In summary, the I_1-imidazoline receptor agonist, moxonidine, suppressed basal gastric acid output in both conscious and anaesthetized rats, decreased pepsin output and decreased ethanol-induced gastric lesions. These studies indicate a potential role for I_1-specific imidazoline receptor agonists in the management of gastroduodenal diseases in addition to their blood pressure lowering activity. The relative contribution of the central and peripheral effects of moxonidine to these gastrointestinal actions remains to be determined.

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References

AL-BEKAIRI, A., AL-RAJHI, A. & TARIQ, M. (1993). Effect of (+)-propranolol and clonidine on stress- and chemically-induced ulcers in rats. *Arch. Int. Pharmacodyn. Ther.*, **323**, 97–113.

BHANDARE, P., DINIZ-D'SOUZA, D., MAINKER, A. & DHUME, V. (1990). Protective effect of propranolol and ethanol-induced gastric lesions in mice. *Eur. J. Pharmacol.*, **191**, 167–172.

CHRISP, P. & FAULDS, D. (1992). Moxonidine: a review of its pharmacology, and therapeutic use in essential hypertension. *Drugs*, **44**, 993–1012.

DEL SOLDATO, P. (1986). Gastric lesion-preventing or potentiating activity of clonidine in rats. *Jpn. J. Pharmacol.*, **41**, 257–259.

DOXEY, J.C., LANE, A.C., ROACH, A.C. & VIRDEE, W.K. (1984). Comparison of the α adrenergic profiles of idazoxan (RX 781094) yohimbine, rauwolscine and corynanthine. *Naunyn-Schmied. Arch. Pharmacol.*, **325**, 136–144.

DUPUY, D. & SZABO, S. (1986). Protection by metals against ethanol-induced gastric mucosal injury in the rat. *Gastroenterology*, **91**, 966–974.

ERNSBERGER, P., DAMON, T.H., GRAFF, L.M., SCHAFER, S.G. & CHRISTEN, M.O. (1993). Moxonidine, a centrally acting antihypertensive agent, is a selective ligand for I_1-imidazoline sites. *J. Pharmacol. Exp. Ther.*, **264**, 172–182.

ERNSBERGER, P., WESTBROOK, K.L., CHRISTEN, M.O. & SCHAFER, S.G. (1992). A second generation of centrally acting antihypertensive agents act on putative I_1-imidazoline receptors. *J. Cardiovasc. Pharmacol.*, **20**, (Suppl. 4) S1–S10.

FONDACARO, J., MCCAFFERTY, G., KOLPAK, D. & SMITH, P. (1989). Antidiarrheal activity of alpha-2 adrenoceptor agonist SK & F 35886. *J. Pharmacol. Exp. Ther.*, **249**, 221–228.

GLAVIN, G. & HALL, A. (1992). Brain-gut relationships: gastric mucosal defense is also important. *Acta Physiol. Hung.*, **80**, 107–115.

HAMILTON, C. (1992). The role of imidazoline receptors in blood pressure regulation. *Pharmacol. Ther.*, **54**, 231–248.

IRZYNIEC, T., MALL, G., GREBER, D. & RITZ, E. (1992). Beneficial effect of nifedipine and moxonidine on glomerulosclerosis in spontaneously hypertensive rats. A micromorphometric study. *Am. J. Hypertens.* **5**, 437–443.

KIRK, R. (1982). *Experimental Design.* Belmont: Brooks/Cole.

KUNCHANDY, J., KHANNA, S. & KULKARNI, K. (1985). Effects of alpha$_2$ agonists clonidine, guanfacine and B-HT 920 on gastric acid secretion and ulcers in rats. *Arch. Int. Pharmacodyn. Ther.*, **275**, 123–138.

LI, P. & SMYTH, D.D. (1993). Suppressed renal response to 2,6 dimethyl clonidine but not clonidine in one kidney – one clip hypertensive rats. *J. Pharmacol. Exp. Ther.*, **267**, 1395–1400.

MICHEL, M.C., BRODDE, O.E., SCHNEPEL, B., BEHRENDT, J., TSCHADA, R., MOTULSKY, H.J. & INSEL, P.A. (1989). [^{3}H] idazoxan and some other alpha-2 adrenergic drugs also bind with a high affinity to a non-adrenergic site. *Mol. Pharmacol.*, **35**, 324–330.

MICHEL, M.C., REGAN, J.W., GERHARDT, M.A., NEUBIG, R.R., INSEL, P.A. & MOTULSKY, H.J. (1990). Nonadrenergic [^{3}H] idazoxan binding sites are physically distinct from alpha-2 adrenergic receptors. *Mol. Pharmacol.*, **37**, 65–68.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keep an eye on the I-site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13**, 369–370.

MITANA, S., YOSHIDA, J., SAITOH, M., ASAKA, M., MATSUSHIMA, T. & MIYAZAKI, T. (1989). Study of the relationship between portal hypertension and gastroduodenal mucosal lesions. *J. Gastroenterol. Hepatol.*, **4**, (suppl. 1) 146–150.

PARÉ, W., ISOM, K., VINCENT, G. & GLAVIN, G. (1977). A technique for chronic gastric fistula preparation in the rat. *Lab. Animal Sci.*, **27**, 244–247.

PARÉ, W. & SCHIMMEL, G. (1986). Stress ulcer in normotensive and spontaneously hypertensive rats. *Physiol. Behav.*, **36**, 699–705.

RAY, A., HENKE, P., GULATI, K. & SEN, P. (1993). The amygdaloid complex, corticotropin releasing factor and stress-induced gastric ulcerogenesis in rats. *Brain Res.*, **624**, 286–290.

REIS, D.J., REGUNATHAN, S., WANG, H., FEINSTEIN, D.L. & MEELY, M.P. (1992). Imidazoline receptors in the nervous system. *Fundam. Clin. Pharmacol.*, **6**, (Suppl. 1), 23s–29s.

ROBERT, A., NEZAMIS, J., LANCASTER, C. & HANCHAR, A. (1979). Cytoprotection by prostaglandins. *Gastroenterol*, **77**, 433–443.

SANKARY, H., SARFEH, I., TARNAWSKI, A., MAEDA, R., IVEY, K. & MASON, G. (1986). Propranolol reduces ethanol-induced gastric mucosal damage in portal hypertensive rats. *Dig. Dis. Sci.*, **31**, 162–165.

SHICHIJO, K., ITO, M. & SEKINE, L. (1991). The mechanism of low susceptibility to stress in gastric lesions of spontaneously hypertensive rats. *Life Sci.*, **49**, 2023–2029.

SMYTH, D.D., LI, P., BLANDFORD, D.E. & PENNER, S.B. (1992). Opposite rank order of potency for clonidine, 2,6 dimethyl clonidine and UK 14,304 on water and solute excretion in the rat. Two sites and/or receptors? *J. Pharmacol. Exp. Ther.*, **261**, 1080–1086.

TAZI-SAAD, K., CHARIOT, J., DEL TACCA, M. & ROZE, C. (1992). Effect of α_2-adrenoceptor agonists on gastric pepsin and acid secretion in the rat. *Br. J. Pharmacol.*, **106**, 846–852.

TIBIRICA, E., FELDMAN, J., MERMET, C., GONON, F. & BOUSQUET, P. (1991). An imidazoline-specific mechanism for the hypotensive effect of clonidine: study with yohimbine and idazoxan. *J. Pharmacol. Exp. Ther.*, **256**, 606–613.

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Effects of *Bordetella pertussis* toxin pretreatment on the antiarrhythmic action of ischaemic preconditioning in anaesthetized rats

[1]L. Piacentini, [2]C.L. Wainwright & J.R. Parratt

Department of Physiology & Pharmacology, University of Strathclyde, Glasgow G1 1XW

1 *Bordetella pertussis* toxin, which catalyses the ADP-ribosylation of certain guanine nucleotide binding proteins (G proteins), thus functionally uncoupling them from associated receptors, was examined to determine whether it modified the antiarrhythmic effect of ischaemic preconditioning in anaesthetized rats.

2 *Pertussis toxin* (25 μg kg^{-1}, i.p., 48 h prior to heart isolation) attenuated the negative chronotropic effect of acetylcholine (ACh) in rat isolated Langendorff perfused hearts. ACh (10 μM) reduced heart rate by 4% in hearts taken from *pertussis* toxin-treated animals, compared to a reduction of 57% in hearts taken from animals treated only with vehicle.

3 In anaesthetized rats, ischaemic preconditioning (a single 3 min occlusion of the left main coronary artery followed by 10 min reperfusion) had a pronounced antiarrhythmic effect during a subsequent 30 min period of regional myocardial ischaemia. Compared to hearts receiving only a 30 min period of left coronary occlusion, there was a reduced mortality (67% and 0% for control and preconditioned groups, respectively; $P<0.01$) and decreased incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF). Pretreatment with *pertussis* toxin (25 μg kg^{-1}, i.p., 48 h previously) did not modify the arrhythmias associated with a 30 min period of regional myocardial ischaemia, neither did it modify the reduction in mortality (from 56% to 0%; $P<0.05$) associated with preconditioning. Furthermore, the decrease in total ventricular premature beat count induced by preconditioning seen in controls (from 427 ± 130 to 95 ± 45) was also seen in *pertussis* toxin-treated rats (from 252 ± 190 to 57 ± 25).

4 These results suggest that receptor coupling to *pertussis* toxin-sensitive G proteins is not necessary for the antiarrhythmic effect of ischaemic preconditioning in this model.

Keywords: Preconditioning; antiarrhythmic; *pertussis* toxin-sensitive G proteins; myocardial ischaemia; coronary artery occlusion; rat isolated hearts

Introduction

Ischaemic preconditioning is a phenomenon whereby a short period of myocardial ischaemia and reperfusion renders the heart more tolerant to a subsequent more prolonged ischaemic episode (Murry *et al.*, 1986). This cardioprotection is characterized by a reduction in infarct size (Murry *et al.*, 1986), an improvement in post-ischaemic function (Asimakis *et al.*, 1992) and attenuation of arrhythmias associated with ischaemia and reperfusion (Vegh *et al.*, 1992). The mechanisms underlying preconditioning have yet to be fully elucidated. In most species studied, it is thought that some of the cardioprotective effects of preconditioning are mediated by adenosine receptors (Liu *et al.*, 1991; Schwarz *et al.*, 1992; Auchampach & Gross, 1993), possibly of the A_1 subtype. In rats this does not appear to be the case since the improvement in post-ischaemic functional recovery (Asimakis *et al.*, 1993; Cave *et al.*, 1993) and the infarct size limiting (Liu & Downey, 1992) and antiarrhythmic (Piacentini *et al.*, 1992) effects associated with preconditioning occur independently of adenosine receptor stimulation. It remains possible that in the rat heart the initial hormonal response differs from that seen in other species but thereafter employs a similar underlying signal transduction system. In this regard, in both rat (Speechly-Dick *et al.*, 1994) and rabbit (Ytrehus *et al.*, 1994) models, induction of cytosolic protein kinase C to its activated membrane state has been shown to occur as a consequence of the preconditioning stimulus.

It has been demonstrated that the infarct size-limiting effect of preconditioning in rabbits is partly dependent (Thornton *et al.*, 1993b) on *pertussis* toxin-sensitive guanine nucleotide binding proteins (G proteins). However, conflicting results surround the involvement of such G proteins in rats. We have found previously that the application of a single preconditioning cycle exerted an antiarrhythmic effect which was *pertussis* toxin-sensitive in crystalloid perfused rat isolated hearts (Piacentini *et al.*, 1993). However, the antiarrhythmic and infarct size-limiting effects of multiple preconditioning cycles in blood perfused rat hearts (Lawson *et al.*, 1993) and anaesthetized rats (Liu & Downey, 1993), respectively, appear to be *pertussis* toxin-insensitive. These opposing results may be a consequence of the different experimental models and preconditioning protocols employed and of the end-points measured. Thus, since the main differences in the models used to date are the presence of blood and the application of differing numbers of preconditioning cycles, the aim of this study was to examine whether the antiarrhythmic effect of a single preconditioning cycle, which is *pertussis* toxin-sensitive in an *in vitro* model, is also *pertussis* toxin-sensitive in an *in vivo* anaesthetized rat model.

Methods

Animal preparation

Male Sprague-Dawley rats (240–340 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and maintained under anaesthesia with sodium pentobarbitone (3 mg, i.v.) as required. Following anaesthesia, the right femoral vein and artery were cannulated (with heparinised catheters) for the administration of additional anaesthetic and for the

[1] Present address: Department of Cardiology, University of Heidelberg, Germany.
[2] Author for correspondence.

measurement of arterial blood pressure, respectively. A precalibrated steel thermistor probe was inserted into the rectum to measure core temperature which was maintained at 37–38°C with the aid of a heating lamp.

To allow for artificial respiration, a tracheostomy was performed. The rats were then prepared for coronary artery ligation by the method described by Clark *et al.* (1980). The chest was opened using a left thoracotomy performed between the fourth and fifth ribs approximately 3 mm from the sternum. Artificial respiration with room air was immediately initiated with a respirator (C.F. Palmer, U.K., 48 strokes min^{-1}, 1.5 ml 100 g^{-1} animal weight). The two ribs above the thoracotomy were sectioned and the pericardium incised to allow access to the heart. The heart was then exteriorised by application of gentle pressure to either side of the ribcage and a 6/0 silk suture on a curved taper needle (Mersilk, Ethicon) was placed under the left main coronary artery immediately below the tip of the left atrial appendage. The heart was then replaced with the thoracic cavity and the animal was allowed to stabilize for 15 min before onset of experimental protocols. Any animal in which this procedure caused persistent arrhythmias (i.e. more than a few premature ventricular ectopic beats after manipulation of the heart) or a sustained fall in mean arterial blood pressure below 70 mmHg was excluded from the study. Although direct measurement of area at risk, the size of which may contribute to arrhythmia severity, was not made in these studies, we know from our previous studies that ligation of the coronary artery at this site results in approximately 40–45% of the free left ventricular wall being ischaemic (Garjani *et al.*, unpublished). When animals were to be subjected to the preconditioning protocol, the ends of the suture were threaded through a length of polyethylene tubing during the stabilization period. This then formed a snare which, when pressed against the heart, occluded the coronary vessels rendering the associated area ischaemic. Reperfusion could then be allowed by loosening this snare. When ischaemia without reperfusion was performed, the ligature was tied with a single knot. Successful ligation was assumed for each individual animal by the presence of regional cyanosis, a slight drop in arterial blood pressure and characteristic electrocardiographic changes, notably an increase in the height of the R-wave.

Systemic arterial blood pressure was monitored from the catheter inserted into the right femoral artery, connected to a Spectramed P23KL transducer. The electrocardiogram (ECG) was recorded from standard limb leads placed under the skin. Heart rate was calculated from the ECG over a 10 s period and expressed as beats min^{-1}. Both the ECG and mean arterial blood pressure were recorded continuously with a Gould Easygraf Recorder, model TA240.

Isolated hearts

Rats were anaesthetized as outlined above, heparinised with 100 units of heparin sulphate (i.p.) and the hearts were rapidly removed and transferred to a Langendorff apparatus where retrograde perfusion at a constant flow (10 ml min^{-1}) was started. The perfusate was a modified Krebs-Henseleit solution containing (in mmol l^{-1}): NaCl 118, KCl 3.2, $CaCl_2$ 2.52, $MgSO_4$ 1.66, $NaHCO_3$ 26.88, KH_2PO_4 1.18, glucose 5.55, and sodium pyruvate 2.0, adjusted to pH 7.4, aerated with 95% O_2/5% CO_2, filtered and maintained at 37°C. Fine platinum subdermal electrodes (Grass type E2) were placed on the right atrium and the apex of the left ventricle allowing an epicardial electrocardiogram to be recorded, using the metal aortic cannula as a reference electrode. The ECGs were recorded continuously on a Grass model 79 recorder. Heart rate (beats min^{-1}) was calculated from the ECG trace over a 10 s period. Hearts were allowed to stabilize for 30 min before experimental protocols began.

Experimental protocols

Toxin from *Bordetella pertussis* (Sigma) was diluted with normal saline to give a final concentration of 10 μg ml^{-1}. Rats received either *pertussis* toxin (25 μg kg^{-1}, i.p.) or an equivalent volume of the vehicle (a saline solution containing 10% glycerol and 10 mM sodium phosphate). Experimental protocols, both *in vivo* and *in vitro*, were initiated 48 h after the *pertussis* toxin or vehicle pretreatment.

Following the stabilization period, anaesthetized rats were randomised to receive one of two protocols outlined in Figure 1. The preconditioning cycle consisted of a 3 min occlusion of the left main coronary artery and 10 min reperfusion which was then followed by a 30 min period of coronary artery occlusion (Vegh *et al.*, 1992). Rats subjected to this preconditioning regimen (vehicle $n = 9$; *pertussis* toxin $n = 9$) were compared to rats receiving a single 30 min period of ischaemia (vehicle $n = 9$; *pertussis* toxin $n = 9$). The myocardium was not reperfused after the 30 min period of ischaemia.

Isolated hearts (vehicle $n = 5$; *pertussis* toxin $n = 4$) were allowed to stabilize for 30 min and then each heart was given two 5 min infusions of acetylcholine (1 and 10 μM) each separated by 30 min drug-free perfusion.

Arrhythmia analysis

Ventricular arrhythmias were analysed during periods of ischaemia and reperfusion by (i) counting the total number of ventricular premature beats (VPBs), which included single ventricular premature beats, couplets and triplets (termed salvos), and ventricular tachycardia (defined as four or more consecutive premature beats), in accordance with the Lambeth Conventions (Walker *et al.*, 1988), and (ii) determining the incidences of ventricular tachycardia (VT), ventricular fibrillation (VF) and mortality.

Statistical analysis

Except for the incidences of VT and VF, all results are expressed as means with the standard error of the means (s.e.mean). To compare the number of VPBs the Kruskal-Wallis non-parametric test was used, followed, when appropriate, by Dunn's multiple comparison procedure with an experimentwise error rate of 0.05 (Hollander & Wolfe, 1973). For comparisons of incidences of events, the Fisher Irwin

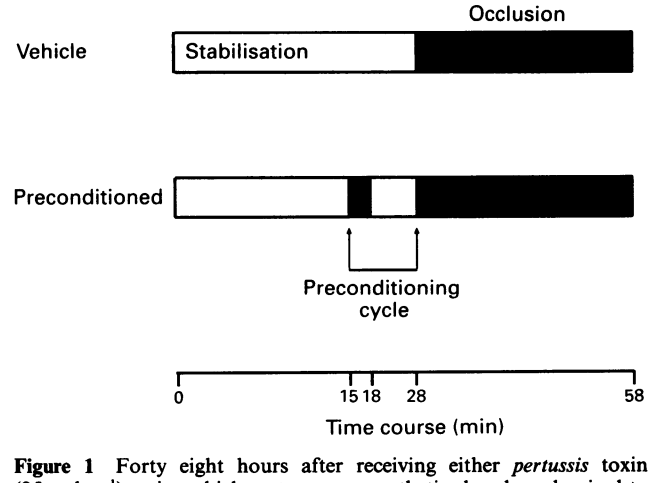


Figure 1 Forty eight hours after receiving either *pertussis* toxin (25 μg kg^{-1}) or its vehicle, rats were anaesthetized and randomised to one of two protocols. All rats were subjected to a 30 min occlusion of the left coronary artery. Rats in the preconditioned group were first subjected to a preconditioning cycle of 3 min ischaemia and 10 min reperfusion. Non-preconditioned rats were allowed a further 13 min period of stabilization before being subjected to the 30 min occlusion period. For each of the four groups, $n = 9$.

(Chi^2 with Yates' correction) test was used. For normally distributed data, i.e. mean arterial blood pressure and heart rate, comparisons between groups were made by ANOVA followed by Tukeys *post hoc* test. Direct comparisons between two groups, when necessary, were made with Student's *t* test (unpaired, two tailed). For comparisons at different time points within a single group, a repeated measure ANOVA was carried out, followed if $P<0.05$, by Dunnets *post hoc* test. When necessary, Student's *t* test (paired, two tailed) was used for comparisons at two different time points, within a single group. The differences in the duration of ventricular tachycardia between groups was analyzed by the Mann Whitney U-test.

Results

Haemodynamic effects of pertussis toxin pretreatment in anaesthetized rats before and after coronary artery ligation

Pretreatment with *pertussis* toxin resulted in a reduced mean arterial blood pressure with the result that six rats were excluded from the study with a mean arterial pressure of less than 70 mmHg after the stabilization period. However, these rats were replaced immediately to ensure that *n* numbers were equal in each group. The mean arterial pressure of the *pertussis* toxin-treated rats was approximately 10 mmHg lower than in vehicle-treated rats (81 ± 4 and 92 ± 6 mmHg for *pertussis* toxin and vehicle treated rats, respectively). As a consequence of the preconditioning occlusion, pressure initially decreased in both vehicle and *pertussis* toxin treated groups. After 10 min reperfusion, whilst pressure recovered to pre-occlusion levels (92 ± 7 mmHg) in the vehicle-treated rats, recovery was not complete in the *pertussis* toxin-treated rats (77 ± 5 mmHg). Similarly, blood pressure dropped sharply upon onset of the sustained ischaemic episode in all four treatment groups, recovering gradually over the sustained occlusion period (Table 1).

Baseline heart rates were similar for rats pretreated with vehicle and *pertussis* toxin and remained stable throughout the preconditioning occlusion and reperfusion period in both treatment groups. During the sustained 30 min ischaemic period there was a slight increase (not significant) in the heart rates measured in vehicle-treated rats, whether preconditioned or not, which was not apparent in rats which had been pretreated with *pertussis* toxin (Table 1).

Arrhythmias following coronary artery occlusion in control and preconditioned anaesthetized rats

A 30 min occlusion of the left coronary artery in anaesthetized rats not previously subjected to the preconditioning protocol resulted in the development of severe arrhythmias with the majority of animals succumbing to terminal VF (Figure 2). The distribution of ventricular premature beats (VPBs) was similar in both vehicle- and *pertussis* toxin-treated rats, although the latter displayed greater ectopic activity (total VPB count 2022 ± 797 compared to 730 ± 671 in vehicle treated controls; $P>0.2$). This apparent difference is due to small *n* numbers of rats surviving the 30 min occlusion ($n=3$ and 4 for vehicle and *pertussis*-treated, respectively) and *pertussis*-treated rats surviving for a longer time and experiencing longer bursts of VT (43 ± 37 and 134 ± 61 s for 9 out of 9 vehicle and 4 out of 9 *pertussis*-treated rats respectively).

Although VPBs occurring as a consequence of the preconditioning occlusion were few (8 ± 2 for both vehicle- and *pertussis* toxin-treated groups), there was a significant increase in the total VPB count in the intervening reperfusion

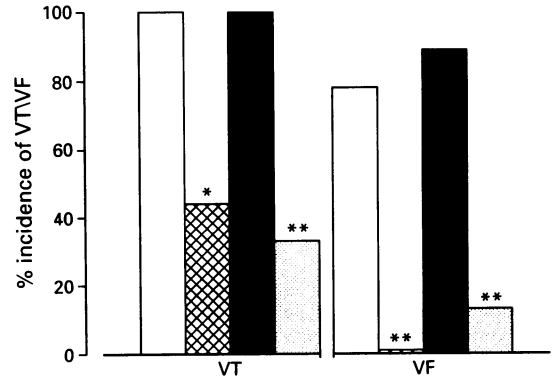


Figure 2 The incidences of Ventricular tachycardia (VT) and ventricular fibrillation (VF) occurring in anaesthetized rats during a 30 min occlusion of the left coronary artery. Values obtained from rats subjected only to a 30 min occlusion (open and solid columns for vehicle- and *pertussis*-toxin treated rats, respectively) are compared to the incidences in rats first subjected to the preconditioning cycle (cross-hatched and stippled columns for vehicle- and *pertussis* toxin-treated, respectively). The preconditioning cycle consisted of a 3 min occlusion and 10 min reperfusion. For each of the four groups, $n=9$. ** $P<0.05$ compared to same treatment control.

Table 1 Blood pressure and heart rate in anaesthetized rats, pretreated with either *pertussis* toxin or its vehicle, during a 30 min occlusion of the left coronary artery

			Occlusion time course (min)				
Group	n	*Pre-occlusion*	*1*	*3*	*10*	*20*	*30*
MABP (mmHg)							
Vehicle-treated							
Control	9	90 ± 7	67 ± 7	76 ± 7	85 ± 16 (4)	87 ± 11 (3)	95 ± 8 (3)
Preconditioned	9	92 ± 7	81 ± 5	91 ± 7	93 ± 8	91 ± 7	93 ± 8
pertussis toxin-treated							
Control	9	82 ± 5	72 ± 4	76 ± 5	89 ± 6 (7)	78 ± 7 (4)	81 ± 11 (4)
Preconditioned	9	77 ± 6	74 ± 5	77 ± 7	76 ± 10	76 ± 6	75 ± 5
Heart rate (beats min^{-1})							
Vehicle-treated							
Control	9	419 ± 16	426 ± 11	433 ± 11	456 ± 29 (4)	452 ± 30 (3)	453 ± 26 (3)
Preconditioned	9	419 ± 19	427 ± 18	433 ± 20	435 ± 21	433 ± 21	427 ± 21
pertussis toxin							
Control	9	392 ± 16	388 ± 16	405 ± 18	402 ± 33 (7)	385 ± 25 (4)	371 ± 28 (4)
Preconditioned	9	420 ± 16	425 ± 16	422 ± 17	421 ± 13	419 ± 17	418 ± 11

n numbers decreased with time as a consequence of animal death. Thus *n* represents the initial number of hearts with the reduced values shown in parentheses where appropriate. MABP = mean arterial blood pressure.

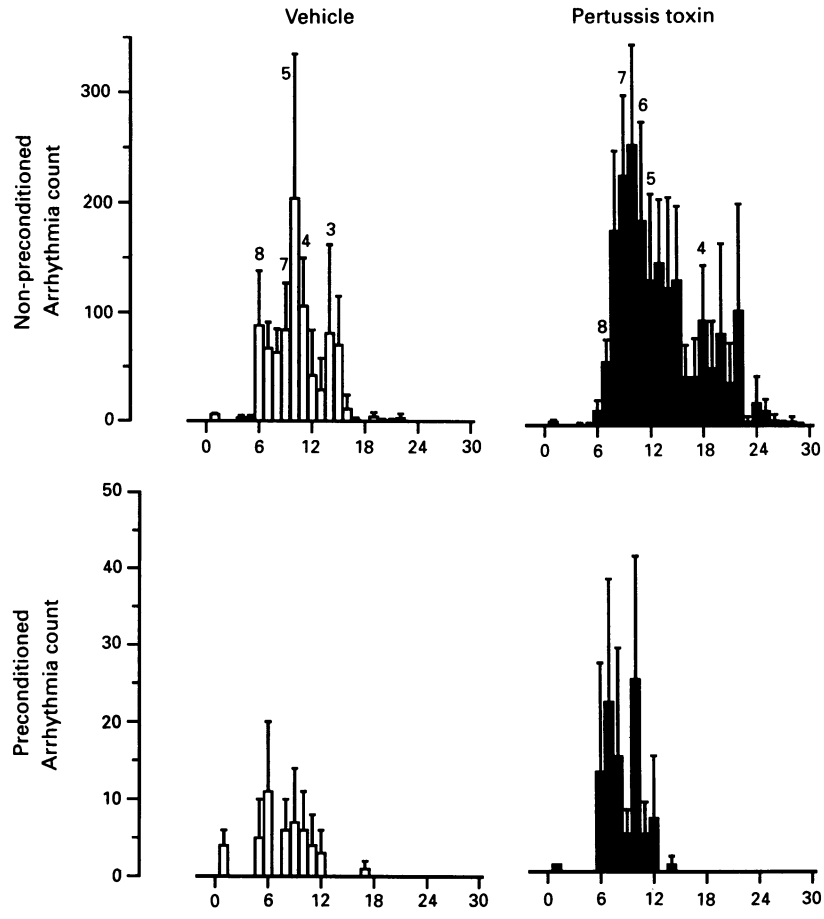


Figure 3 Distribution of arrhythmias over a 30 min period of coronary artery occlusion in anaesthetized rats without (upper panel) and with (lower panel) preconditioning induced by a 3 min occlusion and 10 min reperfusion, before onset of the sustained occlusion. Rats were pretreated with either vehicle (left-hand open columns) or *pertussis* toxin (right-hand solid columns). Each column represents the mean ± s.e.mean number of ventricular premature beats (VPBs) occurring over each min of the sustained occlusion. All groups had an initial $n = 9$; reductions in the n number as a consequence of terminal VF are shown when they occur.

Table 2 Heart rate responses before and 5 min after the administration of acetylcholine (ACh) to isolated hearts taken from rats pretreated with either *pertussis* toxin (25 μg kg^{-1}, i.p.) or its vehicle (10% glycerol in saline)

		Heart rate (beats min^{-1})			
Pretreatment	n	*Pre-drug*	*ACh* (1 μM) 5 min	*Pre-drug*	*ACh* (10 μM) 5 min
Vehicle	5	367 ± 14	313 ± 48	362 ± 6	154 ± 20***
Pertussis toxin	4	337 ± 23	364 ± 50	332 ± 32	318 ± 26

***$P < 0.001$ compared to pre-drug value (paired t test).

period in *pertussis* toxin-treated rats compared to vehicle-treated rats (90 ± 48 compared with 2 ± 1; $P < 0.05$). Irrespective of whether rats were pretreated with *pertussis* toxin or vehicle, preconditioning the myocardium resulted in attenuation in the number of VPBs and the incidence of VT and VF during a subsequent 30 min ischaemic episode (Figures 2 and 3). All preconditioned rats (i.e. both vehicle- and *pertussis* toxin-treated) survived the ischaemic episode, compared with 67% and 56% mortality in vehicle- and *pertussis* toxin-treated non-preconditioned rats respectively ($P < 0.05$ for both) and the total VPB count during occlusion was low (98 ± 45 and 57 ± 25 VPB for vehicle- and *pertussis* toxin-treated preconditioned animals respectively).

Responses to acetylcholine infusions in isolated hearts removed from rats given pertussis *toxin*

In hearts taken from rats pretreated with vehicle there was a marked negative chronotropic response to ACh infusions (Table 2). As a consequence of a 5 min infusion of ACh (10 μM) there was a significant reduction in heart rate. In contrast, even a high concentration of ACh failed to elicit a reduction in heart rate when hearts were taken from rats pretreated with *pertussis* toxin.

Discussion

Although the cardioprotective effect of preconditioning in rats does not appear to depend upon stimulation of adenosine receptors (Piacentini *et al.*, 1992; Liu & Downey, 1992), it does share some characteristics of preconditioning with other species. For example, in rabbit (Thornton *et al* 1993a; Toombs *et al.*, 1993), dog (Kitakaze *et al.*, 1992; Vegh *et al.*, 1994) and rat (Banerjee *et al.*, 1993; Ravingerova *et al.*, 1994) models, stimulation of α_1-adrenoceptors prior to sustained ischaemia can mimic the cardioprotective effect of preconditioning. A role for the endogenous release of noradrenaline as a mechanism of preconditioning has thus been proposed in dogs (Kitakaze *et al.*, 1992) and rats

(Banerjee *et al.*, 1993) although the evidence from rabbit studies is conflicting (Toombs *et al.*, 1993; Thornton *et al.*, 1993a). α_1-Adrenoceptors can be coupled to *pertussis* toxin-sensitive G-proteins in neonatal rat cardiac myocytes (Barratt *et al.*, 1993) while the A_1 adenosine and M_2 muscarinic receptors are also coupled to some of their effectors by *pertussis* toxin-sensitive G-proteins (Birnbaumer, 1990). Stimulation of muscarinic receptors can mimic preconditioning in rabbits (Thornton *et al.*, 1993b) and dogs (Yao & Gross, 1993) and treatment with *pertussis* toxin reduces the infarct size limiting effect in rabbits (Thornton *et al.*, 1993b). Thus, in light of these similarities, we speculated that a G-protein signal transducer system, rather than the receptor type which triggers it, may be focal to the preconditioning phenomenon.

We have found previously, in a rat isolated heart model, that the antiarrhythmic effect of preconditioning was attenuated when hearts were taken from rats pretreated with *pertussis* toxin (Piacentini *et al.*, 1993). However, studies examining the infarct size limiting (Liu & Downey, 1993) and antiarrhythmic (Lawson *et al.*, 1993) effects of preconditioning in anaesthetized rats and blood-perfused rat hearts, respectively, showed that the cardioprotective effects of preconditioning were still apparent after *pertussis* toxin treatment. Two experimental differences could explain this anomaly. Firstly the presence of blood containing a blood-borne, *pertussis* toxin-insensitive, substance might contribute to this cardioprotective effect. This possibility is not excluded by the results of Lawson *et al.* (1993) since in their model rat isolated hearts were perfused with blood from donor rats not treated with *pertussis* toxin. Second, the application of different numbers of preconditioning cycles may influence the eventual outcome. In our previous study we examined the effects of a single preconditioning cycle whereas both Lui & Downey (1993) and Lawson *et al.* (1993) employed three preconditioning cycles. The number of cycles employed is of importance since, although the antiarrhythmic effect is seen after one preconditioning cycle *in vivo* (present study; Vegh *et al.*, 1992), increasing the number of preconditioning cycles appears to increase the protection against ischaemic arrhythmias *in vitro* (Lawson & Hearse, 1993). Furthermore, while a reduction in infarct size is seen in anaesthetized rats after either three (Liu & Downey, 1992; Li *et al.*, 1992) or one (Yellon *et al.*, 1992) preconditioning occlusion, a single cycle gives the maximal improvement in post-ischaemic function (Asikamis *et al.*, 1992). It could be speculated that more than one mechanism of protection determining the degree and type of protection is important in this species. We hypothesized that the *pertussis* toxin-sensitive cardioprotective effect, as a consequence of a single preconditioning cycle, was overridden by a second *pertussis* toxin-insensitive mechanism when multiple preconditioning cycles were applied. In order to minimize experimental differences further we repeated our previous *in vitro* study using an *in vivo* model to investigate this antiarrhythmic effect in the presence of blood. However, the results of this study indicate that application of a single preconditioning cycle was not *pertussis* toxin-sensitive in anaesthetized rats.

Since an identical preconditioning protocol to that used in our *in vitro* study was employed it must be assumed that either the presence of a *pertussis* toxin-insensitive component(s) of blood plays a role in this model, or that intact autonomic nervous control actively contributes to the antiarrhythmic effect. Although it seems unlikely that blood-borne substances play a role in the infarct size limiting effect of preconditioning in rabbits (Liu *et al.*, 1992), the possibility has not been fully explored in rat models. With regard to the latter possibility, autonomic reflexes are better preserved during ischaemia after preconditioning (Miyazaki & Zipes, 1989).

Differences in the effects of *pertussis* toxin pretreatment between the *in vitro* and *in vivo* models or preconditioning may also lie in dissimilarities in the mechanisms underlying the antiarrhythmic effect of preconditioning in each model as might the application of different exclusion criteria. In the previous *in vitro* study (Piacentini *et al.*, 1993) *pertussis* toxin had an antiarrhythmic effect; *in vivo*, if anything, *pertussis* toxin enhanced arrhythmogenesis. It cannot be excluded that a low VPB count, *in vitro*, masked the antiarrhythmic effect of preconditioning. Furthermore, in the present *in vivo* study, 25% of *pertussis* toxin-treated rats were excluded with a low arterial blood pressure. Since blood pressure before heart isolation was not measured, the same exclusion criteria could not be applied to hearts in the *in vitro* study. The low pressure was probably a consequence of a combination of *pertussis* toxin effects, both related and unrelated to its ability to ADP-ribosylate certain G_α subunits (Nogimori *et al.*, 1984). It is unclear whether the rats with a low blood pressure which had to be excluded were more successfully ADP-ribosylated and, although unlikely, it cannot be excluded that the hearts of the rats included in the study were not sufficiently modified by *pertussis* toxin pretreatment. Unfortunately, since the role of G-protein activation in preconditioning was the process under study, we were unable to determine the extent of inhibition of ACh-induced bradycardia by pretreatment with *pertussis* toxin in our *in vivo* rats, since this would have confounded the subsequent arrhythmia analysis. However, in our earlier study we found that this concentration of *pertussis* toxin consistently attenuated the negative chronotropic effect of acetylcholine (Piacentini *et al.*, 1993). We have demonstrated this once again in this present study by determining the effects of pretreatment, with the same batch of *pertussis* toxin given to the *in vivo* rats, on the bradycardic response to ACh in hearts isolated from a separate group of rats. This indicates that in this series of experiments *pertussis* toxin-sensitive proteins, at least in atrial tissue, were no longer able to couple with associated receptors. We have assumed that the *pertussis* toxin-sensitive G proteins in the ventricular tissue are similarly uncoupled; indeed, successful ADP-ribosylation of G proteins in rat ventricular tissue can be demonstrated after pretreatment with a similar concentration of *pertussis* toxin (Anand-Srivastava, 1989).

The results of this study do not exclude a role for G_i/G_o proteins in the antiarrhythmic effect of preconditioning, since phosphorylation at a non-hormonal site, as a consequence of protein kinase C activation, can modify the activity of these proteins (Pyne *et al.*, 1989). Furthermore, the functional modification of G_i which occurs as a consequence of ischaemia in canine ventricular tissue (Niroomand *et al.*, 1992) is not apparent after preconditioning (Weiss *et al.*, 1993).

In conclusion, we have found that the antiarrhythmic effect of preconditioning in anaesthetized rats is still apparent after treatment with *pertussis* toxin. This indicates that in this model functional coupling of receptors to *pertussis* toxin-sensitive G proteins is not necessary for cardioprotection.

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References

ANAND-SRIVASTAVA, M.B. (1989). Angiotensin II receptors negatively coupled to adenylate cyclase in rat myocardial sarcolemma. *Biochem. Pharmacol.*, **38**, 489–496.

ASIMAKIS, G.A., INNERS-MCBRIDE, K. & CONTI, V.R. (1993). Attenuation of postischaemic dysfunction by ischaemic preconditioning is not mediated by adenosine in the isolated rat heart. *Cardiovasc. Res.*, **27**, 1522–1530.

ASIMAKIS, G.K., INNERS-MCBRIDE, K., MEDELLIN, G. & CONTI, V.R. (1992). Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat hearts. *Am. J. Physiol.*, **263**, H887–H894.

AUCHAMPACH, J.A. & GROSS, G.J. (1993). Adenosine A_1 receptors, K_{ATP} channels, and ischemic preconditioning. *Am. J. Physiol.*, **264**, H1327–H1336.

BANERJEE, A., LOCKEWINTER, C., ROGERS, K.B., BREW, E.C., CAIRNS, C.B., BENSARD, D.D. & HARKEN, A.H. (1993). Preconditioning against myocardial dysfunction after ischemia and reperfusion by an α_1-adrenergic mechanism. *Circ. Res.*, **73**, 656–670.

BARRATT, S., HONBO, N. & KARLINER, J.S. (1993). Alpha$_1$-adrenoceptor-mediated inhibition of cellular cAMP accumulation in neonatal rat ventricular myocytes. *Naunyn. Schmied. Arch. Pharmacol.*, **347**, 384–393.

BIRNBAUMER, L. (1990). G proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 675–705.

CAVE, A.C., COLLIS, C.S., DOWNEY, J.M. & HEARSE, D.J. (1993). Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc. Res.*, **27**, 663–668.

CLARK, C., FOREMAN, M.I., KANE, K.A., McDONALD, F.M. & PARRATT, J.R. (1980). Coronary artery ligation in anaesthetized rats as a method for the production of experimental dysrhythmias and for the determination of infarct size. *J. Pharmacol. Methods*, **3**, 357–368.

HOLLANDER, M. & WOLFE, D.A. (1973). *Non Parametric Statistical Methods*. New York: John Wiley & Sons Inc..

KITAKAZE, M., TAKASHIMA, S., MORIOKA, T., SATO, H. & MORI, H. (1992). α_1-adrenoceptor activation limits infarct size by augmenting adenosine production and 5′ nucleotidase activity in ischemic preconditioning. *Circulation*, **86** (suppl I), I-213.

LAWSON, C.S., COLTART, D.J. & HEARSE, D.J. (1993). The antiarrhythmic action of ischaemic preconditioning in rat hearts does not involve functional Gi proteins. *Cardiovasc. Res.*, **27**, 681–687.

LAWSON, C.S. & HEARSE, D.J. (1993). The antiarrhythmic effect of ischaemic preconditioning: true protection or temporal effect? *J. Mol. Cell Cardiol.*, **25** (suppl. I), S.33.

LI, Y., WHITTAKER, P. & KLONER, R.A. (1992). The transient nature of the effect of ischaemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am. Heart J.*, **123**, 346–352.

LIU, Y. & DOWNEY, J.M. (1992). Ischemic preconditioning protects against infarction in rat heart. *Am. J. Physiol.*, **263**, H1107–H1112.

LIU, Y. & DOWNEY, J.M. (1993). Preconditioning against infarction in the rat heart does not involve a *pertussis* toxin sensitive G protein. *Cardiovasc. Res.*, **27**, 608–611.

LIU, G.S., STANLEY, A.W.H. & DOWNEY, J.M. (1992). Ischaemic preconditioning is not dependent on neutrophils or glycolytic substrate at reperfusion in rabbit heart. *Cardiovasc. Res.*, **26**, 1195–1198.

LIU, G.S., THORNTON, J., VAN WINKLE, D.M., STANLEY, A.W.H., OLSSON, R.A. & DOWNEY, J.M. (1991). Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation*, **84**, 350–356.

MIYAZAKI, T. & ZIPES, D.P. (1989). Protection against autonomic denervation following acute myocardial infarction by preconditioning ischaemia. *Circ. Res.*, **64**, 437–448.

MURRY, C.E., JENNINGS, R.B. & REIMER, K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*, **74**, 1124–1136.

NIROOMAND, F., BANGERT, M., BEYER, T. & RAUCH, B. (1992). Reduced adenylyl cyclase inhibition by carbachol and GTP during acute myocardial ischaemia. *J. Mol. Cell Cardiol.*, **24**, 471–475.

NOGIMORI, K., TAMURA, M., YAJIMA, M., ITO, K., NAKAMURA, T., KAJIKAWA, N., MARUYAMA, Y. & UI, M. (1984). Dual mechanisms involved in development of diverse biological activities of islet-activating protein, *pertussis* toxin, as revealed by chemical modification of lysine residues in the toxin molecule. *Biochim. Biophys. Acta.*, **801**, 232–243.

PIACENTINI, L., WAINWRIGHT, C.L. & PARRATT, J.R. (1992). The antiarrhythmic effect of preconditioning, in rat isolated hearts, does not involve A_1 adenosine receptors. *Br. J. Pharmacol.*, **107**, 137P.

PIACENTINI, L., WAINWRIGHT, C.L. & PARRATT, J.R. (1993). The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a *pertussis* toxin sensitive mechanism. *Cardiovasc. Res.*, **27**, 674–680.

PYNE, N.J., MURPHY, G.J., MILLIGAN, G. & HOUSLAY, M.D. (1989). Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein G_i. *FEBS Lett.*, **243**, 77–82.

RAVINGEROVA, T., PYNE, N.J. & PARRATT, J.R. (1994). Adrenergic stimulation protects rat hearts against severe arrhythmias: relevance to ischeamic preconditioning. *J. Mol. Cell. Cardiol.*, **26**, CXII, Abstract 467.

SCHWARZ, E.R., MOHRI, M., SACK, S., ARRAS, M. & SCHAPER, W. (1992). Preconditioning by ischemia lasts for only 30 min and is inhibited by adenosine antagonists. *J. Mol. Cell Cardiol.*, **24** (suppl. I), S.93.

SPEECHLY-DICK, M.E., BAXTER, G.F. & YELLON, D.M. (1994). Ischaemic preconditioning protects hypertrophied myocardium. *Cardiovasc. Res.*, **28**, 1025–1029.

THORNTON, J.D., DALY, J.F., COHEN, M.V., YANG, X.M. & DOWNEY, J.M. (1993a). Catecholamines can induce adenosine receptor-mediated protection of the myocardium but do not participate in ischemic preconditioning in the rabbit. *Circ. Res.*, **73**, 649–655.

THORNTON, J., LIU, G.S. & DOWNEY, J.M. (1993b). Pretreatment with *pertussis* toxin blocks the protective effects of preconditioning: evidence for a G protein mechanism. *J. Mol. Cell Cardiol.*, **25**, 311–320.

TOOMBS, C.F., WILTSE, A.L. & SHEBUSKI, R.J. (1993). Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium. *Circulation*, **88**, 2351–2358.

VEGH, A., KOMORI, S., SZEKERES, L. & PARRATT, J.R. (1992). Antiarrhythmic effects of preconditioning in anaesthetized dogs and rats. *Cardiovasc. Res.*, **26**, 487–495.

VEGH, A., PAPP, G.Y. & PARRATT, J.R. (1994). Intracoronary noradrenaline suppresses ischaemia-induced arrhythmias in anaesthetized dogs. *J. Mol. Cell. Cardiol.*, **26**, CXII, Abstract 343.

WALKER, M.J.A., CURTIS, M.J., HEARSE, D.J., CAMPBELL, R.W.F., JANSE, M.J., YELLON, D.M., COBBE, S.M., COKER, S.J., HARNESS, J.B., HARRON, D.W.G., HIGGINS, A., JULIAN, D.J., LAB, M.J., MANNING, A.S., NORTHOVER, B.J., PARRATT, J.R., RIEMERSMA, R.A., RIVA, E., RUSSELL, D.C., SHERIDAN, D.C., WINSLOW, E. & WOODWARD, B. (1988). The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. *Cardiovasc. Res.*, **22**, 447–455.

WEISS, A., ZEIFANG, F., RAUCH, B. & NIROOMAND, F. (1993). A functional modification of G_i proteins as the underlying mechanism of ischaemic preconditioning. *Circulation*, **88**, I-631.

YAO, Z. & GROSS, G.J. (1993). Role of nitric oxide, muscarinic receptors, and the ATP-sensitive K + channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. *Circ. Res.*, **73**, 1193–1201.

YELLON, D.M., ALKHULAIFI, A.M., BROWNE, E.E. & PUGSLEY, W.B. (1992). Ischaemic preconditioning limits infarct size in the rat heart. *Cardiovasc. Res.*, **25**, 983–987.

YTREHUS, K., LIU, G.S. & DOWNEY, J.M. (1994). Preconditioning protects the ischaemic rabbit heart by protein kinase C activation. *Am. J. Physiol.*, **266**, H1145–H1152.

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Pharmacokinetic modelling of the haemodynamic effects of the A_{2a} adenosine receptor agonist CGS 21680C in conscious normotensive rats

R.A.A. Mathôt, A. Cleton, W. Soudijn, A.P. IJzerman & [1]M. Danhof

Divisions of Pharmacology and Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands

1 The aim of the present investigation was to determine the relationship between the blood concentration and haemodynamic effects of the adenosine A_{2a} receptor agonist, CGS 21680C (the sodium salt of 2-*p*-(2-carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine) in conscious normotensive rats.

2 Chronically cannulated rats were randomly assigned to three groups which received 300, 1000 or 3000 $\mu g\ kg^{-1}$ (0.56, 1.9 or 5.6 $\mu mol\ kg^{-1}$) of CGS 21680C intravenously over 15 min. The mean arterial blood pressure (MAP) and heart rate (HR) were monitored continuously during the experiment and serial arterial blood samples were taken for analysis of drug concentration. The ratio MAP/HR was also calculated, which may reflect changes in total peripheral resistance on the assumption that no changes in stroke volume occur.

3 For each individual rat the reduction in mean arterial pressure was related to the blood concentration according to the sigmoidal E_{max} model. The concentration-effect relationships were consistent for the different treatment groups. The potency based on free drug concentrations ($EC_{50,u}$) was 5.8 $ng\ ml^{-1}$ (11 nM) (mean ± s.e.; $n = 19$) and correlated well with the reported adenosine A_{2a} receptor affinity (K_i 19 nM). In comparison with the reduction in blood pressure, CGS 21680C exhibited a greater potency for the reduction of the ratio MAP/HR.

4 It is concluded that estimates can be obtained for the potency and intrinsic activity of adenosine A_{2a} receptor agonists *in vivo* by pharmacokinetic-pharmacodynamic analysis of mean arterial pressure data in a rat model. In future studies, total peripheral resistance may also be useful as a pharmacodynamic parameter for A_{2a} activation, provided that possible changes of the stroke volume are also assessed.

Keywords: Adenosine A_{2a} receptor; cardiovascular effects; CGS 21680 (2-*p*-(2-carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine); pharmacokinetic-pharmacodynamic modelling

Introduction

Adenosine exerts its cardiovascular effects through interaction with at least two subtypes of extracellular adenosine receptors: A_1 and A_{2a} (Van Calker *et al.*, 1979; Daly, 1982). Stimulation of the A_{2a} receptor by adenosine induces coronary and peripheral vasodilatation (Kusachi *et al.*, 1983; Webb *et al.*, 1990), while the A_1 receptor mediates cardiac depression (Olsson & Pearson, 1990). During the past few years selective and potent agonists and antagonists have been described for both receptor subtypes (Jacobson *et al.*, 1992; Shimada *et al.*, 1992).

In dose-response studies of adenosine receptor agonists, correlations have been reported between the affinity for the receptor and the cardiovascular potency *in vitro* and *in vivo* (Mertz *et al.*, 1984; Hamilton *et al.*, 1987; Oei *et al.*, 1988; Abiru *et al.*, 1991). However, discrepancies in the rank orders of affinity and potency have been reported in an *in vivo* study with structurally distinct adenosine A_{2a} receptor agonists, which were suggested to be the result of differences in pharmacokinetic properties of the ligands (Webb *et al.*, 1991). When evaluating potency and activity *in vivo*, the relationship between the dose and the pharmacological effect is determined by both pharmacokinetic processes (absorption, distribution, metabolism and excretion) and pharmacodynamic processes (drug receptor interaction, second messengers, etc.). Furthermore, the assessment of concentration-cardiovascular effect relationships could be complicated by several factors such as the formation of active metabolites, the development of acute tolerance or the activation of physiological control mechanisms (Guyton *et al.*, 1972; Dingemanse *et al.*, 1988).

Recently, we have developed a pharmacokinetic-pharmacodynamic model for the quantitative characterization of the cardiovascular effects of agonists for the adenosine A_1 receptor in normotensive rats (Mathôt *et al.*, 1994). In these studies it was shown that, although the adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA) evoked both bradycardia and hypotension, the reduction in heart rate was the more relevant pharmacodynamic end-point for activation of the adenosine A_1 receptor *in vivo*. The haemodynamic effects of agonists for the adenosine A_{2a} receptor have generally been characterized in spontaneously hypertensive rats by relating the administered dose to the hypotensive effect (Webb *et al.*, 1991; Abiru *et al.*, 1991). The reduction in blood pressure is probably caused predominantly by adenosine A_{2a} receptor-mediated systemic vasodilatation (Webb *et al.*, 1991).

The purpose of the present study was to determine the relationship between blood concentrations of an adenosine A_{2a} receptor agonist and its cardiovascular effects in individual rats. CGS 21680C (the sodium salt of 2-*p*-(2-carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine) was selected as a model compound because of its high affinity for the adenosine A_{2a} receptor ($K_i = 19$ nM) and its 74 fold A_{2a}/A_1 selectivity (Hutchison *et al.*, 1990). Conscious normotensive rats received a single administration of CGS 21680C, while the time courses of blood concentration, heart rate and blood pressure were monitored. As an additional cardiovascular effect parameter, the time profile of the ratio of mean arterial pressure over heart rate (MAP/HR) was derived, which may reflect changes in total peripheral resistance, assuming that no changes in stroke volume occur. The concentration-haemodynamic effect relationships were assessed

[1] Author for correspondence.

for each individual rat by a pharmacokinetic-pharmacodynamic modelling procedure. The consistency of the concentration-response relationships was determined following the administration of different doses of CGS 21680C.

Methods

Animals and catheter implantation

All experiments were performed in normotensive male SPF Wistar rats (weight 200–250 g), while conscious and unrestrained. The rats were housed individually in plastic cages, at a constant temperature of 21°C, and with a controlled 12 h light-dark cycle (light: 7 h 00 min to 19 h 00 min). Food (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and tap water were available *ad libitum*.

Two days before experimentation, indwelling cannulae were implanted under light ether anaesthesia, as has been described recently (Mathôt *et al.*, 1994). The abdominal aorta was cannulated by an approach through the left and the right femoral arteries for the measurement of the arterial blood pressure and the collection of serial blood samples, respectively. The right jugular vein was cannulated for administration of the compound. The cannulae were passed subcutaneously to emerge on the nape of the neck.

Cardiovascular monitoring

Arterial blood pressure was measured from the left femoral catheter in the abdominal aorta using a miniature strain gauge P10EZ transducer, equipped with a TA 1017 CritiFlo diaphragm dome (Viggo-Spectramed BV, Bilthoven, The Netherlands). During the experiment the diaphragm dome was flushed with heparinized saline (20 iu ml^{-1}) at a rate of 500 $\mu l\ h^{-1}$ (Harvard infusion pump 22, Plato, Diemen, The Netherlands) to avoid clotting of the cannula. The pressure transducer was placed at the level of the animal's heart and connected to a polygraph amplifier console (RMP6018, Nihon Kohden Corp., Tokyo, Japan). The heart rate was determined from the pressure signal, which was coupled to a tachograph. Signals were recorded on a polygraph and concurrently converted in a CED1401 interface (Cambridge Electronics Design Ltd., Cambridge, England) and fed into a 80387 computer (Philips, Eindhoven, The Netherlands). The data were stored on hard disk for off-line analysis. Data acquisition and reduction were performed with Spike2 computer software (Cambridge Electronics Design Ltd.). The ratio of mean arterial blood pressure over heart rate (MAP/HR) was calculated off-line. All experiments were started between 10 h 00 min and 11 h 00 min in order to exclude diurnal variation in base-line cardiovascular responses. Recording started 30 min prior to the beginning of the infusion.

Animal studies

The relationships between cardiovascular effects and blood concentrations of CGS 21680C were investigated in 19 individual rats, which were randomly allocated to three groups that received an intravenous infusion of 300, 1000 or 3000 $\mu g\ kg^{-1}$ (0.56, 1.9, 5.6 $\mu mol\ kg^{-1}$) of CGS 21680C over 15 min (Braun infusion pump, Melsungen, Germany). Depending on the dose, 15 serial arterial blood samples with volumes of 50, 100 or 200 μl were taken over a period of 0–62 or 0–92 min. The maximal total volume of blood withdrawn was 1400 μl. The blood samples were haemolyzed immediately in 400 μl ice cold water and stored at −35°C until analysis.

The effects of the injection volume and the volume of blood withdrawn on both base-line and drug-induced cardiovascular responses were investigated in two groups of 7 rats. One group of rats received an intravenous infusion of the vehicle (saline) for 15 min, with a blood sampling schedule identical to the administration of 300 $\mu g\ kg^{-1}$, the other group received a 15 min infusion of 300 $\mu g\ kg^{-1}$ (0.56 $\mu mol\ kg^{-1}$) CGS 21680C without blood sampling.

The plasma-to-blood ratio (P/B) and the extent of plasma protein binding (f_u) of CGS 21680C were determined in a separate group of 6 rats which had received 3000 $\mu g\ kg^{-1}$ (5.6 $\mu mol\ kg^{-1}$) of CGS 21680C during a 15 min infusion. P/B and f_u were determined at two CGS 21680C blood concentrations in order to examine a potential concentration-dependency of both parameters. At 14.5 and 30 min after starting infusion, blood samples with a volume of 1.5 ml were withdrawn and directly transferred to heparinized tubes on ice. An aliquot of 50 μl was haemolyzed in 400 μl distilled water (0°C) for measurement of total blood concentration. The rest of the blood sample was centrifuged for 10 min at 10,000 *g* at 4°C to separate blood cells. The plasma was separated and a sample of 50 μl was retained for analysis. A volume of 500 μl of the supernatant was subjected to ultrafiltration within 10 min at 1090 *g* at 37°C to separate free CGS 21680C from plasma protein bound CGS 21680C using the Amicon Micropartition System with an YMT ultrafiltration membrane (Amicon Division, Danvers, MA, U.S.A.). The concentration of free CGS 21680C was determined in 100 μl of the ultrafiltrate. The samples were stored at −35°C until analysis.

Analysis of drug concentration

The concentrations of CGS 21680C in blood, plasma and ultrafiltrate were determined according to the selective and sensitive high performance liquid chromatography (h.p.l.c.) procedure described by Irth *et al.* (1994), with some modifications. Calibration standards were prepared by adding 100 μl of solutions of CGS 21680C to 100 μl blood haemolyzed in 400 μl water to produce a blood concentration range of 2.5 to 250 ng ml^{-1}. Standard solutions were prepared in water and were stable over a period of 2 months when stored at 4°C. N^6-cyclohexyladenosine (CHA) (0.5 μg in 20 μl methanol) was added to samples with concentrations of CGS 21680C lower than 250 ng ml^{-1}; increased amounts (5 μg in 200 μl methanol) were added to samples with concentrations higher than 250 ng ml^{-1}. The samples were diluted with 200 μl 0.1 M citrate buffer pH 4.0 and extracted for 60 s with 5 ml of ethyl acetate on a vortex mixer. After centrifugation for 15 min at 2000 *g* the organic layer was transferred to another tube and evaporated to dryness under reduced pressure at 45°C. The extracts were subjected to liquid-solid extraction on Bond-Elut silica bound phenylboronic acid cartridges (Analytichem, Harbor City, CA, U.S.A.). Following preconditioning with 1.5 ml methanol and 1 ml water, the cartridges were loaded with the sample, which had been reconstituted in 0.25 ml 0.25 M ammonium acetate buffer, pH 8.8. After consecutive washing with 0.35 ml ammonium acetate buffer and 0.25 ml water, the compounds were eluted with 1.0 ml 0.1 M HCl. Following the addition of 25 μl 4.0 M NaOH, the eluate was evaporated to dryness under reduced pressure. The residue was reconstituted in 150 μl of the mobile phase and 100 μl was injected into the h.p.l.c. system; a volume of 10 μl was injected for blood samples with concentrations higher than 250 ng ml^{-1}.

The liquid chromatographic system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, U.S.A.), a WISP-712B autosampler (Millipore-Waters, Milford, MA, U.S.A.), a LS 240 fluorescence detector (Perkin Elmer, Beaconsfield, Buckinghamshire, U.K.) and a Spectroflow 757 variable-wavelength u.v. detector (Applied Biosystems, Ramsey, NJ, U.S.A.). Chromatography was performed at room temperature using a stainless-steel Microsphere C18 3-μm cartridge column (100 mm × 4.6 mm i.d.) (Chrompack Nederland BV, Bergen Op Zoom, The Netherlands) equipped with a guard column (20 mm × 2 mm i.d.) (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with

C18 (particle size 20–40 μm) (Chrompack Nederland BV). CGS 21680C and the internal standard (CHA) were detected by fluorescence (λ-excitation 262 nm and λ-emission 345 nm) and u.v. absorption (λ 269 nm), respectively. Data processing was performed with a Chromatopack C-R3A reporting integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of a 23/77 (v/v) mixture of acetonitrile and 65 mM acetate buffer (pH 5.0) with a flow rate of 0.5 ml min^{-1}. Retention times were 6 and 12 min for CGS 21680C and CHA, respectively. Peak height ratios of CGS 21680C/CHA were calculated and calibration curves were constructed by weighted linear regression (weight factor: 1/(peak-height-ratio)). The calibration curves were linear ($r > 0.995$) and the intra- and inter-assay variations were 7, 8 and 5% and 13, 4 and 6% for blood concentrations of 10, 100 and 1000 ng ml^{-1}, respectively ($n = 6$). The limit of detection was 2.5 ng ml^{-1} for a sample of 100 μl of blood as determined by a signal-to-noise ratio of 3.

Chemicals

CGS 21680C (the sodium salt of 2-*p*-(2-carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine) was a gift from Dr R.L. Webb (Ciba-Geigy Corp., Summit, NJ, U.S.A.). N^6-cyclohexyladenosine (CHA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Ethyl acetate was purchased from Baker Chemicals (Deventer, The Netherlands) and distilled prior to use. Acetonitrile (h.p.l.c. grade) was obtained from Westburg (Leusden, The Netherlands). Water was drawn from a Milli-Q system (Millipore SA, Molsheim, France). All the other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

Data analysis

For each individual animal the pharmacokinetic and pharmacodynamic data were analysed separately. Curve fitting of the blood concentration-versus-time and the cardiovascular response-versus-concentration profiles was performed by using the non-linear leasts squares-regression programme, Siphar (Simed S.A., Creteil, France).

The blood concentration-time profiles following intravenous infusion were described by a poly-exponential function:

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \times T} \times (1 - e^{-\lambda_i \cdot t}) \qquad t \leq T \quad (1A)$$

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \times T} \times (e^{-\lambda_i \cdot (t-T)} - e^{-\lambda_i \cdot t}) \qquad t > T \quad (1B)$$

In this equation C(t) is the blood concentration of CGS 21680C at time t, C_i is the coefficient associated with the i^{th} exponent λ and T is the infusion duration. Coefficients and exponents were optimally fitted to the experimental data based upon the Akaike information criterion (Akaike, 1974; Yamaoka *et al.*, 1978). In the fitting procedure a weight of 1/y was used. Pharmacokinetic parameters such as the area under the blood concentration-time curve (AUC), the systemic clearance (CL), the mean residence time (MRT), the volume of distribution at steady-state (V_{ss}) and the terminal half-life ($t_{\frac{1}{2},n}$) were calculated following standard procedures using the coefficients and exponents of the fitted functions (Gibaldi & Perrier, 1982):

$$AUC = \sum_{i=1}^{n} \frac{C_i}{\lambda_i} \quad (2)$$

$$CL = \frac{D}{AUC} \quad (3)$$

$$MRT = \frac{1}{AUC} \times \sum_{i=1}^{n} \frac{C_i}{\lambda_i^2} \quad (4)$$

$$V_{ss} = CL \times MRT \quad (5)$$

$$t_{\frac{1}{2},n} = \frac{0.693}{\lambda_n} \quad (6)$$

where D is the intravenous dose. Clearances and volumes of distribution were normalized for body weight.

The poly-exponential pharmacokinetic fit was used to generate the CGS 21680C blood concentration at the time of the averaged cardiovascular effect measurement. Time-effect points were obtained by averaging 60–300 s of consecutive heart rate, blood pressure or MAP/HR data. Data were collected more frequently at the time of rapid change in drug concentration. For each individual rat, concentrations were correlated to cardiovascular responses according to the sigmoidal E_{max} pharmacodynamic model with a fixed no-drug value (Holford & Sheiner, 1981):

$$E = E_0 + \frac{E_{max} \cdot C^{n_H}}{EC_{50}^{n_H} + C^{n_H}} \quad (7)$$

where E is the cardiovascular response, E_0 is the no-drug response, EC_{50} is the CGS 21680C concentration that corresponds to 50% of the maximum effect (E_{max}) and n_H expresses the sigmoidicity of the curve (Hill factor). EC_{50} values based on free drug concentrations ($EC_{50,u}$) were obtained after correction for binding of the compound to blood cells and plasma proteins. Values of the no-drug response were obtained by averaging the cardiovascular effect from 165 to 195 min, after the start of the administration.

To compare the cardiovascular responses and the pharmacokinetic and pharmacodynamic parameter estimates obtained after the administration of different doses, a one-way analysis of variance (ANOVA) or a non-parametric Kruskal-Wallis test, if more appropriate, was performed. The P/B and f_u values were subjected to a Student's *t* test to detect a potential concentration-dependency. The values for EC_{50} and E_{max} obtained for the decrease in blood pressure were compared to the corresponding values for the reduction in MAP/HR by using a paired *t* test. For all tests a significance level of 5% was selected. Unless otherwise indicated, all data are reported as the mean ± s.e.

Results

Pharmacokinetics

The averaged concentration-time profiles after the administration of 300, 1000 and 3000 μg kg^{-1} (0.56, 1.9 and 5.6 μmol kg^{-1}) of CGS 21680C are shown in Figure 1. For the three different doses a bi-exponential function satisfactorily described the time course of blood concentration. The pharmacokinetic parameters are summarized in Table 1 and no physiological relevant differences between the values were observed for the different infusion rates. Averaged over the three infusion rates, the total blood clearance, the mean residence time, the apparent volume of distribution at steady-state and the terminal half-life were 59 ± 3 ml min^{-1} kg^{-1}, 6.7 ± 0.4 min, 390 ± 20 ml kg^{-1} and 10 ± 1 min, respectively ($n = 19$).

The plasma-to-blood ratio (P/B) and the extent of plasma protein binding (f_u) were assessed *in vivo* as 0.71 ± 0.03 and 68 ± 3% ($n = 12$), respectively, at two different CGS 21680C blood concentrations: 2800 ± 300 and 210 ± 40 ng ml^{-1} ($n = 6$), with no significant concentration-dependent differences.

Pharmacokinetic-pharmacodynamic relationship

The averaged changes in mean arterial pressure as a function of time after the administration of different doses of CGS

Table 1 Pharmacokinetic parameters obtained after intravenous infusion of different doses of CGS 21680C in 15 min to conscious normotensive rats

Dose ($\mu g\ kg^{-1}$)	n	C_1 ($\mu g\ ml^{-1}$)	λ_1 (min^{-1})	C_2 ($\mu g\ ml^{-1}$)	λ_2 (min^{-1})	*AUC* ($\mu g\ min\ ml^{-1}$)	*CL* ($ml\ min^{-1}\ kg^{-1}$)	*MRT* (min)	V_{ss} ($ml\ kg^{-1}$)	$t_{1,n}$ (min)
300	7	1.6 ± 0.3	0.48 ± 0.09	0.24 ± 0.04	0.084 ± 0.005	6.2 ± 0.5	56 ± 4	7.1 ± 0.5	400 ± 40	8.3 ± 0.5
1000	6	6.1 ± 0.6	0.49 ± 0.05	0.35 ± 0.09	0.072 ± 0.007	17 ± 1	63 ± 5	5.4 ± 0.7*	330 ± 30	10 ± 1
3000	6	16 ± 2	0.44 ± 0.09	1.0 ± 0.2	0.058 ± 0.004**	56 ± 2	59 ± 2	7.5 ± 0.3	440 ± 40	12 ± 1**

The individual concentration-time courses were described with a bi-exponential pharmacokinetic function with coefficients C_1 and C_2 and exponents λ_1 and λ_2 (equation 1). These coefficents and exponents were used to calculate the area under the blood concentration-time curve (*AUC*), the systemic clearance (*CL*), the mean residence time (*MRT*), the volume of distribution at steady-state (V_{ss}) and the terminal half-life ($t_{1,n}$) (equations 2–6). Values are reported as mean ± s.e. Data were analysed statistically by a one-way ANOVA or a Kruskal-Wallis test, if more appropriate.
*Significantly different from 300 and 1000 $\mu g\ kg^{-1}$ ($P<0.05$).
**Significantly different from 300 $\mu g\ kg^{-1}$ ($P<0.01$).

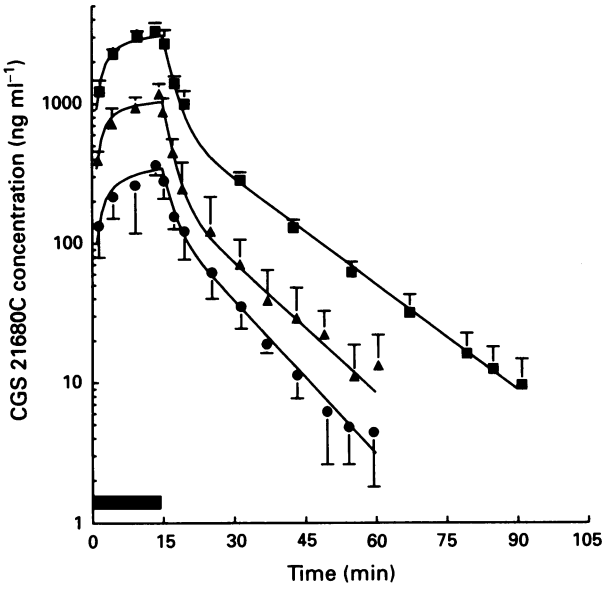


Figure 1 Concentration of CGS 21680C in the blood of rats after intravenous infusion of 300 (●), 1000 (▲) and 3000 (■) $\mu g\ kg^{-1}$ (0.56, 1.9 and 5.6 $\mu mol\ kg^{-1}$) over 15 min. The data are presented as mean ± s.d. and the fitted solid lines are the averages of 6 (1000 and 3000 $\mu g\ kg^{-1}$) or 7 (300 $\mu g\ kg^{-1}$) curves.

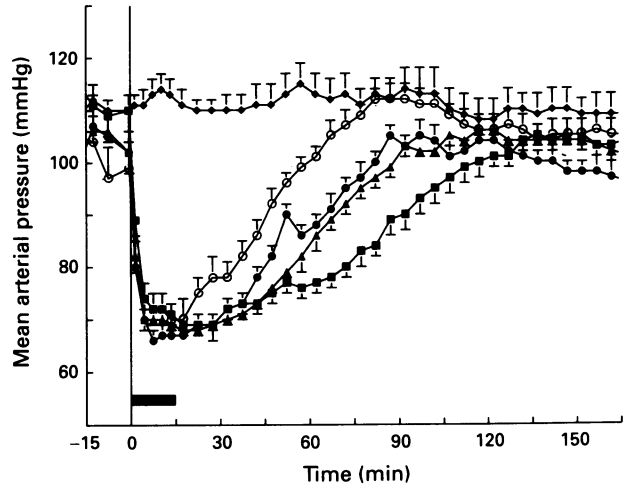


Figure 2 Average time profile of mean arterial blood pressure for rats given an intravenous infusion of 300 (●; $n = 7$), 1000 (▲; $n = 6$) or 3000 (■; $n = 6$) $\mu g\ kg^{-1}$ (0.56, 1.9 and 5.6 $\mu mol\ kg^{-1}$) of CGS 21680C over 15 min. Control rats had received saline with blood sampling (◆; $n = 7$) or 300 $\mu g\ kg^{-1}$ CGS 21680C in 15 min (○; $n = 7$) without blood sampling. Data are presented as mean ± s.e. For each time point data were analysed by one-way ANOVA to test for differences between the treatment groups and the saline group.

21680C are depicted in Figure 2. During the infusion a rapid fall in blood pressure was observed. After the infusion was terminated, the effect returned gradually to a baseline. The blood pressure of the drug-treated groups was not significantly different from the saline-treated group after 110 min. The volume of the infusion and the volume of blood withdrawn had no effect on the base-line blood pressure as shown in Figure 2. The blood pressure profiles of the groups which received 300 $\mu g\ kg^{-1}$ CGS 21680C either with or without blood sampling were significantly different in the period from 55 to 90 min, suggesting a more rapid recovery in the absence of blood sampling.

Figure 3a shows the time courses of blood concentration and mean arterial pressure in an individual rat after the intravenous administration of 3000 $\mu g\ kg^{-1}$ CGS 21680C during 15 min. The bi-exponential pharmacokinetic function was used to calculate the blood concentrations at the time points of blood pressure measurement. In the plots of blood concentration versus hypotensive response no difference could be detected between the rising and the falling phases of drug concentrations. Since no time-delay or hysteresis was observed, the pharmacological response was directly related to CGS 21680C blood concentrations as shown in Figure 3b. For each rat the relationship between blood concentration and the reduction in mean arterial pressure was quantified according to the sigmoidal E_{max} model. The averaged pharmacodynamic parameters are presented in Table 2. The mean values of the parameters as calculated from the three groups were: $E_0 = 100 \pm 1$ mmHg, $E_{max} = -32 \pm 2$ mmHg, $EC_{50} = 12 \pm 2\ ng\ ml^{-1}$, $n_H = 1.6 \pm 0.2$ and $EC_{50,u} = 5.8 \pm 1.0\ ng\ ml^{-1}$ ($n = 19$).

Following the infusion of different doses of CGS 21680C, an increase in heart rate was observed (Figure 4) which did not appear to be related to the pharmacokinetic profile. The heart rate increased within the first 5 min of the infusion and then decreased during the rest of the dose administration. The heart rate returned slowly after the infusion to a baseline, which was significantly higher than that of the placebo-treated group. The positive chronotropic effect in the different doses was related to the decrease in mean arterial pressure as depicted in Figure 5. The heart rate-blood pressure relationships were not different for doses of 300 and 1000 $\mu g\ kg^{-1}$ CGS 21680C, while for the dose of 3000 $\mu g\ kg^{-1}$ the magnitude of the tachycardia was reduced in comparison with the other doses.

For the different administrations, the time profiles of the ratio of mean arterial pressure over heart rate (MAP/HR) are shown in Figure 6. After the start of the infusion, MAP/HR decreased rapidly to levels which were approximately 50% of the pre-infusion levels. The effect rapidly reached a

Table 2 Pharmacodynamic parameter estimates for the reduction in mean arterial pressure after intravenous infusion of different doses of CGS 21680C to conscious normotensive rats

Dose ($\mu g\ kg^{-1}$)	n	E_0 (mmHg)	E_{max} (mmHg)	(%)	EC_{50} ($ng\ ml^{-1}$)	n_H	$EC_{50,u}$ ($ng\ ml^{-1}$)
300	7	97 ± 2	−30 ± 3	(−30 ± 3)	11 ± 2	1.8 ± 0.3	5.3 ± 1.0
1000	6	101 ± 1	−33 ± 2	(−32 ± 2)	11 ± 4	1.7 ± 0.2	5.3 ± 1.9
3000	6	104 ± 2**	−34 ± 3	(−33 ± 3)	14 ± 3	1.3 ± 0.3	6.8 ± 1.4

For each rat the CGS 21680C blood concentration-mean arterial pressure relationship was fitted to the sigmoidal E_{max} model, yielding estimates for the baseline response (E_0), intrinsic activity (E_{max}), potency (EC_{50}), Hill factor (n_H) and potency, based on free concentrations ($EC_{50,u}$). Values are reported as mean ± s.e. Data were analysed statistically by a one-way ANOVA or a Kruskal-Wallis test, if more appropriate.
**Significantly different from 300 $\mu g\ kg^{-1}$ ($P<0.01$).

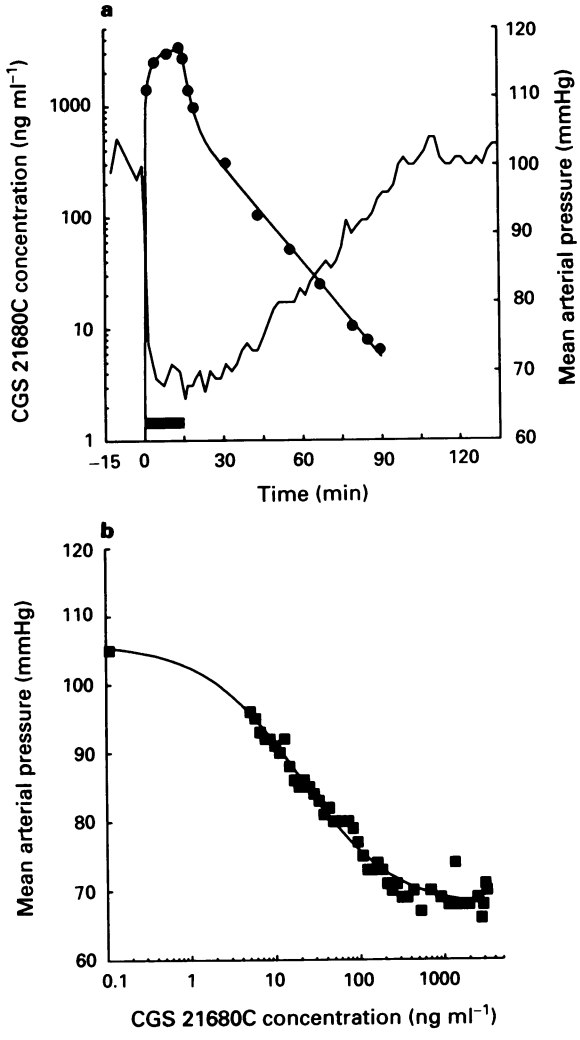


Figure 3 (a) Time course of blood concentration and mean arterial pressure for a rat after an intravenous infusion of 3000 $\mu g\ kg^{-1}$ (5.6 $\mu mol\ kg^{-1}$) of CGS 21680C over 15 min. The solid line represents the predicted blood concentration on fitting a bi-exponential function to the concentration-time data (●). (b) Concentration-blood pressure relationship for the same rat. The solid line represents the sigmoidal E_{max} model fitted to the concentration-effect data (■). E_0 was fixed to a value of 106 mmHg.

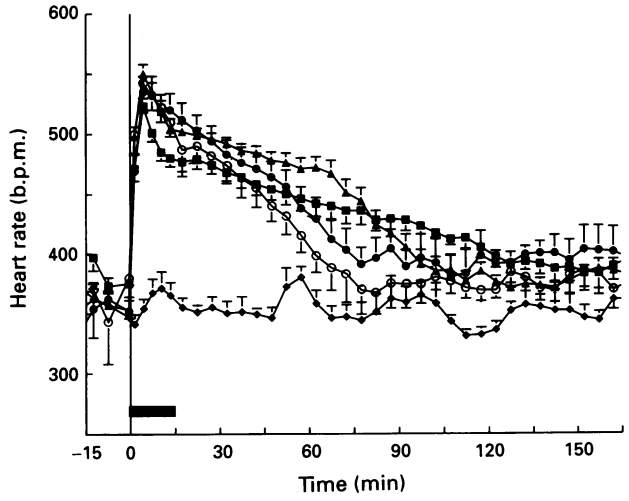


Figure 4 Average heart rates of rats given an intravenous infusion of 300 (●; $n = 7$), 1000 (▲; $n = 6$) or 3000 (■; $n = 6$) $\mu g\ kg^{-1}$ (0.56, 1.9 and 5.6 $\mu mol\ kg^{-1}$) of CGS 21680C over 15 min. Control rats had received saline with blood sampling (◆; $n = 7$) or 300 $\mu g\ kg^{-1}$ CGS 21680C in 15 min (○; $n = 7$) without blood sampling. Data are presented as mean ± s.e. For each time point data were analysed by one-way ANOVA to test for differences between the treatment groups and the saline group.

maximum and maintained that level during the infusion. After the end of the infusion MAP/HR values gradually increased to plateau levels, which were significantly lower than those of the placebo-treated rats. Figure 7a shows the time courses of blood concentration and MAP/HR after the intravenous administration of 300 $\mu g\ kg^{-1}$ CGS 21680C during 15 min. No time delay was observed and blood concentrations were correlated directly to the pharmacological response according to the sigmoidal E_{max} model (Figure 7b). The pharmacodynamic parameters, as obtained by the kinetic-dynamic modelling procedure, are given in Table 3 and there were no significant differences between the doses except that after the administration of 3000 $\mu g\ kg^{-1}$ CGS 21680C the Hill factor was significantly lower than for the infusion of 300 $\mu g\ kg^{-1}$. After combining the results of the three groups, the average pharmacodynamic parameters of CGS 21680C for the effect parameter MAP/HR were: $E_0 = 26 \pm 1 \times 10^{-2}$ mmHg/b.p.m., $E_{max} = -13 \pm 1 \times 10^{-2}$ mmHg/b.p.m., $EC_{50} = 7.2 \pm 1.0\ ng\ ml^{-1}$, $n_H = 0.97 \pm 0.07$ and $EC_{50,u} = 3.5 \pm 0.5\ ng\ ml^{-1}$ ($n = 19$).

The individual values for the EC_{50} and E_{max} as obtained with both blood pressure and MAP/HR as pharmacodynamic endpoints, are shown in Figure 8. In comparison with the hypotensive effect, the EC_{50} for the decrease in MAP/HR was significantly lower (7.2 ± 1.0 vs. 12 ± 2 $ng\ ml^{-1}$, $P<0.01$; $n = 19$), while the maximal reduction (E_{max}) in MAP/HR was significantly higher (−48 ± 2% vs. −32 ± 2%, $P<0.001$; $n = 19$).

Discussion

Recently, experimental strategies have been developed to model the pharmacokinetics of the cardiovascular effects of

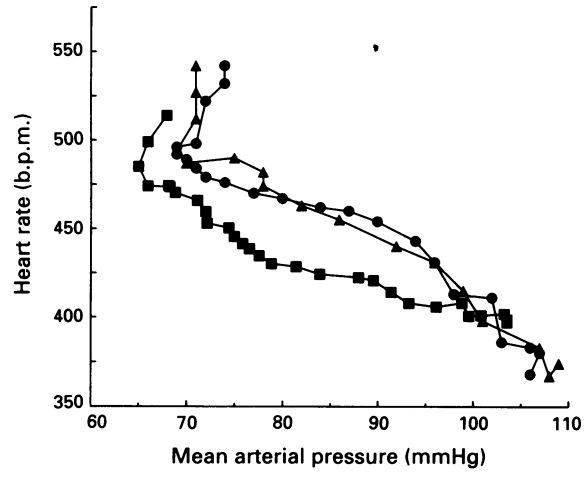


Figure 5 Relationship between mean arterial pressure and heart rate after intravenous infusion of 300 (●), 1000 (▲) and 3000 (■) $\mu g\ kg^{-1}$ (0.56, 1.9 and 5.6 $\mu mol\ kg^{-1}$) over 15 min. The data are presented as mean ± s.e. of 7 (300 $\mu g\ kg^{-1}$) or 6 (1000 and 3000 $\mu g\ kg^{-1}$) rats.

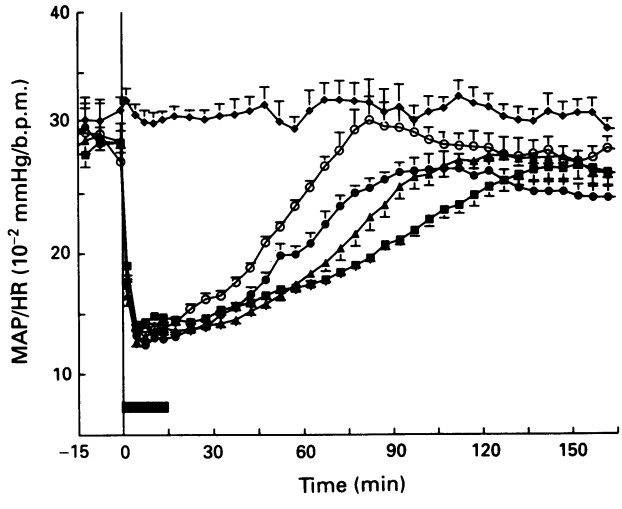


Figure 6 Average profiles of the ratio of mean arterial blood pressure over heart rate (MAP/HR). Rats had received an intravenous infusion of 300 (●; $n = 7$), 1000 (▲; $n = 6$) and 3000 (■; $n = 6$) $\mu g\ kg^{-1}$ (0.56, 1.9 and 5.6 $\mu mol\ kg^{-1}$) of CGS 21680C over 15 min. Control rats had received saline with blood sampling (◆; $n = 7$) or 300 $\mu g\ kg^{-1}$ CGS 21680C in 15 min (○; $n = 7$) without blood sampling. Data are presented as mean ± s.e. For each time point data were analysed by one-way ANOVA to test for differences between the treatment groups and the saline group.

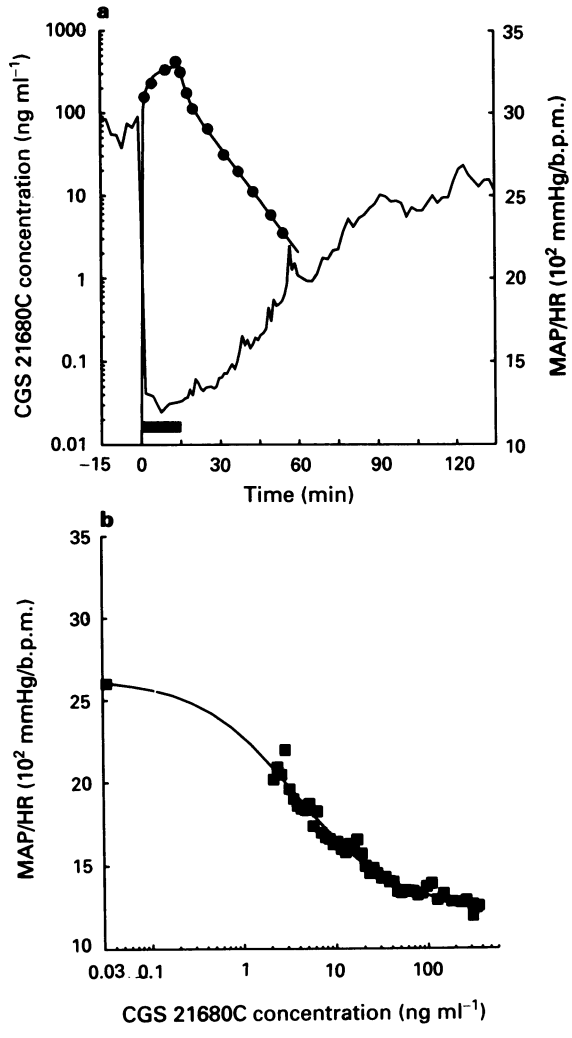


Figure 7 (a) Time course of blood concentration and the ratio of mean arterial pressure over heart rate (MAP/HR) for a rat after an intravenous infusion of 300 $\mu g\ kg^{-1}$ (0.56 $\mu mol\ kg^{-1}$) of CGS 21680C in 15 min. The solid line represents the predicted blood concentration on fitting a bi-exponential function to the concentration-time data (●). (b) Concentration-blood pressure relationship for the same rat. The solid line represents the sigmoidal E_{max} model fitted to the concentration-effect data (■). E_0 was fixed to a value of 26.3×10^{-2} mmHg/b.p.m.

agonists for the adenosine A_1 receptor (Mathôt *et al.*, 1994). It has been shown that estimates for the *in vivo* potency and intrinsic activity could be obtained in individual rats by relating the concentrations in blood to the induced negative chronotropic effect. While the A_1 receptor mediates cardiac depression, activation of the adenosine A_{2a} receptor has been shown to provoke hypotension, mainly through peripheral vasodilatation (Webb *et al.*, 1991).

Following intravenous infusion of the adenosine A_{2a} agonist, CGS 21680C, the time course of drug concentrations in blood was described adequately by a bi-exponential pharmacokinetic function (Figure 1). CGS 21680C is eliminated rapidly with a terminal half-life of 10 min, due to a high blood clearance of 59 ml $min^{-1}\ kg^{-1}$ and a low apparent volume of distribution at steady-state of 390 ml kg^{-1} (Table 1). The clearance of CGS 21680C was found to approximate the liver blood flow. The low apparent volume of distribution could be explained by the hydrophilic nature of the compound. The observed pharmacokinetic parameters were in accordance with previously reported values (Chovan *et al.*, 1992).

Intravenous infusion of CGS 21680C produced a reduction in blood pressure (Figure 2), which corresponded to reported observations for adenosine A_{2a} receptor activation in the rat (Abiru *et al.*, 1991; Webb *et al.*, 1991; Milavec-Krizman *et al.*, 1991). The hypotensive effect of CGS 21680C was reversible and the blood pressures of drug-treated rats were not significantly different from those of vehicle-treated rats after 110 min. The no-drug responses (E_0) were fixed to post-dose values (165–195 min). This approach has the advantage that possible baseline changes, which are caused by diurnal variation, can be taken into account. For each individual rat, correlation of the hypotensive effect to concentrations in blood yielded a sigmoidal relationship (Figure 3b), which was independent of the dose administered (Table 2). The low value for the EC_{50} (12 ng ml^{-1}) demonstrates the high hypotensive potency of CGS 21680C *in vivo*. In the concentration-effect plots, the absence of hysteresis indicates that no equilibrium delay was present between the sampling site and the site of action. In view of this, it is justified to compare the blood

Table 3 Pharmacodynamic parameter estimates for the reduction in the ratio of mean arterial pressure over heart rate (MAP/HR) after administration of different doses of CGS 21680C to conscious normotensive rats

Dose ($\mu g\ kg^{-1}$)	n	E_0 (10^{-2} mmHg/b.p.m.)	E_{max} (10^{-2} mmHg/b.p.m.)	(%)	EC_{50} ($ng\ ml^{-1}$)	n_H	$EC_{50,u}$ ($ng\ ml^{-1}$)
300	7	25 ± 1	−12 ± 1	(−48 ± 3)	8.4 ± 2.1	1.1 ± 0.1	4.1 ± 1.0
1000	6	27 ± 1	−14 ± 1	(−51 ± 2)	4.8 ± 1.1	1.1 ± 0.1	2.3 ± 0.5
3000	6	27 ± 1	−12 ± 2	(−45 ± 5)	8.1 ± 1.3	0.7 ± 0.1**	3.9 ± 0.6

For each rat the reduction in the MAP/HR ratio was related to blood concentrations of CGS 21680C according to the sigmoidal E_{max} model, yielding estimates for the baseline response (E_0), intrinsic activity (E_{max}), potency (EC_{50}), Hill factor (n_H) and potency, based on free concentrations ($EC_{50,u}$). Values are reported as mean ± s.e. Data were analysed statistically by a one-way ANOVA or a Kruskal-Wallis test, if more appropriate.
**Significantly different from 300 and 1000 $\mu g\ kg^{-1}$ ($P<0.01$).

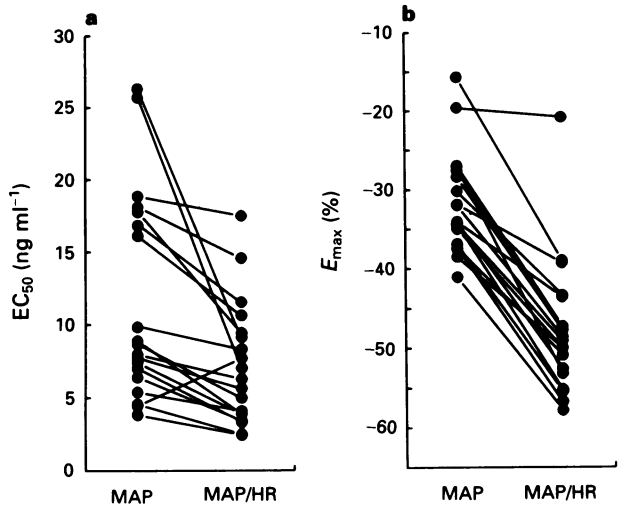


Figure 8 Relationship between the individual EC_{50} (a) and relative E_{max} (b) values after pharmacokinetic-pharmacodynamic modelling of the effects with mean arterial blood pressure (MAP) and the ratio of mean arterial pressure over heart rate (MAP/HR) and as pharmacological endpoint. The corresponding values were 12 ± 2 and 7.2 ± 10 $ng\ ml^{-1}$ ($P<0.01$, paired t test) and −32 ± 2 and −48 ± 2 and % ($P<0.001$, paired t test).

concentration of CGS 21680C, which produces 50% of the maximal fall in blood pressure, with the affinity for the adenosine A_{2a} receptor, as determined in binding studies. The observed value for the $EC_{50,u}$, the EC_{50} based on free CGS 21680C concentrations, was 5.8 $ng\ ml^{-1}$ (11 nM) and correlated well with the A_{2a} affinity (K_i: 19 nM), as has been found in *in vitro* binding studies (Hutchison *et al.*, 1990).

In addition to the hypotensive effect, an increase in heart rate was observed, which could not be related directly to the concentration in blood. This tachycardia, which has been reported to be accompanied by an increase in cardiac output, has been postulated to be partially the result of baroreceptor reflex activation of cardiac sympathetic nerves (Webb *et al.*, 1991). The triggering of the baroreceptor reflex depends both on the level of the mean arterial pressure and on its rate of change (Franchetou *et al.*, 1993). The rapid increase and subsequent decrease in heart rate during the continuing infusion of CGS 21680C may therefore be explained by the rapid initial fall in blood pressure, which causes a transient and additional activation of the baroreceptor reflex. Furthermore, after administration of 3000 $\mu g\ kg^{-1}$ CGS 21680C, the reflex increase of heart rate to a reduction in blood pressure was slightly diminished in comparison with the lower doses (Figure 5). This small decrease in sensitivity could be explained by the presence of higher blood concentrations of CGS 21680C, which, despite the 74 fold selectivity of the compound, could activate the adenosine A_1 receptor and, consequently, induce a reduction in heart rate.

Although the reduction in blood pressure proved to be reversible, heart rate remained elevated after the infusion of CGS 21680C. Therefore, the tachycardiac response to CGS 21680C may not merely be the result of a baroreceptor-mediated reflex increase in heart rate secondary to the decrease in blood pressure. Comparable observations have been made by Webb *et al.* (1991), who have shown that blockade of the β-adrenoceptor produced only a partial reduction of the tachycardic response to CGS 21680C. Furthermore, Jackson *et al.* (1993) have reported recently that the adenosine A_{2a} receptor-mediated hypotensive response to CGS 21680C was blocked by the selective antagonist KF 17837, whereas the tachycardic response was not affected. Nevertheless, the mechanism for the prolonged tachycardia has yet to be addressed specifically.

Cardiovascular homeostatic mechanisms, activated by the primary effect of the compound, are responsible for the development of acute tolerance. The triggering of these mechanisms, which include neural reflexes, endocrine or renal mechanisms, may cause time-dependent discrepancies between drug concentration and cardiovascular effect (Struyker Boudier, 1992; Franchetou *et al.*, 1993). Under physiological conditions activation of the baroreceptor reflex causes arterial pressure to rise to normal level through an increase of cardiac output. However despite the time- and dose-dependent reflex increases of heart rate, no marked differences were found for the concentration-hypotensive effect relationships between the different doses (Table 2). The absence of acute tolerance towards the hypotensive effect of CGS 21680C is also reflected by the plot of the intensity of the effect versus the concentration, which showed no proteresis (Figure 3a).

After administration of CGS 21680C to spontaneously hypertensive rats, peripheral vasodilatation causes a decrease in blood pressure, which is subsequently attenuated by a reflex increase in cardiac output (Webb *et al.*, 1991). Consequently, since vasodilatation appears to be the primary cardiovascular response, total peripheral resistance might be a more sensitive pharmacodynamic end-point for *in vivo* activation of the adenosine A_2 receptor than blood pressure. In the present study, changes in the total peripheral resistance may be reflected by the ratio of mean arterial blood pressure over heart rate (MAP/HR), assuming that no significant change in stroke volume occurs. Webb *et al.* (1991) have reported that stroke volume remained constant after intravenous infusion of CGS 21680C. However, it is uncertain whether these observations can be related to the present experimental conditions, since, in that study, CGS 21680C was administered to spontaneously hypertensive rats with a different rate of infusion. In our experiments, the MAP/HR ratio gradually returned after the infusion of CGS 21680C to levels, which were lower than those of placebo-treated rats. This observation can be explained by the sustained increase in heart rate after the administration. However, the time profile of the blood pressure indicated that adenosine A_{2a} receptor-mediated vasodilatation was absent after 110 min. Consequently, it was decided to eliminate the confounding influence of the prolonged tachycardia on the concentration-MAP/HR relationship by fixation

of the no-drug response to post-dose values. The relationship between blood concentration and the decrease in the MAP/HR ratio was adequately described with the sigmoidal E_{max} model for each individual rat (Figure 7b). The concentration-response relationship was independent of the infused dose, yielding consistent pharmacodynamic parameter estimates (Table 3). Both the potency and the intrinsic activity *in vivo* of the MAP/HR ratio were significantly greater than the corresponding parameters for the hypotensive effect (Figure 8), apparently indicating a greater sensitivity of the MAP/HR ratio for activation of the adenosine A_{2a} receptor *in vivo*. The greater sensitivity of the MAP/HR ratio may imply that the parameter reflects the total peripheral resistance, which has been indicated to be the primary cardiovascular response after infusion of CGS 21680C (Webb *et al.*, 1991). Therefore, the activation of adenosine A_{2a} receptors *in vivo* may be characterized most effectively by relating the blood concentration to the reduction of total peripheral resistance, provided that effects on stroke volume are assessed.

Finally, this study has shown that estimates of the *in vivo* potency and the intrinsic activity of CGS 21680C can be obtained in individual rats by correlating the hypotensive effect to concentrations in blood. The pharmacodynamic response estimates were independent of the dose administered, which demonstrates that the reduction in blood pressure is a relevant pharmacodynamic end-point for activation of the adenosine A_{2a} receptor *in vivo*. The developed pharmacokinetic-pharmacodynamic model offers the possibility of evaluating the potency and intrinsic activity of new adenosine A_{2a} receptor agonists *in vivo*.

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References

ABIRU, T., YAMAGUCHI, T., WATANABE, Y., KOGI, K., AIHARA, K. & MATSUDA, A. (1991). The antihypertensive effect of 2-alkynyladenosines and their selective affinity for adenosine A_2 receptors. *Eur. J. Pharmacol.*, **196**, 69–76.

AKAIKE, H. (1974). A new look at the statistical model identification. *IEEE Trans. Automat. Control*, **AC-19**, 716–723.

CHOVAN, J.P., ZANE, P.A. & GREENBERG, G.E. (1992). Automated-extraction, high-performance liquid chromatographic method and pharmacokinetics in rats of a highly A_2-adenosine agonist, CGS 21680. *J. Chrom. Biomed. Appl.*, **578**, 77–83.

DALY, J.W. (1982). Adenosine receptors. Targets for future drugs. *J. Med. Chem.*, **15**, 61–69.

DINGEMANSE, J., DANHOF, M. & BREIMER, D.D. (1988). Pharmacokinetic-pharmacodynamic modelling of CNS drug effects. An overview. *Pharmacol. Ther.*, **38**, 1–52.

FRANCHETOU, P., STEIMER, J.L., MERDJAN, H., GUERRET, M. & DUBRAY, C. (1993). A mathematical model for cardiovascular drug action: application to intravenous dihydropyridines in healthy volunteers. *J. Pharmacokin. Biopharm.*, **21**, 489–514.

GIBALDI, M. & PERRIER, D. (1982). Multicompartment models. In *Pharmacokinetics*. ed. Gibaldi, M. & Perrier, D. pp. 409–424. Marcel Dekker, Inc.,: New York.

GUYTON, A.C., COLEMAN, T.G. & GRANGER, H.J. (1972). Circulation: overall regulation. *Annu. Rev. Physiol.*, **34**, 13–46.

HAMILTON, H.W., TAYLOR, M.D., STEFFEN, R.P., HALEEN, S.J. & BRUNS, R.F. (1987). Correlation of adenosine receptor affinities and cardiovascular activity. *Life Sci.*, **41**, 2295–2302.

HOLFORD, N.H.G. & SHEINER, L.B. (1981). Understanding of the dose-effect relationship: clinical application of pharmacokinetic-pharmacodynamic models. *Clin. Pharmacokin.*, **6**, 429–453.

HUTCHISON, A.J., WILLIAMS, M., DE JESUS, R., YOKOYAMA, R., OEI, H.H., GHAI, G.R., WEBB, R.L., ZOGANAS, H.C., STONE, G.A. & JARVIS, M.F. (1990). 2-(Arylalkylamino)adenosin-5'-uronamides: a new class of highly selective adenosine A_2 receptor ligands. *J. Med. Chem.*, **33**, 1919–1924.

IRTH, H., CLETON, A., MATHÔT, R.A.A., DANHOF, M., YZERMAN, A.P., TJADEN, U.R. & VAN DER GREEF, J. (1994). Liquid chromatographic determination of the adenosine receptor agonist CGS 21680 in blood using on-line solid-phase extraction on a phenylboronic acid support and fluorescence detection. *J. Chromatogr.*, **658**, 207–212.

JACKSON, E.K., HERZER, W.A. & SUZUKI, F. (1993). KF17837 is an A_2 adenosine receptor antagonist *in vivo*. *J. Pharmacol. Exp. Ther.*, **267**, 1304–1310.

JACOBSON, K.A., VAN GALEN, P.J.M. & WILLIAMS, M. (1992). Adenosine receptors: structure-activity relationships, and therapeutic potential. *J. Med. Chem.*, **35**, 407–422.

KUSACHI, S., THOMPSON, R.D. & OLSSON, R.A. (1983). Ligand selectivity of dog coronary adenosine receptor resembles that of adenylate cyclase stimulatory (R_a) receptors. *J. Pharmacol. Exp. Ther.*, **227**, 316–321.

MATHÔT, R.A.A., VAN SCHAICK, E.A., LANGEMEIJER, M.W.E., SOUDIJN, W., BREIMER, D.D., IJZERMAN, A.P. & DANHOF, M. (1994). Pharmacokinetic-pharmacodynamic relationship of the cardiovascular effects of the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) in the rat. *J. Pharmacol. Exp. Ther.*, **268**, 616–624.

MERTZ, T.E., BRUNS, R.F., GERMAIN, L. & EVANS, D.B. (1984). Cardiovascular and receptor-binding selectivity of adenosine agonists in rats. *Pharmacologist*, **26**, 143.

MILAVEC-KRIZMAN, M., WAGNER, H., KRALJ, A., EVENOU, J.P. & GADIENT, F. (1991). Cardiovascular effects of adenosine derivatives in conscious normotensive rats. *Nucleos. Nucleot.*, **10**, 1127–1129.

OEI, H.H., GHAI, G.R., ZOGANAS, H.C., STONE, G.A., ZIMMERMAN, M.B., FIELD, F.P. & WILLIAMS, M. (1988). Correlation between binding affinities for brain A_1 and A_2 receptors of adenosine agonists and antagonists and their effects on heart rate and coronary vascular tone. *J. Pharmacol. Exp. Ther.*, **247**, 882–888.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.*, **70**, 761–845.

SHIMADA, J., SUZUKI, F., NONAKA, H. & ICHIKAWA, S. (1992). (E)-1,3-Dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthines: potent and selective adenosine A_2 antagonists. *J. Med. Chem.*, **35**, 2342–2345.

STRUYKER BOUDIER, H.A.J. (1992). Kinetics and dynamics of cardiovascular drug action: Introduction. In *The in vivo Study of Drug Action*. ed. van Boxtel, C.J., Holford, N.H.G. & Danhof, M. pp. 217–229. Elseviers Science Publishers: Amsterdam.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

WEBB, R.L., BARCLAY, B.W. & GRAYBILL, S.C. (1991). Cardiovascular effects of adenosine A_2 agonists in the conscious spontaneously hypertensive rat: a comparative study of three structurally distinct ligands. *J. Pharmacol. Exp. Ther.*, **259**, 1203–1212.

WEBB, R.L., MCNEAL, R.B., BARCLAY, B.W. & YASAY, G.D. (1990). Hemodynamic effects of adenosine agonists in the conscious spontaneously hypertensive rat. *J. Pharmacol. Exp. Ther.*, **254**, 1090–1099.

YAMAOKA, K., NAKAGAWA, T. & UNO, T. (1978). Application of Aikaike's information criterion (AIC) in the evaluation of linear pharmacokinetics. *J. Pharmacokin. Biopharm.*, **6**, 165–175.

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Characterization of muscarinic receptors mediating relaxation and contraction in the rat iris dilator muscle

Yutaka Masuda, Natsuki S. Yamahara, Masako Tanaka, Shinwon Ryang, Tomoyuki Kawai, Yuji Imaizumi & [1]Minoru Watanabe

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, 467 Nagoya, Japan

1 The characteristics of muscarinic receptors mediating relaxation and/or contraction in the rat iris dilator muscle were examined.

2 Relaxation was induced in a dilator muscle by application of acetylcholine (ACh) at low doses (3 μM or less) and contraction was induced by high doses. Methacholine and carbachol also showed biphasic effects similar to those of ACh; in contrast, bethanechol, arecoline, pilocarpine and McN-A-343 induced mainly relaxation but no substantial contraction.

3 After parasympathetic denervation by ciliary ganglionectomy, the relaxant response to muscarinic agonists disappeared upon nerve stimulation. Application of McN-A-343 and pilocarpine induced only small contractions in denervated dilator muscles, indicating that these are partial agonists for contraction.

4 pA_2 values of pirenzepine, methoctramine, AF-DX 116, himbacine, and 4-DAMP for antagonism to pilocarpine-induced relaxation in normal dilator muscles and those for antagonism to ACh-induced contraction in denervated dilator muscles were determined. The pA_2 values for antagonism to relaxation of all these antagonists were most similar to those for M_3-type muscarinic receptors.

5 Although pA_2 values for contraction of these antagonists, except for methoctramine, were very close to those for relaxation, contraction was not significantly antagonized by methoctramine. Contraction might be mediated by M_3-like receptors which have a very low affinity for methoctramine.

6 In conclusion, ACh-induced biphasic responses in rat iris dilator muscles were clearly distinguished from each other by specific muscarinic agonists and parasympathetic denervation, whereas muscarinic receptors could not be subclassified according to the pA_2 values of 5 specific antagonists only.

Keywords: Iris dilator; iris sphincter; muscarinic receptor; parasympathetic nerve; ciliary ganglion; iris smooth muscle (rat)

Introduction

It is generally recognized that iris dilator smooth muscle is predominantly innervated by adrenergic nerves (Ehinger & Falck, 1966). The functional significance of cholinergic innervation was originally suggested in *in vitro* studies in feline dilator muscle after sympathetic denervation (Ehinger *et al.*, 1968) and was clearly shown *in vitro* in normal dilator muscles of the rat (Narita & Watanabe, 1982a), ox (Suzuki *et al.*, 1983), and dog (Yoshitomi & Ito, 1986). It is important to note that this is also true of human dilator muscles (Yoshitomi *et al.*, 1985).

Iris dilator muscles of these species have inherent tone and show a biphasic response to transmural nerve stimulation: a fast contraction followed by a relaxation. Sympathetic innervation contributes to fast contraction in these species and also to relaxation at least in part in the feline, canine, and bovine but not in rat or human dilator muscles. Atropine-sensitive biphasic responses to nerve stimulation have been observed in dilator muscles in all these species after application of α- and β-adrenoceptor blockers; in contrast, the response is most clearly demonstrated in the rat and human even in the absence of adrenoceptor blockade. Correspondingly, relaxation and contraction are induced by exogenously applied acetylcholine (ACh) at low and high concentrations, respectively, in rat dilator muscle (Narita & Watanabe, 1982a; Hasegawa *et al.*, 1988). It has also been shown that the response to ACh is not affected by adrenoceptor blockade (Narita & Watanabe, 1982a) or by sympathetic denervation (Shibata *et al.*, 1989).

The mechanisms of ACh-induced relaxation in dilator muscle, however, still remain to be determined. It is not even clear whether relaxation is due to release of a relaxing factor from somewhere in the dilator region or is directly mediated by muscarinic receptor activation in smooth muscle cells. If release of a relaxing factor is involved, it is not noradrenaline, nitric oxide, or cyclo-oxygenase products of arachidonic acid (Ryang *et al.*, 1990). The receptor subtypes mediating muscarinic relaxation and contraction have not been clarified, since both responses are susceptible to various muscarinic antagonists with similar pA_2 values. To determine the mediating receptor subtypes and subsequent intracellular signal transduction, it is necessary to separate the relaxation from contraction by some means.

The present study was undertaken: (1) to distinguish the 2 responses from each other using specific agonists and (2) to produce parasympathetic denervation by ciliary ganglionectomy in order to analyse receptor subtypes. It has been reported that parasympathectomy abolishes relaxation induced not only by nerve stimulation but also by exogenously applied ACh; in contrast, the contractile response to ACh remains almost unchanged (Hasegawa *et al.*, 1988). Preliminary results of the present study were reported at the 64th Annual Meeting of the Japanese Pharmacological Society (Suzuki *et al.*, 1991).

Methods

Preparation

Both eyes were dissected from male Wistar rats (350 to 500 g) which had been stunned by a blow to the head and

[1] Author for correspondence.

killed by exsanguination. Strip preparations of iris dilator and sphincter muscle, and tension measurements were carried out as described in detail previously (Narita & Watanabe, 1981; 1982a). Briefly, about one sixth of the whole iris (about 3 mm in width at the outer edge of the iris) was dissected from the isolated eye for the dilator preparation. Sphincter strips were prepared by cutting the dilator region. Whole sphincters were dissected for the ring preparations.

The dilator muscle strip was mounted horizontally in a 4 ml organ bath and was perfused with bathing solution. The pupillary end of the preparation was pinned on a sheet of silicone rubber which formed the bottom of the bath. The other end was fixed to an isometric transducer lever. The ring preparation of the rat sphincter muscle was mounted in a similar manner, except that one side of the sphincter was pinned. At the start of the experiment, resting tone was adjusted to 10 mg and 50 mg in the dilator and sphincter muscles, respectively.

Denervation

Ciliary ganglionectomy was performed according to the method of Marmfors & Nilsson (1964) as modified by Hasegawa *et al.* (1987) and Hashimoto *et al.* (1993). Since the changes in drug-sensitivity reached steady levels about 2 to 3 days after ciliary ganglionectomy, the denervated dilator muscles were used in experiments 1 week after the operation (Hasegawa *et al.*, 1988). The success of the operation was confirmed in each preparation by a lack of relaxation response to nerve stimulation and to exogenously applied 3 μM ACh (Hasegawa *et al.*, 1988). Since sham operation did not affect the sensitivity to nerve stimulation (NS) or drugs (Hashimoto *et al.*, 1993), it was not carried out in the present study.

Recording

Isometric tension was measured by force displacement transducers which were made in the laboratory for small tension measurements. The transducers used to measure tension in sphincter muscles were suitable for measuring exact tension in a range of 0.5 mg to 150 mg. Those for dilator muscles were capable of measuring exact tension in a range of 0.1 to 50 mg. The electrical signal was amplified by an amplifier (U350, Unipulse), and recorded on a pen-recorder (FBR252A, TOA Electronics Ltd., Japan).

NS was performed to confirm the success of parasympathetic denervation by field stimulation via a pair of platinum electrodes which were placed at either side of the strip. A train of 100 pulses (0.1 or 0.2 ms in duration in dilator or sphincter, respectively, 20 Hz, and supramaximal voltage) was applied once every 2 min. The stimulator used was made in the laboratory and had a high output current up to 250 mA.

Solutions

The bathing solution was a modified Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 14.0. Solutions were bubbled with 95% O_2 and 5% CO_2 and kept at 37°C and pH 7.4.

Drugs

Acetylcholine chloride (ACh), pilocarpine hydrochloride, and papaverine hydrochloride were obtained from Wako Pure Chemical Industry (Japan). Carbamylcholine chloride and methacholine chloride were from Tokyo Kasei Ltd. (Japan). Arecoline hydrochloride and bethanechol chloride were from Nakarai Chemicals Ltd. (Japan) and Yoshitomi (Japan), respectively. McN-A-343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride), methoctramine and 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide) were from Research Biochemicals Inc. (U.S.A.). Pirenzepine was from Sigma (U.S.A.). AF-DX 116 ((±)-[11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-[pyrido(2,3-b)(1,4)benzodiazepine-6-one]) and himbacine were gifts from Boehringer Ingelheim, Japan, and from Professor W.C. Taylor, Dept. of Organic Chem., Sydney University (Australia), respectively.

Statistics

Results were expressed as mean ± s.e. Statistical significance was determined according to Student's *t* test (*$P<0.05$, **0.01 or ***0.001). When data were obtained from denervated and control (contralateral) eyes from the same rat, paired *t* tests were used to detect significant differences. pA_2

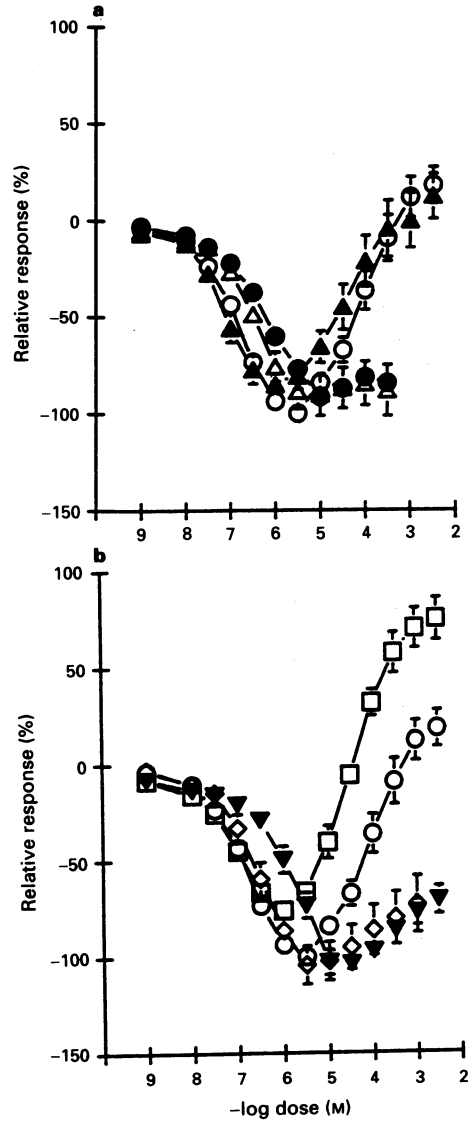


Figure 1 Dose-response relationships for 7 muscarinic agonists in the rat iris dilator muscle. In each preparation, the response to 3 μM acetylcholine (ACh) which gave the maximum relaxation was observed first and then the relationship for one of the agonists was determined. Abscissae: negative logarithms of the molar concentrations of agonists. Ordinates: relative magnitude of muscle relaxation and contraction. The resting tone and the maximum relaxation induced by 3 μM ACh were taken as 0 and −100%, respectively. Symbols show mean values with s.e.mean. Number of experiments is 5 for each agonist. (a) ACh (○), carbachol (▲), pilocarpine (●) and McN-A-343 (△). (b) ACh (○), methacholine (□), arecoline (◇), bethanechol (▼).

values of muscarinic antagonists for receptors were obtained using a Schild plot (Arunlakshana & Schild, 1959).

Results

Effects of muscarinic agonists

Rat iris dilator muscles have an inherent tone of approximately 10 mg (Narita & Watanabe, 1982a,b). When ACh at doses of 0.01 to 3.0 μM was applied cumulatively, dilator muscle relaxed and contracted at doses of 10 μM to 3 mM. Figure 1 shows dose-response relationships of 7 muscarinic agonists. The magnitude of maximum relaxation induced by 3 μM ACh was taken as −100% in each preparation. Carbachol showed a biphasic effect similar to that of ACh in almost the same concentration-range (Figure 1a). It is notable that the responses to pilocarpine and McN-A-343 were only relaxation and were not converted to contraction when higher concentrations of these 2 agonists were applied. Bethanechol and arecoline also did not induce significant contractions (Figure 1b). On the other hand, methacholine contracted the muscle more effectively than ACh. The maximum level of methacholine-induced contraction is much higher than the resting tone. It is clear that some muscarinic agonists such as McN-A-343 and pilocarpine selectively induce relaxation in rat iris dilator.

The effects of muscarinic agonists were, then, examined in dilator muscles dissected 1 week after ciliary ganglionectomy (Figure 2). All agonists examined induced only contraction as has been reported for ACh (Hasegawa *et al.*, 1988). Responses to 1 mM ACh were taken as 100%. Methacholine and carbachol showed dose-response relationships similar to that of ACh. The maximum responses to pilocarpine and McN-A-343 were markedly smaller than those to ACh, indicating that these are partial agonists for contraction.

pD_2 values and maximum relaxation and contraction in normal and denervated preparations shown in Figures 1 and 2 are summarized in Table 1. The increase in tension from maximum relaxation was taken as contraction. pD_2s were determined as apparent pD_2s, assuming the maximum magnitude of relaxation or contraction observed in each preparation was 100%, respectively. The overlap of relaxation and contraction at the dose where the response was changed from relaxation to contraction was ignored to simplify the analysis. There was a 10 fold increase in affinity for ACh, carbachol, and methacholine in denervated muscles compared to control values.

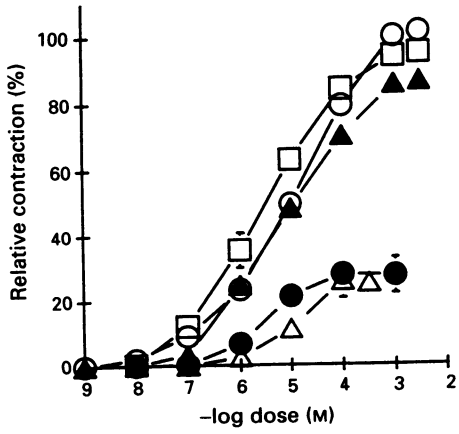


Figure 2 Dose-response relationships for 5 muscarinic agonists in iris dilator muscle dissected 1 week after ciliary ganglionectomy. The response to 1 mM acetylcholine (ACh) was taken as 100% in each preparation. Number of experiments was 10 for ACh and 5 for each of the other agonists. ACh (○), methacholine (□), carbachol (▲), pilocarpine (●), McN-A-343 (△).

Table 1 pD_2 values for rat iris dilator muscle (control and denervated)

	(a) Control					(b) Denervated				
	Relaxation			Contraction		Contraction				
Compound	n	pD_2	*Max response*[1] (%)	pD_2	*Max response*[1] (%)	n	pD_2	*Max response*[2] (%)		
Acetylcholine	(5)	7.01 ± 0.10	102.0 ± 7.5	4.08 ± 0.01	119.6 ± 11.3	(10)	5.05 ± 0.01	101.4 ± 0.24		
Methacholine	(5)	7.33 ± 0.12	76.1 ± 2.6*	4.43 ± 0.02	151.8 ± 8.5	(5)	5.59 ± 0.02	95.0 ± 2.5		
Carbachol	(5)	7.31 ± 0.16	85.0 ± 6.0	4.27 ± 0.16	108.4 ± 12.7	(5)	5.20 ± 0.12	86.4 ± 3.4*		
Bethanechol	(5)	5.96 ± 0.24	102.8 ± 6.4	3.60 ± 0.08	34.3 ± 6.4***		ND	ND		
Arecoline	(5)	6.79 ± 0.13	104.2 ± 9.8	4.08 ± 0.06	31.6 ± 6.7***		ND	ND		
Pilocarpine	(5)	6.55 ± 0.13	84.9 ± 6.9		No response	(5)	ND	27.7 ± 4.6***		
McN-A-343	(5)	6.73 ± 0.12	90.4 ± 10.7		No response	(5)	ND	25.1 ± 4.3***		

n: Number of experiments. Values are means ± s.e.means.
ND: Not determined.
[1]The magnitude of 3 μM ACh-induced relaxation which was obtained before determining the concentration-response relationship was taken as 100% in each preparation.
[2]The magnitude of contraction induced by 1 mM ACh which was obtained before determining the concentration-response relationship was taken as 100% in each preparation.
*$P<0.05$ or ***$P<0.001$ vs ACh in each column.

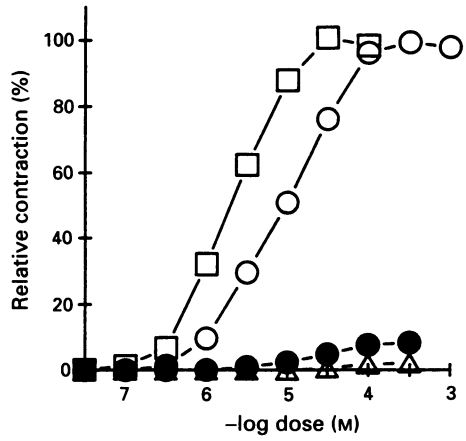


Figure 3 Dose-response relationships for 4 muscarinic agonists in rat iris sphincter muscles. The maximum response to acetylcholine (ACh) was taken as 100% in each preparation. Number of experiments was 5. Symbols indicate the agonists as follows: ACh (○), methacholine (□), pilocarpine (●) and McN-A-343 (△).

The effects of some muscarinic agonists were examined in rat iris sphincter muscles (not denervated). Application of ACh and methacholine induced only contraction. As in dilator muscles, application of pilocarpine or McN-A-343 did not induce substantial contraction. Figure 3 shows dose-response relationships for these 4 agonists. The maximum response to ACh was taken as 100% in each preparation. The pD_2 values for ACh and methacholine were 5.07 ± 0.03 and 5.68 ± 0.02 ($n = 5$ for each), respectively. The pD_2s for pilocarpine and McN-A-343 could not be determined.

Effects of muscarinic antagonists

Effects of muscarinic antagonists on pilocarpine-induced relaxation and ACh-induced contraction were examined in normal and parasympathectomized dilator muscles, respectively. Pirenzepine was used as a selective M_1 blocker. Methoctramine and AF-DX 116 are considered to be relatively specific antagonists, at M_2 receptors. 4-DAMP has high pA_2 values for M_1 and M_3 receptors. Himbacine has

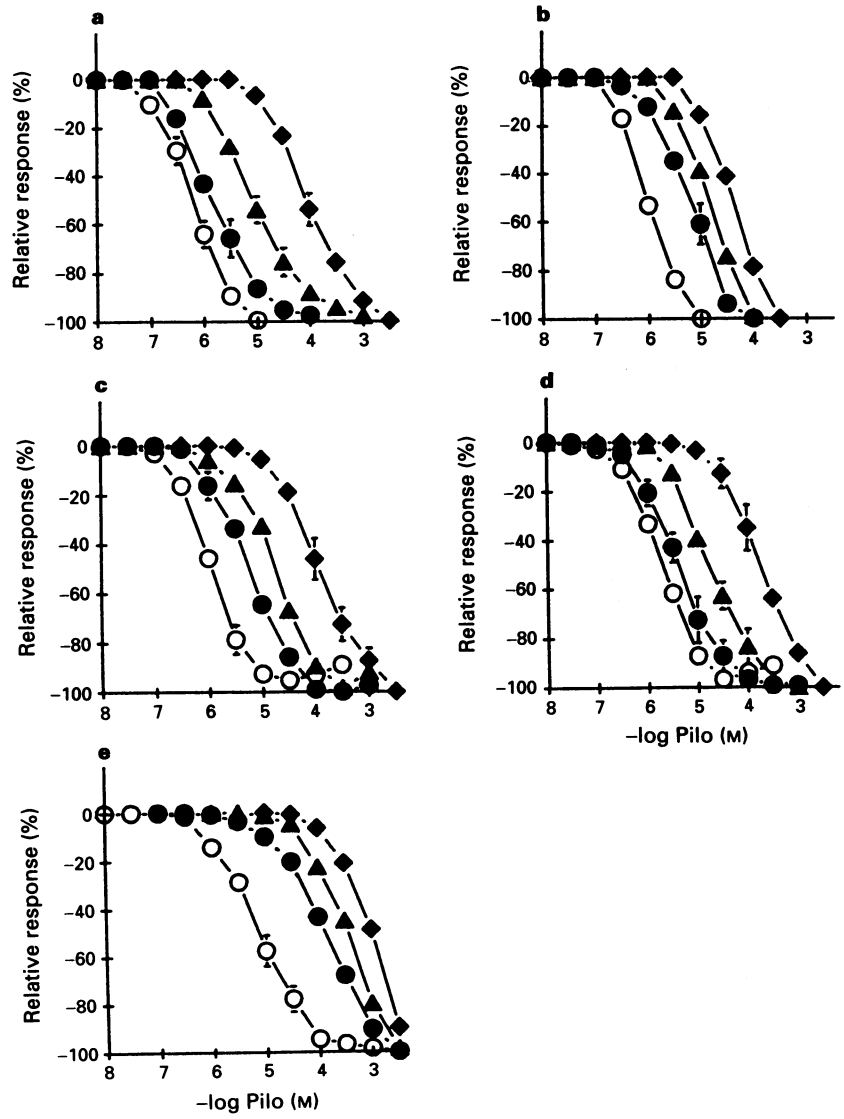


Figure 4 Dose-response relationships for pilocarpine(pilo)-induced relaxation of rat iris dilator muscles in the absence or presence of muscarinic antagonists. The maximum relaxation induced by pilocarpine was taken as −100% in each preparation. Antagonist was added about 10 min prior to the second cumulative application of pilocarpine. (a) Effects of pirenzepine: ○, ●, ▲, and ◆ indicate control ($n = 5$), the presence of 0.1 μM ($n = 5$), 1 μM ($n = 5$), and 10 μM pirenzepine ($n = 5$), respectively. (b) Effects of AF-DX 116: ○, ●, ▲, and ◆ indicate control ($n = 4$), the presence of 1 μM ($n = 4$), 3 μM ($n = 4$), and 10 μM AF-DX 116 ($n = 4$), respectively. (c) Effects of methoctramine: ○, ●, ▲, and ◆ indicate control ($n = 13$), the presence of 1 μM ($n = 4$), 3 μM ($n = 4$), and 10 μM methoctramine ($n = 5$), respectively. (d) Effects of 4-DAMP: ○, ●, ▲, and ◆ indicate control ($n = 12$), the presence of 1 nM ($n = 4$), 10 nM ($n = 4$), and 100 nM 4-DAMP ($n = 4$), respectively. (e) Effects of himbacine: ○, ●, ▲, and ◆ indicate control ($n = 7$), the presence of 1 μM, 3 μM and 10 μM himbacine ($n = 7$).

higher selectivity for M_4 than M_2. When these antagonists were applied, the inherent tone of the normal dilator muscles tended to increase by about 10% at the maximum. The dose-response relationship for pilocarpine in the presence of an antagonist was determined assuming the tone after addition of antagonist to be zero. The maximum relaxation by pilocarpine which was obtained in each preparation was taken as 100%.

Figure 4 illustrates the dose-response relationships for pilocarpine-induced relaxation in the absence or presence of these antagonists (a: pirenzepine, b: AF-DX 116, c: methoctramine, d: 4-DAMP, e: himbacine) in normal dilator muscles. Any antagonist used here shifted the relationship to the right in a dose-dependent manner. pA_2s were determined from Schild plots of the data for 5 antagonists in Figure 4a, b, c, d and e and are shown in Table 2. The slopes of these plots were not significantly different from unity ($P > 0.05$).

Figure 5 shows the effects of these antagonists on the relationships for ACh-induced contraction in parasympathectomized dilator muscles. It is surprising that addition of 3 or 10 μM methoctramine did not shift the relationship significantly (Figure 5c), whereas pirenzepine, AF-DX 116, 4-DAMP, and himbacine did in a dose-dependent manner. (Figure 5a, b, d and e). pA_2s determined from Schild plots for pirenzepine, AF-DX 116, 4-DAMP, and himbacine are shown in Table 2. The slopes of these plots were not significantly different from unity ($P > 0.05$).

In Table 2, pA_2 values for these antagonists determined in other studies in various preparations and shown in reviews by Hulme *et al.* (1990) and by Caulfield (1993) and in an article by Lambrecht *et al.* (1989) are also presented for comparison. It is clear that the pA_2 values for these antagonists against pilocarpine-induced relaxation are close to those for M_3 antagonism in other studies. Moreover, the pA_2s against ACh-induced contraction are also close to those for M_3 antagonism; in contrast, methoctramine did not affect contraction. It seems clear that neither relaxation nor contraction is mediated by M_2 receptors.

Discussion

The present study uses muscarinic agonists and parasympathetic denervation to demonstrate that a biphasic response

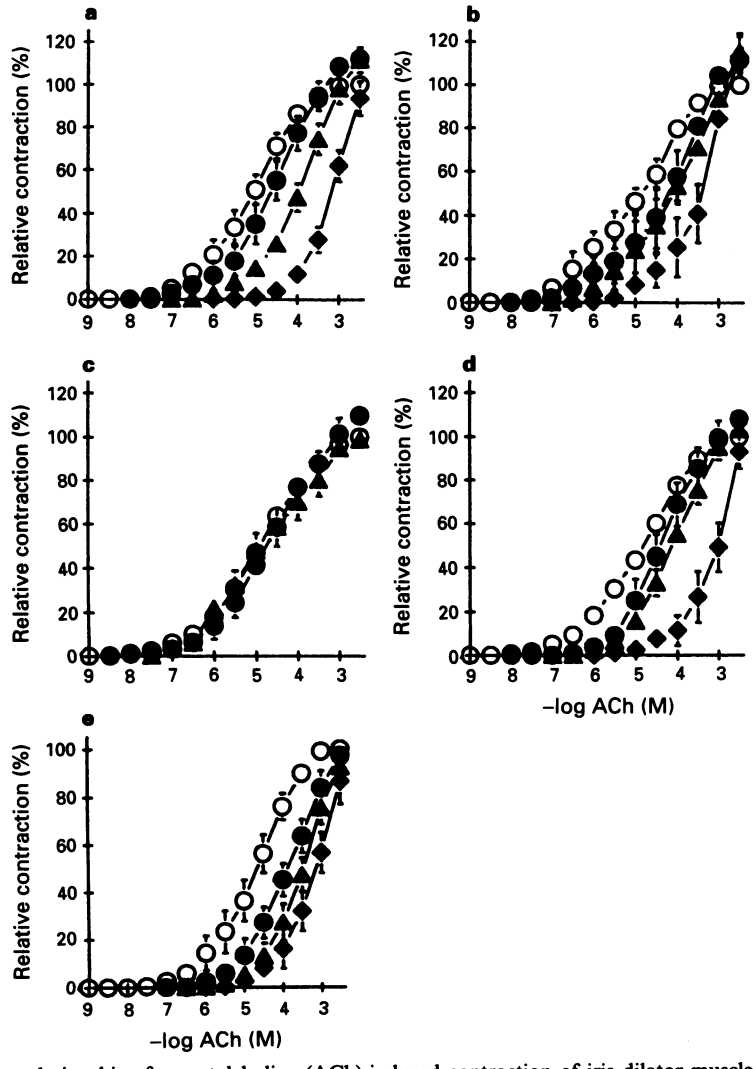


Figure 5 Dose-response relationships for acetylcholine (ACh)-induced contraction of iris dilator muscles from ciliary ganglionectomized rats in the absence or presence of muscarinic antagonists. The maximum contraction induced by ACh in the control was taken as 100% in each preparation. (a) Effects of pirenzepine: ○, ●, ▲, and ◆ indicate control ($n = 5$), the presence of 0.1 μM ($n = 5$), 1 μM ($n = 5$), and 10 μM pirenzepine ($n = 5$), respectively. (b) Effects of AF-DX 116: ○, ●, ▲, and ◆ indicate control ($n = 3$), the presence of 1 μM ($n = 3$), 3 μM ($n = 3$), and 10 μM AF-DX 116 ($n = 3$), respectively. (c) Effects of methoctramine: ○, ●, and ▲ indicate control ($n = 7$), the presence of 3 μM ($n = 4$) and 10 μM methoctramine ($n = 3$), respectively. (d) Effects of 4-DAMP: ○, ●, ▲, and ◆ indicate control ($n = 11$), the presence of 1 nM ($n = 4$), 10 nM ($n = 5$), and 100 nM 4-DAMP ($n = 4$), respectively. (e) Effects of himbacine: ○, ●, ▲, and ◆ indicate control ($n = 4$), the presence of 1 μM ($n = 4$), 3 μM ($n = 4$) and 10 μM ($n = 4$) himbacine.

Table 2 pA_2 values of muscarinic antagonists in the rat iris dilator

	Relaxation (Pilo)	*Contraction denervated* (ACh)	M_1	M_2	M_3	M_4
Pirenzepine	7.22 ± 0.12 (1.00 ± 0.14)	7.21 ± 0.24 (0.86 ± 0.25)	~8.1	~6.5	~6.8	~8.0
AF-DX 116	6.73 ± 0.10 (0.91 ± 0.11)	6.48 ± 0.30 (1.00 ± 0.68)	~6.9	~7.2	~6.4	~7.0
Methoctramine	6.39 ± 0.15 (1.25 ± 0.41)	no effect	~6.9	~7.8	~6.2	~7.6
Himbacine	6.94 ± 0.12 (0.98 ± 0.24)	6.94 ± 0.12 (0.83 ± 0.21)	~7.2	~8.2	~7.3	~8.7
4-DAMP	8.97 ± 0.18 (1.00 ± 0.21)	8.92 ± 0.16 (0.97 ± 0.19)	~9.1	~7.9	~9.0	~9.4

pA_2 values were obtained from Schild plots shown in Figure 4 (relaxation) and 5 (contraction). Numbers in parentheses are means of slope ± the 95% confidence interval of the slope.
M_1, M_2, M_3: pA_2 values are from Hulme *et al.* (1990) except for the value for M_1-methoctramine which is from Lambrecht *et al.* (1989) and M_1-himbacine which is from Caulfield (1993).
M_4: pA_2 values are from Caulfield (1993).

to ACh in rat iris dilator muscle can be separated into 2 monophasic responses, relaxation and contraction. Although ACh, methacholine, and carbachol showed similar large biphasic responses in normal dilator muscles, arecoline and bethanechol induced large relaxation and small contraction responses. Moreover, pilocarpine and McN-A-343 induced only relaxation in normal dilator muscles. In parasympathecomized dilator muscles, the relaxant response to exogenously applied ACh disappeared and contraction remained almost unchanged (Hasegawa *et al.*, 1988). Since contractions induced by pilocarpine and McN-A-343 in denervated muscles were about one fourth of that induced by ACh, these are full agonists as relaxants but weak partial agonists for contraction.

On the other hand, 5 specific muscarinic antagonists could not distinguish between the receptors mediating these 2 responses. It has been reported that pirenzepine binds M_1 and M_4 receptors with high affinity (pA_2 values of 8.1 and 8.0, respectively). The pA_2 values of pirenzepine for antagonism to pilocarpine-induced relaxation and ACh-induced contraction in normal and denervated dilator muscles, respectively, were both about 7.2, indicating that these responses may not be mediated by M_1 or M_4 receptors. The pA_2 values of himbacine and 4-DAMP for antagonism to both responses were apparently smaller than those for M_4 receptor antagonism. In the present study, the pA_2 values of 5 antagonists are apparently different from those reported for M_2. In conclusion, the pA_2 values of 5 antagonists to relaxation may be closest to those for M_3. The pA_2 values of pirenzepine, AF-DX 116, himbacine, and 4-DAMP for antagonism to ACh-induced contraction in denervated dilator muscles were very close to those for pilocarpine-induced relaxation in normal dilator muscles (Table 2). Therefore, ACh-induced contraction might also be mediated by activation of M_3-like receptors. It is, however, notable that contraction was not significantly antagonized by methoctramine.

Although 5 different types of DNA sequences encoding muscarinic receptors (m1-m5) have been determined, 4 subtypes of functional muscarinic receptors (M_1, M_2, M_3, and M_4) have been pharmacologically identified (see Buckley, 1990; Hulme *et al.*, 1990; Caulfield, 1993). The m1, m2, and m3 receptors cloned from c-DNA correspond most closely to the pharmacologically defined M_1, M_2, and M_3 receptors respectively. Activation of M_1, M_3, and m5 receptors stimulates phospholipase C and enhances inositolphosphates metabolism (PI turnover) in various types of cells (see Hulme *et al.*, 1990; Caulfield, 1993). The produced IP_3 releases Ca^{2+} from intracellular storage sites and consequently elicits many kinds of biological responses such as secretion in glandular cells and contraction in smooth muscle cells (see Abdel-Latif, 1986).

The muscarinic receptor subtype predominant in iris sphincter muscles of the guinea-pig (Bognar *et al.*, 1990) and the rabbit (Fuder *et al.*, 1989) has been pharmacologically characterized as the M_3 type by use of antagonists, whereas the presynaptic muscarinic receptors involved in autoinhibition of ACh release from nerve endings are the M_2 type (Bognar *et al.*, 1989). The finding that pilocarpine and McN-A-343, which do not activate phospholipase C (Leiber *et al.*, 1990; Eglen *et al.*, 1993), did not elicit contraction in sphincter muscles or in dilator muscles suggests that the receptor subtype mediating contraction may be common in dilator and sphincter muscles of the rat. Moreover, m-RNA encoding m3 receptors have been predominantly found in bovine iris sphincter and ciliary processes (Honkanen *et al.*, 1990). Minor amounts of m2 receptor m-RNA have also been detected in iris sphincter and ciliary processes and tracer amounts of m4 receptor m-RNA have been found in ciliary processes. These facts may indicate that the contraction induced by muscarinic agonists in rat iris dilator muscles is mediated by activation of the M_3 receptor subtype and formation of IP_3 as shown in sphincter muscles of other species (see Abdel-Latif, 1986; Honkanen & Abdel-Latif, 1989).

Muscarinic relaxation in iris dilator muscles, which may have important physiological functions *in situ* as has been shown clearly *in vitro* in the human (Yoshitomi *et al.*, 1985) as well as in the rat (Narita & Watanabe, 1982a), is a unique and somewhat mysterious response. The mechanism underlying the inherent tone is also unknown. Relaxation induced by a muscarinic agonist is abolished in dilator muscles denervated by parasympathectomy but is not changed by sympathectomy (Hasegawa *et al.*, 1988; Shibata *et al.*, 1989). Moreover, application of a muscarinic antagonist to normal dilator muscle slightly increases the tone as mentioned in the Results. These findings may suggest that spontaneous ACh release from parasympathetic nerve endings partly relaxes the muscle and that muscarinic receptors involved in relaxation are not located at the presynaptic region of sympathetic or parasympathetic nerve endings.

Interestingly, relaxation similar to that induced by ACh is observed by application of high K^+ solution in the presence of atropine, phentolamine, propranolol, tetrodotoxin, and nifedipine (Ryang *et al.*, 1990). Therefore, it is possible that a relaxing factor is released from certain cells in the iris region that affects dilator smooth muscle cells (Ryang *et al.*, 1990) such as has been shown in vascular smooth muscle endothelium (Furchgott & Zawadzki, 1980). The increase in

intracellular Ca^{2+} concentration via production of IP_3 by M_3 receptor activation or depolarization by high K^+ might result in the release of a relaxing factor somewhere in the tissue. The factors, if any, are neither nitric oxide (NO) nor arachidonic acid metabolites (Ryang *et al.*, 1990).

An alternative possibility is that both relaxation and contraction are induced by activation of M_3 type muscarinic receptors in dilator smooth muscle cells. In the rat atria, arecoline induces biphasic responses, i.e. a depression of basal inotropy at low doses and positive inotropic effect at higher doses (Kenakin & Boselli, 1991). Both responses are considered to be mediated by activation of the M_2 receptor subtype. It has been assumed that a single receptor subtype may couple with 2 distinctive GTP binding proteins (G proteins) and consequently operate 2 signal transduction pathways in cells (Kenakin, 1989). Antagonists may simply bind to the receptors but agonists can dose-dependently select separate effector G proteins and corresponding signal transductions. This hypothesis is an attractive one for explaining the present results. It may be possible that M_3 receptor stimulation at a high affinity site causes relaxation via activation of pertussis toxin (PTX)-sensitive G protein (Yamahara *et al.*, 1995); at a low affinity site it results in contraction via activation of PTX-insensitive G protein. Stimulation of the receptor by pilocarpine or McN-A-343 may mainly couple with the former G protein. This function of the PTX-sensitive G protein may possibly be maintained by parasympathetic innervation.

A simpler possibility, that the antagonists used in the present study are not adequate to distinguish 2 receptor subtypes which mediate the 2 responses, respectively, cannot be discarded. Actually, muscarinic contraction in rabbit iris sphincter was reported as mediated by smooth muscle type M_2 receptors when M_3 antagonists had not been developed (Akhtar *et al.*, 1987; Honkanen & Abdel-Latif, 1988a,b). More recent results using several new muscarinic antagonists have suggested that the receptor is different from M_1, M_2, M_3, and M_4 subtypes (Bognar *et al.*, 1992). These results may indicate the serious limitation of the muscarinic receptor subclassification in iris muscles using these selective antagonists.

In conclusion, a biphasic response to muscarinic stimulation in the rat iris dilator muscle was distinguished into 2 monophasic responses by specific agonists and parasympathetic denervation but not by pirenzepine, AF-DX 116, methoctramine, himbacine, or 4-DAMP.

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References

ABDEL-LATIF, A.A. (1986). Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol. Rev.*, **35**, 227–272.

AKHTAR, R.A., HONKANEN, R.E., HOWE, P.H. & ABDEL-LATIF, A.A. (1987). M2 muscarinic receptor subtype is associated with inositol trisphosphate accumulation, myosin light chain phosphorylation and contraction in sphincter smooth muscle of rabbit iris. *J. Pharmacol. Exp. Ther.*, **243**, 624–632.

ARUNLAKSHANA, O. & SCHILD, H. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BOGNAR, I.T., ALTES, U., BEINHAUSER, C., KESSLER, I. & FUDER, H. (1992). A muscarinic receptor different from the M1, M2, M3, M4 subtypes mediates the contraction of the rabbit iris sphincter. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 611–618.

BOGNAR, I.T., BAUMANN, B., DAMMANN, F., KNÖLL, B., MEINCKE, M., PALLAS, S. & FUDER, H. (1989). M_2 muscarinic receptors on the iris sphincter muscle differ from those on iris noradrenergic nerves. *Eur. J. Pharmacol.*, **163**, 263–274.

BOGNAR, I.T., WESNER, M.T. & FUDER, H. (1990). Muscarine receptor types mediating autoinhibition of acetylcholine release and sphincter contraction in the guinea-pig iris. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 22–29.

BUCKLEY, N.L. (1990). Molecular pharmacology of cloned muscarinic receptors. In *Transmembrane Signalling, Intracellular Messengers and Implications for Drug Development*. ed. Nahorski, S.R. pp. 11–30, New York: John Wiley & Sons Ltd.

CAULFIELD, M.P. (1993). Muscarinic receptors-characterization, coupling and function. *Pharmacol. Ther.*, **58**, 319–379.

EGLEN, R.M., SHARIF, N.A. & TO, Z.P. (1993). Muscarinic M3 receptors mediate total inositol phosphate accumulation in murine HSDM1C1 fibro sarcoma cells. *Eur. J. Pharmacol.*, **244**, 49–55.

EHINGER, B. & FALCK, B. (1966). Concomitant adrenergic and parasympathetic fibers in rat iris. *Acta Physiol. Scand.*, **67**, 201–207.

EHINGER, B., FALCK, B. & PERSSON, H. (1968). Function of cholinergic nerve fibers in the cat iris dilator. *Acta Physiol. Scand.*, **72**, 139–147.

FUDER, H., SCHÖPF, J., UNCKELL, J., WESNER, M.TH., MELCHIORRE, C., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1989). Different muscarinic receptors mediate the prejunctional inhibition of [^{3}H]-noradrenaline release in rat or guinea-pig iris and the contraction of the rabbit iris sphincter muscle. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 597–604.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373.

HASEGAWA, N., IMAIZUMI, Y. & WATANABE, M. (1987). Parasympathetic denervation supersensitivity in the rat iris sphincter muscle: an in vitro study. *Jpn. J. Pharmacol.*, **43**, 143–151.

HASEGAWA, N., IMAIZUMI, Y. & WATANABE, M. (1988). Parasympathetic denervation abolishes acetylcholine induced relaxation in the rat iris sphincter. *Eur. J. Pharmacol.*, **156**, 291–294.

HASHIMOTO, Y., HASEGAWA, N., SUZUKI, N., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1993). Effects of ciliary ganglionectomy on contractile responses in the dilator muscle of the rat iris. *Exp. Eye Res.*, **56**, 135–141.

HONKANEN, R.E. & ABDEL-LATIF, A.A. (1988a). AFDX 116 discriminate between M_2 receptors of the heart and iris smooth muscle. *Mol. Cell. Biochem.*, **82**, 131–135.

HONKANEN, R.E. & ABDEL-LATIF, A.A. (1988b). Characterization of cholinergic muscarinic receptors in the rabbit iris. *Biochem. Pharmacol.*, **37**, 2575–2583.

HONKANEN, R.E. & ABDEL-LATIF, A.A. (1989). Muscarinic-agonist and guanine nucleotide stimulation of myo-inositol trisphosphate formation in membranes isolated from bovine iris sphincter muscle: effects of short-term cholinergic desensitization. *Membr. Biochem.*, **8**, 39–59.

HONKANEN, R.E., HOEARD, E.F. & ABDEL-LATIF, A.A. (1990). M_3-muscarinic receptor subtype predominates in the bovine iris sphincter smooth muscle and ciliary process. *Inv. Ophthal. Visual Sci.*, **31**, 590–593.

HULME, E.C., BIRDSALL, N.L.M. & BUCKLEY, N.L. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

KENAKIN, T.P. (1989). Challenges for receptor theory as a tool for drug and drug receptor classification. *Trends Pharmacol. Sci.*, **10**, 18–22.

KENAKIN, T.P. & BOSELLI, C. (1991). Biphasic dose-response curves to arecoline in rat atria-mediation by a single promiscuous receptor or two receptor subtypes? *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 201–205.

LAMBRECHT, G., FEIFEL, R., WAGNER-RÖDER, M., STROHMANN, C., ZILCH, H., TACKE, R., WAELBROECK, M., CHRISTOPHE, J., BODDEKE, H. & MUTSHLER, E. (1989). Affinity profiles of hexahydro-siladifenidol analogues at muscarinic receptor subtypes. *Eur. J. Pharmacol.*, **168**, 71–80.

LEIBER, D., MARC, S. & HARBON, S. (1990). Pharmacological evidence for distinct muscarinic receptor subtypes coupled to the inhibition of adenylate cyclase and to the increased generation of inositol phosphates in the guinea pig myometrium. *J. Pharmacol. Exp. Ther.*, **252**, 800–809.

MARMFORS, T. & NILSSON, O. (1964). Parasympathetic postganglionic denervation of the iris and parotid gland in the rat. *Acta Morphol. Neerl. Scand.*, **6**, 81–85.

NARITA, S. & WATANABE, M. (1981). Response of the isolated rat iris sphincter to cholinergic agents and electrical stimulation. *Life Sci.*, **29**, 285–294.

NARITA, S. & WATANABE, M. (1982a). Response of isolated rat iris dilator to adrenergic and cholinergic agents and electrical stimulation. *Life Sci.*, **30**, 1211–1218.

NARITA, S. & WATANABE, M. (1982b). Effects of calcium blockers and Mn^{2+} on the response of isolated rat iris sphincter and dilator muscles to agonists and Ca^{2+}. *J. Pharm. Dyn.*, **5**, 285–294.

RYANG, S., TAKEI, S., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1990). Atropine-resistant relaxation by high K^+ in iris dilator muscle of the rat and pig. *Br. J. Pharmacol.*, **100**, 401–406.

SHIBATA, K., TAKEI, S., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1989). Decrease in neuronal uptake of noradrenaline simply explains the supersensitivity after sympathectomy in the rat iris dilator. *Jpn. J. Pharmacol.*, **50**, 19–29.

SUZUKI, N., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1991). Subtypes of muscarinic receptors mediating relaxation and contraction in the rat iris dilator. *Jpn. J. Pharmacol.*, **55**, Suppl. 174P.

SUZUKI, R., OSA, T. & KOBAYASHI, S. (1983). Cholinergic inhibitory response in the bovine iris dilator muscle. *Invest. Ophthalmol. Vis. Sci.*, **24**, 760–765.

YAMAHARA, S.N., TANAKA, M., IMAIZUMI, Y. & WATANABE, M. (1995). Pertussis toxin sensitive muscarinic relaxation in the rat iris dilator muscle. *Br. J. Pharmacol.*, **114**, 777–784.

YOSHITOMI, T., ITO, Y. & INOMATA, H. (1985). Adrenergic excitatory and cholinergic inhibitory innervations in the human iris dilator. *Exp. Eye Res.*, **40**, 453–459.

YOSHITOMI, T. & ITO, Y. (1986). Double reciprocal innervations in dog iris sphincter and dilator muscles. *Invest. Ophthalmol. Vis. Sci.*, **27**, 83–91.

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Pertussis toxin-sensitive muscarinic relaxation in the rat iris dilator muscle

Natsuki S. Yamahara, Masako Tanaka, Yuji Imaizumi & [1]Minoru Watanabe

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, 467 Nagoya, Japan

1 The effects of pertussis toxin (PTX) on contraction and/or relaxation induced by agonists or transmural nerve stimulation (TNS) were examined in the rat iris dilator and sphincter muscles.

2 TNS in the presence of phentolamine induced an atropine-sensitive biphasic response: initial contraction followed by relaxation in dilator muscles. Exogenously applied acetylcholine (ACh) elicited a large relaxation at low doses (3 μM or less) and a concentration at high doses.

3 Only the ACh-induced relaxation was affected by injection of PTX (10 ng) into the anterior eye chamber. Relaxation was decreased 12 h after injection and had completely disappeared after 24 h. Relaxation recovered in part 3 weeks and almost completely 8 weeks after PTX treatment. A gradual decrease in muscarinic relaxation in a dilator muscle was also observed *in vitro* after addition of PTX to the bathing solution.

4 The pA_2 values of muscarinic blockers, pirenzepine, AF-DX 116, 4-DAMP, and himbacine for competitive antagonism to ACh-induced contraction were 7.14, 6.53, 9.03, and 6.80, respectively, in PTX-pretreated dilator muscles. These values are comparable to those obtained in parasympathectomized dilator muscles and may indicate involvement of M_3 or M_3-like receptors in muscle contraction.

5 Pretreatment with PTX did not significantly affect contraction induced by noradrenaline or 5-hydroxytryptamine or the relaxation induced by isoprenaline in dilator muscles.

6 In conclusion, among several agonist-induced responses in the rat iris dilator and sphincter muscles, only muscarinic relaxation in dilator muscle occurs via activation of PTX-sensitive GTP binding proteins.

Keywords: Iris dilator muscle (rat); iris sphincter muscle; pertussis toxin; islet activating protein; muscarinic receptor; parasympathetic nerve

Introduction

The activation of muscarinic receptors by exogenously applied acetylcholine (ACh) elicits unique responses in rat iris dilator smooth muscle: relaxation at low doses and contraction at high doses. Although the mechanisms of ACh-induced relaxation in vascular smooth muscle have been extensively studied (Furchgott & Zawadzki, 1980; Furchgott & Vanhoutte, 1989), signal transduction of that in the iris dilator is totally unclear. It is possible that an unknown relaxing factor is released from certain cells in the tissue (Ryang *et al.*, 1990). In contrast to vascular tissues, however, activation of guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase by a relaxing factor such as NO may not be involved in the rat iris dilator (Ryang *et al.*, 1990). Moreover, muscarinic antagonists relatively specific to M_1, M_2 or M_3 receptor subtypes cannot inhibit relaxation or contraction selectively. The subtypes mediating the two responses have, therefore, not been separated by use of antagonists (Masuda *et al.*, 1995).

However, it has been found that the muscarinic agonists, pilocarpine or McN-A-343, can induce relaxation but not contraction (Masuda *et al.*, 1995). This finding suggests that agonists may distinguish between the receptor subtypes mediating the two responses. Alternatively, ACh may activate a single muscarinic receptor which is coupled to two distinctive GTP binding proteins mediating relaxation and contraction. Activation of this receptor by selective agonists may stimulate coupling with one of these GTP binding proteins as has been suggested for cardiac muscarinic responses (Kenakin, 1989; Kenakin & Boselli, 1991).

The present study examines the effects of pertussis toxin (PTX), which selectively inactivates one family of GTP binding proteins (G protein) by ADP-ribosylation (Katada & Ui, 1982a,b), on ACh-induced biphasic responses. A great advantage of using PTX in iris muscles is that treatment with PTX can be performed by a microinjection into the anterior chamber of the eye.

Methods

Preparation and tension measurement

Both eyes were dissected from male Wistar rats (330 to 450 g) which had been stunned by a blow to the head and killed by exsanguination. Strip preparations of iris dilator and sphincter muscle, and tension measurements were carried out as described in detail previously (Narita & Watanabe, 1981; 1982; Masuda *et al.*, 1995). Transmural nerve stimulation (TNS) consisted of 100 pulses per train at supramaximal voltage and 20 Hz for 0.1 ms or 0.2 ms in duration in the dilator or sphincter, respectively, using a pair of platinum electrodes.

Solutions

The bathing solution was a modified Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, and glucose 14.0. Solutions were bubbled with 95% O_2 and 5% CO_2 and kept at 37°C and pH 7.4. To prepare Ca^{2+}-free solution $CaCl_2$ was removed and EGTA 0.1 mM was added.

[1] Author for correspondence.

Treatment with PTX

For application of PTX *in vivo*, rats were anaesthetized with an i.p. injection of thiopentone sodium (25 mg kg^{-1}) and amobarbitone sodium (50 mg kg^{-1}). Injection of PTX into the anterior chamber of the eye was performed by slow injection of 10 ng PTX in 10 µl saline with a sterilized 26 gauge injection needle and a Hamilton syringe. At times ranging from 6 h to 8 weeks after injection, the eye was dissected and the iris was obtained for tension measurements. Control responses were obtained in 3 ways: normal dilator muscles were dissected from saline-injected eyes (1), non-injected eyes of the rats whose contralateral eyes were not injected (2), or PTX-injected (3). Since significant differences were not observed between the three types of controls, the third method was used in all experiments except those shown in Figure 2a.

Drugs

The following drugs were used; noradrenaline bitartrate (NA, Wako, Japan), acetylcholine chloride (ACh, Wako, Japan), 5-hydroxytryptamine creatine sulphate (5-HT, Wako, Japan), atropine sulphate (Atr, Wako, Japan), phentolamine mesylate (Phent, Ciba-Geigy, Japan), isoprenaline (Iso, Wako, Japan), EGTA (Dojin, Japan), papaverine (Wako, Japan), pertussis toxin (PTX, List Biological Laboratories, Inc., U.S.A.).

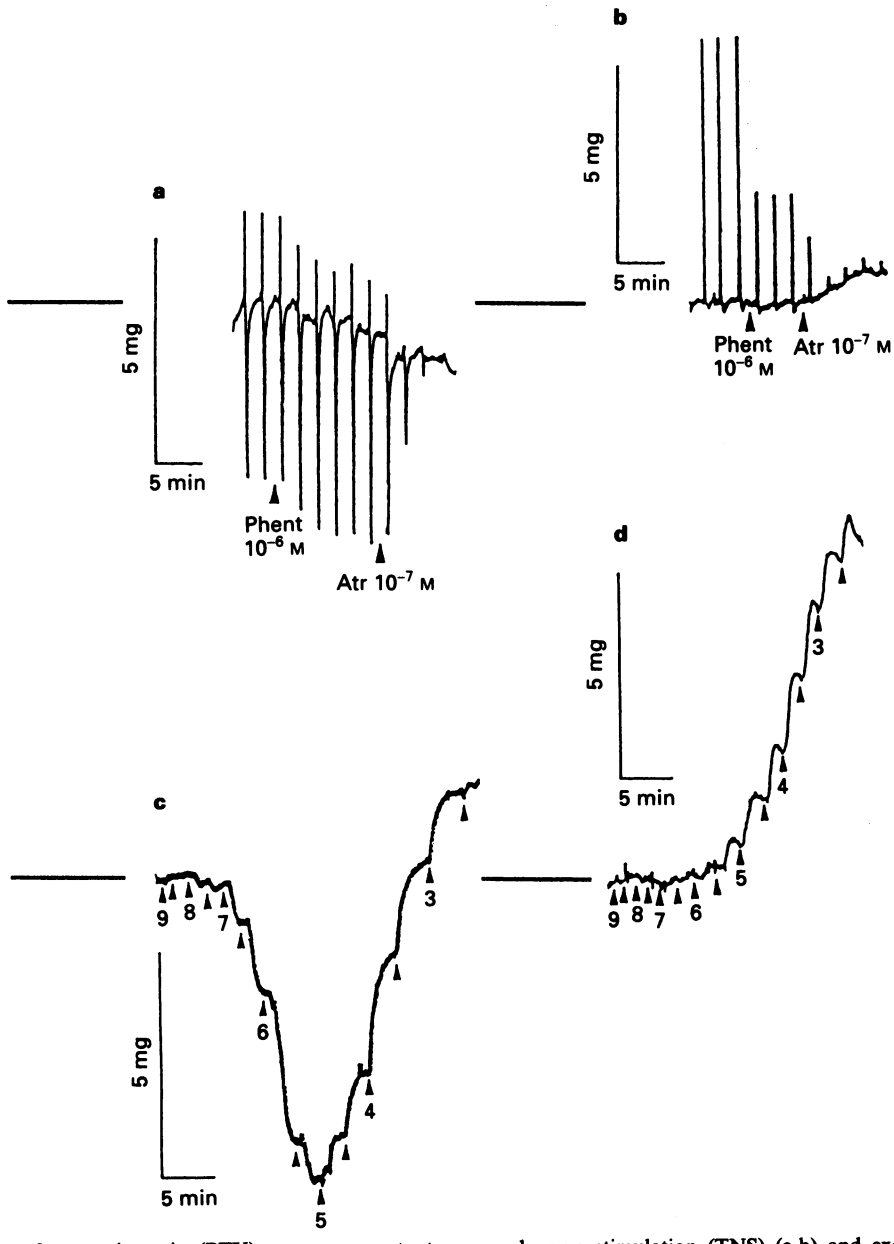


Figure 1 Effects of pertussis toxin (PTX) on responses to transmural nerve stimulation (TNS) (a,b) and exogenously applied acetylcholine (ACh) (c,d) in rat iris dilator muscles. PTX-treated or normal dilator muscles were dissected from eyes injected with 10 ng PTX or from contralateral non-injected ones, respectively, approximately 24 h after injection. (a,b) In normal dilators (a), TNS elicited a biphasic response: an initial contraction followed by a relaxation. Application of 1 µM phentolamine (Phent) decreased contraction and increased relaxation. Further addition of 0.1 µM atropine (Atr) almost abolished the remaining response. In a PTX-pretreated dilator (b), relaxation disappeared. Contraction was inhibited by 1 µM phentolamine and 0.1 µM atropine. (c,d) Cumulative addition of ACh induced relaxation at concentrations less than 10 µM and contraction at higher concentrations in the normal dilator (c). Relaxation completely disappeared in the PTX-pretreated dilator (d). The numbers with arrows indicate the final concentrations of ACh (− logarithm of Molar concentrations). The arrows without numbers indicate that the final concentration is 3 times higher than the preceding concentration. The horizontal line indicates the level of resting tension in each preparation. The resting tension consisting of inherent and passive tone was 14.0, 16.3, 17.7, and 14.1 mg from zero tension level in (a), (b), (c), and (d), respectively.

Statistics

Results were expressed as mean ± s.e. Statistical significance was tested according to Student's t test ($P<$ *0.05, **0.01 or ***0.001). When data were obtained from PTX-treated and control (contralateral) eyes from the same rat, paired t tests were used to detect significant differences. The affinity of antagonists for muscarinic receptors was examined by measuring pA_2 values using Schild plot (Arunlakshana & Schild, 1959).

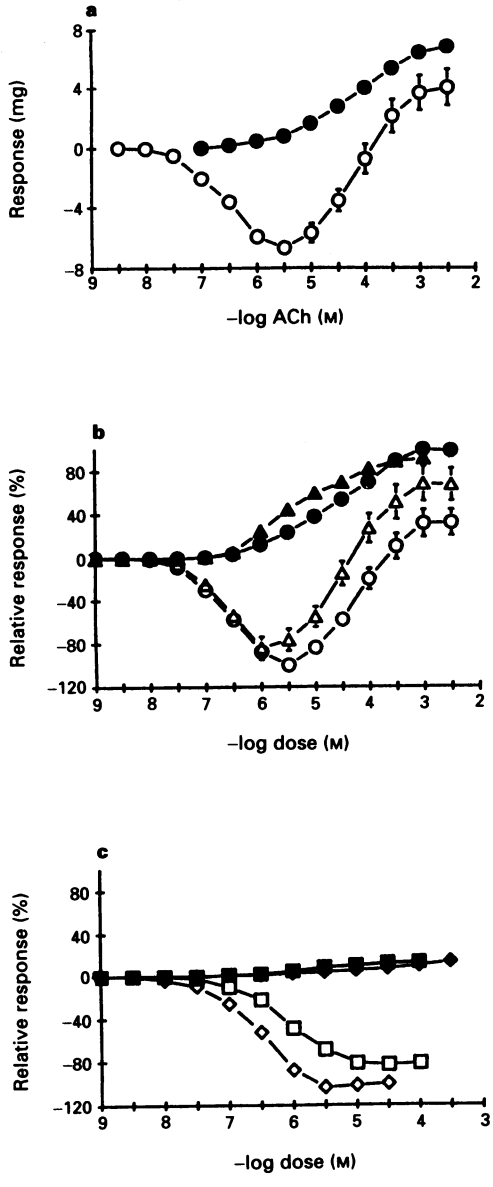


Figure 2 Effects of pertussis toxin (PTX) on concentration-response relationships for acetylcholine (ACh) and other muscarinic agonists. Dilator muscles were isolated about 24 h after injection of 10 ng PTX. Open or closed symbols indicate the data obtained from the normal and PTX-pretreated dilators, respectively. (a) Summarized data from experiments shown in Figure 1c,d. Number of preparations was 6 for each. Symbols indicate mean values with s.e.mean obtained in normal (○) and PTX-pretreated (●) dilators. Magnitudes of relaxations or contractions are shown as absolute values taking the resting level as 0 mg. Normal dilators were obtained from rats injected with saline (PTX was not added to either eye). (b) Effects of PTX on responses to methacholine (△, ▲) were compared with those to ACh (○, ●). The maximum relaxation induced by 3 μM ACh was taken as −100% in each preparation (b and c). Number of preparations was 10 for ACh and 5 for methacholine. Normal dilators were obtained from eyes contralateral to those injected with PTX. (c) Effects of PTX on responses to pilocarpine (□, ■) and McN-A-343 (◇, ◆). $n = 5$.

Results

Effects of PTX on relaxant responses in rat iris dilator muscles

TNS elicited a biphasic response: an initial contraction followed by a relaxation in normal rat dilator muscle (Narita & Watanabe, 1982; Hasegawa *et al.*, 1988). Rat iris dilator muscle has an inherent tone and, therefore, can show relaxation. Application of 1 μM phentolamine significantly reduced contraction and enhanced relaxation. The remaining contraction and relaxation were almost completely blocked by 1 μM atropine (Figure 1a). In dilator muscles, which had been pretreated with 10 ng PTX *in vivo* and isolated for tension measurement 1 day after treatment, relaxation in response to TNS was abolished and contraction was enhanced by 170.1 ± 60.1% in comparison with the contralateral dilator ($n = 10$, $P<0.05$) (Figure 1b). The amplitude of the TNS-induced contraction and relaxation in normal dilator was reduced by 51.9 ± 5.2% ($n = 4$, $P<0.01$) and increased by 22.5 ± 2.1% ($n = 4$, $P<0.01$), respectively, by addition of 1 μM phentolamine. The amplitude of the TNS-induced contraction in PTX-pretreated dilators was decreased by 61.7 ± 3.3% ($n = 3$, $P<0.01$) and the remaining component was almost completely blocked by 1 μM atropine. Occasionally, a small contractile component remained in the presence of both phentolamine and atropine (Figure 1b); it was blocked by 1 μM tetrodotoxin. This may be a tachykinergic component, but it was not examined systematically.

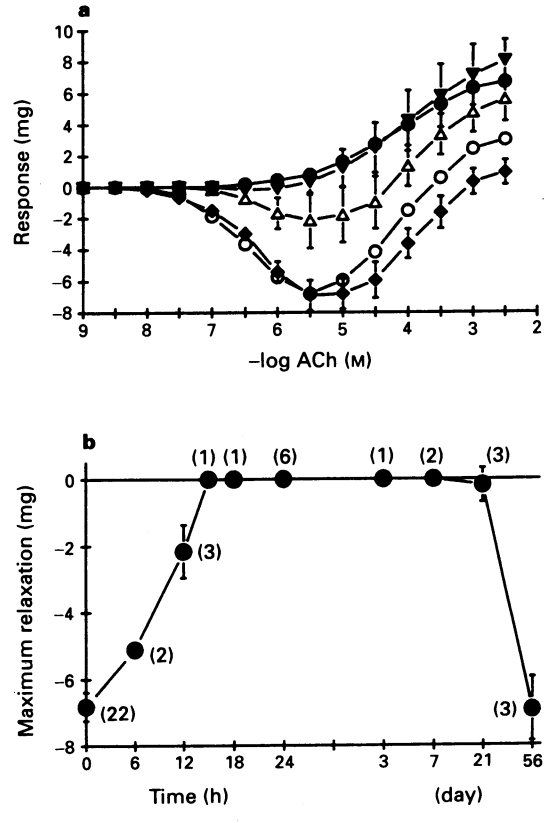


Figure 3 Time-course of changes in the relaxant response in dilators to acetylcholine (ACh) after injection of pertussis toxin (PTX). (a) Concentration-response relationships for ACh in dilators isolated at specific times after PTX-injection. Symbols indicate mean values obtained without injection (control; ○) and 12 h (△), 1 day (●), 3 weeks (▼), and 8 weeks (◆) after injection. Number of preparations is shown in parentheses in (b). The amplitudes of relaxations and contractions are shown as absolute values taking the resting level as 0 mg. (b) Maximum relaxation induced by ACh in experiments shown in (a) was plotted against time after PTX-injection.

Figure 1c and d show responses to exogenous ACh, added cumulatively, to obtain dose-response relationships in normal and PTX-pretreated dilators, respectively. Normal dilator muscle was obtained from the contralateral eye to that treated with 10 ng PTX 24 h before. Normal dilator muscles relaxed at low concentrations of ACh (30 nM–3 μM) and contracted at higher concentrations. It is notable that relaxation disappeared and contraction started at 1 μM ACh in PTX-pretreated muscle, but relaxation changed to contraction with 10 μM ACh in the normal dilator, as has been reported previously (Narita & Watanabe, 1982). When the amount of injected PTX was tested over a range of 0.1 ng to 100 ng per eye, the effect of PTX was dose-related. ACh-induced relaxation observed after 24 h was markedly reduced and completely abolished by injection of 1 ng and 10 ng PTX, respectively. The dose of PTX (10 ng) which abolished the response was used in most of the present study.

Figure 2a shows the dose-response relationships to exogenously applied ACh in control and PTX-treated dilator muscles. In the experiments shown in Figure 2a, normal dilator muscles were obtained from rats which had been injected with saline (but not PTX) 1 day before isolation. The relationship for normal dilator muscles overlapped for dilator muscles from eyes without saline injection over the whole dose-range (not shown to simplify the figure). In PTX-treated dilator muscles (24 h after injection), relaxation in response to ACh consistently disappeared and contraction was elicited by 1 μM or higher concentrations of ACh. Figure 2b shows concentration-response relationships for ACh and methacholine in normal and PTX-pretreated dilator muscles. In PTX-pretreated dilator muscles, methacholine induced only a large contraction as did ACh. In the experiments shown in Figure 2b normal dilator muscles were obtained from eyes contralateral to the PTX-treated ones. The relationship for ACh in normal dilator muscle almost completely overlapped that shown in Figure 2a. It indicates that the PTX-treatment of an eye did not significantly affect the response of the dilator muscle from the contralateral eye. Application of pilocarpine and McN-A-343 induced only relaxation in normal dilator muscle and very small contractions in PTX-pretreated dilator muscle (Figure 2c).

The negative logarithms of doses ($-\log ID_{50}$) of ACh, methacholine, pilocarpine, and McN-A-343 giving half-maximum relaxation in normal dilator muscles were 6.69 ± 0.05 ($n = 10$), 6.72 ± 0.07 ($n = 5$), 6.13 ± 0.08 ($n = 5$), and 6.63 ± 0.14 ($n = 5$), respectively. When maximum relaxation induced by ACh in normal dilators was taken as −100%, those by methacholine, pilocarpine, and McN-A-343 were 83.7 ± 10.6 ($P < 0.05$ vs ACh), 83.9 ± 3.3 ($P < 0.001$ vs ACh), and 104.6 ± 4.5%, respectively. The concentrations of ACh and methacholine giving half-maximum contraction ($-\log ED_{50}$) were 4.18 ± 0.05 ($n = 10$) and 4.39 ± 0.05 ($n = 5$) in normal dilators, and 4.70 ± 0.08 ($n = 10$) and 5.32 ± 0.09 ($n = 5$) in PTX-treated dilators. Pilocarpine and McN-A-343 elicited no detectable contractions in normal dilators and small but significant contractions (13.8 ± 2.0 and 14.0 ± 2.4%, respectively) in PTX-pretreated dilators.

Figure 3a shows the concentration-response relationships for ACh obtained in PTX-pretreated dilators from 6 to 24 h and from 1 day to 8 weeks after treatment. Figure 3b shows the time course of changes in the maximum relaxation measured in the concentration-response curves for ACh. A tendency to decreased relaxation was found within 6 h of treatment. Relaxation was abolished after 15 h. The changes in the concentration-response relationships were maximal after 18 h and were maintained at that level for 3 weeks. The control values shown in Figure 3 were the averages of responses in dilators obtained from the eyes contralateral to the PTX-treated ones.

Rat iris dilator muscle has an inherent tone which cor-

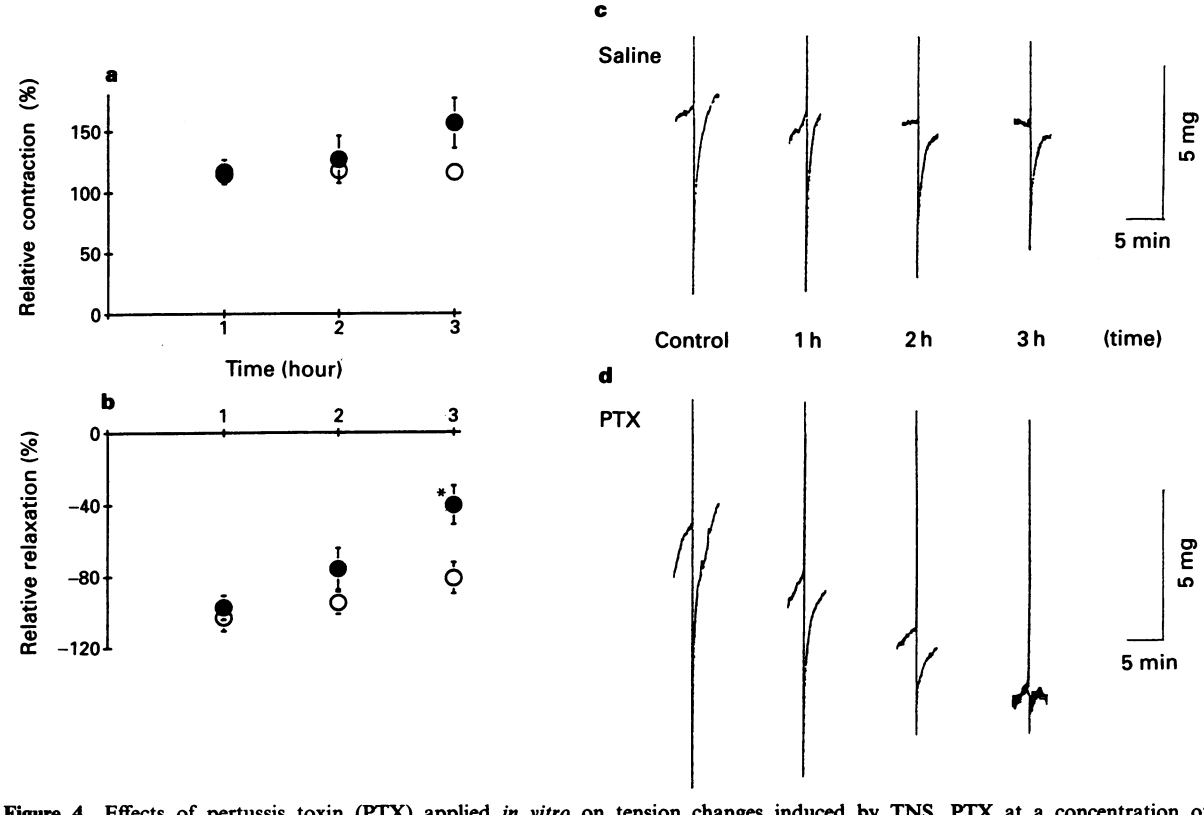


Figure 4 Effects of pertussis toxin (PTX) applied *in vitro* on tension changes induced by TNS. PTX at a concentration of 1 μg ml^{-1} was added to the bathing solution. (a,b) Changes in magnitude of TNS-induced contraction (a) and relaxation (b) with time after addition of saline (○) or PTX (●). The magnitude of contraction or relaxation just before PTX addition was taken as 100 or −100%, respectively. Number of observations was 4 for each treatment. (c,d) Typical recordings of TNS-induced responses in dilators before (control) and 1, 2, and 3 h after addition of saline (c) or PTX (d). Note that relaxation markedly decreased and contraction increased 3 h after PTX addition.

relates well with extracellular Ca^{2+} concentration but appears not to be dependent upon innervation (Narita & Watanabe, 1982). This inherent tone depends strongly upon extracellular Ca^{2+} and was, therefore, not present in Ca^{2+}-free Krebs solution. Both normal and PTX-treated muscles relaxed completely in the Ca^{2+} free-Krebs solution. The remaining tension (about 5 mg) was not significantly affected by 0.3 mM Cd^{2+} or 100 μM papaverine and, therefore, was a passive tone. The inherent tone measured by removal of external Ca^{2+} was 9.2 ± 2.0 mg ($n = 8$) in PTX-treated dilator muscle and was smaller than that in normal dilator (12.3 ± 1.9 mg, $n = 8$) but the difference was not statistically significant ($P > 0.05$). The abolition of ACh-induced relaxation, however, cannot be explained by the 25% decrease in tone, since addition of 100 μM papaverine relaxed PTX-treated dilator (7.2 ± 1.3 mg, $n = 3$, 78% decrease in tone) to a similar extent to normal dilator (9.8 ± 2.2 mg, $n = 3$, $P > 0.05$ vs. PTX, 80% decrease in tone).

When PTX was added to the bathing solution at a concentration of 1 μg ml^{-1}, the responses to TNS in normal dilator changed with time as shown in Figure 4. Three changes were prominent; a decrease in relaxation, an increase in contraction, and a decrease in inherent tone. Therefore, the changes in dilator muscles after treatment with PTX *in vivo* can be also observed *in vitro*. Although the responses to TNS did not significantly change for about 3 h in normal dilators in the absence of PTX, relaxation and inherent tone tended to decrease 3 h after the experiments started.

pA_2 values of muscarinic antagonists in PTX-treated dilators

Effects of muscarinic antagonists, pirenzepine, methoctramine, AF-DX 116, 4-DAMP, and himbacine, were examined in dilator muscles pretreated with PTX for 3 days, where the relaxant response to ACh disappeared. ACh in the concentration range of 1 nM to 3 mM was added cumulatively to obtain a concentration-response relationship. When the effect of an antagonist was examined, it was added 10 minutes prior to the addition of ACh. Except for methoctramine, these antagonists competitively antagonized the ACh-induced contraction (Figure 5). The slopes and pA_2 values obtained from Schild plots were, respectively, 0.92 ± 0.20 and 7.14 ± 0.19 for pirenzepine ($n = 5$); 0.97 ± 0.18 and 6.53 ± 0.32 for

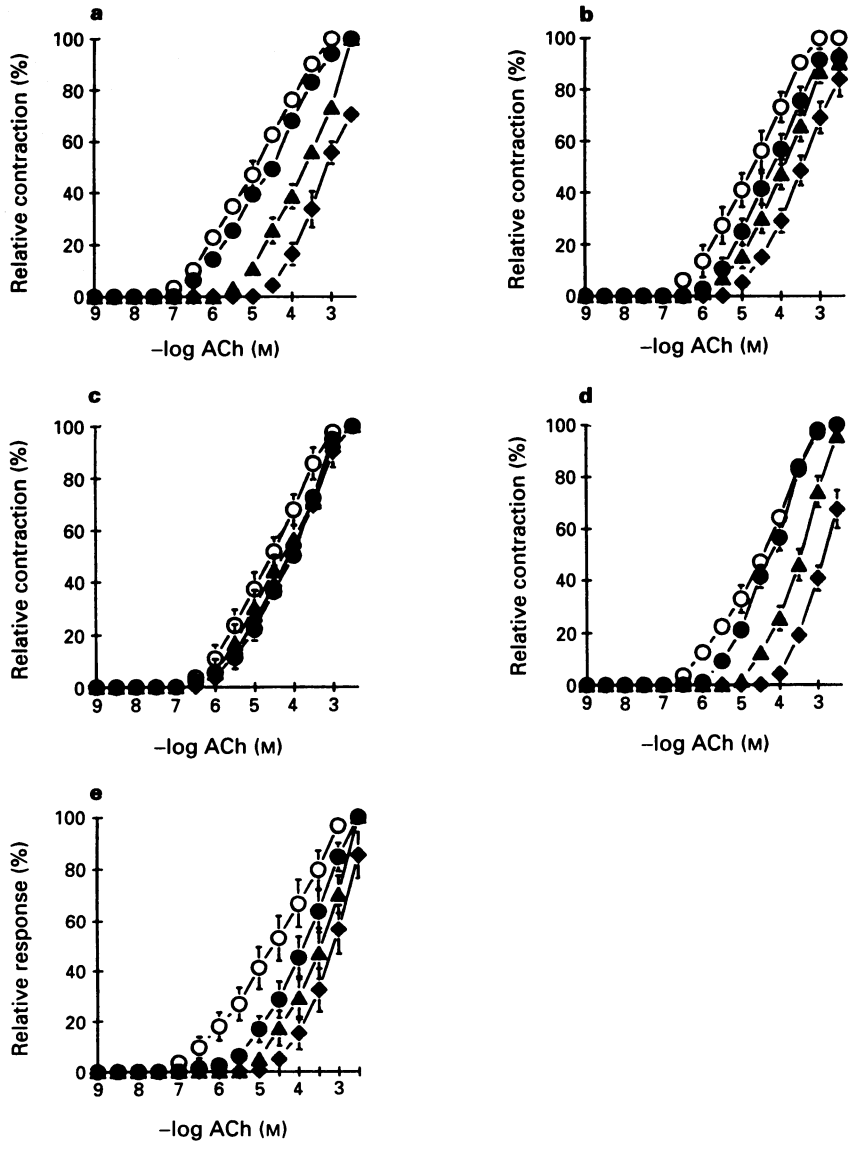


Figure 5 Effects of muscarinic antagonists on concentration-response relationships for acetylcholine (ACh) in dilators pretreated with pertussis toxin (PTX). $n = 5$ for pirenzepine, AF-DX 116, methoctramine, and 4-DAMP and $n = 4$ for himbacine at each concentration. (a) Control (○), pirenzepine 0.1 μM (●), 1 μM (▲) and 10 μM (◆); (b) control (○), AF-DX 116 1 μM (●), 3 μM (▲), 10 μM (◆); (c) control (○), methoctramine 1 μM (●), 3 μM (▲), 10 μM (◆); (d) control (○), 4-DAMP 1 nM (●), 10 nM (▲), 100 nM (◆); (e) control (○), himbacine 1 μM (●), 3 μM (▲), 10 μM (◆).

AF-DX 116 ($n = 5$); 0.89 ± 0.11 and 9.03 ± 0.10 for 4-DAMP ($n = 5$); and 0.85 ± 0.33 and 6.80 ± 0.20 for himbacine. Application of methoctramine at concentrations of 1, 3, and 10 μM did not shift the concentration-response curves for ACh significantly (only by 3 times at the maximum).

Effects of PTX on the responses to NA, 5-HT, and Iso in dilator muscles

Application of NA or 5-HT induced concentration-dependent contractions in rat iris dilator (Figure 6a and b). The effects of PTX were examined in dilators isolated 1 day after treatment with 10 ng PTX. The maximal contraction (control 12.8 ± 1.2 mg, $n = 7$) and the sensitivity (pD_2 5.79 ± 0.05) to NA were not changed significantly by pretreatment with PTX (16.0 ± 1.8 mg, 5.68 ± 0.04, $n = 7$, $P > 0.05$ vs. control, respectively). 5-HT, applied in the presence of 1 μM atropine to avoid the influence of ACh release from nerve endings, also induced concentration-dependent contractions in rat iris dilator. The maximal contractions (control 7.2 ± 0.7 mg, $n = 8$) and sensitivity (pD_2 6.35 ± 0.04) to 5-HT were not changed significantly by PTX pretreatment (6.0 ± 0.6 mg, 6.45 ± 0.06, $n = 8$, $P > 0.05$ vs. control). Application of Iso in a concentration-dependent manner relaxed dilator muscles (Figure 6c). Maximum relaxation (control 3.5 ± 0.7 mg, $n = 3$) and sensitivity to Iso (pD_2 7.19 ± 0.14, $n = 3$) were not significantly affected by PTX pretreatment (3.4 ± 0.6 mg, 7.31 ± 0.17, $n = 3$, $P > 0.05$ vs. control, respectively).

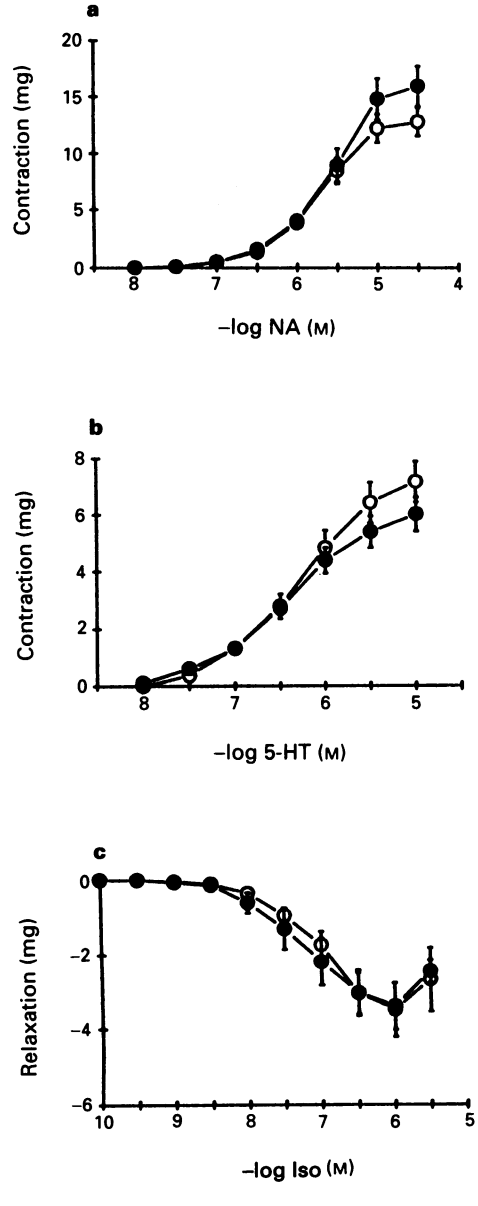


Figure 6 Effects of pertussis toxin (PTX) on concentration-response relationships for noradrenaline (NA) (a) 5-hydroxytryptamine (5-HT) (b) and isoprenaline (Iso) (c). Open and closed symbols indicate the mean values of data obtained from normal dilators and those pretreated with PTX, respectively. Number of preparations is 7 in (a), 8 in (b), and 3 in (c) for each of the control and PTX-treated dilators. Note that the absolute values of responses in PTX-pretreated dilators are not significantly different from those in normal dilators ($P > 0.05$) at any concentration of these agonists.

Effects of PTX in rat iris sphincter

The response to TNS in rat iris sphincter muscles was the only contraction to be almost completely blocked by addition of 1 μM atropine. Occasionally, a small atropine-resistant component to the contraction which was sensitive to capsaicin remained (Banno *et al.*, 1985). The contractile responses to TNS in sphincters from eyes injected with PTX 1 day before isolation did not change from control values (control 19.6 ± 2.8 mg; PTX 19.7 ± 1.4 mg, $n = 6$, $P > 0.05$).

Application of ACh or 5-HT induced concentration-dependent contractions in normal and PTX-treated sphincters in a similar manner. The maximum responses and sensitivity in terms of pD_2 (−logarithm concentration of an agonist giving a half-maximal contraction) to ACh were 72.0 ± 3.4 mg and 4.83 ± 0.08 ($n = 6$), respectively, in normal sphincters and 78.0 ± 3.5 mg ($n = 6$; $P > 0.05$ vs control) and 4.89 ± 0.03 ($P > 0.05$ vs control), respectively, in PTX-pretreated sphincters. The maximum responses and sensitivity (pD_2) to 5-HT were also not affected by PTX-treatment (34.1 ± 3.0 mg, 6.39 ± 0.05 in control sphincters, $n = 6$; 35.2 ± 2.9 mg, 6.39 ± 0.07 in PTX-pretreated sphincters, $n = 6$, $P > 0.05$ vs corresponding control values).

Discussion

The present study clearly shows that only the ACh-induced relaxation of biphasic responses to ACh in dilator muscles is sensitive to PTX. PTX is an ADP-ribosyltransferase (Katada & Ui, 1982a,b) and leads to an ADP-ribosylation of some kinds of cellular guanine nucleotide-binding proteins (G proteins) resulting in elimination of the regulatory function of the G proteins. The family of G protein alpha-subunits has been subclassified as G_s, G_i, G_o, G_k, and G_p, according to their function. In the last few years, however, based upon the amino acid identity of the alpha subunit, G proteins have been classified into 4 groups: G_s, G_i, G_q, and G_{12} (Simon *et al.*, 1991; Hepler & Gilman, 1992). G_s and G_i are susceptible to cholera toxin and PTX, respectively, whereas G_q and G_{12} are insensitive to both toxins.

The possibility that PTX-treatment increased contraction and masked relaxation in response to ACh can be ruled out for two reasons. First, the concentration of ACh initiating detectable contraction changed only from 10 μM to 1 μM after treatment with PTX, whereas relaxation in normal dilators was elicited by ACh at concentrations between 30 nM and 3 μM. Second, application of pilocarpine or McN-A-343 which induces only relaxation in normal dilator muscles (Masuda *et al.*, 1995) elicited neither a relaxation nor an apparent contraction in PTX-pretreated dilators. Moreover, the inhibition of muscarinic relaxation is not due to non-specific mechanisms, since neither β-adrenoceptor-mediated nor papaverine-induced relaxations were affected significantly by PTX-pretreatment. The inherent tone was not affected either. Therefore, it can be concluded that muscarinic relaxation is mediated by a PTX-sensitive G protein, whereas muscarinic, α-adrenoceptor, and 5-HT receptor-mediated contractions, and β-adrenoceptor-mediated relaxation are not.

It is interesting that treatment with PTX abolished the relaxant response in a similar manner to parasympathetic denervation by ciliary ganglionectomy (Hasegawa *et al.*, 1988). Even the time course of the decrease in relaxation was similar for these two different treatments. When 10 ng PTX is injected into an anterior eye chamber, the initial PTX concentration may be approximately 400 ng ml^{-1} assuming the volume of the eye chamber to be 25 μl. A significant decrease in ACh-induced relaxation was observed 6 to 12 h after injection as it is after ciliary ganglionectomy (Hasegawa *et al.*, 1988). The effects of PTX were, however, not due to direct damage to parasympathetic nerves, since the contractions, induced by the stimulation of parasympathetic nerves as well as sympathetic ones, remained almost unchanged and actually rather increased in amplitude after PTX-treatment (see Figure 1a,b). The addition of 1 μg ml^{-1} PTX to isolated dilators *in vitro* decreased TNS-induced relaxation within 2 h but did not inhibit the response completely even after 3 h. The results indicate that application of PTX to the eye chamber is an excellent way to ribosylate G protein selectively without additional undesirable damage.

The major difference in pharmacological changes in dilators pretreated with PTX and those parasympathectomized is that the effect of PTX was very selective for muscarinic relaxation, whereas denervation results in nonspecific supersensitivity. Contractile responses to 5-HT or NA were not affected by PTX-treatment in either dilator or sphincter muscles. After ciliary ganglionectomy, there is increased responsiveness to a variety of agonists in both rat iris sphincter and dilator muscles. The mechanism of nonspecific supersensitivity remains unclear (Banno *et al.*, 1987; Hasegawa *et al.*, 1987; 1988; Hashimoto *et al.*, 1993). Although it is possible that blockade of muscarinic relaxation after parasympathectomy is due to changes in G protein, this possibility was not examined directly in the present study. After treatment with PTX, muscarinic relaxation did not recover substantially for over 3 weeks but was clearly observed after 8 weeks indicating that the turnover of ADP-ribosylated G protein took a period of several weeks. On the other hand, relaxation did not recover even one year after denervation (Hasegawa, unpublished observation).

It may be reasonable that the contractile responses to ACh, NA, or 5-HT in both dilator and sphincter muscles were not affected by PTX-treatment, since these responses are considered to be elicited by activation of a G protein which couples with phospholipase C (PLC) and is not susceptible to PTX. A group of G proteins coupling with PLC is called G_p from a functional basis, or more recently G_q (Smrcka *et al.*, 1991; Simon *et al.*, 1991), and they are not substrates for PTX. While some reports indicate that PTX ADP-ribosylates some G_ps (Ashkenazi *et al.*, 1989), IP_3 formation subsequent to the receptor activation by agonists in smooth muscle tissues, including muscarinic M_3 receptor activation in bovine iris sphincter (Honkanen & Abdel-Latif, 1989), is, in general, considered to be PTX-insensitive. Similarly, it is also reasonable that Iso-induced relaxation was not sensitive to PTX. Since PTX-sensitive α subunits possess specific cystein residues near the carboxyl terminus and belong to $(\alpha)_i$ group, the G protein mediating muscarinic relaxation in dilators may be one of the G_i proteins.

As reported in the preceding study (Masuda *et al.*, 1995), subtypes of the muscarinic receptor mediating relaxation and contraction cannot be distinguished from each other by use of selective muscarinic receptor antagonists. According to pA_2 values for pirenzepine as a M_1 antagonist, AF-DX 116 and methoctramine as M_2 antagonists, 4-DAMP as a M_3 antagonist, and himbacine as a M_4 antagonist, it appears that both muscarinic receptor relaxation and contraction may be mediated by M_3 or M_3-like receptors (Masuda *et al.*, 1995). pA_2 values obtained in the present study for antagonism of ACh-induced contraction in PTX-pretreated dilators were comparable to those obtained in parasympathectomized dilators, indicating that the affinity of antagonists for M_3 receptors was not affected by PTX-treatment or by denervation.

The activation of muscarinic receptors expressed by m_1, m_3, and m_5 genes stimulates IP_3 turnover whereas that by m_2 and m_4 genes decreases cyclic AMP production (Bonner, 1989; Hulme *et al.*, 1990; Dörje *et al.*, 1990; Buckley, 1990). The latter signal is transduced by PTX-sensitive G proteins (G_i family) (Hulme *et al.*, 1990). The m_2 receptor also mediates activation of a K current in cardiac myocytes via a G_K protein functionally (a family of G_i) (Birnbaumer, 1990). In rat left atria, arecoline also produces a biphasic response, i.e., a depression of basal inotropy at low doses and a positive inotropic effect at higher doses (Kenakin & Boselli, 1991). Although both responses are mediated by the same subtype of muscarinic receptors (M_2) in accordance with classification by antagonists, negative inotropy is selectively abolished by treatment with PTX. Therefore, it has been postulated that a single receptor can couple with two distinct G proteins which transduce distinct intracellular signals. Agonists may interact with and recognize different cytosolic G-protein coupling domains (third cytoplasmic loop; Bonner, 1989, Wess *et al.*, 1989) thus stimulating different intracellular signals. On the other hand, antagonists which interact with binding sites in the external domain of the receptor protein cannot differentiate between couplings with 2 G proteins. Classification of muscarinic receptors by antagonist affinity would not account for the different cellular functions which are mediated by activation of receptors. The previous (Masuda *et al.*, 1995) and present results may be favourable to this hypothesis in dilator muscles.

In conclusion, relaxation of the biphasic response to ACh was mediated by PTX-sensitive G protein in rat iris dilator. Contractions induced by agonists, including ACh, in both dilator and sphincter muscles are mediated by PTX-insensitive G protein.

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References

ARUNLAKSHANA, O. & SCHILD, H. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.*, **14**, 48–58.

ASHKENAZI, A., PERALTA, E.G., WINSLOW, J.W., RAMACHANDRAN, J. & CAPON, D.J. (1989). Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth. *Trends Pharmacol. Sci.* (supplement), 16–22.

BANNO, H., IMAIZUMI, Y. & WATANABE, M. (1985). Pharmacomechanical coupling response to acetylcholine and substance P in the smooth muscle of the rat iris sphincter. *Br. J. Pharmacol.*, **85**, 905–911.

BANNO, H., IMAIZUMI, Y. & WATANABE, M. (1987). Cellular mechanisms of supersensitivity to acetylcholine and potassium ion after ciliary ganglionectomy in the rat iris sphincter muscle. *Jpn. J. Pharmacol.*, **43**, 153–163.

BIRNBAUMER, L. (1990). G proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 675–705.

BONNER, T.I. (1989). New subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* (supplement), 11–15.

BUCKLEY, N.J. (1990). Molecular pharmacology of cloned muscarinic receptors. In *Transmembrane Signalling; Intracellular Messengers and Implications for Drug Development.* ed. Nahorski, S.R. p. 13. New York: John Wiley.

DÖRJE, F., FRIEBE, T., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1990). Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea-pig uterus. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 284–289.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373.

HASEGAWA, N., IMAIZUMI, Y. & WATANABE, M. (1987). Parasympathetic denervation supersensitivity in the rat iris sphincter muscle: an *in vitro* study. *Jpn. J. Pharmacol.*, **43**, 143–151.

HASEGAWA, N., IMAIZUMI, Y. & WATANABE, M. (1988). Parasympathetic denervation abolishes acetylcholine induced relaxation in the rat iris dilator. *Eur. J. Pharmacol.*, **156**, 291–294.

HASHIMOTO, Y., HASEGAWA, N., SUZUKI, N., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1993). Effects of ciliary ganglionectomy on contractile responses in the dilator muscle of the rat iris. *Exp. Eye Res.*, **56**, 135–141.

HEPLER, J.R. & GILMAN, A.G. (1992). G proteins. *Trends Biochem.*, **17**, 383–387.

HONKANEN, R.E. & ABDEL-LATIF, A.A. (1989). Muscarinic-agonist and guanine nucleotide stimulation of myo-inositol trisphosphate formation in membranes isolated from bovine iris sphincter smooth muscle: effects of short-term cholinergic desensitization. *Membr. Biochem.*, **8**, 39–59.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

KATADA, T. & UI, M. (1982a). Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3129–3133.

KATADA, T. & UI, M. (1982b). ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.*, **257**, 7210–7216.

KENAKIN, T.P. (1989). Challenges for receptor theory as a tool for drug and drug receptor classification. *Trends Pharmacol. Sci.*, **10**, 18–22.

KENAKIN, T.P. & BOSELLI, C. (1991). Biphasic dose-response curves to arecholine in rat atria-mediation by a single promiscuous receptor or two receptor subtypes? *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 201–205.

MASUDA, Y., YAMAHARA, S.N., RYANG, S., TANAKA, M., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1995). Characterization of muscarinic receptors mediating relaxation and contraction in the rat iris dilator. *Br. J. Pharmacol.*, **114**, 769–776.

NARITA, S. & WATANABE, M. (1981). Response of the isolated rat iris sphincter to cholinergic agents and electrical stimulation. *Life Sci.*, **29**, 285–294.

NARITA, S. & WATANABE, M. (1982). Response of isolated rat iris dilator to adrenergic and cholinergic agents and electrical stimulation. *Life Sci.*, **30**, 1211–1218.

RYANG, S., TAKEI, S., KAWAI, T., IMAIZUMI, Y. & WATANABE, M. (1990). Atropine-resistant relaxation by high K^+ in iris dilator muscle of the rat and pig. *Br. J. Pharmacol.*, **100**, 401–406.

SIMON, M.I., STRATHMANN, M.P. & GAUTAM, N. (1991). Diversity of G proteins in signal transduction. *Science*, **252**, 802–808.

SMRCKA, A.V., HEPLER, J.R., BROWN, K.O. & STERNWEIS, P.C. (1991). Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science*, **251**, 804–807.

WESS, J., BRANN, M.R. & BONNER, T.I. (1989). Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. *FEBS Lett.*, **258**, 133–136.

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Characterization of endothelin receptors in rat renal artery *in vitro*

[1]Kenneth L. Clark & Lisa Pierre

Pharmacology 2, Glaxo Research and Development Limited, Ware SG12 0DJ, Hertfordshire, U.K.

1 The aim of this study was to investigate the function and characteristics of endothelin receptors in rat main branch renal artery *in vitro*.

2 Endothelin(ET)-1 (mean EC_{50} = 9.8 nM) was approximately 12 fold more potent than ET-3 (mean EC_{50} = 120 nM) as a contractile agonist and produced a greater maximum response. In contrast, neither of the ET_B receptor-selective agonists, alanine[1,3,11,15]ET-1 nor sarafotoxin S6c, (0.1 nM–1 μM), induced any contractile effect, or any relaxant effect in endothelium-intact preparations pre-contracted with the thromboxane A_2 mimetic, U-46619. Sarafotoxin S6c (30 nM) also failed to induce any further contraction in tissues pre-contracted with an EC_{50} concentration of ET-1.

3 The ET_A receptor-selective antagonist, BQ123, behaved as a weak and variable antagonist of the contractile effects of ET-1 (mean pA_2 estimates in the range 5.8–6.3). In contrast, BQ123 antagonized ET-3 with a potency (mean pA_2 = 7.6) consistent with its affinity for ET_A receptors. Co-incubation of BQ123 (3 μM) with the putative ET_B receptor-selective antagonist, IRL1038 (10 μM), produced no greater antagonism of ET-1 responses than was induced by BQ123 (3 μM) alone.

4 In conclusion, ET_B receptors do not appear to be present in rat main branch renal artery. The contractile effects of ET-3 in this tissue seem to be mediated by ET_A receptors. While ET_A receptors partly mediate the contractile effects of ET-1, these data raise the possibility that a population of novel BQ123-insensitive endothelin receptors may also contribute to this response.

Keywords: Rat renal artery *in vitro*; endothelin; sarafotoxin S6c; BQ123; ET_A receptor; ET_B receptor; receptor subtypes

Introduction

The endothelins represent a class of recently discovered potent vasoactive 21 amino acid peptides (Yanigasawa *et al.*, 1988). There is evidence to support the existence of discrete pathways for the production of three closely related peptides, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3).

Administered intravenously, the most prevalent effect of the endothelins is vasoconstriction, although a vasodilator component often precedes the vasoconstriction (Wright & Fozard, 1988; Lippton *et al.*, 1988). Current knowledge supports the existence of two main classes of endothelin receptors, termed ET_A and ET_B, which have been successfully cloned and expressed (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Elshourbagy *et al.*, 1993). It was initially thought that ET_A receptors mediate the vasoconstrictor effects of the endothelins, while ET_B receptors mediate their vasodilator effects. This view has recently been challenged following the demonstration of ET_B receptor-mediated vasoconstrictor responses (Sumner *et al.*, 1992). Whether the same subtype of ET_B receptor mediates both constrictor and dilator responses remains to be clarified. Moreover, although only two subtypes of mammalian endothelin receptor have as yet been cloned, many groups have observed atypical agonist and antagonist potency profiles in radioligand binding and isolated tissue studies, raising the possibility that further subtypes of endothelin receptors occur (Sokolovsky *et al.*, 1993; Bodelsson & Stjernquist, 1993; Bax *et al.*, 1993a,b; Eglezos *et al.*, 1993; Warner *et al.*, 1993).

Although much studied, the physiological and pathophysiological significance of endothelin remains largely undetermined. Interestingly, the renal vasculature appears particularly sensitive to the vasoconstrictor effects of endothelin (Pernow *et al.*, 1988: Lanese *et al.*, 1992), and ET-1 can be produced in several renal cell types (Kon & Badr, 1991; Perico & Remuzzi, 1993). Since several forms of acute renal failure involve intense renal vasoconstriction, it is not surprising that endothelin has been postulated as a potential mediator. Indeed, studies in rat models support some potential involvement of endothelin in both ischaemia and cyclosporine-induced renal failure (Kon & Badr, 1991). In addition, there is some evidence to support a role for endothelin in animal models of chronic renal disease, possibly as a consequence of its mitogenic effects and through interactions with other mediators of inflammation (Benigni *et al.*, 1993; Perico & Remuzzi, 1993).

The possible pathophysiological involvement of endothelin in the kidney has provoked excitement in the possibility that endothelin receptor antagonists may have efficacy in the treatment of renal disease. However, much remains to be learned regarding the pharmacology of renal endothelin receptors. In terms of the renal vasculature, there is evidence from studies in the rat to suggest that both ET_A and ET_B receptors contribute to vasoconstrictor responses *in vivo* (Wellings *et al.*, 1993; Pollock & Opgenorth, 1993). While perfused kidney preparations or studies *in vivo* are invaluable in allowing microvascular responses to endothelin to be studied, they make it difficult to control drug concentrations and hence quantitatively characterize receptors. The aim of the present study was to use an *in vitro* test system, where conditions could be closely controlled, in order to characterize functional endothelin receptors in rat main branch renal artery. A preliminary account of these findings has been presented to the British Pharmacology Society (Pierre & Clark, 1994).

Methods

General

Male Sprague-Dawley rats (250–350 g) were killed by cervical dislocation. The main branch renal artery to the left

[1] Author for correspondence at present address: Department of Pharmacology, Rhône-Poulenc Rorer Central Research, 500 Arcola Road, P.O. Box 1200, Collegeville, PA 19426-0107, U.S.A.

kidney was carefully removed along with a portion of the abdominal aorta so that handling of the vessel was minimized. The artery was placed into ice cold gassed (95% O_2, 5% CO_2) Krebs-Henseleit solution, pH 7.4. Indomethacin (30 μM) was also present in the solution to prevent the formation of cyclo-oxygenase products. The vessel was cut into rings, approximately 1 mm in length and 400 μm in diameter. Where required, the endothelium was removed by gently rolling the rings on a steel wire 100 μm in diameter. The arterial rings were suspended between stainless steel wires, 100 μm in diameter, in 10 ml glass tissue baths containing gassed Krebs-Henseleit solution at 37°C. Changes in isometric tension were measured with isometric force transducers (Gould-Statham UC3) and displayed on an eight channel chart recorder (Electromed, MT8).

Arterial rings were put under an initial tension of 0.4 g and left to equilibrate for 20 min. KCl (80 mM) was then added to test the contractile viability of the preparations. Once any contractile response had plateaued, the tissues were washed, allowed to relax and the tension was readjusted to 0.4 g. The peptidase inhibitors, phosphoramidon (1 μM) and bestatin (10 μM), were added 45 min before the construction of agonist concentration-response curves in an attempt to prevent any peptide degradation. Where required, the resting tension was readjusted to 0.4 g, 15 min prior to construction of agonist concentration-response curves.

Effects of agonists: relaxant responses

A 10 fold cumulative contractile concentration-response curve to the thromboxane A_2 mimetic, U-46619 (0.1 nM–10 μM), was constructed and the EC_{80} concentration determined (concentration required to produce 80% of the maximum response). The tissues were then washed four times, at 5 min intervals and the tension re-adjusted to 0.4 g. After 15 min, the tissues were contracted with an EC_{80} concentration of U-46619. After the contraction had stabilized, acetylcholine (1 μM) was added. A marked relaxant response (at least 80% relaxation of the induced tone) to acetylcholine was taken as evidence of a functional endothelium (Furchghott & Zawadzki, 1980) and preparations were discarded if this was not achieved.

The tissues were then washed four times, at 5 min intervals. After a further 45 min, the tissues were again contracted with an EC_{80} concentration of U-46619. Once the contraction had stabilized, a 10 fold cumulative concentration-response curve to either of the ET_B receptor-selective agonists, sarafotoxin S6c (S6c) or alanine$^{[1,3,11,15]}$ET-1 ([Ala^4]ET-1) (both 0.1 nM–1 μM) or vehicle was constructed.

Effects of agonists: contractile responses

Endothelium-denuded preparations were contracted with an approximate EC_{80} concentration (0.1 μM) of U-46619 and tissues which showed greater than 10% relaxation to a subsequent acetylcholine challenge (1 μM) were discarded. Tissues were then washed four times at 5 min intervals; 45 min later, cumulative three fold concentration-response curves to an endothelin isopeptide were constructed. In some tissues, after a maximum response to ET-3 had been attained, a single maximal concentration (1 μM) of ET-1 was added to determine whether any additional contractile response could be achieved.

Agonist interaction studies

Endothelium-denuded preparations were contracted with an approximate EC_{50} concentration of ET-1 (concentration required to evoke 50% of the maximum response). When the contraction had stabilized, the ET_B receptor-selective agonist, S6c (30 nM) was added and any response observed was compared to that which occurred in a time-matched vehicle control tissue.

Antagonist studies

Initial attempts to wash out ET-1 following the construction of a concentration-response curve were unsuccessful, so consecutive reproducible concentration-response curves could not be obtained. Therefore, for a given endothelium-denuded preparation, contractile responses to ET-1 could not be studied in the absence and subsequent presence of an antagonist. Hence, antagonists were added 45 min (or in come cases 15 min) prior to the construction of a 3 fold cumulative curve to either ET-1 or ET-3 and responses compared with those observed in time-matched vehicle-treated control preparations.

Expression of results

Agonist studies Agonist-induced contractile responses were expressed as a percentage of the response to the initial U-46619 challenge and concentration-response curves were plotted. Where appropriate, geometric mean EC_{50} values with 95% confidence intervals were determined for each agonist.

Antagonist studies Agonist concentration-ratios in the absence and presence of antagonist were calculated at the EC_{50} level. Antagonist potencies were expressed as estimated pA_2 values. These were derived from single concentrations of antagonist according to Schild (1957), using the following equation and assuming a slope (– n) of unity:

$$\log (x\text{-}1) = \log K - n\ pA_x$$

where, x is the concentration-ratio, and K is the antagonist affinity constant. Estimated mean pA_2 values are presented as arithmetic mean ± s.e.mean.

Where required, differences between groups of data were tested for statistical significance by Student's t test for unpaired data.

Drugs

ET-1, ET-3 and sarafotoxin S6c were purchased from Peptide and Protein Research Consultants (England) or Peptide Institute Inc (Japan). Acetylcholine, phosphoramidon and bestatin were purchased from Sigma Chemical Company Ltd. IRL1038 ([Cys^{11}-Cys^{15}]-endothelin 1(11-21)) was obtained from Cambridge Research Biochemicals (England). U-46619 (15S)-hydroxy-11α, 9α-(epoxymethano) prosta -5z, 13E-dienoic acid), [Ala^4]ET-1 and BQ123 (cyclo(-D-Val-Leu-D-Trp-D-Asp-Pro) were synthesized by Glaxo Research and Development Ltd. All chemicals for the Krebs-Henseleit solution were of Analar grade and obtained from BDH Ltd. (UK). Drugs were dissolved in distilled water with the following exceptions. ET-1, S6c and [Ala^4]ET-1 were dissolved in dimethylsulphoxide, whilst ET-3 and IRL1038 were dissolved in 0.1 M ammonium acetate. U-46619 was dissolved in 0.1 M $NaHCO_3$. All drugs were stored as frozen aliquots (1 mM) and defrosted for use as required. Dilutions of drugs to working concentrations were made with distilled water.

Results

Agonist profile

Cumulative addition of ET-1 (0.1 nM–1 μM) and ET-3 (0.1 nM–3 μM) produced concentration-dependent contractions of endothelium-denuded preparations. ET-3 was less potent than ET-1 (approx. 12 fold) and evoked a significantly smaller maximum response (Figure 1; Table 1). In eight tissues, after constructing a concentration-response curve to ET-3 (0.3–3 μM) and obtaining a maximum response, addition of ET-1 (1 μM) evoked a further significant increase in tension (from 98.9 ± 7.7 to 130.4 ± 8.2% of the initial U-46619-induced contraction).

In contrast to ET-1 and ET-3, the selective ET_B receptor agonists, S6c (Williams *et al.*, 1991) and [Ala^4]ET-1 (Saeki *et al.*, 1991) (0.1 nM–3 μM), had no contractile effect (Figure 1). S6c (30 nM) also failed to evoke any contractile response when added to endothelium-denuded preparations ($n = 5$) pre-contracted with an approximate EC_{50} concentration (10 nM) of ET-1 (data not shown).

In endothelium-intact preparations pre-contracted with an EC_{80} concentration of the thromboxane A_2-mimetic, U-46619, the selective ET_B receptor agonists, S6c and [Ala^4]ET-1 (0.1 nM–1 μM), failed to elicit any relaxant or contractile response (Figure 2).

Antagonist studies

Effects of antagonists on ET-1-induced contractile responses Incubation with the ET_A receptor-selective antagonist, BQ123 (Ihara *et al.*, 1991), at 0.3, 1 and 3 μM caused a parallel rightward displacement of the concentration-response curves to ET-1, with no significant change in the maximum response (Figure 3). However, Figure 3 also illustrates that BQ123 was a rather weak and inconsistent antagonist of the effects of ET-1, and consequently the antagonism appeared poorly concentration-related. Estimates of the pA_2 were made at each concentration of antagonist and were in the range 5.8 to 6.3 (Table 2). When the incubation period with BQ123 was reduced from 45 to 15 min ($n = 4$), the same profile of antagonism was observed and the potency of BQ123 was not altered (data not shown).

The putative ET_B receptor antagonists, IRL1038 (Urade *et al.*, 1992), at a concentration of 10 μM, caused no rightward displacement of the ET-1 concentration-response curve and did not affect the maximum response observed ($n = 4$; data not shown). Furthermore, a combination of IRL1038 (10 μM) with BQ123 (3 μM) resulted in no greater antagonism than that exerted by BQ123 (3 μM) alone (Figure 4). The mean EC_{50} for ET-1 in the presence of IRL1038 and BQ123 (12.7, 5.6–29 nM) was not significantly different from that observed in the presence of BQ123 alone (43, 12.3–152 nM).

Effects of BQ123 on ET-3-induced contractile responses At a single concentration of 0.3 μM, BQ123 caused a marked rightward displacement of the ET-3 concentration-response curve without reducing the maximum response (Figure 5), and a mean pA_2 estimate of 7.6 ± 0.2 ($n = 5$) was calculated. Thus, BQ123 was approximately 20–60 fold more potent as an antagonist of ET-3 in comparison with ET-1-induced contractions.

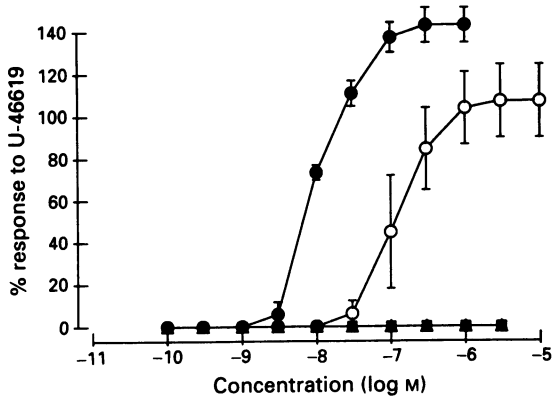


Figure 1 Contractile effects of endothelin-1 (●), endothelin-3 (○), [Ala^4]ET-1 (▲) and sarafotoxin S6c (■) on rat endothelium-denuded renal artery rings. Values are arithmetic mean ± s.e.mean ($n = 5$–6).

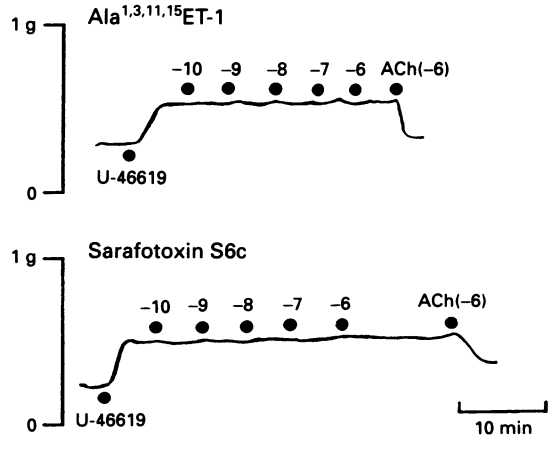


Figure 2 Example trace showing the effects of the selective ET_B receptor agonists, [Ala^{1,3,11,15}]ET-1 and sarafotoxin S6c, in rat endothelium-intact renal artery rings pre-contracted with U-46619 (0.1 μM).

Table 1 Contractile potencies of endothelins and their analogues in endothelium-denuded rat renal artery rings ($n = 5$–6)

Compound	*EC_{50}* (nM)	*95% confidence limits*	*Maximum response* (% U-46619)	*s.e.mean*
ET-1	9.8	7.6–12.5	143	8.4
ET-3	120*	56.4–251	107*	7.8
[Ala^4]ET-1	Inactive up to 1 μM			
S6c	Inactive up to 1 μM			

*$P < 0.05$ ET-1 vs ET-3, Student's unpaired t test.

Table 2 Mean concentration-ratios (CR) and estimated pA_2 values for the ET_A receptor-selective antagonist, BQ123, versus endothelium-1 in endothelium-denuded rat renal artery rings

	BQ123 (μM) 0.3	1.0	3.0
CR	1.5 ± 0.6	3.8 ± 0.9	4.9 ± 1.4
Estimated pA_2	< 6.5	6.3 ± 0.2	5.8 ± 0.3

Values are mean ± s.e.mean; $n = 4$–6.

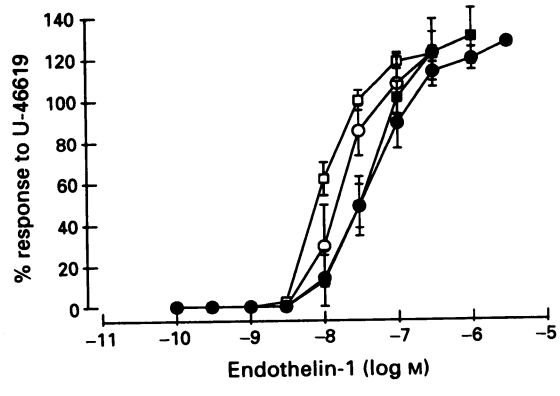


Figure 3 Effects of the ET_A receptor-selective antagonist, BQ123, (control, □; BQ123 0.3 ○, 1 ■ and 3 μM ●) on contractile responses to endothelin-1 in rat endothelium-denuded renal artery rings. Values are arithmetic means ± s.e.mean ($n = 4$–6).

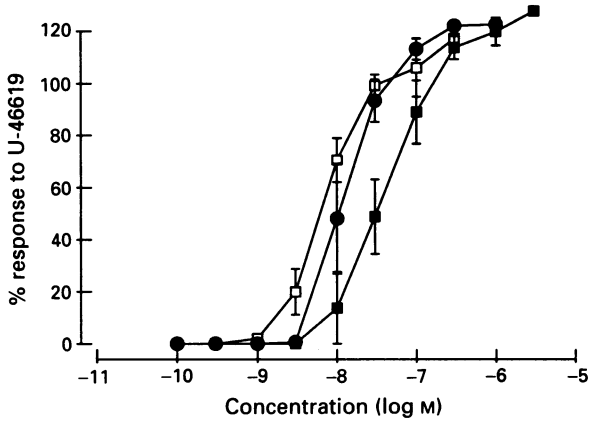


Figure 4 Effects of the ET_A receptor-selective antagonist, BQ123 (3 μM), and a combination of BQ123 (3 μM) and the ET_B receptor selective antagonist, IRL1038 (10 μM), on contractile responses to endothelin-1 in rat endothelium-denuded renal artery rings: (□) control; (■) BQ123 alone; (●) BQ123 & IRL1038. Values are arithmetic mean ± s.e.mean ($n = 5$).

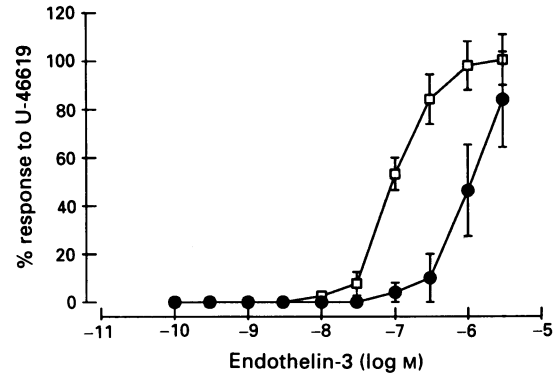


Figure 5 Effects of the ET_A receptor-selective antagonist, BQ123, (control, □; BQ123 0.3 μM, ●) on contractile responses to endothelin-3 in rat endothelium-denuded renal artery rings. Values are arithmetic means ± s.e.mean ($n = 5$).

Discussion

There is growing evidence to suggest that endothelin may contribute to the pathology of certain forms of acute and chronic renal disease (Kon & Badr, 1991; Perico & Remuzzi, 1993; Benigini *et al.*, 1993). Consequently, it is possible that endothelin receptor antagonists may find a role in the treatment of these conditions. However, in order to identify appropriate antagonists, it is essential to have an understanding of the location and function of renal endothelin receptors. The aim of this paper was to study the function and characteristics of endothelin receptors in rat main branch renal artery *in vitro*; a test system which should allow for quantitative receptor characterization.

The initial approach taken was to compare the potencies of various endothelin receptor agonists. The results indicate that ET-1 and ET-3 both contract rat renal artery, although ET-1 is more potent (~12 fold) than ET-3 and induces a slightly but significantly greater maximal response. This profile of agonist action is consistent with that seen in other ET_A receptor containing tissues, such as rabbit carotid artery and rat thoracic aorta (Moreland *et al.*, 1992; Sumner *et al.*, 1992). In contrast to these two agonists, neither of the ET_B receptor-selective agonists, sarafotoxin S6c nor [Ala^4]ET-1, induced any contractile effect at concentrations up to 30 μM, nor did they induce relaxation of pre-contracted endothelium-intact renal arteries. Thus, it appeared that neither ET_B relaxant nor contractile receptors were present. The agonist profile, therefore, suggested that rat renal artery contains exclusively ET_A receptors. However, classification of receptors solely on the basis of rank orders of agonist potencies can be misleading. Consequently, we went on to test our preliminary hypothesis using the ET_A receptor-selective antagonist, BQ123 (Ihara *et al.*, 1991).

It has previously been demonstrated by several groups (Ihara *et al.*, 1991; Sumner *et al.*, 1992; Moreland *et al.*, 1992) that in ET_A receptor containing tissues (e.g. rabbit carotid artery, pig coronary artery, rat thoracic aorta) BQ123 competitively antagonizes the contractile effects of ET-1, with pA_2 values in the range 6.9–7.5. However, in the present study BQ123 was a relatively weak and inconsistent antagonist of ET-1; pA_2 estimates were in the range 5.8–6.3. It seems unlikely that the weak antagonism exerted by BQ123 was due to breakdown or uptake of the cyclic peptide antagonist since (a) all experiments were carried out in the presence of the peptidase inhibitors, phosphoramidon and bestatin, and (b) BQ123 still exerted weak and inconsistent antagonism when the contact time was reduced from 45 to 15 min.

Previous workers (LaDouceur *et al.*, 1993) reported that BQ123 was a weak antagonist of ET-1-induced contractions of rabbit pulmonary artery and demonstrated that this was a result of the tissue containing a mixed population of ET_A and ET_B receptors. Thus, an alternative hypothesis to account for the low potency of BQ123 in the present study could be that a small population of contractile ET_B receptors are present in rat renal artery. Although the agonist profile suggested that ET_B receptors were not in this tissue (since the selective ET_B receptor agonists tested failed to evoke a contractile or relaxant response), we questioned whether it was possible that ET_B receptors were present, but unable to elicit a response under basal conditions. Hence, we postulated that when ET_A receptors were stimulated and the tissue partially contracted, ET_B receptor stimulation may also trigger a contractile response. This scenario could explain the low potency of BQ123, since even when ET_A receptors were blocked, ET_B receptors could continue to mediate the contractile effects of ET-1. To test this possibility, we examined whether co-incubation with BQ123 and the putative ET_B receptor antagonists, IRL1038 (Urade *et al.*, 1992), resulted in greater antagonism of the effects of ET-1 than that observed in the presence of BQ123 alone. This was not the case, although it is worth noting that Urade and co-workers (1994) have recently raised concerns regarding variation between experimental batches in the affinity or IRL1038 for ET_B receptors. Nevertheless, further evidence that ET_B receptors are absent from rat renal artery was derived from the observation that when tissues were pre-contracted with an approximate EC_{50} concentration of ET-1, the ET_B receptor-selective agonist, S6c, failed to elicit any further contractile response. Thus, we feel it is unlikely that the weak antagonism exerted by BQ123 in this tissue can be explained by the argument that a small population of ET_B contractile receptors are present.

Given these data, we begin to consider the speculative but intriguing possibility that rat renal artery contains both ET_A receptors and a novel subtype of contractile endothelin receptor which is BQ123-insensitive. To investigate this possibility further, we went on to study the ability of BQ123 to antagonize another endothelin receptor agonist, ET-3. Interestingly, BQ123 was more potent (approximately 20–60 fold) as an antagonist of ET-3 and the pA_2 value (7.6) obtained was consistent with an action at ET_A receptors. The increased potency of BQ123 as an antagonist of ET-3 relative to ET-1 supports the possibility of receptor heterogeneity in this tissue since in a single receptor system the potency of an antagonist should be independent of the agonist studied. Thus, the data can be explained by the hypothesis that ET-3 contracts rat renal artery solely via an action on ET_A recep-

tors, while ET-1 may stimulate both ET_A receptors and a novel endothelin receptor subtype which has low affinity for BQ123. Consistent with this view is the observation that ET-1 can evoke an additional contractile response when added on top of a maximal ET-3-induced contraction. Moreover, although carried out using a modified Krebs solution, we have also found (unpublished observations) that while ET-1 causes cross desensitization in rat renal artery, ET-3 desensitizes responses to itself, but does not alter sensitivity to ET-1. These data support the hypothesis that ET-1 recognises a subtype of endothelin receptor not bound by ET-3.

Alternatively, it could be argued that the weak antagonism of ET-1 by BQ123 may be due to the kinetics of the interaction of ET-1 with ET_A receptors. Radioligand binding studies indicate that ET-1 binds almost irreversibly to ET_A receptors in vascular smooth muscle cells (Hirata *et al.*, 1988). Thus, the weak antagonism by BQ123 of ET-1-induced contractions in rat renal artery could reflect an inability of the antagonist to displace bound ET-1 from rat vascular ET_A receptors. However, this seems unlikely since studies on rat aorta (Sumner *et al.*, 1992), under almost identical conditions to the present work, show that BQ123 competitively antagonizes ET-1-induced contractions with a pA_2 of 6.9. Thus, we feel that the present data raise the possibility that rat renal artery contains a novel subtype of BQ123-resistant endothelin receptor. Since the concentration-response curve to ET-1 in the presence of BQ123 was not biphasic, this suggests that the putative population of BQ123-resistant receptors must be able to mediate a full contractile response to ET-1. A question of interest is whether a similar receptor subtype may occur in the human renal vasculature. Present evidence suggests this is unlikely. Maggi and co-workers (1990) reported that human renal artery showed an agonist selectivity profile consistent with it containing ET_A receptors while a very recent study by Maguire *et al.* (1994) found no evidence to suggest that human renal artery contains a novel BQ123-insensitive endothelin receptor.

The possible existence of a novel contractile endothelin receptor subtype in rat renal artery is supported by reports that a similar BQ123-resistant receptor subtype may occur in other vascular and non-vascular tissues including rat vas deferens (Eglezos *et al.*, 1993), guinea-pig iliac artery (Schoeffter *et al.*, 1993) and rabbit renal artery (Moreland *et al.*, 1994). Moreover, recent evidence suggests that a novel BQ123-resistant receptor may occur in human vascular smooth muscle preparations including saphenous vein (Bax *et al.*, 1993a), coronary artery (Bax *et al.*, 1993b; Godfraind *et al.*, 1993) and umbilical artery (Bodelsson & Stjernquist, 1993). Assessment in these tissues of the antagonist potencies of recently reported non-peptide ET_A receptor antagonists, such as Ro 46-2005 (Clozel *et al.*, 1993) and BMS182,874 (Stein *et al.*, 1994), may help define whether they truly possess a novel endothelin receptor subtype. Similarly, investigations at the molecular level should help determine whether an additional contractile endothelin receptor subtype is present in smooth muscle.

While studies using isolated conductance arteries *in vitro* allow for quantitative characterization of receptors it cannot be assumed that the same subtypes of receptor occur in the microvasculature. Interestingly, the ineffective antagonism by BQ123 or ET-1-induced contractions in rat main branch renal artery has also been observed in the rat renal microvasculature by workers using perfused kidneys or anaesthetized rat preparations (Wellings *et al.*, 1993; Cristol *et al.*, 1993; Pollock & Opgenorth, 1993). However, in contrast to the present results from main branch renal artery, these workers (Wellings *et al.*, 1993; Cristol *et al.*, 1993) found that ET_B receptors play an important role in mediating the contractile effects of ET-1 in renal microvasculature. If confirmatory evidence can be found for a BQ123-insensitive endothelin receptor subtype, then it will be important to determine whether it is present in renal microvessels and plays a role in regulating glomerular blood flow.

In conclusion, we have shown that rat main branch renal artery does not contain ET_B receptors mediating either relaxation or contraction. The contractile effects in this tissue of ET-3, and to some extent ET-1, appear to be mediated by ET_A receptors. However, the weak antagonism exerted by the ET_A receptor-selective antagonist, BQ123, against the contractile effects of ET-1 raises the possibility that in rat renal artery, ET-1 may stimulate a novel subtype of BQ123-insensitive endothelin receptor.

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References

ARAI, H., ARAMORI, I., HIROAKI, O. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BAX, W.A., BOS, E. & SAXENA, P.R. (1993a). Heterogeneity of endothelin/sarafotoxin receptors mediating contraction of the human isolated saphenous vein. *Eur. J. Pharmacol.*, **239**, 267–268.

BAX, W.A., PETTERSON, R.W.G., INAN, T., BOS, E. & SAXENA, P.R. (1993b). Heterogeneity of endothelin/sarafotoxin receptors mediating contractions of human isolated coronary artery. *Br. J. Pharmacol.*, **111**, 15P.

BENIGNI, A., ZOJA, C., CORNA, D., ORISIO, S., LONGARETTI, L., BERTANI, T. & REMUZZI, G. (1993). A specific endothelin subtype A receptor antagonist protects against injury in renal disease progression. *Kidney Int.*, **44**, 440–444.

BODELSSON, G. & STJERNQUIST, M. (1993). Characterization of endothelin receptors and localization of ^{125}I-endothelin-1 binding sites in human umbilical artery. *Eur. J. Pharmacol.*, **249**, 299–305.

CLOZEL, M., BREU, V., BURRI, K., CASSAL, J.M., FISCHLI, W., GRAY, G., HIRTH, G., LOFFLER, B.M., MULLER, M., NEIDHART, W. & RAMUZ, H. (1993). Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature*, **365**, 759–761.

CRISTOL, J.P., WARNER, T.D., THIEMERMANN, C. & VANE, J.R. (1993). Modulation via different receptors of the vasoconstrictor effects of endothelins and sarafotoxins in the systemic circulation and renal vasculature of the anaesthetized rat. *Br. J. Pharmacol.*, **108**, 776–779.

EGLEZOS, A., CUCCHI, P., PATACCHINI, R., QUARTARA, L., MAGGI, C.A. & MIZAHRI, J. (1993). Differential effects of BQ123 against endothelin-1 and endothelin-3 on the rat vas deferens: evidence for an atypical endothelin receptor. *Br. J. Pharmacol.*, **109**, 736–738.

ELSHOURBAGY, N.A., KORMAN, D.R., WU, H.L., SYLVESTER, D.R., LEE, J.A., NUTHALGANTI, P., BERGSMA, D.J., KUMAR, C.S. & NAMBI, P. (1993). Molecular characterization and regulation of the human endothelin receptors. *J. Biol. Chem.*, **268**, 3873–3879.

FURCHGOTT, R.F. & ZAWADSKI, J.V. (1980). The obligatory role of the endothelium in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GODFRAIND, T. (1993). Evidence for heterogeneity of endothelin receptor distribution in human coronary artery. *Br. J. Pharmacol.*, **110**, 1201–1205.

HIRATA, Y., YOSHIMI, H., TAKATA, S., WATANABE, T.X., KUMAGAI, S., NAKAJIMA, K. & SAKAKIBARA, S. (1988). Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **154,** 868–875.

IHARA, M., NOGUCHI, K., TOSHIHIKO, S., FUKORODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, S., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1991). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50,** 247–255.

KON, V. & BADR, K.F. (1991). Biological actions and pathophysiologic significance of endothelin in the kidney. *Kidney Int.*, **40,** 1–12.

LADOUCEUR, D.M., FLYNN, M.A., KEISER, J.A., REYNOLDS, E. & HALEEN, S.J. (1993). ET_A and ET_B receptors coexist on rabbit pulmonary artery vascular smooth muscle mediating contraction. *Biochem. Biophys. Res. Commun.*, **196,** 209–215.

LANESE, D.M., YUAN, B.H., MCMURTY, I.F. & CONGER, J.D. (1992). Comparitive sensitivities of isolated rat renal arterioles to endothelin. *Am. J. Physiol.*, **263,** F894–F899.

LIPPTON, H., GOFF, J. & HYMAN, A. (1988). Effects of endothelin in the systemic and renal vascular beds *in vivo*. *Eur. J. Pharmacol.*, **155,** 197–199.

MAGGI, C.A., GIULIANI, S., PATACCHINI, R., BARBANTI, G., TURINI, D. & MELI, A. (1990). Contractile responses of the human urinary bladder, renal pelvis and renal artery to endothelins and sarafotoxin S6b. *Gen Pharamcol.*, **21,** 247–249.

MAGUIRE, J.J., KUC, R.E., O'REILLY, G.O. & DAVENPORT, A.P. (1994). Vasoconstrictor endothelin receptors characterized in human renal artery and vein *in vitro*. *Br. J. Pharmacol.*, **113,** 49–54.

MORELAND, S., MCMULLEN, D.M., DELANEY, C.L., LEE, V.G. & HUNT, J.T. (1992). Venous smooth muscle contains vasoconstrictor ET_B-like receptors. *Biochem. Biophys. Res. Commun.*, **184,** 100–106.

MORELAND, S., MCMULLEN, D., ABBOA-OFFEI, B. & SEYMOUR, A. (1994). Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature. *Br. J. Pharmacol.*, **112,** 704–708.

PERICO, N. & REMUZZI, G. (1993). Role of endothelin in glomerular injury. *Kidney Int.*, **43,** S76–S80.

PERNOW, J., BOUTIER, J.F., FRANCO-CERECEDA, A., LACROIX, J.S., MATRAN, R. & LUNDBERG, J.M. (1988). Potent selective vasoconstrictor effects of endothelin in the pig kidney *in vivo*. *Acta Physiol. Scand.*, **134,** 573–574.

PIERRE, L. & CLARK, K.L. (1994). Characteristics of endothelin receptors in rat main branch renal artery. *Br. J. Pharmacol.*, **112,** 163P.

POLLOCK, D.M. & OPGENORTH, T.J. (1993). Evidence for endothelin-induced renal vasoconstriction independent of ET_A receptor activation. *Am. J. Physiol.*, **264,** R222–R226.

SAEKI, T., IHARA, M., FUKURODA, T., YAMAGIWA, M. & YANO, M. (1991). [$Ala^{1,3,11,15}$]Endothelin-1 analogs with ET_B agonist activity. *Biochem. Biophys. Res. Commun.*, **179,** 286–292.

SUKURAI, T., YANIGASAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348,** 732–735.

SCHILD, H.O. (1957). Drug antagonism and pA_x. *Pharmacol. Rev.*, **9,** 242–246.

SCHOEFFTER, P., RANDRIANTSOA, A., JOST, B. & BRUTTEL, K. (1993). Comparitive effects of the two endothelin ET_A receptor antagonists, BQ123 and FR139317, on endothelin-1-induced contraction in guinea pig iliac artery. *Eur. J. Pharmacol.*, **241,** 165–169.

SOKOLOVSKY, M., AMBAR, I. & GALRON, R. (1993). A novel subtype of endothelin receptor. *J. Biol. Chem.*, **267,** 2052–2054.

STEIN, P.D., HUNT, J.T., FLOYD, D.M., MORELAND, S., DICKINSON, K.E.J., MITCHELL, C., LIU, E.C.K., WEBB, M.L., MURUGESAN, N., DICKEY, J., MCMULLEN, D., ZHANG, R., LEE, V.G., SERAFINO, R., DELANEY, C., SCHAEFFER, T.R. & KOZLOWSKI, M. (1994). The discovery of sulfonamide endothelin antagonists and the development of the orally active ET_A antagonist 5-(dimethylamino)-N-(3,4-dimethyl-5-ispxazolyl)-1-naphthalenesulfonamide. *J. Med. Chem.*, **37,** 329–331.

SUMNER, M.J., CANNON, T.R., MUNDIN, J.W., WHITE, D.G. & WATTS, I.S. (1992). Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. *Br. J. Pharmacol.*, **107,** 858–860.

URADE, Y., FUJITANI, Y., ODA, K., WATAKABE, T., UMEMURA, I., TAKAI, M., OKADA, T., SAKATA, K. & KARAKI, H. (1992). An endothelin B receptor-selective antagonist: IRL1038, [Cys^{11}-Cys^{15}]-endothelin-1(11–21). *FEBS Lett.*, **311,** 12–16.

URADE, Y., FUJITANI, Y., ODA, K., WATAKABE, T., UMEMURA, I., TAKAI, M., OKADA, T., SAKATA, K. & KARAKI, H. (1994). Retraction concerning an endothelin B receptor-selective antagonist. *FEBS Lett.*, **342,** 103.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol.*, **110,** 777–782.

WELLINGS, R.P., WARNER, T.D., CORDER, R., CRISTOL, J.P., THIEMERMANN, C. & VANE, J.R. (1993). Vasoconstriction in the rat kidney induced by endothelin-1 is blocked by PD145065. *J. Cardiovasc. Pharmacol.*, **22,** S103–S106.

WILLIAMS, D.L., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESCMIDT, B.V. (1991). Sarafotoxin S6c: An agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.*, **175,** 556–561.

WRIGHT, C.E. & FOZARD, J.R. (1988). Regional vasodilatation is a prominent feature of the haemodynamic response to endothelin in anaesthetized, spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155,** 201–203.

YANIGASAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332,** 411–415.

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Effects of portal hypertension on responsiveness of rat mesenteric artery and aorta

Teresa Cawley, *J. Geraghty, *H. Osborne & [1]J.R. Docherty

Department of Physiology, Royal College of Surgeons in Ireland, Dublin 2 and *Department of Surgery, Beaumont Hospital, Dublin 9, Ireland.

1 We have examined the effects of pre-hepatic portal hypertension on the responsiveness of rat small mesenteric arteries and aorta. Rats were made portal hypertensive by creating a calibrated portal vein stenosis, or sham-operated.

2 In rat mesenteric arteries, there was no significant difference between portal hypertensive and sham-operated animals in the contractile potency of noradrenaline (NA), but the maximum contractile responses to NA, U46619 and KCl were significantly increased in vessels from portal hypertensive animals. This altered maximum contractile response was not due to alterations in smooth muscle mass.

3 In rat mesenteric arteries, there were no significant differences between portal hypertensive and sham-operated animals in endothelium-dependent relaxations to acetylcholine (ACh). The difference between portal hypertensive and sham-operated rats in the maximum response to U46619 was maintained following a combination of methylene blue (1 μM) and N^G-monomethyl-L-arginine (100 μM), suggesting that any differences in endothelial function do not explain differences in the response to vasoconstrictors.

4 In rat aorta, there were no significant differences between portal hypertensive and sham-operated animals in the contractile response to NA or KCl or in the endothelium-dependent relaxations to ACh.

5 In pithed rats, there was no difference between portal hypertensive and sham-operated animals in the pressor potency of NA.

6 It is concluded that portal hypertension produces an increase in the contractile response to the vasoconstrictors NA, U46619 and KCl in rat mesenteric arteries but not in the aorta. This suggests that the diminished responsiveness to vasoconstrictors reported in portal hypertensive rats *in vivo* is not due to a diminished responsiveness at the level of the vascular smooth muscle.

Keywords: Portal hypertension; noradrenaline; endothelium-dependent relaxations; mesenteric artery; acetylcholine; U46619; N^G-monomethyl-L-arginine (L-NMMA)

Introduction

The pre-hepatic portal hypertensive rat is a widely used model of human portal hypertension characterized by the development of portal-systemic shunting (Sikuler *et al.*, 1985; Geraghty *et al.*, 1989; Bosch *et al.*, 1992). It is generally accepted that shunts develop in response to the increased portal pressure, but that a hyperdynamic circulatory state follows, characterized by increased cardiac output and increased splanchnic blood flow. The increased cardiac output and splanchnic blood flow may be a result of diminished responsiveness to vasoconstrictors and/or to the increased entry of gastrointestinal vasodilator hormones into the circulation due to shunting, and vasodilator substances such as glucagon have been implicated (Benoit *et al.*, 1984).

A component of the increased splanchnic blood flow found in portal hypertension may be due to increased function of the vascular endothelium (Valance & Moncada, 1991) or decreased mesenteric vascular responsiveness to vasoconstrictors. Indeed, many authors have reported decreased vascular responsiveness to vasoconstrictors such as noradrenaline (NA) (Kiel *et al.*, 1985; Pizcueta *et al.*, 1990) in the mesenteric vascular bed of portal hypertensive rats *in situ*. Similar findings have been reported for angiotensin but not NA (Murray & Paller, 1985; Villamediana *et al.*, 1988) in the mesenteric vascular bed of cirrhotic rats *in situ*. In contrast, Joh *et al.* (1993) report a diminished vasodilator response to α-adrenoceptor antagonists in the mesenteric bed of portal hypertensive rats, suggesting diminished sympathetic drive: diminished release of noradrenaline might be expected to cause up-regulation of receptors and increased responsiveness to exogenous noradrenaline. However, the presence of circulating vasodilators in these *in situ* studies probably influenced the actions of vasoconstrictors. Other authors have examined isolated blood vessels. Leehey (1993) found a decreased pressor sensitivity to angiotensin *in vivo*, but no change in the contractile response to angiotensin in isolated aorta from cirrhotic rats, although Castro *et al.* (1993) found decreased responsiveness to angiotensin in aorta. Rat aortic response to NA is reported to be increased or decreased by portal hypertension of 10 or 21 day duration, respectively (Bomzon & Blendis, 1987; Bomzon *et al.*, 1991). Cummings *et al.* (1986) found an increased maximum contraction to 5-hydroxytryptamine (5-HT) and vasopressin, but not to NA, in mesenteric veins from portal hypertensive rats, and no change in the responsiveness to NA in mesenteric arteries. Hence, whereas most studies of pressor responses *in vivo* report a diminished responsiveness to vasoconstrictors in portal hypertension, conflicting results have been obtained *in vitro*.

The object of the present study was to investigate whether responses to vasoconstrictors are altered by portal hypertension by examining isolated mesenteric arteries from portal hypertensive and sham-operated rats in their responsiveness to NA, the thromboxane-mimetic, U46619 and KCl in the absence of the influence of vasodilators. Responses to vasoconstrictors in mesenteric arteries might be expected to be reduced or even increased depending on whether these alterations were responsible for, or in response to, the hyper-

[1] Author for correspondence.

dynamic circulation. Responses in mesenteric arteries were compared with responses obtained in the aorta, and in the pithed rat preparation. Secondly, endothelium-dependent responses were investigated to assess whether altered endothelial function occurred. Some of these results have been published in abstract form (Cawley *et al.*, 1994).

Methods

Male Wistar rats (200–400 g) were obtained from Trinity College, Dublin, and tissues were obtained from portal hypertensive or sham-operated animals.

Surgical preparation

Animals were anaesthetized with ether, a midline incision was made in the abdomen, and the portal vein exposed. A 21 gauge needle was placed alongside the portal vein and a suture was tied around both; the needle was then removed resulting in a calibrated stenosis. The abdomen was then closed and animals were examined 14 days after surgery. In sham-operated animals, the portal vein was exposed but no suture was placed around it.

Recording of pressures

Animals were anaesthetized with ether, and maintained with pentobarbitone (Nembutal, 10% solution) injected into the jugular vein following cannulation. The ileocaecal vein and carotid artery were cannulated and connected to blood pressure transducers for pressure measurements.

Radioactive microspheres

Animals were anaesthetized with ether and the ileocaecal vein was cannulated. Radioactive microspheres (^{57}Co, 15 μm diameter, approximately 100,000) were suspended in 10% dextran with 0.01% of the surfactant Tween 80 to prevent clumping, and injected in a volume of 1 ml kg^{-1}. After 10 min, animals were killed and the lung, liver and kidney removed, chopped up and counted in a gamma scintillation counter. The degree of shunting was assessed from the equation

$$\% \text{ shunting} = \text{lung c.p.m.} \times 100/(\text{lung c.p.m.} + \text{liver c.p.m.}).$$

Kidney radioactivity was sampled to assess leaching of microspheres, but radioactivity in kidney samples was not significantly different from background so that no significant leaching occurred.

Pithed rat preparation

Animals were anaesthetized with ether and a tracheal cannula was placed. The rats were then pithed and respired artificially with 100% O_2 (Gillespie *et al.*, 1970; Docherty, 1988). The carotid artery was cannulated for the recording of blood pressure. Following a 30 min equilibration period, a dose-response curve was carried out to NA (10 ng kg^{-1}–100 μg kg^{-1}), injected into the jugular vein in 0.5 log unit increments at intervals of 3–5 min.

Aorta

Following stunning and exsanguination, aortic rings of 3–5 mm in length were attached to myograph transducers under 1 g tension in organ baths at 37°C in Krebs-Henseleit solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0 and EDTA 0.03. Additionally, cocaine (3 μM), propranolol (3 μM) and indomethacin (10 μM) were present. Tissues were contracted with KCl (40 mM), exposed to ACh (10 μM) to test for endothelium-dependent relaxations (EDR), and washed. Bathing fluid was then changed every 15 min for the next hour. Tissues were then contracted with noradrenaline (NA) (1 μM), with KCl (40 mM). and when contraction had reached a maximum, ACh (or an equivalent volume of vehicle) was administered cumulatively in 1 log unit increments beginning with 10 nM. After the final concentration of ACh, sodium nitroprusside (SNP) (100 μM) was administered. In some experiments, cumulative concentration-response curves were carried out to NA in 0.5 log unit increments, beginning with 1 nM.

Mesenteric arteries

Following stunning and exsanguination, the intestines and mesentery were removed and placed in Krebs-Henseleit solution of the same composition as above. By use of a dissecting microscope, a segment of rat small mesenteric artery, approximately 1.5 mm in length, corresponding to a first or second order branch of the superior mesenteric artery, was carefully dissected free from its vein. The artery was mounted in a small vessel myograph with 40 μm tungsten wires (Mulvany & Halpern, 1977). Data were recorded on a dual channel electronic display recorder (Dual Myograph MK-4) and pen recorder. Vessels were allowed to equilibrate at 37°C and gassed with 5% CO_2 in O_2. The vessel was set to a tension generated at 0.9 times the diameter of the vessel at 100 mmHg transmural pressure (Mulvany & Warshaw, 1977). Experiments were carried out exactly as described for rat aorta, except that vessels were contracted with a higher concentration of NA (10 μM). The maximum response to the thromboxane-mimetic, U46619, was also obtained, the highest concentration employed was 20 μM, due to cost of this agent, although this concentration gave no further increase in contractile response over that obtained to 1–10 μM. Responses to U46619 were also obtained following inhibition of nitric oxide synthase and guanylate cyclase with a combination of L-NMMA (100 μM) and methylene blue (1 μM) for 1 h. Administration of L-NMMA sometimes produced a contraction, but this appeared to be due to the presence of small amounts of the previously administered vasoconstrictor after washout. Tension returned to baseline before the second administration of U46619 (see also Rees *et al.*, 1990).

Microscopy

Wall thickness of mesenteric arteries was measured by light microscopy and a micrometer eyepiece with the vessel diameter at 0.9 times the diameter at 100 mmHg transmural pressure, and was the average of up to 10 determinations for each vessel.

Drugs

The following were used: acetylcholine chloride (Sigma, Poole, U.K.); cocaine hydrochloride (Sigma); indomethacin (Sigma); methylene blue (Aldrich); L-NMMA (N^G-monomethyl-L-arginine acetate: Sigma); U46619 (9,11-dideoxy-11α, 9α-epoxy-methanoprostaglandin $F_{2\alpha}$: Sigma); noradrenaline bitartrate (Sigma); propranolol hydrochloride (Sigma); sodium nitroprusside (Sigma). Drugs were dissolved in distilled water, except for indomethacin (100% ethanol).

Statistics

Values are mean ± s.e.mean. Agonist potency was expressed as a $-\log EC_{50}$. Differences between groups were compared by Student's *t* test for unpaired data.

Results

Radioactive microspheres

Effectiveness of surgery was assessed by measuring the degree of portal-systemic shunting using the radioactive microsphere

technique. Degree of shunting was 59.6±7.2% (range 10–98%) ($n = 5$) and 0.08 ± 0.05% (range 0.02–0.24%) ($n = 5$) in 14 day portal hypertensive and 14 day sham rats, respectively (Student's t test: $P<0.05$).

Portal and systemic arterial pressures

Systolic blood pressure was 117.6±3.8 mmHg ($n = 9$), and 130.8 ± 8.0 mmHg ($n = 6$), and diastolic blood pressure was 80.5±3.0 mmHg and 93.7±6.0 mmHg, in anaesthetized portal hypertensive and sham-operated animals, respectively (no significant differences). Portal pressure was 9.5±0.7 mmHg ($n = 5$) in sham-operated animals (14 days), 25.3±3.4 mmHg ($n = 5$) after acute ligation ($P<0.01$) and 14.2±0.6 mmHg ($n = 4$) in 14 day portal hypertensive animals ($P<0.01$ from sham-operated).

Pithed rat preparation

In pithed rats, resting diastolic blood pressure was 41.8±4.0 mmHg ($n = 4$) and 40.0±3.4 mmHg ($n = 4$) in portal hypertensive and sham-operated animals, respectively (no significant difference). NA (10 ng kg^{-1}–100 μg kg^{-1}) produced dose-dependent increases in diastolic blood pressure, but there were no significant differences between portal hypertensive and sham-operated animals in the pressor response (Figure 1).

Aorta

In rat aortic rings, NA produced concentration-dependent contractions with $-\log EC_{50}$ values of 7.61±0.18 and 7.70±0.16 in tissues from portal hypertensive and sham-operated animals, respectively (no significant difference). There were no significant differences between tissues from portal hypertensive and sham-operated animals in the maximum contraction to NA, nor in the contraction to KCl (40 mM) (Table 1).

In tissues pre-contracted with KCl (40 mM), ACh produced relaxations with $-\log EC_{50}$ values of 6.36 ± 0.15 ($n = 6$) and 6.18 ± 0.23 ($n = 6$), and in tissues pre-contracted with NA (1 μM) ACh produced relaxations with $-\log EC_{50}$ values of 6.79±0.18 ($n = 4$) and 6.68±0.17 ($n = 4$), in tissues from portal hypertensive and sham-operated animals, respectively (no significant differences). There were no significant differences between tissues from portal hypertensive and sham-operated animals in the maximum relaxation to ACh, when NA or KCl was used as the contractile agent (Table 2).

Mesenteric arteries

Potency of NA was not significantly altered by portal hypertension (NA $-\log EC_{50}$; portal hypertensive: 6.14±0.07, $n = 11$; sham: 6.0±0.10, $n = 9$), but the maximum contraction to NA was significantly greater in mesenteric arteries from portal hypertensive rats than in vessels from sham-operated rats (see Table 1 and Figure 2). Likewise, the contraction to KCl (40 mM) was significantly greater in mesenteric arteries from portal hypertensive rats than in vessels from sham-operated rats (Table 1 and Figure 3). The maximum contraction to U46619 was also significantly greater in arteries from portal hypertensive rats and this difference was maintained following blockade of the actions of endogenous NO with methylene blue (1 μM) and L-NMMA (100 μM) (Table 1 and Figure 3).

In tissues pre-contracted with KCl (40 mM), ACh produced relaxations with $-\log EC_{50}$ values of 6.77±0.20 ($n = 10$) and 6.86 ± 0.17 ($n = 7$), and in tissues pre-contracted with NA (1 μM) ACh produced relaxations with $-\log EC_{50}$ values of 6.92±0.12 ($n = 10$) and 7.15±0.11 ($n = 7$), in tissues from portal hypertensive and sham-operated animals, respectively (no significant differences). There were no significant differ-

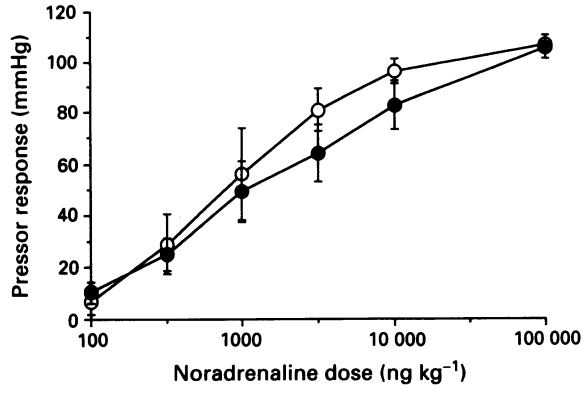


Figure 1 Dose-response curves for the increase in diastolic pressure produced by noradrenaline in pithed sham-operated (○) and portal hypertensive rats (●). Values are the mean ± s.e.mean of 4 experiments.

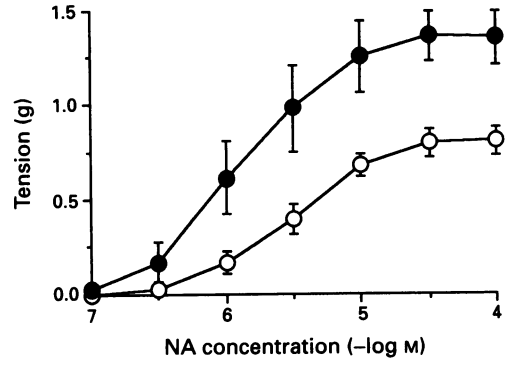


Figure 2 Concentration-response curves to noradrenaline in mesenteric arteries from sham-operated (○) and portal hypertensive rats (●). Values are the mean ± s.e.mean of 9–11 experiments. The maximum contraction to noradrenaline was significantly greater in portal hypertensive animals with no significant change in potency.

Table 1 Maximum contractile response to noradrenaline (NA) and U46619 and contractile response to KCl (40 mM) in rat aorta and mesenteric artery from portal hypertensive and sham-operated animals

	Portal hypertensive	*Sham-operated*
Aorta		
NA	1.35 ± 0.21 g ($n = 6$)	1.17 ± 0.22 g ($n = 6$)
KCl	0.79 ± 0.29 g ($n = 4$)	0.63 ± 0.28 g ($n = 4$)
Mesenteric artery		
NA	1.36 ± 0.12 g** ($n = 11$)	0.82 ± 0.07 g ($n = 9$)
KCl	0.58 ± 0.07 g* ($n = 11$)	0.40 ± 0.02 g ($n = 13$)
U46619	0.92 ± 0.12 g* ($n = 7$)	0.57 ± 0.03 g ($n = 6$)
U46619 (following methylene blue and L-NMMA)	1.26 ± 0.16 g** ($n = 6$)	0.72 ± 0.08 g ($n = 6$)

Responses to U46619 were also obtained following exposure to methylene blue (1 μM) and L-NMMA (100 μM).
Values are mean ± s.e.mean, with n the number of experiments. Asterisks denote responses in tissues from portal hypertensive animals significantly different from responses in tissues from sham-operated animals (Student's t test. *$P<0.05$; **$P<0.01$).

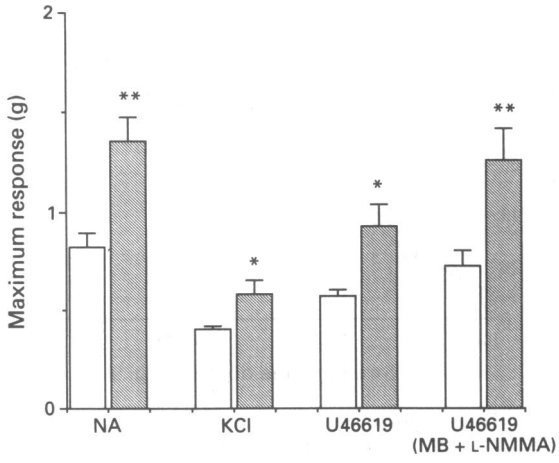


Figure 3 Maximum responses to noradrenaline (NA), KCl (40 mM) and U46619 in mesenteric arteries from sham-operated (open columns) or portal hypertensive (shaded columns) animals. Responses to U46619 in tissues exposed to methylene blue (MB) (1 μM) and N^G-monomethyl-L-arginine (L-NMMA, 100 μM) are also shown. Values are mean with s.e. of mean from at least 6 experiments. Asterisks denote significant difference between responses obtained in tissues from portal hypertensive and sham-operated animals (Student's *t* test: $*P<0.05$; $**P<0.01$).

Table 2 Maximum relaxation to acetylcholine (ACh) in rat aorta and mesenteric artery from portal hypertensive and sham-operated animals, contracted with noradrenaline (NA) (aorta: 1 μM; mesenteric artery: 10 μM) or KCl (40 mM)

	Portal hypertensive	*Sham-operated*
Aorta		
NA	51.5 ± 12.3% ($n=4$)	50.8 ± 7.3% ($n=5$)
KCl	42.8 ± 12.3% ($n=4$)	44.2 ± 12.6% ($n=4$)
Mesenteric artery		
NA	58.6 ± 7.5% ($n=10$)	49.9 ± 5.2% ($n=7$)
KCl	50.7 ± 6.9% ($n=10$)	44.4 ± 5.7% ($n=7$)

Values are mean ± s.e.mean, with *n* the number of experiments. There were no significant differences.

ences between tissues from portal hypertensive and sham-operated animals in the maximum relaxation to ACh, when NA or KCl was used as the contractile agent (Table 2).

Maximum relaxation to sodium nitroprusside (SNP) (100 μM) in KCl (40 mM)-contracted tissues was similar in tissues from portal hypertensive (91.0±4.3%, $n=4$) and sham-operated animals (78.0±5.0%, $n=4$), respectively (no significant difference).

Microscopy

In mesenteric arteries set to a diameter 0.9 times the diameter of the vessel at 100 mmHg transmural pressure, wall thickness was 31.7±1.5 μm ($n=5$) and 30.5±1.0 μm ($n=6$) with lumen diameters of 312±9.7 μm and 298±18.5 μm in vessels from portal hypertensive and sham-operated animals, respectively (no significant differences). Similarly, wall to lumen ratios did not differ significantly between groups.

Discussion

The major findings from this study are that the maximum contraction to the vasoconstrictors NA, U46619 and KCl is increased in mesenteric arteries but not in aorta from portal hypertensive rats, with no change in endothelium-dependent relaxations. These and other findings will be discussed.

Firstly, it is necessary to consider the effectiveness of portal vein ligation in producing portal hypertension in this study. Portal pressure was significantly increased 14 days after portal vein ligation, as compared with 14 days after sham-operation, and acute ligation more than doubled portal pressure in sham-operated animals. Portal-systemic shunting was effectively nil in sham-operated animals but approximately 60% (range 10–98%) in portal vein-ligated animals, which compares with approximately 60% (range 0–85%) shunting in a previous study using the same methodology (Geraghty *et al.*, 1989). Other authors have injected microspheres into the spleen, rather than into the ileocaecal vein, and reported 95% shunting (range 89–99%) in portal vein ligated animals (Chojkier & Groszmann, 1981). However, this difference may not reflect differing degrees of portal hypertension, but rather an anatomical difference, since we have found similar high degrees of shunting when microspheres were injected into the spleen in our studies (authors, unpublished observations). Overall, we concluded that our portal hypertensive animals had the expected degree of portal systemic shunting and were a useful model of human portal hypertension.

In our studies, we found an increased maximum contraction to NA, U46619 and KCl in mesenteric arteries, but no change in maximum contraction to NA and KCl in aorta, from portal hypertensive animals. An increased maximum response to NA in mesenteric arteries could be due to an increase in number of α_1-adrenoceptors or a post-receptor change in responsiveness. However, an increase in number of receptors might be expected to increase the potency of NA, rather than the maximum response, in a tissue with spare receptors. Furthermore, the contraction to KCl (40 mM) and U46619 was also significantly increased in mesenteric arteries from portal hypertensive animals, and since KCl acts to produce membrane depolarization rather than at the receptor level, it is more likely that the change in contractile responsiveness occurs beyond the receptor level. Increased contractile response could be due to alterations in second messenger systems, or to alterations in smooth muscle mass. It has been previously reported that an increased maximum contraction to NA and angiotensin II in mesenteric veins from portal hypertensive rabbits was due to an increase in media thickness, and thus to an increase in smooth muscle (Jensen *et al.*, 1987), presumably as an adaptive change to increased pressure. Similar hypertrophy of the portal vein has been reported in portal hypertensive rats, with concomitant increased contraction to KCl (Chao *et al.*, 1992). In the present study, no alteration in wall thickness was found, suggesting that the changes in contractile responsiveness seen in this study are not due to changes in smooth muscle mass.

Mesenteric vessels were compared at the same level of transmural pressure (see Mulvany & Halpern, 1977), although it might be argued that mesenteric vessels from sham-operated animals may have experienced a lower mean perfusion pressure *in vivo* than those from the portal hypertensive animals due to decreased mesenteric vascular resistance in the latter group (see Pizcueta *et al.*, 1992). However, we have previously found that there is no significant difference between contractile responses to NA in mesenteric arteries from normal rats when the resting tension is changed from 90% of the diameter at 100 mmHg to 90% of the diameter at 67 mmHg (Breslin, 1993), so that this is probably not a major problem.

The increased contractile responsiveness of the isolated mesenteric artery found in this study contrasts with reports of decreased responsiveness of the *in situ* mesenteric vascular bed of pre-hepatic portal hypertensive rats of cirrhotic rats to

vasoconstrictors such as NA (Kiel *et al.*, 1985; Pizcueta *et al.*, 1990; Villamediana *et al.*, 1988), endothelin-1 (Yu *et al.*, 1992) and angiotensin (Villamediana *et al.*, 1989). Pressor responses to angiotensin also decreased in bile duct-ligated dogs (Finberg *et al.*, 1981). The most likely reason for this apparent discrepancy is that endogenous vasodilators produced in the mesenteric bed enter the systemic circulation through shunt vessels, resulting in increased circulating levels of these vasodilators in portal hypertension (Benoit *et al.*, 1984). These vasodilators act as physiological antagonists of the mesenteric vasoconstrictor response *in vivo*, and may cause an adaptive change to occur in the responsiveness of the mesenteric artery to vasoconstrictors. Such a physiological antagonism by vasodilators would be absent *in vitro*, but the altered contractile responsiveness would be revealed. However, in the present study, responses to the vasoconstrictors NA, U46619 and KCl were increased in mesenteric artery but not aorta, although both tissues were presumably exposed to vasodilators in vivo. This difference may be due to differing susceptibilities to the actions of vasodilators such as glucagon. In other studies of portal hypertension, the contractile response to NA was reported to be increased in the aorta and decreased in the portal vein of portal hypertensive rats (Bomzon *et al.*, 1991), unchanged in femoral artery (Villamediana *et al.*, 1988) from cirrhotic rats, but increased in mesenteric veins from portal vein ligated rabbits (Jensen *et al.*, 1987). Other studies of isolated blood vessels report no change in the contractile response to angiotensin II in aorta (Leehey, 1993) or femoral artery (Villamediana *et al.*, 1989) from cirrhotic rats, and an increased contraction to 5-HT in mesenteric veins from portal hypertensive rats (Cummings *et al.*, 1986).

In the present study, we also examined pressor responses to NA in the pithed rat, a preparation in which vasoconstrictor responses can be examined in the absence of cardiovascular reflexes (Gillespie *et al.*, 1970), and found that these responses were unaffected by portal hypertension. This contrasts with a decreased pressor responsiveness to the α-adrenoceptor agonist, phenylephrine, in conscious portal vein ligated rats, a difference in responsiveness that was abolished by ganglion block. This effect of ganglion blockade suggests that differences in sympathetic drive and baroreflex function contribute to altered pressor responsiveness to NA (Battarbee *et al.*, 1989) and angiotensin II (Villamediana *et al.*, 1989) in conscious or anaesthetized portal vein-ligated and cirrhotic rats, and this complication is avoided by examining the pithed rat preparation. There is evidence for diminished sympathetic tone to mesenteric vessels from portal hypertensive rats, as demonstrated by decreased vasodilatation to α-adrenoceptor antagonists (Joh *et al.*, 1993).

In rat mesenteric arteries, there was no evidence for altered endothelium-dependent relaxation to ACh in tissues from portal hypertensive animals. Furthermore, the increased maximum contraction to U46619 in mesenteric arteries from portal hypertensive animals was not due to altered endothelial function since the difference in maximum response was maintained when actions of NO were blocked by a combination of methylene blue (inhibition of guanylate cyclase, and additional actions: Mayer *et al.*, 1993) and L-NMMA (inhibition of nitric oxide synthase; Martin *et al.*, 1992). Hence, although the vascular endothelium may contribute to the hyperdynamic circulation in portal hypertension and cirrhosis (Valance & Moncada, 1991; Pizcueta *et al.*, 1992; Sieber & Groszmann, 1992; Castro *et al.*, 1993), there is no evidence for increased function *in vitro*, at least in terms of relaxations to ACh.

In conclusion, portal hypertension produces an increase in the contractile response to the vasoconstrictors NA, U46619 and KCl in rat mesenteric arteries but not in the aorta or in the pithed rat preparation. No alterations in endothelium-dependent responses to ACh were found. The diminished responsiveness to vasoconstrictors reported in portal hypertensive rats *in vivo* is not due to a diminished responsiveness at the level of the vascular smooth muscle.

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References

BATTARBEE, H.D., FARRAR, G.E. & SPEARS, R.P. (1989). Pressor responses in conscious rats with chronic portal venous hypertension. *Am. J. Physiol.*, **257**, G773–G781.

BENOIT, J.N., BARROWMAN, J.A., HARPER, S.L., KVIETYS, P.R. & GRANGER, D.N. (1984). Role of humoral factors in the intestinal hyperemia associated with chronic portal hypertension. *Am. J. Physiol.*, **247**, G486–G493.

BOMZON, A. & BLENDIS, L.M. (1987). Vascular reactivity in experimental portal hypertension. *Am. J. Physiol.*, **252**, G158–G162.

BOMZON, A., JACOB, G., LEE, S.S. & MEDDINGS, J. (1991). In vitro vascular responsiveness to norepinephrine in experimental portal hypertension. *Clin. Invest. Med.*, **14**, 63–71.

BOSCH, J., PIZCUETA, P., FEU, F., FERNANDEZ, M. & GARCIA-PAGAN, J.C. (1992). Pathophysiology of portal hypertension. *Gastroenterol. Clin. N. Am.*, **21**, 1–14.

BRESLIN, E. (1993). Factors affecting vascular function in human and animal tissues. *Ph.D. Thesis, National University of Ireland.*

CASTRO, A., JIMENEZ, W., CLARIA, J., ROS, J., MARTINEZ, J.M., BOSCH, M., ARROYO, V., PIULATS, J., RIVERA, F. & RHODES, J. (1993). Impaired vascular responsiveness to angiotensin II in experimental cirrhosis; role of nitric oxide. *Hepatology*, **18**, 367–372.

CAWLEY, T., GERAGHTY, J., OSBORNE, H. & DOCHERTY, J.R. (1994). Effects of portal hypertension on responsiveness of rat mesenteric arteries and aorta. *J. Physiol.*, **475**, 45P.

CHAO, T.-W., YU, P.-C., KUO, J.-S., PANG, P.K.T. & YANG, M.C.M (1992). Responsiveness to synthetic parathyroid hormone in the portal vein of hypertensive rats. *J. Hepatol.*, **16**, 326-331.

CHOJKIER, M. & GROSZMANN, R.J. (1981). Measurement of portal-systemic shunting in the rat by using g-labeled microspheres. *Am. J. Physiol.*, **240**, G371-G375.

CUMMINGS, S.A., GROSZMANN, R.J. & KAUMANN, A.J. (1986). Hypersensitivity of mesenteric veins to 5-hydroxytryptamine- and ketanserin- induced reduction of portal pressure in portal hypertensive rats. *Br. J. Pharmacol.*, **89**, 501–513.

DOCHERTY, J.R. (1988). The effects of ageing on vascular α-adrenoceptors in pithed rat and rat aorta. *Eur. J. Pharmacol.*, **146**, 1–5.

FINBERG, J.P.M., SYROP, H.A. & BETTER, O.S. (1981). Blunted pressor response to angiotensin and sympathomimetic amines in the bile duct ligated dog. *Clin. Sci.*, **61**, 535–539.

GERAGHTY, J.G., ANGERSON, W.J. & CARTER, D.C. (1989). Portal vein pressure and portasystemic shunting in experimental portal hypertension. *Am. J. Physiol.*, **257**, G52–G57.

GILLESPIE, J.S., MACLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. *Br. J. Pharmacol.*, **40**, 257–267.

JENSEN, L.S. JUHL, C.O. & MULVANY, M.J. (1987). Mechanical, morphological and pharmacological properties of oesophageal varices and small mesenteric veins in portal hypertensive rabbits. *Acta Physiol. Scand.*, **130**, 649–656.

JOH, T., GRANGER, D.N. & BENOIT, J.N. (1993). Endogenous vasoconstrictor tone in intestine of normal and portal hypertensive rats. *Am. J. Physiol.*, **264**, H171–H177.

KIEL, J.W., PITTS, V., BENOIT, J.N., GRANGER, D.N. & SHEPHERD, A.P. (1985). Reduced vascular sensitivity to norepinephrine in portal-hypertensive rats. *Am. J. Physiol.*, **248**, G192–G195.

LEEHEY, D.J. (1993). Dissociation between pressor sensitivity in vivo and contractile reactivity in vitro to angiotensin II in rats with experimental cirrhosis. *Clin. Sci.*, **84**, 525–529.

MARTIN, G.R., BOLOFO, M.L. & GILES, H. (1992). Inhibition of endothelium-dependent vasorelaxation by arginine analogues, a pharmacological analysis of agonist and tissue dependence. *Br. J. Pharmacol.*, **105**, 643–652.

MAYER, B., BRUNNER, F. & SCHMIDT, K. (1993). Inhibition of nitric oxide synthesis by methylene blue. *Biochem. Pharmacol.*, **45**, 367–374.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19-25.

MULVANY, M.J. & WARSGAW, D.M. (1977). The active tension-length of vascular smooth muscle related to its cellular components. *J. Gen. Physiol.*, **74**, 85–104.

MURRAY, B.M. & PALLER, M.S. (1985). Decreased pressor reactivity to angiotensin II in cirrhotic rats. Evidence for a post-receptor defect in angiotensin action. *Circ. Res.*, **57** 424–431.

PIZCUETA, M.P., CASAMITJANA, R., BOSCH, J. & RODES, J. (1980). Decreased systemic vascular sensitivity to norepinephrine in portal hypertensive rats: role of hyperglucagonism. *Am. J. Physiol.*, **258**, G191–G195.

PIZCUETA, M.P., PIQUE, J.M., BOSCH, J., WHITTLE, B.R.J. & MONCADA, S. (1992). Effects of inhibiting nitric oxide biosynthesis on the systemic splanchnic circulation of rats with portal hypertension. *Br. J. Pharmacol.*, **105**, 184–190.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

SIEBER, C.C. & GROSZMANN, R.J. (1992). Nitric oxide mediates hyporeactivity to vasopressors in mesenteric vessels of portal hypertensive rats. *Gastroenterology*. **103**, 235–239.

SIKULER, E., KRAVETZ, D. & GROSZMANN, R.J. (1985). Evolution of portal hypertension and mechanisms involved in its maintenance in a rat model. *Am. J. Physiol.*, **248**, G618–G625.

VALANCE, P. & MONCADA, S. (1991). Hyperdynamic circulation in cirrhosis: a role for nitric oxide? *Lancet*, **337**, 776–778.

VILLAMEDIANA, L.M., DIEGUEZ, G., SANTOS, J.C., GARCIA-VILLALON, A.L., CARAMELO, C. & LOPEZ-NOVOA, J.M. (1988). Vascular reactivity to norepinephrine in rats with cirrhosis of the liver. *Can. J. Physiol. Pharmacol.*, **66**, 567–572.

VILLAMEDIANA, L.M., GARCIA-VILLALON, A.L., CARAMELO, C. & LOPEZ-NOVOA, J.M. (1989). Effect of dietary sodium intake on the pressor reactivity to angiotensin II in rats with experimental cirrhosis of the liver. *Can. J. Physiol. Pharmacol.*, **67**, 1506–1511.

YU, P.-C., KUO, J.-S., LIN, H.-C. & YANG, M.C.M. (1992). Effects of endothelin in portal hypertensive rats. *Clin. Sci.*, **83**, 165–170.

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Modulation of NMDA effects on agonist-stimulated phosphoinositide turnover by memantine in neonatal rat cerebral cortex

Rajendra Mistry, *Rolf Wilke & [1]R.A. John Challiss

Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN and *Merz & Co., Eckenheimer Landstrasse 100-104, 60318 Frankfurt am Main, Germany

1 The ability of memantine (1-amino-3,5-dimethyladamantane) to antagonize the modulatory effects of N-methyl-D-aspartate (NMDA) on phosphoinositide turnover stimulated by muscarinic cholinoceptor- and metabotropic glutamate receptor-agonists has been examined in neonatal rat cerebral cortex slices.

2 Memantine antagonized the inhibitory effect of NMDA (100 μM) on both total [^{3}H]-inositol phosphate ([^{3}H]-InsP_x) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) mass accumulations stimulated by carbachol (1 mM) with EC_{50} values of 21 and 16 μM respectively.

3 Memantine concentration-dependently antagonized (IC_{50} 24 μM) the ability of NMDA (10 μM) to potentiate [^{3}H]-InsP_x accumulation in response to a sub-maximal concentration of the metabotropic glutamate receptor agonist, 1S,3R-ACPD (10 μM).

4 The small (approx. 3 fold), concentration-dependent increase in [^{3}H]-InsP_x accumulation stimulated by NMDA was completely antagonized by the prototypic NDMA receptor-channel blocker, MK-801 (1 μM) at all concentrations of NDMA studied (1-1000 μM). In contrast, antagonism by memantine (100 μM) was observed only at low concentrations of NMDA (1-10 μM), whilst [^{3}H]-InsP_x accumulation stimulated by high concentrations of NMDA (300-1000 μM) was markedly enhanced by memantine.

5 Assessment of the incorporation of [^{3}H]-inositol into inositol phospholipids revealed that memantine (100 μM) caused an approximate 2 fold increase in the labelling of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate.

6 H.p.l.c. separation of [^{3}H]-inositol (poly)phosphates demonstrated that whilst memantine (100 μM) alone had no significant effect on the accumulation of any isomer, it substantially altered the profile of accumulation stimulated by NMDA (1 mM), greatly facilitating accumulation of Ins(1,4,5)P_3 and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4).

7 These data provide evidence that memantine can antagonize the actions of NMDA in neonatal rat cerebral cortex slices in a manner consistent with this agent acting as a NMDA receptor-channel blocker. In addition, at least two further actions of memantine can be proposed. Memantine increases the rate of [^{3}H]-inositol incorporation into the cellular inositol phospholipid fraction, without significantly stimulating phosphoinositide turnover. Furthermore, memantine can substantially alter patterns of inositol (poly)phosphates stimulated by NMDA, promoting the accumulation of the established and putative second messengers Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 which are not increased by NMDA in the absence of memantine. It is unknown whether these latter loci of memantine action contribute to known therapeutic actions of this agent.

Keywords: NMDA-receptor; memantine (1-amino-3,5-dimethyladamantane); metabotropic glutamate receptor; muscarinic cholinoceptor; phosphoinositide turnover; inositol 1,4,5-trisphosphate; cerebral cortex (neonatal rat)

Introduction

Memantine (1-amino-3,5-dimethyladamantane) is used clinically in the treatment of Parkinson's disease and neurogenic motor disorders such as spasticity (Grossman & Schutz, 1982; Wesemann *et al.*, 1983; Schneider *et al.*, 1984). Evidence is also accruing to suggest that memantine may be a useful therapeutic agent in cerebrovascular diseases and dementia-related cognitive deficits (Wesemann *et al.*, 1983; Ditzler, 1991; Goertelmeyer & Erbler, 1992).

Although initial data pointed to memantine being an 'enhancer' of dopaminergic transmission (Maj *et al.*, 1974), recent work has demonstrated that memantine displaces [^{3}H]-MK-801 binding (Kornhuber *et al.*, 1989), blocks NMDA-receptor activation (Bormann, 1989) and antagonizes the consequences of NMDA-receptor activation (Erdö & Schaefer, 1991; Keilhoff & Wolf, 1992; Lupp *et al.*, 1992). These findings have been confirmed by patch clamp methodologies which have shown that memantine is an uncompetitive open channel blocker of the NMDA-receptor cation channel, effective at low micromolar concentrations and exhibiting weaker use-dependency and stronger voltage-dependency compared to MK-801 (Chen *et al.*, 1992; Parsons *et al.*, 1993).

Memantine has also been reported to exert a number of effects in neuronal preparations which are unlikely to be a consequence of NMDA-receptor blockade. In particular, memantine has been reported to stimulate inositol phosphate accumulation in retinal cell cultures and slices prepared from a number of brain regions (Osborne & Quack, 1992).

We have recently investigated the complex modulatory actions of NMDA on muscarinic cholinoceptor- and metabotropic glutamate receptor-stimulated phosphoinositide turnover in neonatal rat cerebral cortex (Challiss *et al.*, 1994a,b). In the present study we have assessed under what conditions memantine antagonizes NMDA modulatory effects on agonist-stimulated phosphoinositide turnover and whether memantine alone can elicit this second messenger response. Our data provide evidence that memantine can

[1] Author for correspondence.

antagonize the actions of NMDA in this preparation and also demonstrate that memantine can affect phosphoinositide turnover by apparently novel mechanisms.

Methods

Incubation methods and extract preparation

Cerebral cortex slices from 7–8 day old neonatal rats (Wistar strain, either sex) were prepared and incubated as previously described (Challiss *et al.*, 1994a,b). Where inositol phospholipids were labelled with [^{3}H]-inositol, slices (25 µl gravity-packed) were incubated in 250 µl KHB, containing 0.5 µCi [^{3}H]-inositol, at 37°C for a period of 60 min. At the end of this period, LiCl (5 mM final concentration) was added. For inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) mass studies, the labelling period and LiCl addition were omitted from the protocol. In all cases, incubations were terminated by addition of 300 µl ice-cold 1 M trichloroacetic acid (TCA) and immediately transferred to an ice-bath. Samples were neutralized for subsequent analysis as described previously (Challiss *et al.*, 1994a,b).

[^{3}H]-InsP_x measurement and separation of [^{3}H]-inositol (poly)phosphate isomers

The total [^{3}H]-inositol phosphate fraction ([^{3}H]-InsP_x) was recovered and quantified as reported previously (Challiss *et al.*, 1994a,b). Alternatively the total [^{3}H]-InsP_x fraction, from each experimental condition performed in triplicate, was pooled and injected onto a Partisil (10 µm) SAX analytical high performance liquid chromatography (h.p.l.c.) column equipped with a pre-column packed with Whatman pellicular anion-exchange resin. The column was washed to remove [^{3}H]-inositol and gradient elution of [^{3}H]-inositol (poly)-phosphate isomers was performed as described previously (Batty *et al.*, 1989).

Recovery and separation of [^{3}H]-inositol phospholipids

Slice pellets were sequentially washed with 5% TCA/1 mM EDTA and H_2O before phospholipid extraction as described by Downes & Wusteman (1983) and determination of either total [^{3}H]-inositol phospholipids ([^{3}H]-PtdIns(P_x)) in the chloroform phase, or deacylation and separation of [^{3}H]-glycerophosphoinositol (phosphates) ([^{3}H]-GroPIns/[^{3}H]-GroPIns(4)P/[^{3}H]-GroPIns(4,5)P_2) using Dowex-1 (formate-form) columns as previously described (Simpson *et al.*, 1987). In other experiments slice pellets were washed with 2 ml 0.9% NaCl and digested in 0.5 M NaOH for subsequent protein determination by the Lowry method.

Measurement of Ins(1,4,5)P_3 and PtdIns(4,5)P_2 mass

Mass assay of Ins(1,4,5)P_3 was performed as described previously (Challiss *et al.*, 1988). In parallel, the total phospholipid extract was processed as described previously (Challiss & Nahorski, 1993) with the Ins(1,4,5)P_3, released by alkaline hydrolysis of PtdIns(4,5)P_2, determined by mass assay.

Materials

Myo-[2-^{3}H]-inositol (17–20 Ci mmol^{-1}) and [^{3}H]-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3; 17–20 Ci mmol^{-1}) were obtained from DuPont NEN (DuPont U.K. Ltd., Stevenage, Herts., U.K.). D-Ins(1,4,5)P_3 was purchased from Rhode Island University (Kingston, Rhode Island, U.S.A.). 1-Aminocyclopentane-1S,3R-dicarboxylic acid (ACPD) was obtained from Tocris Neuramin (Langford, Bristol, U.K.). N-methyl-D-aspartate (NMDA) and Dowex AG 1-X8 (100–200 mesh, Cl^- form) were from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Dowex AG 1-X8 (200–400 mesh, formate form) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate) was a gift from Merck Sharp & Dohme Laboratories (Harlow, Essex, U.K.). Memantine (1-amino-3,5-dimethyladamantane) was supplied by Merz & Co. (Frankfurt am Main, Germany).

Data analysis

All values are presented as means ± s.e.mean for the indicated number of separate experiments. A computer programme (GraphPad, ISI) was used to generate EC_{50}/IC_{50} values from concentration-response data; EC_{50}/IC_{50} values are given as means with 95% confidence limits in parentheses. Statistical comparisons were performed by Student's *t* test for unpaired observations.

Results

Effects of memantine on NMDA modulation of carbachol-stimulated phosphoinositide turnover

We have previously demonstrated that NMDA has a complex modulatory effect upon carbachol-stimulated [^{3}H]-InsP_x accumulation in neonatal cerebral cortex slices (Challiss *et al.*, 1994a). Data entirely consistent with previous observations are shown in Figure 1. Exposure of slices to 1 mM carbachol for 15 min in the presence of 5 mM LiCl resulted in a 36 fold increase in [^{3}H]-InsP_x accumulation: co-addition of 10 µM NMDA caused a small (18%), but significant ($P<0.05$; unpaired Student's *t* test) increase in the agonist-stimulated response, whilst 100 µM NMDA profoundly inhibited the response (by 71%; $P<0.001$).

Neither memantine (100 µM) nor MK-801 (1 µM) affected carbachol-stimulated [^{3}H]-InsP_x accumulation, although memantine caused a small, but significant (32%; $P<0.05$) increase in basal [^{3}H]-InsP_x accumulation (Figure 1). The inhibitory effect of 100 µM NMDA on carbachol-stimulated [^{3}H]-InsP_x accumulation was completely prevented by memantine and MK-801 suggesting that like MK-801, memantine also antagonizes the effects of NMDA. However, whilst MK-801 significantly attenuated the enhancement of the agonist-stimulated [^{3}H]-InsP_x accumulation by 10 µM NMDA, memantine was without effect.

This effect of memantine was also seen with respect to carbachol-stimulated Ins(1,4,5)P_3 mass accumulation in neonatal cerebral cortex slices (Figure 2). Addition of carbachol (1 mM) caused a prompt increase in Ins(1,4,5)P_3 accumulation which reached about 6 fold over basal levels 15 s after agonist challenge and subsequently decreased to an elevated plateau level. In agreement with previous work (Challiss *et al.*, 1994a), the presence of 100 µM NMDA significantly enhanced the initial carbachol-stimulated increase in Ins(1,4,5)P_3 accumulation (by 33%; $P<0.02$), whilst with increasing time of exposure the presence of NMDA significantly suppressed agonist-stimulated Ins(1,4,5)P_3 accumulation 5 and 10 min after challenge (Figure 2). Pre-addition of 100 µM memantine had no effect on the initial enhancement of carbachol + NMDA-stimulated Ins(1,4,5)P_3 accumulation, but completely prevented the time-dependent development of the inhibitory phase. Indeed, 5 and 10 min after carbachol + NMDA addition, memantine appeared to enhance significantly the Ins(1,4,5)P_3 accumulation compared to that observed in the presence of carbachol only. These data suggest that memantine blocks the NMDA effect by 90–95%, reducing the effective NMDA concentration to a range over which only a facilitatory effect of NMDA is observed (see Discussion).

The effects of increasing concentrations of NMDA on basal and 10 min carbachol-stimulated Ins(1,4,5)P_3 accumulations were established in the absence and presence of 100 μM memantine (Figure 3). Low concentrations of NMDA significantly enhanced the carbachol-stimulated Ins(1,4,5)P_3 response (by 43% ($P<0.05$) and 78% ($P<0.001$) at 3 and 10 μM NMDA respectively), whilst high concentrations (100–1000 μM NMDA) profoundly inhibited both the basal and agonist-stimulated accumulations of Ins(1,4,5)P_3. In the presence of 100 μM memantine the bi-modal modulatory effect of NMDA was essentially lost. Thus, memantine prevented the inhibitory effect of high concentrations of NMDA on both basal and agonist-stimulated responses and appeared to shift the enhancement of carbachol-stimulated Ins(1,4,5)P_3 accumulations to higher concentrations of NMDA.

The ability of memantine to reverse the inhibitory action of NMDA (100 μM) on carbachol-stimulated [^{3}H]-InsP_x and Ins(1,4,5)P_3 accumulation is shown in Figure 4. Memantine concentration-dependently and fully reversed the inhibitory effect of NMDA; the concentration of memantine calculated to cause 50% reversals of the NMDA effect on carbachol-stimulated [^{3}H]-InsP_x and Ins(1,4,5)P_3 accumulations were respectively observed as 21.2 (95% confidence limits 17.7–25.3) and 16.0 (14.3–17.8) μM.

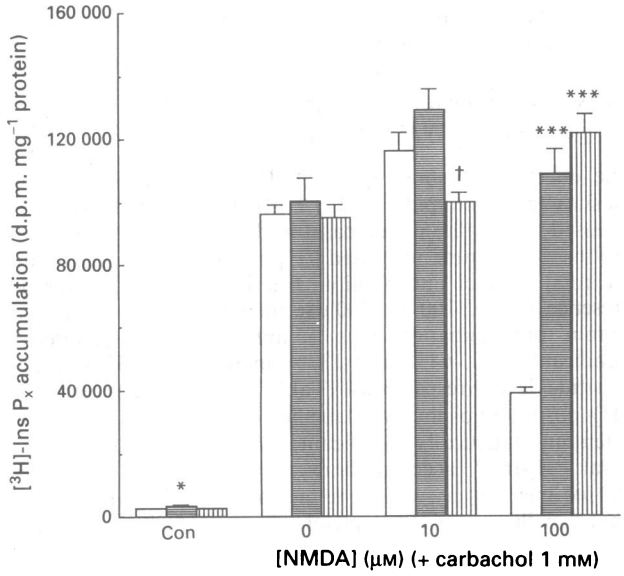


Figure 1 Effects of memantine and MK-801 on the modulation of carbachol-stimulated [^{3}H]-InsP_x accumulation by N-methyl-D-aspartate (NMDA). Either 100 μM memantine (horizontally-hatched columns), 1 μM MK-801 (vertically lined columns), or vehicle (open columns) was added 15 min prior to simultaneous addition of 1 mM carbachol and 0, 10 or 100 μM NMDA. No further agent additions are designated as Con. Values are presented as means ± s.e.mean for 4 separate experiments performed in triplicate. Statistically significant effects of memantine or MK-801 pretreatment on the subsequent response are indicated as $*P<0.05$ and $***P<0.001$ for increases over respective values and $\dagger P<0.05$ for decreases below respective control values.

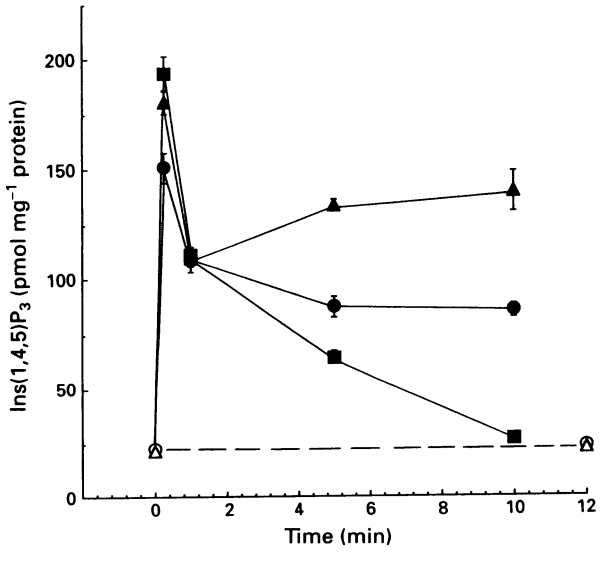


Figure 2 Effect of memantine on the time-dependent modulations of carbachol-stimulated Ins(1,4,5)P_3 mass accumulation by N-methyl-D-aspartate (NMDA). Additions of 100 μM memantine (△, ▲) or vehicle (○, ●, ■) were made 15 min before addition of 1 mM carbachol in the absence (●) or presence (■, ▲) of 100 μM NMDA. Values are presented as means ± s.e.mean for 4 separate experiments performed in triplicate.

Effects of memantine on NMDA modulation of 1S,3R-ACPD-stimulated phosphoinositide turnover

In agreement with a previous study (Challiss *et al.*, 1994b), data in Figure 5 illustrate that addition of either 10 μM 1S,3R-ACPD or 10 μM NMDA (for 15 min in the presence of 5 mM LiCl) caused only modest increases in [^{3}H]-InsP_x accumulation over basal levels (control 3323 ± 317; +10 μM 1S,3R-ACPD 13394 ± 762; +10 μM NMDA 9015 ± 887 d.p.m. mg^{-1} protein), however, co-addition of 1S,3R-ACPD + NMDA greatly potentiated the observed response (to 67267 ± 3578 d.p.m. mg^{-1} protein). Figure 5 also

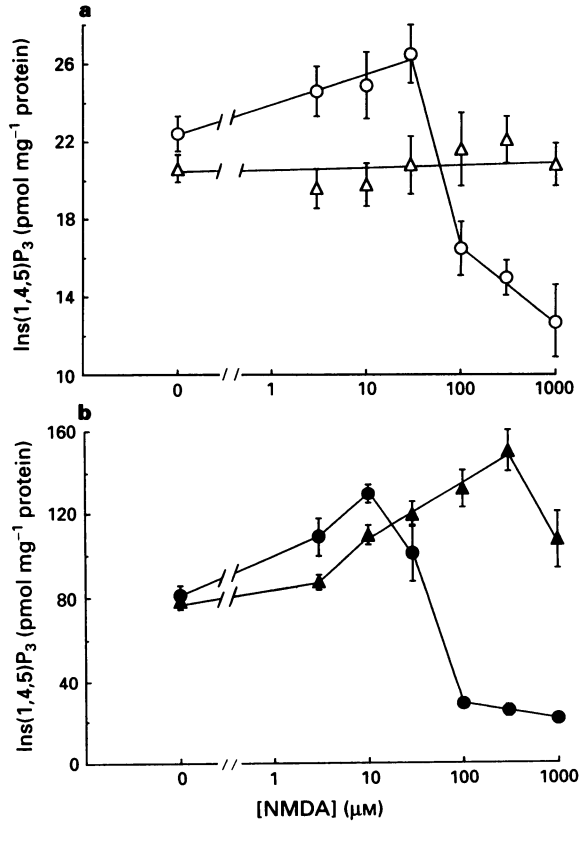


Figure 3 Effects of memantine on N-methyl-D-aspartate (NMDA) modulation of basal (a) and carbachol-stimulated (b) Ins(1,4,5)P_3 mass accumulation. Where indicated, additions of 100 μM memantine (△, ▲) or vehicle (○, ●) were made 15 min prior to addition of the indicated concentrations of NMDA in the absence (a) or presence (b) of 1 mM carbachol. Values are presented as means ± s.e.mean for 3 separate experiments performed in triplicate. Note the difference in ordinate scales between (a) and (b).

illustrates the abilities of memantine and MK-801 to antagonize the NMDA-mediated potentiation of 1S,3R-ACPD-stimulated [^{3}H]-InsP$_x$ accumulation. MK-801 fully reversed the NMDA potentiation with a 50% effect being observed at 68.1 (53.9–86.0) nM; memantine only appeared to cause an 80% reversal of the NMDA potentiation, with a half-maximal effect being observed at 24.1 (22.4–26.0) μM.

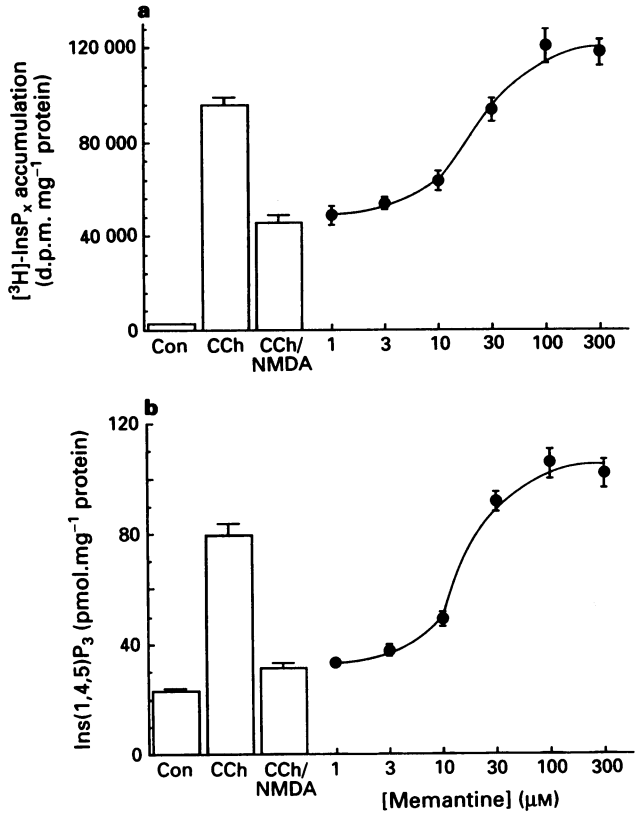


Figure 4 Concentration-dependent reversals of N-methyl-D-aspartate (NMDA)-inhibition of carbachol-stimulated [^{3}H]-InsP$_x$ (a) and Ins(1,4,5)P$_3$ (b) accumulations. The indicated concentrations of memantine were added 15 min prior to addition of 1 mM carbachol + 100 μM NMDA. The histograms on the left of each panel show the effects of vehicle (Con), 1 mM carbachol (CCh) and 1 mM carbachol + 100 μM NMDA (CCh/NMDA). Values are presented as means ± s.e.mean for 3 separate experiments performed in triplicate.

These studies all point towards memantine exerting an effect similar to MK-801, albeit with a 300–400 fold lower potency. However, memantine appears to possess properties distinct from MK-801. As reported above, NMDA caused a small concentration-dependent accumulation of [^{3}H]-InsP$_x$ (Figure 6), which was largely antagonized by MK-801 (1 μM). In contrast, whilst memantine (100 μM) appeared to antagonize [^{3}H]-InsP$_x$ accumulations stimulated by low concentrations of NMDA, it potentiated the response observed at high concentrations of NMDA (Figure 6).

Does memantine affect phosphoinositide turnover independently of NMDA-receptor antagonism?

The effects of memantine on [^{3}H]-inositol labelling of the inositol phospholipids and [^{3}H]-InsP$_x$ accumulation were assessed in parallel under basal and NMDA-stimulated conditions. Memantine increased [^{3}H]-inositol incorporation into the total inositol phospholipid fraction (Table 1). Thus, the presence of 30, 100 or 300 μM memantine for 30 min (i.e. 15 min pre-incubation plus 15 min ± NMDA) resulted in significant 30%, 64% and 137% increases in [^{3}H]-phosphoinositide levels respectively (Table 1). The enhancement of [^{3}H]-inositol phospholipid labelling was reflected to a lesser extent in statistically significant 25% and 84% increases in basal [^{3}H]-InsP$_x$ accumulations in the presence of 100 and 300 μM memantine, whilst the memantine-evoked increases in lipid labelling could completely account for the respective observed increases in NMDA-stimulated [^{3}H]-InsP$_x$ accumulation (Table 1).

The enhancement of [^{3}H]-inositol phospholipid labelling by memantine appeared to be independent of the ability of this agent to antagonize NMDA-receptor activation. Thus, pre-incubation with MK-801 inhibited NMDA-stimulated [^{3}H]-InsP$_x$ accumulation by 65% in the absence, and by 69% in the presence of 100 μM memantine, whilst having no effect on [^{3}H]-inositol phospholipid labelling. The time-course of the enhancement of [^{3}H]-inositol incorporation into the phospholipid fraction evoked by 100 μM memantine is shown in Figure 7. In this series of experiments memantine was added 30 min after initiation of labelling to allow the effects of this agent to be followed over a longer (90 min) time-course. Under basal conditions a linear rate of [^{3}H]-inositol labelling of phospholipids was observed over the 120 min time course. MK-801 (1 μM) had no effect on the rate of phospholipid

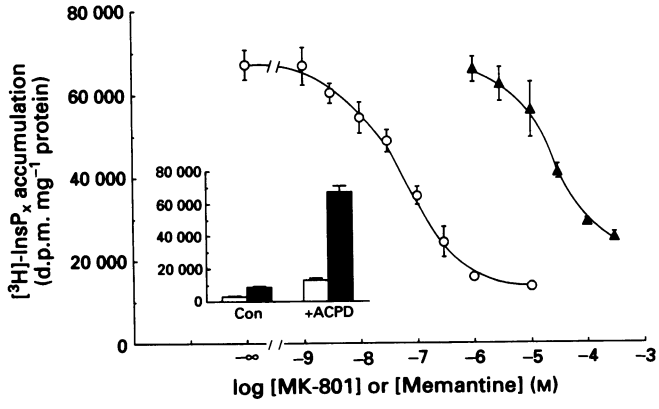


Figure 5 Concentration-dependent inhibitions of N-methyl-D-aspartate (NMDA)-potentiated 1-aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD)-stimulated [^{3}H]-InsP$_x$ accumulation by memantine and MK-801. The indicated concentrations of memantine (▲) or MK-801 (○) were added 15 min prior to simultaneous addition of 10 μM 1S,3R-ACPD and 10 μM NMDA. The inset shows the basal (Con) and 10 μM 1S,3R-ACPD-stimulated (ACPD) [^{3}H]-InsP$_x$ accumulation in the absence (open columns) or presence (solid columns) of 10 μM NMDA. In all cases values are presented as means ± s.e.mean for 3 separate experiments performed in triplicate.

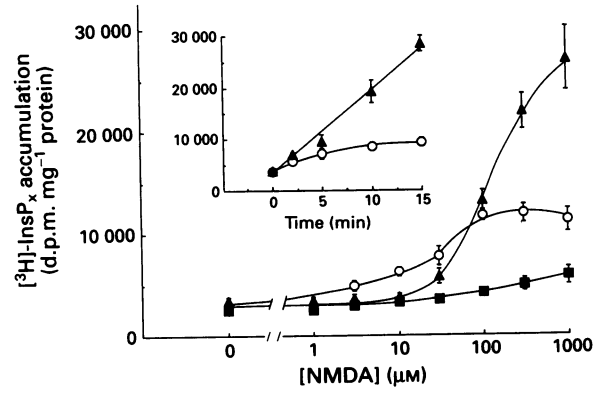


Figure 6 Effect of memantine and MK-801 on N-methyl-D-aspartate (NMDA)-stimulated [^{3}H]-InsP$_x$ accumulation. Memantine (100 μM; ▲), MK-801 (1 μM; ■) or vehicle (○) was added 15 min prior to addition of the indicated concentrations of NMDA. The inset shows the time-courses of [^{3}H]-InsP$_x$ accumulation stimulated by 1 mM NMDA in the absence (○) or presence (▲) of 100 μM memantine. In all cases, values are presented as means ± s.e.mean of 3 separate experiments performed in triplicate (main figure), or one typical experiment performed in triplicate (inset figure).

Table 1 Effects of memantine on total [^{3}H]-phosphoinositide labelling and [^{3}H]-InsP$_x$ accumulation under basal and N-methyl-D-aspartate (NMDA)-stimulated conditions

± NMDA (1 mM)	Memantine (μM)	[^{3}H]-PtdIns(P$_x$) (d.p.m. per 25 μl slices)	[^{3}H]-InsP$_x$	Ratio
−	0	76656 ± 1135	3647 ± 164	0.048
−	10	80792 ± 2981	3699 ± 341	0.046
−	30	99576 ± 4171[a]	4211 ± 511	0.042
−	100	125917 ± 3241[b]	4553 ± 241[a]	0.036
−	300	181603 ± 1873[b]	6695 ± 306[b]	0.037
+	0	65728 ± 3339[c]	10764 ± 106[d]	0.164
+	10	68978 ± 3651[c]	11039 ± 532[d]	0.160
+	30	87915 ± 2231[a]	14284 ± 181[a,d]	0.162
+	100	120728 ± 4068[b]	21094 ± 498[b,d]	0.175
+	300	155563 ± 6528[b,c]	28661 ± 1815[b,d]	0.184
+*	0	69879 ± 2292	6102 ± 833[e]	0.087
+*	100	118271 ± 2106[b]	9632 ± 641[f]	0.073

Where indicated *MK-801 (1 μM) was added 5 min prior to memantine (and 20 min prior to NMDA) addition. Total inositol phospholipid ([^{3}H]-PtdIns(P$_x$)) and inositol phosphate ([^{3}H]-InsP$_x$) fractions were recovered as described in the Methods section. Data are presented as means ± s.e.mean for a single experiment performed in quadruplicate. Essentially similar data were obtained in one further experiment. The final column gives the ratio of [^{3}H]-InsP$_x$ to [^{3}H]-PtdIns(P$_x$) under each condition. Statistically significant differences are indicated as [a]$P<0.05$, [b]$P<0.001$ increases over respective control (−memantine) values; [c]$P<0.05$ decrease in presence of NMDA compared to respective −NMDA value; [d]$P<0.001$ increase in the presence of NMDA compared to respective −NMDA value; [e]$P<0.01$, [f]$P<0.001$ attenuation caused by MK-801 compared to respective conditions in its absence.

labelling, whereas addition of memantine (100 μM) for 30, 60 or 90 min increased labelling by 83 ± 7%, 76 ± 5% and 74 ± 9% respectively, relative to basal incorporation rates (over 3 experiments).

Memantine increases [^{3}H]-inositol incorporation into all phosphoinositides

Memantine (100 μM) significantly increased [^{3}H]-inositol-labelling of PtdIns, PtdInsP and PtdInsP$_2$ (Table 2). It should be noted that this appears to be due to memantine affecting the rate of inositol phospholipid labelling rather than any effect on the absolute levels. Thus, exposure of slices to 100 μM memantine for 60 min, which in parallel experiments caused a 60% increase in [^{3}H]-PtdInsP$_2$ labelling, had no effect on PtdIns(4,5)P$_2$ mass (−memantine, 3138 ± 154; +memantine, 3082 ± 197 pmol per 25 μl slices: values for 3 experiments performed in duplicate).

In the absence of memantine, challenge with NMDA (1 mM) for 15 min resulted in similar levels of [^{3}H]-PtdIns labelling, but significantly decreased levels of both PtdInsP ($P<0.001$) and PtdInsP$_2$ ($P<0.01$) labelling, whilst in the presence of memantine (100 μM) enhanced levels of labelling of all inositol phospholipid species were well-maintained (Table 2). In both the absence and presence of memantine, carbachol stimulated [^{3}H]-inositol incorporation into all inositol phospholipids, reducing the differential between inositol phospholipid labelling observed ± memantine (Table 2). The increased levels of [^{3}H]-inositol phospholipid labelling observed in the presence of memantine led to a concomitant and proportional increase in NMDA-, but not carbachol-stimulated [^{3}H]-InsP$_x$ accumulation. Although it is possible that the increase in inositol phospholipid labelling seen in the presence of carbachol alone may have obscured any enhancement of [^{3}H]-InsP$_x$ accumulation in the presence of memantine, construction of carbachol concentration-response curves provided no evidence for this explanation (data not shown).

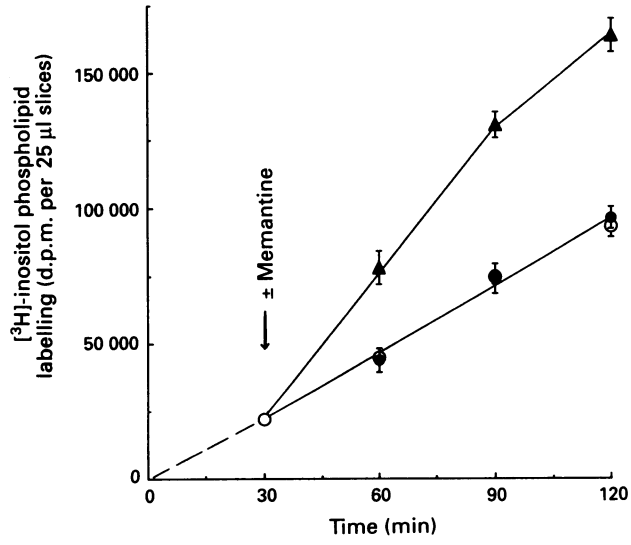


Figure 7 Effect of memantine and MK-801 on the time-courses of [^{3}H]-inositol incorporation into inositol phospholipids. Neonatal rat cerebral cortex slices were added to medium containing [^{3}H]-inositol for the indicated times. After 30 min, memantine (100 μM;▲), MK-801 (1 μM; ●) or vehicle (○) was added and incubations continued for the indicated times. Values are presented as means ± s.e.mean for a single representative experiment performed in triplicate. Similar effects of memantine and MK-801 were observed in two other experiments.

Taken together, these data suggest that NMDA and carbachol may stimulate phospholipase C activities which have differential access to the basal and memantine-enhanced [^{3}H]-inositol phospholipid pools.

Effect of memantine on basal and NMDA-stimulated isomer profiles of inositol (poly)phosphate accumulation

[^{3}H]-Ins(1/3)P$_1$ (by 187%), [^{3}H]-Ins(4)P$_1$ (by 983%) and [^{3}H]-Ins(1,4)P$_2$ (by 456%) accumulations were all increased by NMDA (1 mM), whilst the [^{3}H]-Ins(1,4,5)P$_3$ level decreased to below basal values. The latter finding is in good agreement with the effect of NMDA on basal Ins(1,4,5)P$_3$ mass levels shown in Figure 3a. Memantine (100 μM) did not significantly affect this isomer profile. NMDA evoked greater increases in [^{3}H]-Ins(1/3)P$_1$, [^{3}H]-Ins(4)P$_1$ and [^{3}H]-Ins(1,4)P$_2$ in the presence, compared to the absence of memantine (Table 3). Moreover, a significant NMDA-evoked increase in [^{3}H]-Ins(1,4,5)P$_3$ accumulation was observed and other [^{3}H]-inositol (poly)phosphate isomers were markedly increased in the presence of memantine, particularly those generated by activation of the 3-kinase route of Ins(1,4,5)P$_3$ metabolism. Thus, the increases in Ins(1,3,4,5)P$_4$ (14 fold), Ins(1,3,4)P$_3$ (3 fold), Ins(3,4)P$_2$ (3.5 fold) and Ins(1,3)P$_2$ (14 fold) were all greater than the approximate 2 fold increase which might be anticipated on the basis of differences in the [^{3}H]-inositol phospholipid fractions.

Discussion

Recent studies have provided considerable evidence to support the hypothesis that the therapeutic effects of memantine are mediated, at least in part, through the ability of this agent to inhibit transmembrane cation fluxes activated upon glutamate binding to NMDA-receptors (Kornhuber *et al.*, 1989; Bormann, 1989; Chen *et al.*, 1992; Parsons *et al.*, 1993). Here we have examined the ability of memantine to block the modulations of muscarinic cholinoceptor- and metabotropic glutamate receptor-stimulated phosphoinositide turnover by NMDA in neonatal rat cerebral cortex slices.

Table 2 Effects of memantine (100 μM) on basal and agonist-stimulated changes in phosphoinositide labelling and [^{3}H]-InsP$_x$ accumulation

Addition	*± memantine* (100 μM)	*[^{3}H]-PtdIns*	*[^{3}H]-PtdInsP*	*[^{3}H]-PtdInsP$_2$*	*[^{3}H]-InsP$_x$*
			(d.p.m. per 25 μl slices)		
–	–	56542 ± 1642	6881 ± 107	7929 ± 339	3674 ± 450
–	+	93036 ± 5157**	12495 ± 779***	11273 ± 925*	4440 ± 545
+ NMDA (1 mM)	–	63449 ± 2038	5051 ± 106	4740 ± 301	13359 ± 1688
	+	113085 ± 8502**	14894 ± 823***	11883 ± 1686**	26593 ± 2558**
+ CCh (1 mM)	–	88036 ± 3740	11719 ± 459	14486 ± 691	103237 ± 8437
	+	105454 ± 8023	15311 ± 852*	16498 ± 1515	99334 ± 7242

Data are presented as means ± s.e.mean for 3 separate experiments performed in triplicate. Indications of statistical significance are only shown for ± memantine comparisons with differences indicated as $*P<0.05$, $**P<0.01$ and $***P<0.001$. CCh = carbachol.

Table 3 Effect of memantine (100 μM) on basal and N-methyl-D-aspartate (NMDA)-stimulated accumulations of individual inositol (poly)phosphate isomers separated by h.p.l.c.

Isomer	*No addition*		*+NMDA* (1 mM)	
	– memantine	*+ memantine*	*– memantine*	*+ memantine*
Ins(1/3)P$_1$	7131 ± 599	8699 ± 1842	20464 ± 1356^c	50327 ± 4234^d
Ins(4)P$_1$	1722 ± 106	1792 ± 273	18657 ± 1372^c	39354 ± 2875^d
Ins(1,3)P$_2$	71 ± 13	73 ± 12	71 ± 4	1038 ± 143^d
Ins(1,4)P$_2$	1087 ± 261	1074 ± 115	6041 ± 567^b	13534 ± 575^e
Ins(3,4)P$_2$	46 ± 13	48 ± 6	61 ± 12	276 ± 40^d
Ins(1,3,4)P$_3$	521 ± 78	634 ± 27	425 ± 25	1683 ± 127^e
Ins(1,4,5)P$_3$	1604 ± 240	1982 ± 558	678 ± 59^a	4142 ± 382^e
Ins(1,3,4,5)P$_4$	393 ± 51	525 ± 48	386 ± 39	5752 ± 942^d

Neonatal rat cerebral cortex slices were labelled with [^{3}H]-inositol (2 μCi per vial) for 60 min. Data are presented as means ± s.e.mean for 3 separate experiments performed in triplicate. Indications of statistical significance are shown for +NMDA comparisons with differences indicated as $^aP<0.05$ for a decrease in the presence of NMDA and $^bP<0.01$, $^cP<0.001$ for increases in the presence of NMDA. Similar indications of statistical significance are shown for ± memantine comparisons with differences indicated as $^dP<0.01$, $^eP<0.001$ for increases in the presence of memantine compared to respective values in its absence.

In agreement with our previous work (Challiss *et al.*, 1994b), co-addition of NMDA markedly potentiated metabotropic glutamate receptor-stimulated [^{3}H]-InsP$_x$ accumulation. Thus, 10 μM NMDA increased the [^{3}H]-InsP$_x$ accumulation stimulated by 10 μM 1S,3R-ACPD at least 4 fold more than would be predicted if the responses to NMDA and 1S,3R-ACPD were simply additive (see inset to Figure 5). The potentiation of the metabotropic agonist-stimulated phosphoinositide response by 10 μM NMDA was concentration-dependently inhibited by both MK-801 (IC_{50} 68 nM) and memantine (IC_{50} 24 μM). Thus, like MK-801, memantine can antagonize the NMDA modulation of this response.

In another recent study, we demonstrated that high concentrations of NMDA profoundly inhibit carbachol-stimulated [^{3}H]-InsP$_x$ and Ins(1,4,5)P$_3$ mass accumulations (Challiss *et al.*, 1994a) and proposed that acute treatment of neonatal cerebral cortex slices with high concentrations of NMDA could cause progressive and irreversible damage, at least to cell populations responding to muscarinic cholinoceptor stimulation. Pretreatment of slices with memantine concentration-dependently reversed the inhibitory effect of 100 μM NMDA on carbachol-stimulated [^{3}H]-InsP$_x$ and Ins(1,4,5)P$_3$ mass accumulations, with 50% reversals of the NMDA effects being observed at 21 and 16 μM memantine respectively. If the inhibitory effects of NMDA are indeed causally linked to the excitotoxic actions of this agent, then the fact that memantine can block such effects is entirely consistent with the neuroprotective action of memantine reported by others (Seif el Nasr *et al.*, 1990; Erdö & Schaefer, 1991; Chen *et al.*, 1992; Keilhoff & Wolf, 1992; Weller *et al.*, 1993).

Taken together, these data provide further evidence that memantine can antagonize the actions of NMDA in a complex *in vitro* neuronal preparation. Although the concentrations of memantine required to antagonize the NMDA modulatory effects reported here are higher than values obtained in electrophysiological investigations (Bormann, 1989; Chen *et al.*, 1992; Parsons *et al.*, 1993) and competition binding assays (Kornhuber *et al.*, 1989), they are consistent with values obtained in more complex neuronal preparations such as the cortical wedge (Parsons *et al.*, 1993).

A number of previous studies have demonstrated that memantine may exert effects which might be unconnected with the established NMDA-receptor antagonist action of this agent (Maj, 1982), perhaps the most interesting of these being the ability of memantine to increase [^{3}H]-InsP$_x$ accumulation in a number of neuronal cell and slice preparations (Osborne & Quack, 1992). Such a dual activity (i.e. reducing glutamatergic neurotransmission via NMDA receptor blockade whilst exerting a 'metabotropic' action at the level of phosphoinositide cycle second messenger generation), may endow memantine with a number of additional, perhaps clinically beneficial, effects. For example, co-activation of metabotropic glutamate receptors can ameliorate the neurotoxic effects of inappropriate NMDA receptor activation (Koh *et al.*, 1991; Siliprandi *et al.*, 1992), possibly by inhibition of NMDA-gated cation fluxes through increasing diacylglycerol generation and activation of a protein kinase C-dependent phosphorylation of the NMDA receptor (Courtney & Nicholls, 1992).

In both neonatal and adult (Baird & Nahorski, 1991) rat cerebral cortex slices, NMDA alone can increase [^{3}H]-InsP$_x$ accumulation. That MK-801 can block the NMDA-evoked increase in [^{3}H]-InsP$_x$ accumulation strongly suggests that this effect is mediated through Ca^{2+} entry, via the integral cation channel of the NMDA receptor, leading to activation of Ca^{2+}-dependent phospholipase C activity. Although memantine also blocked [^{3}H]-InsP$_x$ accumulation stimulated by low

concentrations of NMDA it paradoxically potentiated [^{3}H]-$InsP_x$ accumulation stimulated by high concentrations of NMDA. Further experiments demonstrated memantine to have a second effect in the slice preparation, increasing the rate of [^{3}H]-inositol incorporation into the cellular phospholipid fraction. Although NMDA-evoked increases in [^{3}H]-$InsP_x$ accumulation in both the absence and presence of memantine (100 μM) were substantially antagonized by MK-801, this agent had no effect on the ability of memantine to increase [^{3}H]-inositol phospholipid labelling. Such data strongly suggest that the ability of memantine to enhance the rate of [^{3}H]-inositol incorporation into the phospholipid fraction is unrelated to NMDA receptor antagonism. Resolution of the [^{3}H]-inositol phospholipid fraction revealed that memantine increased labelling in all inositol phospholipid species approximately equally. However, these labelling effects were not paralleled by increases in PtdIns(4,5)P_2 mass which remained unaffected by incubation with 100 μM memantine for 60 min.

It might be inferred from these data that differences in agonist-stimulated [^{3}H]-$InsP_x$ accumulation in the absence and presence of memantine are generated by differences in the specific radioactivities of the inositol phospholipids and if it were possible to radiolabel slices with [^{3}H]-inositol to isotopic equilibrium such differences would no longer be observed. However, this is unlikely to explain the results of Osborne & Quack (1992), who assessed memantine-stimulated [^{3}H]-$InsP_x$ accumulations, not only in acutely labelled slice preparations, but also in retinal cell cultures labelled with [^{3}H]-inositol for 16 h to achieve equilibrium labelling. Furthermore, other findings have arisen from the current investigation to suggest that memantine may produce effects over and above those resulting from a simple facilitation of the rate of inositol phospholipid labelling in neonatal cerebral cortex slices. Thus, although the increase in the NMDA-evoked [^{3}H]-$InsP_x$ accumulation was in proportion with the increase in [^{3}H]-inositol phospholipid labelling in the presence of memantine, the same was not true of the carbachol-stimulated response. This discrepancy was also evident when the magnitude of the [^{3}H]-$InsP_x$ accumulations was equalized by adjustment of agonist concentration (1 mM NMDA versus 3 μM carbachol). One interpretation of these data is that memantine selectively facilitates [^{3}H]-inositol incorporation into an inositol phospholipid pool which is accessible to the phospholipase C(s) activated by NMDA, but not by the enzyme activated by carbachol. Such a possibility is not without precedent. Mn^{2+} has been shown to enhance greatly [^{3}H]-inositol incorporation into inositol phospholipids in different neuronal preparations (Yandrasitz & Segal, 1979; Schoepp, 1985). However, although the increased phospholipid radiolabelling is reflected in increased basal [^{3}H]-$InsP_x$ accumulations in cerebral cortex slices, agonist-stimulated responses are largely unaffected suggesting that Mn^{2+} facilitates [^{3}H]-inositol incorporation into a phospholipid pool inaccessible to agonist-stimulated phospholipase C activity (Schoepp, 1985).

Another important observation regarding the possible actions of memantine has been made from the detailed analysis of individual inositol (poly)phosphate isomers generated under basal and agonist-stimulated conditions. In agreement with data presented by Baird & Nahorski (1991), NMDA significantly increased the accumulations of Ins(1,4)P_2, Ins(4)P_1 and Ins(1/3)P_1, whilst having no effect or decreasing the accumulations of higher inositol phosphates. These data suggest that NMDA may cause the selective activation of one or more isozymes of phospholipase C which preferentially hydrolyses PtdIns(4)P and PtdIns. Memantine profoundly affected the profile of NMDA-stimulated inositol (poly)phosphate accumulation. In the presence of memantine, NMDA evoked increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations as well as substantial increases in Ins(1,3,4)P_3, Ins(1,3)P_2 and Ins(3,4)P_2 which were all unchanged from basal values during NMDA challenge in the absence of memantine.

These data suggest that memantine can influence the routes of inositol polyphosphate synthesis and degradation, promoting substantial increases in the concentrations of the established and putative second messengers Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Such a modulatory effect can only be brought about if memantine directly or indirectly causes a substantial shift in the substrate selectivity of NMDA-stimulated phospholipase C activities towards hydrolysis of PtdIns(4,5)P_2. It is possible that activation of the 3-kinase route of Ins(1,4,5)P_3 metabolism might also be affected by memantine; however, the increase in Ins(1,4,5)P_3 concentration in the presence of an NMDA-evoked elevation of $[Ca^{2+}]_i$ may be sufficient to increase the flux through this enzyme (Shears, 1991).

In summary, the present study has demonstrated that memantine concentration-dependently antagonized both stimulatory and inhibitory modulations of agonist-stimulated phosphoinositide turnover by NMDA, consistent with previous reports that memantine is an uncompetitive blocker of the integral cation channel of the NMDA receptor complex. In addition, we have provided evidence for two further actions of memantine. Memantine stimulates the rate at which [^{3}H]-inositol is incorporated into PtdIns, PtdIns(4)P and PtdIns(4,5)P_2. The fact that the enhanced labelling of [^{3}H]-PtdInsP_2 was not reflected in a significant increase in PtdIns(4,5)P_2 mass, suggests that memantine accelerates the rate of inositol incorporation into the phosphoinositide pool rather than perturbing the steady-state levels of these membrane lipids. Whether this reflects the ability of memantine to stimulate selectively or generally inositol:inositol phospholipid exchange, and thus facilitate the recovery of membrane phosphoinositide pools following depletion (Monaco & Adelson, 1991; Thomas *et al.*, 1993) remains to be addressed. Finally, although memantine alone does not increase phosphoinositide turnover, it does dramatically alter the profile of NMDA-stimulated inositol (poly)phosphate generation. Our data strongly argue that memantine not only facilitates NMDA-stimulated [^{3}H]-$InsP_x$ accumulation but also shifts the substrate hydrolysed by the activated phospholipase C(s) from PtdIns and PtdIns(4)P substantially towards PtdIns-(4,5)P_2. Therefore, in addition to the therapeutic advantages memantine has been argued to possess on the basis of studies of the kinetics of NMDA-receptor/channel blockade (Chen *et al.*, 1992; Parsons *et al.*, 1993), further independent loci of action, which may be of significance in the known therapeutic actions and envisaged therapeutic potentials of memantine, should also be taken into account.

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References

BAIRD, J.G. & NAHORSKI, S.R. (1991). Stimulatory and inhibitory effects of N-methyl-D-aspartate on ^{3}H-inositol polyphosphate accumulation in rat cortical slices. *J. Neurochem.*, **57**, 629–635.

BATTY, I.H., LETCHER, A.J. & NAHORSKI, S.R. (1989). Accumulation of inositol polyphosphate isomers in agonist-stimulated cerebral cortex slices. *Biochem. J.*, **258**, 23–32.

BORMANN, J. (1989). Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels. *Eur. J. Pharmacol.*, **166**, 591–592.

CHALLISS, R.A.J., BATTY, I.H. & NAHORSKI, S.R. (1988). Mass measurements of inositol 1,4,5-trisphosphate in rat cerebral cortical slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun.*, **157**, 684–691.

CHALLISS, R.A.J., MISTRY, R., GRAY, D.W. & NAHORSKI, S.R. (1994a). Modulation of muscarinic cholinoceptor-stimulated inositol 1,4,5-trisphosphate accumulation by N-methyl-D-aspartate in neonatal rat cerebral cortex. *Neuropharmacology*, **33**, 15–25.

CHALLISS, R.A.J., MISTRY, R., GRAY, D.W. & NAHORSKI, S.R. (1994b). Modulatory effects of NMDA on phosphoinositide responses evoked by the metabotropic glutamate receptor agonist 1S,3R-ACPD in neonatal rat cerebral cortex. *Br. J. Pharmacol.*, **112**, 231–239.

CHALLISS, R.A.J. & NAHORSKI, S.R. (1993). Measurement of inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, and phosphatidylinositol 4,5-bisphosphate in brain. *Methods Neurosci.*, **18**, 224–244.

CHEN, H.-S.V., PELLEGRINI, J.W., AGGARWAL, S.K., LEI, S.Z., WARACH, S., JENSEN, F.E. & LIPTON, S.A. (1992). Open-channel block of NMDA responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. *J. Neurosci.*, **12**, 4427–4436.

COURTNEY, M.J. & NICHOLLS, D.G. (1992). Interactions between phospholipase C-coupled and N-methyl-D-aspartate receptors in cultured cerebellar granule cells: protein kinase C mediated inhibition of N-methyl-D-aspartate responses. *J. Neurochem.*, **59**, 983–992.

DITZLER, K. (1991). Efficacy and tolerability of memantine in patients with dementia syndrome. *Arzneim. Forsch./Drug Res.*, **41**, 773–780.

DOWNES, C.P. & WUSTEMAN, M.M. (1983). Breakdown of polyphosphoinositides and not phosphatidylinositol accounts for the muscarinic agonist stimulated inositol phospholipid metabolism in rat parotid gland. *Biochem. J.*, **216**, 633–640.

ERDÖ, S.L. & SCHAEFER, M. (1991). Memantine is highly potent in protecting cortical cultures against excitotoxic cell death evoked by glutamate and NMDA. *Eur. J. Pharmacol.*, **198**, 215–217.

GOERTELMEYER, R. & ERBLER, H. (1992). Memantine in the treatment of mild to moderate dementia syndrome. A double-blind placebo-controlled study. *Arzneim. Forsch./Drug Res.*, **42**, 904–913.

GROSSMANN, W. & SCHUTZ, W. (1982). Memantine and neurogenic bladder dysfunction in spastic conditions. *Arzneim. Forsch./Drug Res.*, **32**, 1273–1276.

KEILHOFF, G. & WOLF, G. (1992). Memantine prevents quinolinic acid-induced hippocampal damage. *Eur. J. Pharmacol.*, **219**, 451–454.

KOH, J.-Y., PALMER, E. & COTMAN, C.W. (1991). Activation of the metabotropic glutamate receptor attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9431–9435.

KORNHUBER, J., BORMANN, J., RETZ, W., HUEBERS, M. & RIEDERER, P. (1989). Memantine displaces [^{3}H]MK-801 at therapeutic concentrations in postmortem human frontal cortex. *Eur. J. Pharmacol.*, **166**, 589–590.

LUPP, A., LUECKING, C.H., KOCH, R., JACKISCH, R. & FEUERSTEIN, T.J. (1992). Inhibitory effects of the antiparkinsonian drugs memantine and amantidine on NMDA-evoked acetylcholine release in rabbit caudate nucleus *in vitro*. *J. Pharmacol. Exp. Ther.*, **263**, 717–724.

MAJ, J. (1982). Die Wirkung von Memantin auf zentrale Neurotransmittersysteme. Eine Zusammenfassung der Ergebnisse. *Arzneim. Forsch./Drug Res.*, **32**, 1256–1259.

MAJ, J., SOWINSKA, H., BARAN, L. & SARNEK, J. (1974). Pharmacologic effects of 1,3-dimethyl-5-amino-adamantane, a new adamantane derivative. *Eur. J. Pharmacol.*, **26**, 9–14.

MONACO, M.E. & ADELSON, J.R. (1991). Evidence for coupling of resynthesis to hydrolysis in the phosphoinositide cycle. *Biochem. J.*, **279**, 337–341.

OSBORNE, N.N. & QUACK, G. (1992). Memantine stimulates inositol phosphates production in neurones and nullifies NMDA-induced destruction of retinal neurones. *Neurochem. Int.*, **21**, 329–336.

PARSONS, C.G., GRUNER, R., ROZENTAL, J., MILLAR, J. & LODGE, D. (1993). Patch clamp studies on the kinetics and selectivity of NMDA-receptor antagonism by memantine (1-amino-3,5-dimethyladmantan). *Neuropharmacology*, **32**, 1337–1350.

SCHNEIDER, E., FISCHER, P.A., CLEMENS, R., BALZEREIT, F., FUENFGELD, E.W. & HAASE, H.J. (1984). Effects of oral memantine on symptoms of Parkinson's disease. *Deutsche Med. Wochenzeitschr.*, **109**, 987–990.

SCHOEPP, D. (1985). Manganese stimulates the incorporation of [^{3}H]inositol into a pool of phosphatidylinositol in brain that is not coupled to agonist-induced hydrolysis. *J. Neurochem.*, **45**, 1481–1486.

SEIF EL NASR, M., PERUCHE, B., ROSSBERG, C., MENNEL, H.D. & KRIEGLSTEIN, J. (1990). Neuroprotective effect of memantine demonstrated *in vivo* and *in vitro*. *Eur. J. Pharmacol.*, **185**, 19–24.

SHEARS, S.B. (1991). Regulation of the metabolism of 1,2-diacylglycerols and inositol phosphates that respond to receptor activation. *Pharmacol. Ther.*, **49**, 79–104.

SILIPRANDI, R., LIPARTITI, M., FADDA, E., SAUTTER, J. & MANEV, H. (1992). Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. *Eur. J. Pharmacol.*, **219**, 173–174.

SIMPSON, C.M.F., BATTY, I.H. & HAWTHORNE, J.N. (1987). Physiological responses to receptor activation: phosphoinositide turnover. In *Neurochemistry – a Practical Approach* ed. Turner, A.J. & Bachelard, H.S. pp. 193–224, Oxford: IRL Press.

THOMAS, G.M.T., CUNNINGHAM, E., FENSOME, A., BALL, A., TOTTY, N.F., TRUONG, O., HSUAN, J.J. & COCKCROFT, S. (1993). An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signalling. *Cell*, **74**, 919–928.

WELLER, M., FINIELSMARLIER, F. & PAUL, S.M. (1993). NMDA receptor-mediated glutamate toxicity of cultured cerebellar, cortical and mesencephalic neurons: neuroprotective properties of amantidine and memantine. *Brain Res.*, **613**, 143–148.

WESEMANN, W., SONTAG, K.H. & MAJ, J. (1983). On the pharmacodynamics and pharmacokinetics of memantine. *Arzneim. Forsch./Drug Res.*, **33**, 1122–1134.

YANDRASITZ, J.R. & SEGAL, S. (1979). The effect of $MnCl_2$ on the basal and acetylcholine-stimulated turnover of phosphatidylinositol in synaptosomes. *FEBS Lett.*, **108**, 279–282.

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Effect of the vascular endothelium on noradrenaline-induced contractions in non-pregnant and pregnant guinea-pig uterine arteries

[1]Aleksandar Jovanović, Leposava Grbović & *Sofija Jovanović

Department of Pharmacology, Medical Faculty, P.O. Box 662 and *Department of Anatomy, Faculty of Veterinary Medicine, P.O. Box 370, Belgrade, YU-11000, Yugoslavia

1 The effect of pregnancy on noradrenaline-mediated contraction of guinea-pig uterine artery rings with both intact and denuded endothelium was investigated.

2 Noradrenaline (25 nM–100 μM) induced concentration-dependent contraction of non-pregnant and pregnant guinea-pig uterine arterial rings with intact endothelium with similar pD_2 and maximal response values (non-pregnant: $pD_2 = 5.85 \pm 0.02$, maximal response = 121 ± 8.2%; pregnant: $pD_2 = 5.81 \pm 0.04$, maximal response = 122 ± 9.1%). Removal of endothelium did not affect noradrenaline-induced contractions in non-pregnant guinea-pig uterine artery ($pD_2 = 5.97 \pm 0.02$, maximal response = 119 ± 8.6%). In contrast, in arteries from pregnant guinea-pigs, removal of endothelium shifted concentration-response curve for noradrenaline to the left, without affecting maximal response value ($pD_2 = 6.36 \pm 0.03$, maximal response = 120 ± 9.0%).

3 The pK_A values for noradrenaline were: 5.76 ± 0.09 and 5.82 ± 0.10 for non-pregnant guinea-pig uterine artery with intact and denuded endothelium, respectively and 5.74 ± 0.09 and 5.72 ± 0.07 for pregnant guinea-pig uterine artery with intact and denuded endothelium, respectively.

4 The receptor occupancy-response relationship for noradrenaline was linear for all types of vessels, except for pregnant guinea-pig uterine artery with denuded endothelium, since half-maximal response to noradrenaline was obtained with 44.8 ± 6.9% (non-pregnant guinea-pig uterine artery with intact endothelium), 43.3 ± 6.1% (non-pregnant guinea-pig uterine artery with denuded endothelium) and 44.3 ± 6.3% (pregnant guinea-pig uterine artery with intact endothelium) receptor occupancy. In pregnant guinea-pig uterine artery with denuded endothelium, occupancy-response relationship for noradrenaline was non-linear since half-maximal response to noradrenaline was obtained with 19.7 ± 3.3% receptor occupancy.

5 N^G-monomethyl-L-arginine (100 μM) and indomethacin (10 μM) did not affect concentration-response curve for noradrenaline in guinea-pig uterine arteries, regardless of pregnancy status or endothelial condition.

6 In quiescent preparations, the α-adrenoceptor antagonists, prazosin (5–50 nM) and yohimbine (1–10 μM) produced parallel rightward shifts of the curves for noradrenaline and the slopes of the Schild plots were not significantly different from unity. The plots constrained to a slope of unity gave the following $-\log K_B$ values: prazosin vs. yohimbine 8.78 ± 0.03 vs. 6.41 ± 0.02 for non-pregnant guinea-pig uterine artery with intact endothelium, 8.95 ± 0.03 vs. 6.34 ± 0.02 for non-pregnant guinea-pig uterine artery with denuded endothelium, 8.91 ± 0.01 vs. 6.44 ± 0.03 for pregnant guinea-pig uterine artery with intact endothelium and 9.07 ± 0.01 vs. 6.52 ± 0.03 for pregnant guinea-pig uterine artery with denuded endothelium.

7 It is concluded that initially there is no difference in noradrenaline action between uterine arteries from non-pregnant and pregnant guinea-pigs, but after removal of the endothelium the pregnant guinea-pig uterine artery is more sensitive to noradrenaline, which is related to increased receptor reserve for noradrenaline in this tissue. It is probable that relaxing factor derived from the endothelium mediates this effect, but it is unlikely to be nitric oxide or prostacyclin. Antagonist affinities and affinity of noradrenaline itself suggests that an identical subtype of α-adrenoceptor, probably the α_1 subtype, is involved in the noradrenaline-induced contraction of non-pregnant and pregnant guinea-pig uterine artery with or without endothelium.

Keywords: Noradrenaline; uterine artery; pregnancy; endothelium; α-adrenoceptors

Introduction

It is well known that pregnancy alters reactivity of uterine vasculature to a variety of vasoconstrictor substances (Griendling *et al.*, 1985; McLaughlin *et al.*, 1989; Weiner *et al.*, 1989; 1991; Steele *et al.*, 1993). This vascular alteration probably mediates the increased blood flow to the uterus that is characteristic of pregnancy (Peeters *et al.*, 1980). Since the uterine vasculature is richly innervated by sympathetic nerves (Bell, 1968; 1969; Ekesbo *et al.*, 1991), prior investigations focused on the pregnancy-associated changes in noradrenaline actions on this vascular bed (Barton *et al.*, 1974; Van Nimwegen & Dyer, 1974; Anderson *et al.*, 1977; Assali *et al.*, 1981; Nunotani *et al.*, 1985; Magness & Rosenfeld, 1986; Weiner *et al.*, 1989; 1991; Steele *et al.*, 1993).

However, data in reports regarding changes in noradrenaline-mediated responses in uterine arteries during pregnancy are still confusing. Previous investigations on the effects of noradrenaline on uterine vasculature demonstrated both decreased (Barton *et al.*, 1974; Anderson *et al.*, 1977; Magness & Rosenfeld, 1986; Weiner *et al.*, 1989; 1991) and increased (Van Nimwegen & Dyer, 1974; Assali *et al.*, 1981;

[1] Author for correspondence.

Nunotani *et al.*, 1985; Steele *et al.*, 1993) sensitivity to noradrenaline during pregnancy. Investigations of the mechanisms underlying the observed changes of noradrenaline actions are also controversial. It has been reported that affinity and subtypes of α-adrenoceptors in rat vasculature and rabbit uterus are affected by oestrogen treatment (Hoffman *et al.*, 1981; Colluci *et al.*, 1982). In contrast, Fallgren *et al.* (1988) reported that α-adrenoceptor function in the guinea-pig uterine artery is not altered during pregnancy. On the other hand, Weiner *et al.* (1991) demonstrated that removal of endothelium enhanced the effects of noradrenaline on uterine arteries taken from pregnant, but not on uterine arteries taken from non-pregnant guinea-pigs. Accordingly, it has been hypothesized that changes in sensitivity of uterine arteries to noradrenaline may be due to pregnancy-associated alteration of endothelial function (Weiner *et al.*, 1991; Steel *et al.*, 1993). However, the relationship between the endothelium and underlying receptor function of the noradrenaline actions in non-pregnant and pregnant guinea-pig uterine arteries has not yet been studied.

Therefore the purposes of this study were to (1) examine the influence of endothelium on noradrenaline-induced contractions of non-pregnant and pregnant guinea-pig uterine arteries and (2) to determine whether possible changes in uterine artery sensitivity to noradrenaline during pregnancy are due to altered adrenoceptors or/and endothelial function.

Methods

Vascular preparations

Adult female non-pregnant and pregnant (55 to 60 days' gestation; term, 65–68 days' gestation) guinea-pigs (700–900 g) were used in this study. The animals were stunned and decapitated. The right and left uterine arteries, respectively, were carefully dissected free from surrounding fat and connective tissue and cut into 3-mm long circular segments. All vessel segments were immediately placed in Krebs-Ringer-bicarbonate solution (composition in mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, CaEDTA 0.026, glucose 11.1). The endothelium was removed from the half rings by gently rubbing the intimal surface with stainless steel wire. Ring preparations were mounted between two stainless-steel triangles in an organ bath containing 10 ml Krebs-Ringer-bicarbonate solution (37°C, pH 7.4), aerated with 95% O_2 and 5% CO_2. One of the triangles was attached to a displacement unit allowing the fine adjustment of tension and the other was connected to a force-displacement transducer (Hugo Sachs K30). Isometric tension was recorded on a Hugo Sachs model MC 6621 recorder.

Preparations were allowed to equilibrate for 60 min in Krebs-Ringer-bicarbonate solution. During this period the organ baths were washed with fresh (37°C) buffer solution every 15 min.

After 60 min, each ring was gradually stretched to the optimal resting tension (non-pregnant: 4–5 mN, pregnant: 6–7 mN), as has been previously determined (Jovanović *et al.*, 1994). Once at their optimal length, the segments were allowed to equilibrate for 30 min before experimentation.

Experimental procedure

At the beginning and end of each experiment, the contractile response to 100 mM KCl solution was obtained. Only if the contractile responses to KCl were similar in magnitude (with variation less than 10%), were the data from this particular experiment included in the analysis.

Concentration-response curves for noradrenaline (25 nM–100 μM) were constructed by adding increasing concentrations of noradrenaline when the previous concentration had produced its equilibrium response, or after 5 min if no response was obtained. Experiments followed a multiple curve design since separate experiments in all types of preparations ($n = 7$ for each) demonstrated that first and second concentration-response curves for noradrenaline were not significantly different. At the end of each experiment, in order to confirm the presence or successful denudation of endothelium, the rings precontracted with 100 mM KCl were challenged with acetylcholine (10 μM). On the basis of prior study (Jovanović *et al.*, 1994) relaxation greater than 80% or less than 20% of maximal relaxation evoked by acetylcholine (maximal relaxation represented complete return to the resting tension from the contraction in response to KCl) was indicative of structurally intact or denuded endothelium, respectively, regardless of pregnancy status. The data from a particular tissue were rejected if the relaxation produced by acetylcholine was 20–80%. Additionally, at the end of some experiments (10 of each), the condition of the endothelium was verified by Van Gieson's staining with iron haematoxylin and light microscopic examination of the intimal surface (Disbrey & Rack, 1970). Therefore, the following protocol was used: (1) contraction in response to KCl followed by three washes and a 30 min equilibration period; (2) concentration-response curve with noradrenaline (used as the tissue control), followed by three washes, addition of the antagonist and a 30 N^G-monomethyl-L-arginine (L-NMMA), 40 (indomethacin) or 60 (prazosin and yohimbine) min equilibration period; (3) concentration-response curve with noradrenaline followed by three washes and a 30 min equilibration period; (4) contraction in response to KCl and addition of acetylcholine. When prazosin and yohimbine were used, three different concentrations of antagonist were used in the same day, but with only one concentration of antagonist per ring.

At the beginning of our investigation, in a separate series of experiments, concentration-response curves to noradrenaline (25 nM–100 μM) were made in the presence of 10 μM hydrocortisone, 10 μM desipramine, and 10 μM propranolol to block neuronal and extraneuronal uptake of noradrenaline and β-adrenoceptors, respectively (Furchgott, 1972). Since these compounds did not affect the concentration-response curves for noradrenaline in the vessels studied (see Results), hydrocortisone, desipramine and propanolol were excluded from further study.

In order to determine the dissociation constant (K_A) for noradrenaline, the irreversible antagonist, dibenamine, was used (Furchgott, 1966). After determination of the concentration-response curve for noradrenaline, rings were exposed to dibenamine (50 nM) for 30 min and then rinsed at 10 min intervals for next 60 min. After this period, reproducible concentration-response curves to noradrenaline were obtained which were depressed and shifted to the right with respect to the control.

Treatment of data and statistics

The contraction induced by each concentration of noradrenaline was expressed as a percentage of the contraction due to KCl and was used for making the concentration-response curves. The concentration of noradrenaline eliciting 50% of its own maximum response (EC_{50}) was determined graphically for each curve by linear interpolation. EC_{50} values are presented as pD_2 ($pD_2 = -\log EC_{50}$).

For the assessment of the effect of the α-adrenoceptor antagonists, three different concentrations of the antagonist were examined. The pA_2 values of the antagonists were determined by Schild analysis, as the intercept of the regression line with the abscissa (Arunlakshana & Schild, 1959). The concentration-ratios were also used to calculate a modified Schild plot with a slope of −1, thus an estimate of the −log dissociation constant of the antagonist (−log K_B value) (Tallarida *et al.*, 1979). The significance of the Schild plot

linearity was tested by analysis of variance (Kenakin, 1987). The closeness of the slope to unity was verified by t test and was considered not different from unity if $P > 0.05$.

The noradrenaline dissociation constant (K_A) was calculated according to the procedure described by Furchgott & Bursztyn (1967): after partial receptor inactivation by an irreversible antagonist, dibenamine, equieffective concentrations of noradrenaline before [A] and after treatment [A'] were determined. 1/[A] was plotted against 1/[A']. The slope of the regression line and the y-intercept were used to calculate the noradrenaline dissociation constant (K_A): K_A = (Slope-1)/intercept. K_A values are presented as pK_A ($pK_A = -\log K_A$).

Estimates of receptor reserve were made from K_A/EC_{50} (Ruffolo, 1982; Kenakin, 1987). The fraction of receptors occupied (RA/RT) was calculated from the following equation: [RA]/[RT] = [A]/(K_A + [A]) (Furchgott & Burzstyn, 1967).

The results are expressed as means ± s.e.mean; n refers to the number of experiments. One-way analysis of variance (ANOVA) was used when more. than two groups were analyzed. Statistical significance of differences between two means was determined with Student's t test for paired or unpaired observations where appropriate. A value of $P < 0.05$ was considered to be statistically significant. The least squares method was used for calculating linear regressions.

Drugs used

The following drugs were used: noradrenaline bitartarate, prazosin hydrochloride, yohimbine hydrochloride, indomethacin, acetylcholine chloride, desipramine, hydrocortisone, propranolol (Sigma, U.S.A.); N^G-monomethyl-L-arginine acetate (L-NMMA) (Wellcome, UK); dibenamine hydrochloride (Smith Kline & French, UK). All drug solutions were prepared immediately before the experiment and stored on ice until use. Dibenamine solution was prepared in 99% ethanol. Indomethacin was dissolved in equimolar Na_2CO_3 solution. All other agents were dissolved in distilled water and diluted to the desired concentration with buffer. Previous experiments showed that the solvents used had no effect on preparations at the concentrations applied. All drugs were added directly to the bath in volume of 100 µl and the concentrations given are the calculated final concentration in the bath solution. When potassium chloride was used as a spasmogen, the stated concentration excludes the potassium chloride (4.7 mM) already present in the Krebs-Ringer-bicarbonate solution.

Results

Effect of noradrenaline on non-pregnant and pregnant guinea-pig uterine arteries

Noradrenaline (25 nM–100 µM) induced concentration-dependent contraction of both non-pregnant and pregnant guinea-pig uterine arterial rings with intact endothelium (non-pregnant: $pD_2 = 5.85 \pm 0.02$, maximal response = 121 ± 8.2%, 5.97 ± 0.48 mN, $n = 73$; pregnant: $pD_2 = 5.81 \pm 0.04$, maximal response = 122 ± 9.1%, 6.49 ± 0.59 mN, $n = 75$). The difference between the pD_2 and maximal response values was not statistically significant ($P > 0.05$). The removal of endothelium slightly enhanced contractions of non-pregnant uterine artery evoked by noradrenaline ($pD_2 = 5.97 \pm 0.02$, maximal response = 119 ± 8.6%, $n = 73$, 6.01 ± 0.52 mN, $P > 0.05$). On the contrary, removal of endothelium in pregnant guinea-pig uterine artery significantly shifted concentration-response curve for noradrenaline to the left ($P < 0.01$), without affecting the maximal response value ($pD_2 = 6.36 \pm 0.03$, maximal response 120 ± 9.0%, 7.35 ± 0.66 mN, $n = 75$) (Figure 1).

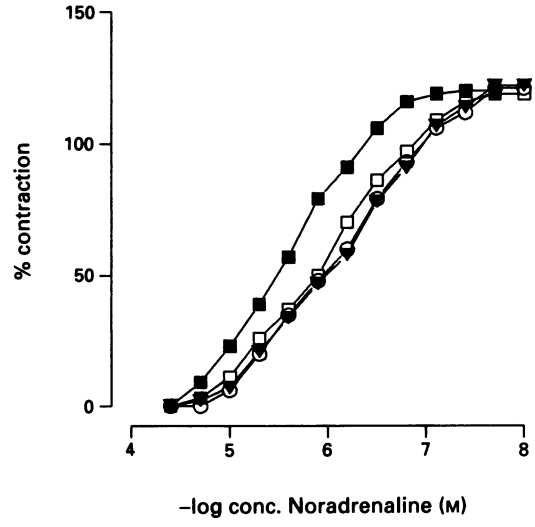


Figure 1 Concentration-response curves for noradrenaline in non-pregnant guinea-pig uterine artery with intact (○) and denuded endothelium (□), and in pregnant guinea-pig uterine artery with intact (▼) and denuded endothelium (■). Each point represents the mean (n = 73–75). Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as a percentage of the contraction induced by KCl (100 mM).

The presence of hydrocortisone, desipramine and propranolol in the bath solution during experimentation did not affect the concentration-response curve for noradrenaline in uterine arteries with either intact or denuded endothelium, irrespective of whether they were taken from non-pregnant or pregnant animals (non-pregnant with intact endothelium: $pD_2 = 5.88 \pm 0.08$, maximal response = 119 ± 9.1%, $n = 7$, in the absence and $pD_2 = 5.84 \pm 0.07$, maximal response = 122 ± 11.2%, $n = 7$, in the presence of these compounds; non-pregnant with denuded endothelium: $pD_2 = 5.91 \pm 0.06$, maximal response = 122 ± 8.6% in the absence and $pD_2 = 5.94 \pm 0.09$, maximal response = 126 ± 12.1% in the presence of these compounds; pregnant with intact endothelium: $pD_2 = 5.83 \pm 0.05$, maximal response = 123 ± 10.2%, $n = 7$, in the absence and $pD_2 = 5.86 \pm 0.08$, maximal response = 125 ± 12.8% in the presence of these compounds; pregnant with denuded endothelium: $pD_2 = 6.33 \pm 0.06$, maximal response = 120 ± 7.9%, $n = 7$, in the absence and $pD_2 = 6.26 \pm 0.10$, maximal response = 126 ± 11.4% in the presence of these compounds) (data not shown).

Dissociation constant of noradrenaline-receptor complex

The dissociation constant (K_A) for noradrenaline was determined by partial alkylation of the adrenoceptor population with the irreversible antagonist, dibenamine (50 nM). The examples of these experiments are presented in Figure 2. The mean pK_A values were: 5.76 ± 0.09 ($n = 11$) and 5.82 ± 0.10 ($n = 11$) for non-pregnant guinea-pig uterine artery with intact and denuded endothelium, respectively; 5.74 ± 0.09 ($n = 11$) and 5.72 ± 0.07 ($n = 11$) for pregnant guinea-pig uterine artery with intact and denuded endothelium, respectively. The concentration-response relationship with noradrenaline (Figure 3a) was replotted as a function of the receptor occupancy using calculated mean K_A value (see Methods) and is shown in Figure 3b. An almost linear stimulus-response relationship was obtained for non-pregnant guinea-pig uterine arteries with both intact and denuded endothelium and for pregnant guinea-pig uterine artery with intact endothelium (Figure 3c,d,e). On the contrary, a non-linear stimulus-response relationship was obtained for pregnant guinea-pig uterine artery with denuded endothelium

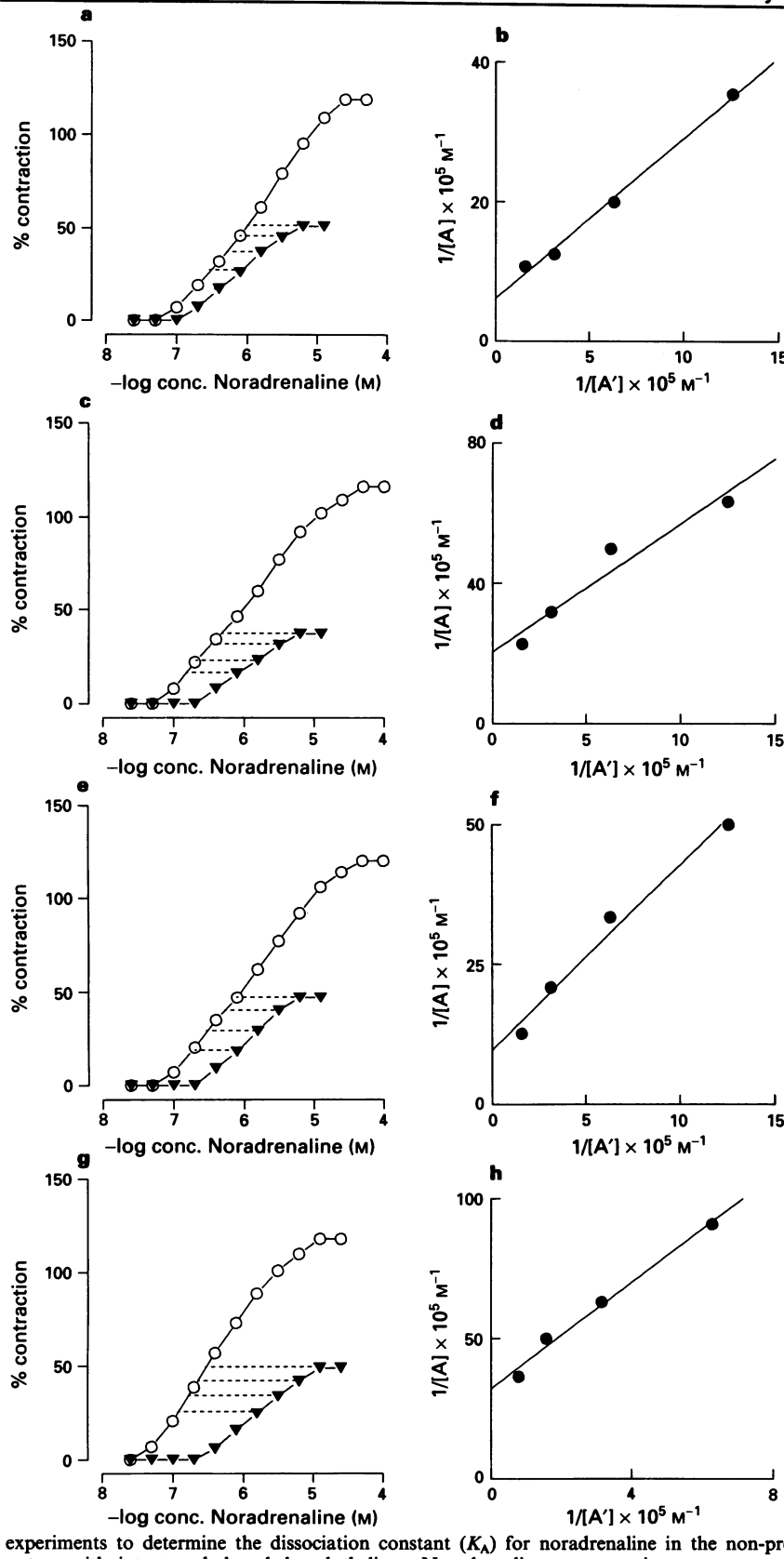


Figure 2 Typical experiments to determine the dissociation constant (K_A) for noradrenaline in the non-pregnant and pregnant guinea-pig uterine artery with intact and denuded endothelium. Noradrenaline concentration-response curves in non-pregnant guinea-pig uterine artery with intact (a) and denuded endothelium (c), and in pregnant guinea-pig uterine artery with intact (e) and denuded endothelium (g) before (○) and after (▼) exposure of the preparation to dibenamine (50 nM). Responses are expressed as percentages of the contraction induced by KCl (100 mM). Equieffective concentrations of noradrenaline were determined graphically by linear interpolation of the concentration-response curves for noradrenaline in the absence and presence of dibenamine. (b) Double-reciprocal plot of equieffective concentrations of noradrenaline before (ordinate scale, 1/[A]) and after (abscissa scale, 1/[A']) treatment with dibenamine, obtained from (A) ($Y = 2.30 \times + 6.13 \times 10^5$, $r = 0.997$; $pK_A = 5.68$). (d) Double-reciprocal plot of equieffective concentrations of noradrenaline before (ordinate scale, 1/[A]) and after (abscissa scale, 1/[A']) treatment with dibenamine, obtained from (c) ($Y = 3.68 \times + 2.01 \times 10^6$, $r = 0.968$; $pK_A = 5.87$). (f) Double-reciprocal plot of equieffective concentrations of noradrenaline before (ordinate scale, 1/[A]) and after (abscissa scale, 1/[A']) treatment with dibenamine, obtained from (E) ($Y = 3.31 \times + 9.60 \times 10^5$, $r = 0.990$; $pK_A = 5.62$). (h) Double-reciprocal plot of equieffective concentrations of noradrenaline before (ordinate scale, 1/[A]) and after (abscissa scale, 1/[A']) treatment with dibenamine, obtained from (G) ($Y = 9.52 \times + 3.20 \times 10^6$, $r = 0.994$; $pK_A = 5.58$).

(Figure 3f). The half-maximal response to noradrenaline needed 44.8 ± 6.9% (non-pregnant guinea-pig uterine artery with intact endothelium, $n = 11$), 43.3 ± 6.1% (non-pregnant guinea-pig uterine artery with denuded endothelium, $n = 11$), 44.3 ± 6.3% (non-pregnant guinea-pig uterine artery with intact endothelium, $n = 11$) and 19.7 ± 3.3% (pregnant guinea-pig uterine artery with denuded endothelium, $n = 11$) receptor occupancy. The receptor reserve expressed as K_A/EC_{50} was 1.22 ± 0.31 (non-pregnant guinea-pig uterine artery with intact endothelium, $n = 11$), 1.32 ± 0.33% (non-

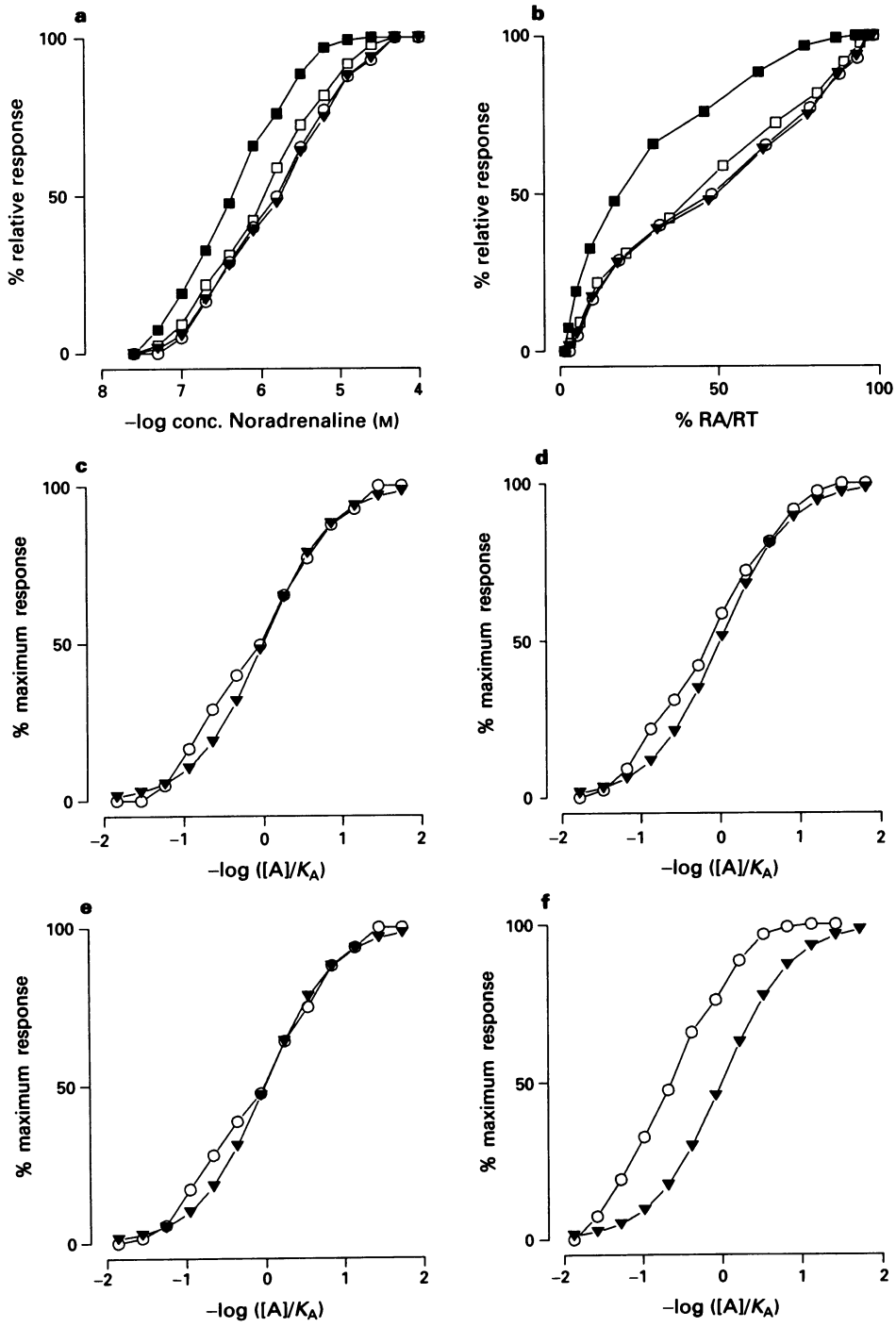


Figure 3 Relationship of the relative response to the −log concentration and the fraction of receptors occupied (RA/RT) in noradrenaline-induced contraction. (a) Concentration-response curve for noradrenaline in non-pregnant guinea-pig uterine artery with intact (○) and denuded endothelium (□), and in pregnant guinea-pig uterine artery with intact (▼) and denuded endothelium (■). Each point represents the mean ($n = 73-75$). Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as percentages of the maximal contraction induced by noradrenaline (100 μM). (b) Replot of data from (a) showing the relative response to noradrenaline in non-pregnant guinea-pig uterine artery with intact (○) and denuded endothelium (□), and in pregnant guinea-pig uterine artery with intact (▼) and denuded endothelium (■) as a function of receptors occupied by noradrenaline. The fraction of receptors occupied was calculated employing the average K_A values for noradrenaline (for non-pregnant guinea-pig uterine artery with intact endothelium: 1.74 μM; for non-pregnant guinea-pig uterine artery with denuded endothelium: 1.51 μM; for pregnant guinea-pig uterine artery with intact endothelium: 1.82 μM; for pregnant guinea-pig uterine artery with denuded endothelium: 1.19 μM). (c, d, e, f) The percentage of maximal responses (○) or receptor occupancy (▼) as a function of log conc. noradrenaline normalized as fractions of average K_A for non-pregnant guinea-pig uterine artery with intact (c) and denuded endothelium (d), and for pregnant guinea-pig uterine artery with intact (e) and denuded (f) endothelium.

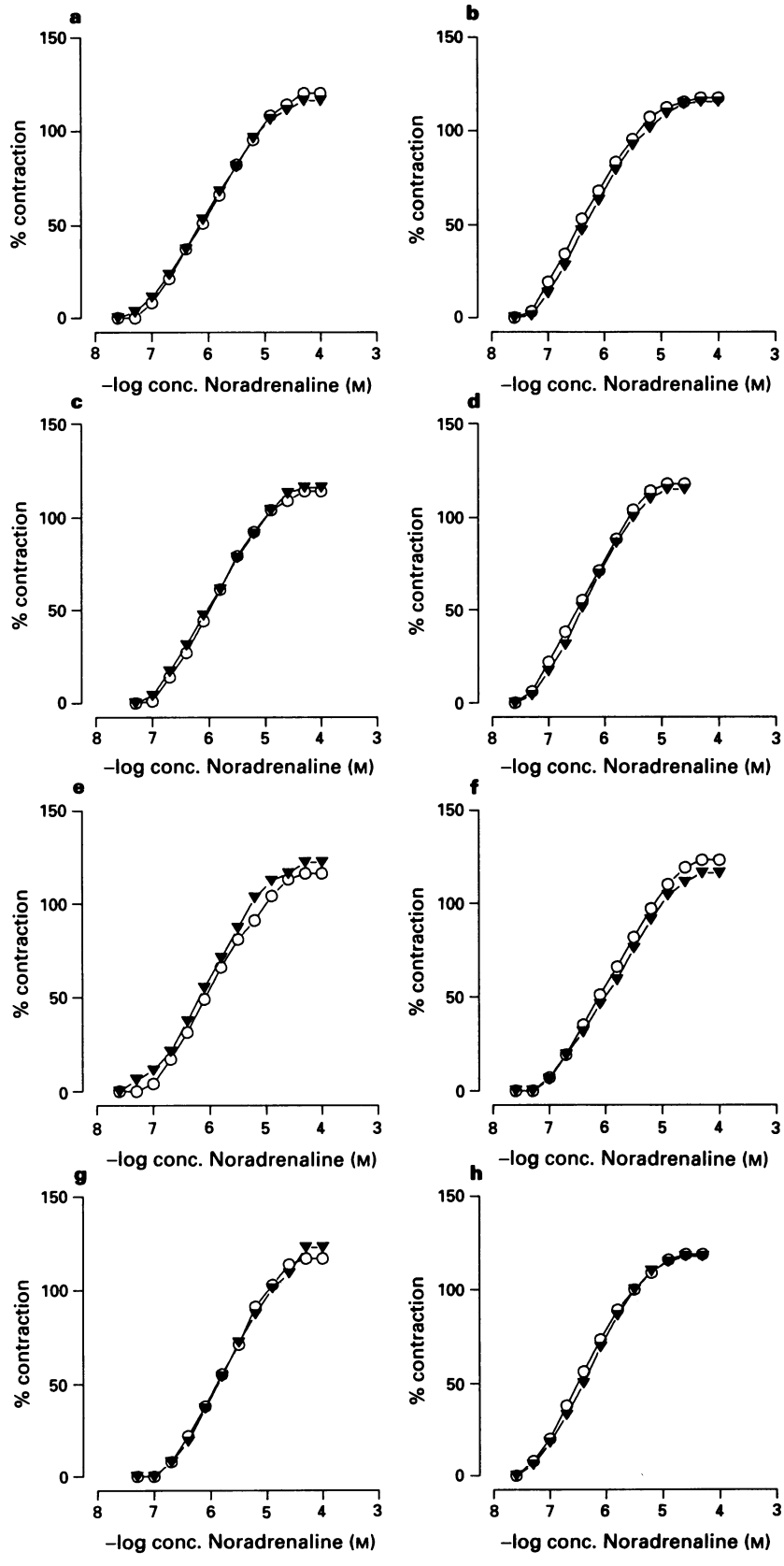


Figure 4 The antagonism of the contraction effects of noradrenaline by indomethacin and N^G-monomethyl-L-arginine (L-NMMA). (a, b, c, d) Concentration-response curves for noradrenaline in non-pregnant guinea-pig uterine artery with intact (a) and denuded (b) endothelium, and in pregnant guinea-pig uterine artery with intact (c) and denuded endothelium (d) in the absence (O) and presence (▼) of 10 μM indomethacin. Each point represents the mean ($n = 7-9$). Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as a percentage of the contraction induced by KCl (100 mM). (e, f, g, h) Concentration-response curves for noradrenaline in non-pregnant guinea-pig uterine artery with intact (a) and denuded (b) endothelium, and in pregnant guinea-pig uterine artery with intact (c) and denuded endothelium (d) in the absence (O) and presence (▼) of 100 μM L-NMMA. Each point represents the mean ($n = 7-9$). Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as a percentage of the contraction induced by KCl (100 mM).

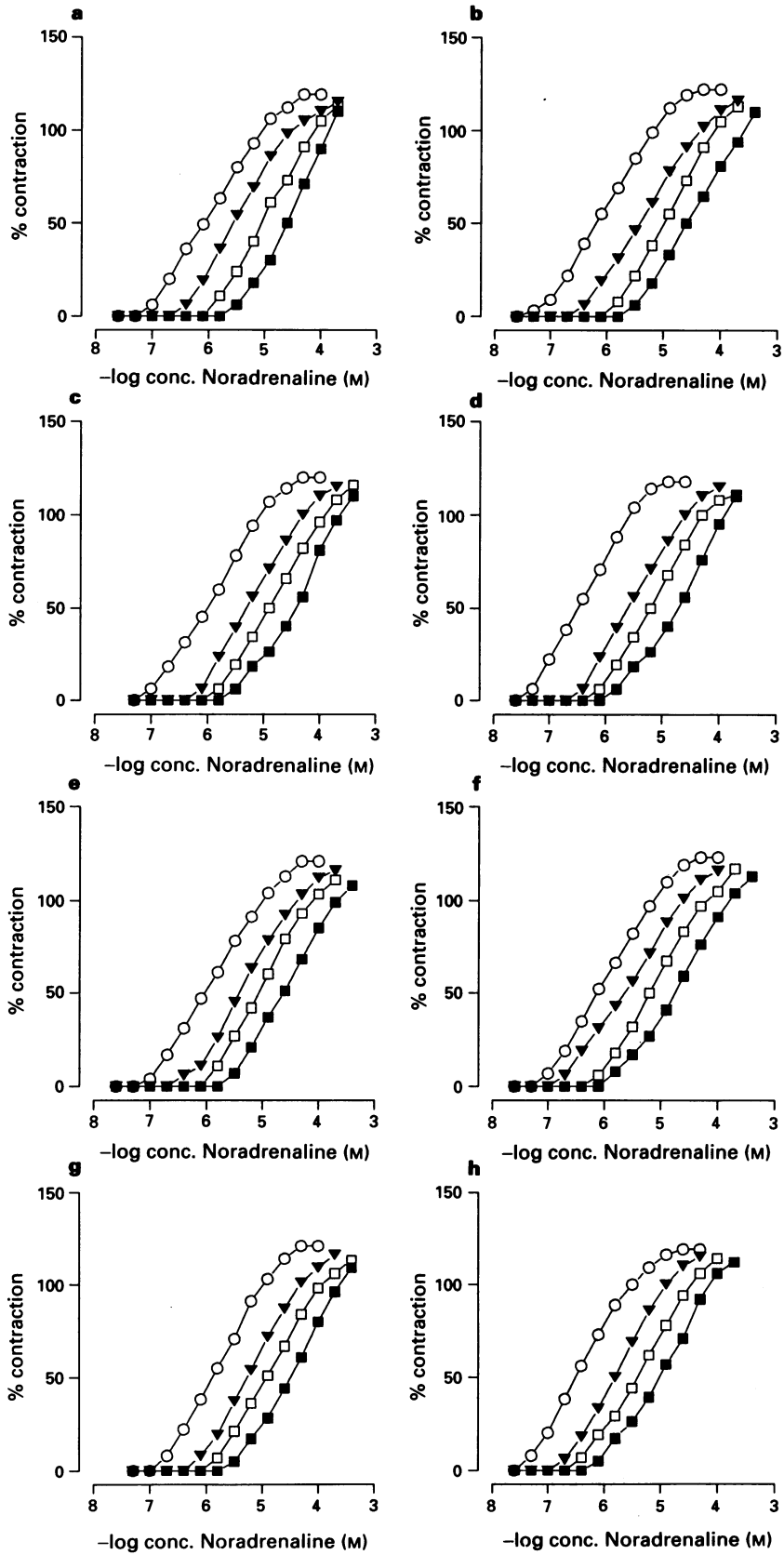


Figure 5 The antagonism of the contraction effects of noradrenaline by antagonists of α-adrenoceptors. (a, b, c, d) Concentration-response curves for noradrenaline in non-pregnant guinea-pig uterine artery with intact (a) and denuded endothelium (b), and in pregnant guinea-pig uterine artery with intact (c) and denuded endothelium (d) in the absence (○) and presence of 5 nM (▼), 15 nM (□) and 50 nM (■) prazosin. Each point represents the mean of 6–23 experiments. Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as percentages of the contraction induced by KCl (100 mM). (e, f, g, h) Concentration-response curves for noradrenaline in non-pregnant guinea-pig uterine artery with intact (e) and denuded endothelium (f), and in pregnant guinea-pig uterine artery with intact (g) and denuded endothelium (h) in the absence of (○) and presence of 1 μM (▼), 3 μM (□) and 10 μM (■) yohimbine. Each point represents the mean of 6–22 experiments. Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as percentages of the contraction induced by KCl (100 mM).

pregnant guinea-pig uterine artery with denuded endothelium, $n = 11$), $1.26 \pm 0.35\%$ (pregnant guinea-pig uterine artery with intact endothelium, $n = 11$) and $4.09 \pm 0.61\%$ (pregnant guinea-pig uterine artery with denuded endothelium, $n = 11$). The difference between the pK_A values was not statistically significant ($P > 0.05$), regardless of pregnancy status or endothelium condition. In contrast, the receptor reserve and receptor occupancy values for pregnant guinea-pig uterine artery with denuded endothelium were significantly greater ($P < 0.01$ for both values) than those values for other three types of arteries.

Effects of L-NMMA and indomethacin on noradrenaline-induced contractions

The pre-addition of indomethacin (10 μM) and L-NMMA (100 μM) did not affect contractions of uterine arteries evoked by noradrenaline, regardless of endothelial condition or pregnancy status ($n = 7-9$) (Figure 4).

Effects of prazosin and yohimbine on noradrenaline-induced contractions

In both non-pregnant and pregnant guinea-pig uterine arteries, with either intact or denuded endothelium, a 'selective' α_1-adrenoceptor antagonist, prazosin (5–50 nM) and a 'selective' α_2-adrenoceptor antagonist, yohimbine (1–10 μM), induced a significant shift to the right in a concentration-dependent manner ($P < 0.01$, for both antagonists studied), without suppression of the maximum of the concentration-response curves for noradrenaline ($P > 0.05$, for both antagonists studied) (Figure 5). The data from the experiments with α-adrenoceptor antagonists were analyzed as described by Arunlakshana & Schild (1959). In both non-pregnant and pregnant guinea-pig uterine arteries with intact and denuded endothelium, the experiments with prazosin and yohimbine yielded straight lines ($P > 0.05$, for both antagonists studied) with mean slope not different from unity (Figure 6, Table 1). The pA_2 and $-\log K_B$ values are shown in Table 1. The $-\log K_B$ values for corresponding antagonists were not significantly different, regardless of the endothelium condition or pregnancy status ($P > 0.05$).

Discussion

Although extensively studied, the effect of pregnancy on noradrenaline-mediated responses in uterine arteries is still controversial. It should be mentioned that the majority of studies were performed before the importance of the endothelium in vascular responsiveness had been established. Consequently, these studies did not take into account the endothelium condition and therefore, unfortunately, they are not comparable to our study.

In prior investigations with verified endothelium condition, it has been shown that sensitivity to noradrenaline is significantly reduced in the pregnant guinea-pig uterine artery with intact endothelium compared to that in the non-pregnant guinea-pig uterine artery with the same endothelial status (Weiner *et al.*, 1989; 1991). On the basis of these results Weiner *et al.* (1989; 1991) suggested that pregnancy-associated changes in endothelial functions decrease the contractile response to noradrenaline in the guinea-pig uterine artery (Weiner *et al.*, 1991). In the current study we did not confirm this hypothesis. We did not find differences in noradrenaline-induced contractions between non-pregnant and pregnant guinea-pig uterine arteries with intact endothelium, but removal of the endothelium in the pregnant guinea-pig uterine artery significantly shifted the concentration-response curve for noradrenaline to the left (relative EC_{50} ratio of endothelium intact: endothelium denuded, 3.44:1), which was not observed in arteries from non-pregnant animals. It is difficult to explain this disagree-

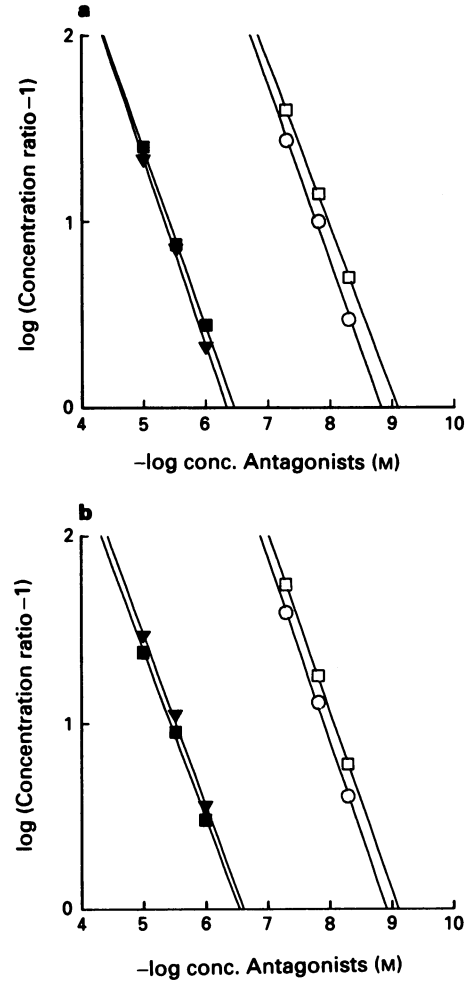


Figure 6 Schild plots of log (concentration ratio − 1) against − log conc. antagonist for noradrenaline-prazosin (○ for arteries with intact and □ for arteries with denuded endothelium) and noradrenaline-yohimbine antagonism (▼ for arteries with intact and ■ for arteries with denuded endothelium) on non-pregnant (a) and pregnant (b) guinea-pig uterine artery. Each point represents the mean ($n = 6-8$).

Table 1 pA_2, $-\log K_B$ values and slopes of Schild plots of the prazosin and yohimbine on the α-adrenoceptors in the non-pregnant and pregnant guinea-pig uterine arteries, determined by their ability to antagonize noradrenaline-induced contraction of vascular segments

Endothelium intact (non-pregnant)	*pA_2*	*Slope*	*$-\log K_B$*
Prazosin	8.83 ± 0.09	0.95 ± 0.08	8.78 ± 0.03
Yohimbine	6.46 ± 0.03	0.95 ± 0.03	6.41 ± 0.02
Endothelium denuded (non-pregnant)			
Prazosin	9.09 ± 0.04	0.89 ± 0.02	8.95 ± 0.03
Yohimbine	6.34 ± 0.05	1.00 ± 0.05	6.34 ± 0.02
Endothelium intact (pregnant)			
Prazosin	8.92 ± 0.06	0.99 ± 0.05	8.91 ± 0.01
Yohimbine	6.56 ± 0.08	0.88 ± 0.06	6.44 ± 0.03
Endothelium denuded (pregnant)			
Prazosin	9.12 ± 0.03	0.96 ± 0.02	9.07 ± 0.01
Yohimbine	6.62 ± 0.09	0.92 ± 0.07	6.52 ± 0.03

The values are expressed as means ± s.e.mean ($n = 6-8$).

ment, but it may be due to differences in experimental design. For example, Weiner *et al.* (1989, 1991) considered 20% relaxation of guinea-pig uterine artery evoked by acetylcholine (100 μM) as indicative of functional endothelium, while our criteria was at least 80% relaxation in response to acetylcholine (10 μM). It should be mentioned that mechanical removal of the endothelium can affect reactivity of vascular smooth muscle to various vasoconstrictors, which could be due to damage caused by this procedure. This methodological error would apparently lead to the wrong conclusion about the endothelium-dependent modulation of vasoconstrictor action. However, the similarity in maximal achieved tension in response to noradrenaline and in contraction evoked by KCl between all types of preparations studied probably excludes this possibility, Moreover, in our laboratory this effect of vascular endothelium removal in pregnant guinea-pig uterine arteries was not observed on prostaglandin $F_{2\alpha}$-induced contractions (Jovanović *et al.*, 1994). It seems that the endothelium-dependent modulation of vasoconstrictor action during pregnancy probably depends on the agonist used. Nevertheless, experimental data from our study are very similar to those obtained with non-pregnant and pregnant human uterine radial arteries (Steele *et al.*, 1993). In addition, the pD_2 and maximal response values for non-pregnant and pregnant guinea-pig uterine artery with intact endothelium obtained from the present investigation are similar to those reported by Fallgren & Edvinsson (1986) and Fallgren *et al.* (1988).

The noradrenaline-induced contraction in all quiescent vessels was not affected by hydrocortisone and desipramine, which suggests that only little neuronal and extraneuronal uptake occurs in the guinea-pig uterine artery, regardless of pregnancy status or endothelial condition. Similarly, the pre-addition of propranolol also did not affect the concentration-response curve for noradrenaline in preparations studied. It appears that there is probably not a significant population of β-adrenoceptors in the guinea-pig uterine artery, irrespective of whether they were taken from non-pregnant animals or whether they were with intact or denuded endothelium. These findings correspond to those reported for non-pregnant and pregnant guinea-pig uterine arteries with intact endothelium (Fallgren & Edvinsson, 1986; Fallgren *et al.*, 1988).

It is established that noradrenaline induces contraction of isolated blood vessels through activation of both α_1- and α_2-adrenoceptor (Timmermans & Van Zwieten, 1981). The pA_2 value for an antagonist in blocking the response to an agonist should be an accurate indication of its affinity for binding to the receptor site if certain criteria were fulfilled (Furchgott, 1972; Ruffolo, 1982; Kenakin, 1987). In order to compare the contribution of different α-adrenoceptor subtypes to the noradrenaline-induced contraction between vessels studied, we used prazosin, a predominantly α_1-adrenoceptor antagonist (Cambridge *et al.*, 1977; Starke, 1981; Skarby & Larsson, 1987) and yohimbine, a predominantly α_2-adrenoceptor antagonist (Doxey *et al.*, 1977; Ruffolo *et al.*, 1981; Goldberg & Robertson, 1983). Although both antagonists are considered as competitive, Alosachie & Godfraind (1986, 1988) reported in rat aorta non-competitive antagonism by prazosin of the noradrenaline concentration-response curve in the presence and competitive antagonism in the absence of the endothelium. We did not observe this deviation of antagonism by prazosin since the slopes of Schild plot for both prazosin and yohimbine were not significantly different from unity, indicating that the antagonism is competitive and therefore that the pA_2 value obtained constrained to unity can be calculated as the $-\log K_B$ value (Arunlakshana & Schild, 1959). It is known that the K_B value for a specific antagonist acting on the same type of receptor in different preparations should be the same (Furchgott, 1972). In non-pregnant guinea-pig uterine arteries, affinity estimates for prazosin and yohimbine were not different from the estimates obtained in pregnant guinea-pig uterine arteries, regardless of the endothelium condition. Therefore, the possibility that different α-adrenoceptor subtypes are involved in noradrenaline-induced contraction of pregnant and non-pregnant uterine arteries with both intact and denuded endothelium was eliminated. Besides, affinity estimates for noradrenaline itself also did not differ between the tissues studied, regardless of pregnancy status or endothelial condition. The $-\log K_B$ values for prazosin obtained in our study (8.78–9.07) are higher than pA_2 values reported for non-pregnant and pregnant guinea-pig uterine arteries with intact endothelium (7.52–7.97, Fallgren & Edvinsson, 1986; Fallgren *et al.*, 1988), but similar to pA_2 values reported for pregnant ovine uterine artery (9.1, Isla & Dyer, 1990) and for arteries supplying the heifers oviduct (9.38, Costa *et al.*, 1992). Nevertheless, the affinities of prazosin for antagonizing the contraction induced by noradrenaline is within the range reported for α_1-adrenoceptor blockade (Agrawal *et al.*, 1984; Skarby & Larsson, 1987), which suggests the presence of contraction-mediating α_1-adrenoceptors in the preparations studied. In contrast, the $-\log K_B$ values of yohimbine observed at receptors mediating contraction of uterine arteries were not analogous to pA_2 or pK_i values for α_2-adrenoceptors obtained in human fat cells (8.37, Berlan & Lafontan, 1980), rat vas deferens (8.32, Kupur & Mottram, 1978), human metacarpal veins (8.41, Stevens & Moulds, 1981) and human digital arteries (8.20, Stevens & Moulds, 1981). The $-\log K_B$ values for yohimbine determined in our study correspond to those values obtained at α_1-adrenoceptors receptors (Ruffolo *et al.*, 1981).

On the basis of these results, it seems reasonable to suggest that in guinea-pig uterine artery noradrenaline induces contractions predominantly via activation of α_1-adrenoceptors, regardless of pregnancy status or endothelial condition. This conclusion is consistent with the concept that in non-pregnant and pregnant guinea-pig uterine arteries with intact endothelium α-adrenoceptors are mainly of the α_1 subtype (Fallgren & Edvinsson, 1986; Fallgren *et al.*, 1988). Further evidence for this hypothesis is the observation that the obtained K_A values for noradrenaline are in the range reported for K_A in other vascular beds in which predominance of α_1-adrenoceptors exists (Oriowo *et al.*, 1989; Isla & Dyer 1990; Costa *et al.*, 1992).

It has been known for some time that removal of vascular endothelium can potentiate responses to exogenously applied noradrenaline (Cocks & Angus, 1983; Lues & Schumann, 1984; Aloschie & Godfraind, 1988). It has been also demonstrated that in some blood vessels removal of endothelium enhanced receptor reserve for noradrenaline (Alosachie & Godfraind, 1988). In the present study, in all types of arteries, except in pregnant guinea-pig uterine artery with denuded endothelium, partial receptor alkylation of α-adrenoceptors with dibenamine revealed a linear relationship between contraction and percentage receptor occupancy for noradrenaline, indicating very low or no receptor reserve for noradrenaline in these tissues. Very similar data for noradrenaline have been obtained with the pregnant ovine uterine artery with intact endothelium (Isla & Dyer, 1990). In contrast, in the pregnant guinea-pig uterine artery with denuded endothelium, the receptor occupancy-response curve manifested a hyperbolic relation between receptor occupation and contractile response, indicating the presence of considerable amount of receptor reserve. Quantification of the receptor reserve by ratio K_A/EC_{50} expresses the efficiency of coupling and/or receptor density (Ruffolo, 1982; Kenakin, 1987). In pregnant guinea-pig with denuded endothelium, this ratio is clearly greater than unity, suggesting that in this tissue, noradrenaline acts as a full agonist. Since the affinity of the contractile mediating α-adrenoceptor is not altered during pregnancy, the greater potency of noradrenaline in pregnant guinea-pig uterine artery with denuded endothelium probably reflects a better coupling of the noradrenaline-receptor complex or/and greater receptor den-

sity in this tissue during pregnancy (Kenakin, 1987). However, the reduced receptor reserve for noradrenaline in the presence of endothelium in this type of preparation, as opposed to the non-pregnant guinea-pig uterine arteries, allowed the supposition that during pregnancy the function of endothelium is probably altered as well. It is likely that increased production of relaxing factor(s) during pregnancy countered the effect of pregnancy on α-adrenoceptors. It has been demonstrated in numerous blood vessels that the modulation by endothelium of the agonist-induced contractile response is related to release of nitric oxide (NO) from the endothelium (Weiner *et al.*, 1991; Vinet *et al.*, 1991; Adeagbo & Triggle, 1993) which in turn could result in a reduction of agonist efficacy and receptor reserve (Aloschie & Godfraind, 1986; Malta *et al.*, 1986; Martin *et al.*, 1986). It has been proposed that NO produced by the endothelium can be considered as an endogenous functional antagonist reducing the apparent efficacy of the adrenoceptor agonist (Alosachie & Godfraind, 1988). On the contrary, in some blood vessels the similar role has been suggested for prostacyclin (Pipili-Synetos *et al.*, 1991). In order to analyze these possibilities, the inhibitor of NO synthesis, L-NMMA (Palmer *et al.*, 1988; Rees *et al.*, 1989) and a cyclo-oxygenase inhibitor, indomethacin (Vane, 1971) were tested. In all types of preparations studied, L-NMMA and indomethacin, in concentrations clearly sufficient to inhibit NO-synthase (Palmer *et al.*, 1988; Rees *et al.*, 1989; Weiner *et al.*, 1991; Vinet *et al.*, 1991; Adeagbo & Triggle, 1993) and cyclo-oxygenase (Toda, 1984), did not affect contractions in response to noradrenaline. These unexpected results suggest that enhanced sensitivity of pregnant guinea-pig uterine artery to noradrenaline and enhanced receptor reserve for this agent in this tissue, after removal of endothelium, is probably not related to release of NO or prostacyclin from the endothelial cells. It is apparent that relaxing factor derived from the endothelium reduces the receptor reserve for noradrenaline in this vessel only during pregnancy, but it is unlikely to be nitric oxide or prostacyclin. At this moment it is not possible to come to a definite conclusion regarding the nature of the relaxing factor produced by endothelium mediating this effect of pregnancy on noradrenaline-induced contraction of uterine artery. Further investigations are necessary to resolve this problem. However, it should be mentioned that these findings are in contrast with some reported previously (Weiner *et al.*, 1991), but in agreement with the results obtained for human uterine radial arteries (Steel *et al.*, 1993).

In conclusion, this study has shown that the noradrenaline-induced contraction of guinea-pig uterine artery is altered during pregnancy. Initially there is no difference between noradrenaline-mediated responses in non-pregnant and pregnant guinea-pig uterine arteries, but after removal of endothelium only the pregnant guinea-pig uterine artery became more sensitive to noradrenaline. In non-pregnant guinea-pig uterine arteries and in pregnant guinea-pig uterine artery with intact endothelium there is a low receptor reserve for noradrenaline, indicating that noradrenaline acts in these tissues as a partial agonist. In contrast, a considerable receptor reserve for noradrenaline exists in pregnant guinea-pig uterine artery with denuded endothelium, indicating that noradrenaline acts in this vessel as a full agonist. The removal of endothelium in the pregnant guinea-pig uterine artery was related to increased receptor reserve for noradrenaline in this tissue, which is not observed in vessels taken from non-pregnant animals. It is possible that relaxing factor derived from endothelium mediates this effect, but it is unlikely to be NO or prostacyclin. On the basis of differential antagonist affinity and affinity of noradrenaline itself, we suggest that the identical subtype of α-adrenoceptors is involved in the noradrenaline-induced contraction of non-pregnant and pregnant guinea-pig uterine artery with both intact or denuded endothelium. It is likely that α-adrenoceptors involved in noradrenaline-induced contraction of non-pregnant and pregnant guinea-pig uterine artery with intact and denuded endothelium belong to the α_1 subtype.

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References

ADEAGBO, A.S. & TRIGGLE, C.R. (1993). Interactions of nitric oxide synthase inhibitors and dexamethasone with alpha-adrenoceptor-mediated responses in rat aorta. *Br. J. Pharmacol.*, **109**, 495–501.

AGRAWAL, D.K., TRIGGLE, C.R. & DANIEL, E.E. (1984). Pharmacological characterization of post-synaptic alpha adrenoceptors in vascular smooth muscle from canine and rat mesenteric vascular beds. *J. Pharmacol. Exp. Ther.*, **229**, 831–838.

ALOSACHIE, I. & GODFRAIND, T. (1986). Role of cyclic GMP in the modulation by endothelium of the adrenolytic action of prazosin in the rat isolated aorta. *Br. J. Pharmacol.*, **89**, 525–532.

ALOSACHIE, I. & GODFRAIND, T. (1988). The modulatory role of vascular endothelium in the interaction of agonists and antagonist with α-adrenoceptors in the rat aorta. *Br. J. Pharmacol.*, **95**, 619–629.

ANDERSON, S.G., STILL, J.G. & GREISS, F.C. (1977). Differential reactivity of the gravid uterine vasculatures: effects of norepinephrine. *Am. J. Obstet. Gynecol.*, **129**, 293–298.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

ASSALI, N.S., NUWAYHID, B., BRINKMAN, C.R., TABSH, K., ERKKOLA, R. & USHIODA, E. (1981). Autonomic control and pelvic circulation: *in vivo* and *in vitro* studies in pregnant and non-pregnant sheep. *Am. J. Obstet. Gynecol.*, **141**, 873–884.

BARTON, M.D., KILLAM, A.P. & MESCHIA, G. (1974). Response of ovine uterine blood flow to epinephrine and norepinephrine. *Proc. Soc. Exp. Biol. Med.*, **145**, 996–1003.

BELL, C. (1968). Dual vasoconstrictor and vasodilator innervation of the uterine arterial supply in the guinea-pig. *Circ. Res.*, **3**, 279–288.

BELL, C. (1969). Evidence for dual innervation of the human extrinsic uterine arteries. *Obstet. Gynecol. Br. Commonwealth*, **76**, 1123–1128.

BERLAN, M. & LAFONTAN, M. (1980). Identification of alpha$_2$-adrenergic receptors in human fat cell membranes by [^{3}H]-clonidine binding. *Eur. J. Pharmacol.*, **67**, 481–484.

CAMBRIDGE, D., DAVEY, M.J. & MASSINGHAM, R. (1977). Prazosin, a selective antagonist of postsynaptic alpha-adrenoceptors. *Br. J. Pharmacol.*, **59**, 514–515.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

COLLUCI, W.S., GIMBRONE, M.A., MCLAUGHLIN, M.K., HALPERN, W. & ALEXANDER, R.W. (1982). Increased vascular catecholamine sensitivity and alpha adrenergic receptor affinity in female and estrogen treated male rats. *Circ. Res.*, **50**, 805–811.

COSTA, G., ISLA, M., GARCIA-PASCUAL, A., JIMENEZ, E., RECIO, P., LABADIA, A. & GARCIA-SACRISTAN, A. (1992). Characterization of postsynaptic α-adrenoceptors in the arteries supplying the oviduct. *Br. J. Pharmacol.*, **105**, 381–387.

DISBREY, B.D. & RACK, J.H. (1970). *Histological Laboratory Methods*. pp. 111–113. Edinburgh and London: E. & S. Livingstone.

DOXEY, J.C., SMITH, C.F.C. & WALKER, J.M. (1977). Selectivity of blocking agents for pre- and postsynaptic alpha-adrenoceptors. *Br. J. Pharmacol.*, **60**, 91–96.

EKESBO, R., ALM, P., EKSTROM, P., LUNDBERG, L.M. & AKERLUND, M. (1991). Innervation of the human uterine artery and contractile responses to neuropeptides. *Gynecol. Obstet. Invest.*, **31**, 30–36.

FALLGREN, B., BJELLIN, L. & EDVINSSON, L. (1988). Effect of pregnancy and sex steroids on α_1-adrenoceptor mechanisms in the guinea-pig uterine vascular bed. *Pharmacol. Toxicol.*, **63**, 375–381.

FALLGREN, B. & EDVINSSON, L. (1986). Characterization of adrenoceptor mechanisms in isolated guinea-pig uterine arteries. *Eur. J. Pharmacol.*, **131**, 163–170.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in determination of dissociation constants of receptor agonist complexes. *Adv. Drug Res.*, **3**, 21–55.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*. Vol. 33, ed. Blaschko, H. & Muscholl, E. pp. 283–335. New York: Springer Verlag.

FURCHGOTT, R.F. & BURSZTYN. P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. New York Acad. Sci.*, **144**, 882–899.

GOLDBERG, M.R. & ROBERTSON, D. (1983). Yohimbine: A pharmacological probe for the study of the α-adrenoceptor. *Pharmacol. Rev.*, **35**, 143–180.

GRIENDLING, K.K., FULLER, E.O. & COX, R.H. (1985). Pregnancy-induced changes in sheep uterine and carotid arteries. *Am. J. Physiol.*, **248**, H658–H665.

HOFFMAN, B.B., LAVIN, T.N., LEWKOWITZ, R.J. & RUFFOLO, Jr, R.R. (1981) Alpha adrenergic receptor subtype in rabbit uterus: mediation of myometrical contraction and regulation by estrogens. *J. Pharmacol. Exp. Ther.*, **219**, 290–295.

ISLA, M. & DYER, D.C. (1990). Characterization of alpha-adrenoceptors in the late pregnant ovine uterine artery. *Eur. J. Pharmacol.*, **178**, 321–331.

JOVANOVIĆ, A., GRBOVIĆ, L., DREKIĆ, D. & NOVAKOVIĆ, S. (1994). Muscarinic receptor function in the guinea-pig uterine artery is not altered during pregnancy. *Eur. J. Pharmacol.*, **258**, 185–194.

KENAKIN, T.P. (1987). *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven Press.

KUPUR, H. & MOTTRAM. D.R. (1978). A comparative study on the pre and postsynaptic alpha blocking activity of a series benzodioxanes. *Biochem. Pharmacol.*, **27**, 1879–1880.

LUES, I. & SCHUMANN, H.H. (1984). Effect of removing the endothelial cells on the reactivity of rat aortic segments to different α-adrenoceptor agonists. *Naunyn-Schmied. Arch. Pharmacol.*, **328**, 160–163.

MAGNESS, R.R. & ROSENFELD, C.R. (1986). Systemic and uterine responses to alpha adrenergic stimulation in non-pregnant and pregnant ewes. *Am. J. Obstet. Gynecol.*, **155**, 897–904.

MALTA, E., SCHINI, V. & MILLER, R.C. (1986). The role of efficacy in the assessment of the actions of α-adrenergic agonists in rat aorta with endothelium. *J. Pharm. Pharmacol.*, **38**, 209–213.

MARTIN, W., FURCGOTT, R.F., VILLANI, G.M. & JOTHIANANDAN, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **237**, 529–538.

MCLAUGHLIN, M.K., KEVE, T.M. & COOKE, R. (1989). Vascular catecholamine sensitivity during pregnancy in the ewe. *Am. J. Obstet. Gynecol.*, **160**, 47–53.

NUNOTANI, T., MATSUURA, S., TAMAI, T., TATSUMI, N., SAGAWA, N. & MORI, T. (1985). The responses of isolated uterine arteries from pregnant sows to vasoconstrictive agents. *Acta Obstet. Gynaecol. Jpn.*, **37**, 15–23.

ORIOWO, M.A., BEVAN, J.A. & BEVAN, R.D. (1989). Variation in sensitivity of six cat and six rat arteries to norepinephrine can be related to differences in agonist affinity and receptor reserve. *J. Pharmacol. Exp. Ther.*, **251**, 16–20.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PEETERS, L.L.H., GRUTTERS, G. & MARTIN, C.R. (1980). Distribution of cardiac output in the unstressed pregnant guinea-pig. *Am. J. Obstet. Gynecol.*, **138**, 1177–1184.

PIPILI-SYNETOS, E., SIDERI, E. & MARAGOUDAKIS, M.E. (1991). Role of endothelium on alpha-adrenoceptor responsiveness and prostacyclin release from the mesenteric arterial bed of the rat. *J. Auton. Pharmacol.*, **11**, 129–137.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

RUFFOLO, Jr, R.R. (1982). Review: important concepts of receptor theory. *J. Auton. Pharmacol.*, **2**, 277–295.

RUFFOLO, Jr, R.R., WADDEL, J.E. & YADEN, E.L. (1981). Postsynaptic alpha adrenergic receptor subtypes differentiated by yohimbine in tissues from the rat. Existence of alpha-2 adrenergic receptors in rat aorta. *J. Pharmacol. Exp. Ther.*, **217**, 235–240.

SKARBY, T.V.C.H. & LARSSON, B. (1987). Theoretical and functional studies on α_1- and α_2-adrenoceptors: an examination using the Schild plot. *J. Auton. Pharmacol.*, **7**, 185–198.

STARKE, K. (1981). α-Adrenoceptor subclassification. *Rev. Physiol. Biochem. Pharmacol.*, **88**, 199–236.

STEELE, S.C., WARREN, A.Y. & JOHNSON, I.R. (1993). Effect of the vascular endothelium on norepinephrine-induced contractions in uterine radial arteries from the nonpregnant and pregnant human uterus. *Am. J. Obstet. Gynecol.*, **168**, 1623–1628.

STEVENS, M.J. & MOULDS, R.F.W. (1981). Heterogeneity of post-junctional α-adrenoceptors in human vascular smooth muscle. *Arch. Int. Pharmacodyn.*, **254**, 43–57.

TALLARIDA, R.J., COWAN, A. & ADLER, M.W. (1979). Minireview: pA_2 and receptor differentiation: A statistical analysis of competitive antagonism. *Life Sci.*, **25**, 637–654.

TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1981). The post-synaptic α_2-adrenoceptor. *J. Auton. Pharmacol.*, **1**, 171–183.

TODA, N. (1984). Endothelium-dependent relaxation induced by angiotensin II and histamine in isolated arteries of dog. *Br. J. Pharmacol.*, **81**, 301–307.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–239.

VAN NIMWEGEN, D. & DYER, D.C. (1974). The action of vasopressors on isolated uterine arteries. *Am. J. Obstet. Gynecol.*, **118**, 1099–1103.

VINET, R., BRIEVA, C., PINARDI, G. & PENNA, M. (1991). Modulation of alpha-adrenergic-induced contractions by endothelium-derived relaxing factor in rat aorta. *Gen. Pharmacol.*, **22**, 137–142.

WEINER, C.P. MARTINEZ, E., CHESTNUT, C.H. & GHODSI, A. (1989). Effect of pregnancy on uterine and carotid artery response to norepinephrine, epinephrine, and phenylephrine in vessels with documented functional endothelium. *Am. J. Obstet. Gynecol.*, **161**, 1605–1610.

WEINER, C., ZHU, L.K., THOMPSON, L., HERRIG, J. & CHESTNUT, D. (1991). Effect of pregnancy on endothelium and smooth muscle: their role in reduced adrenergic sensitivity. *Am. J. Physiol.*, **261**, H1275–H1283.

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A new class of furoxan derivatives as NO donors: mechanism of action and biological activity

R. Ferioli, [1]G.C. Folco, *C. Ferretti, †A.M. Gasco, †C. Medana, †R. Fruttero, **M. Civelli & †A. Gasco

Center for Cardiopulmonary Pharmacology, Institute of Pharmacological Sciences, University of Milano, via Balzaretti 9, 20133 Milano, Italy; *Institute of Pharmacology and Experimental Therapy, University of Torino, via P. Giuria, 13, 10126 Torino, Italy; †Dipartimento di Scienza e Tecnologia del Farmaco, University of Torino, via P. Giuria, 9, 10125 Torino, Italy and **Chiesi Farmaceutici S.p.A., via Palermo 26, 43100 Parma, Italy

1 The mechanism of action and biological activity of a series of R-substituted and di-R-substituted phenylfuroxans is reported.

2 Maximal potency as vasodilators on rabbit aortic rings, precontracted with noradrenaline (1 μM), was shown by phenyl-cyano isomers and by the 3,4-dicyanofuroxan, characterized by a potency ratio 3-10 fold higher than glyceryl trinitrate (GTN). This effect was reduced upon coincubation with methylene blue or oxyhaemoglobin (10 μM).

3 The furoxan derivatives showing maximal potency as vasodilators were also able to inhibit collagen-induced platelet aggregation, with IC_{50} values in the sub-micromolar range.

4 The furoxan derivatives were able to stimulate partially purified, rat lung soluble guanylate cyclase; among the most active compounds, the 3-R-substituted isomers displayed a higher level of stimulatory effect than the 4-R analogues.

5 Solutions (0.1 mM) of all the tested furoxans, prepared using 50 mM phosphate buffer, pH 7.4, (diluting 10 mM DMSO stock solutions) did not release nitric oxide (NO) spontaneously; however in presence of 5 mM L-cysteine, a significant NO-releasing capacity was observed, which correlated significantly with their stimulation of the guanylate cyclase activity.

Keywords: Furoxans; nitric oxide; vasodilatation; haemoglobin; guanylate cyclase; platelet aggregation

Introduction

Since the early observations on the existence of endothelium-dependent relaxation and endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), this field of research has become an important area of biological interest (Ignarro, 1990). In 1987, two groups, independently, forwarded the hypothesis that EDRF could be closely related to nitric oxide (NO) radical (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). These observations were extended in a multiplicity of investigations (Moncada *et al.*, 1991), which led to the present concept of the role of NO as a transduction mechanism and as an effector molecule.

The possibility that there may be more than one type of EDRF cannot be excluded (Rubanyi & Vanhoutte, 1987), but certainly NO provides a comprehensive explanation for the majority of the pharmacological findings on EDRF: NO plays an important role in the vasodilator process (Holtz *et al.*, 1984), is a potent inhibitor of platelet aggregation (Radomski *et al.*, 1987), and is able to kill bacterial cells when released by macrophages (Hibbs *et al.*, 1987).

NO is synthesized from the amino acid L-arginine by the action of an enzyme, NO synthase (Bredt & Snyder, 1990; Bredt *et al.*, 1991), in addition, extension of the original hypothesis by Needleman *et al.* (1973) on the requirement of tissue thiols for the vasodilator action of glyceryl trinitrate (GTN), led to the identification of S-nitrosocysteine, a labile but potent vasorelaxant that works through the action of liberated NO (Ignarro *et al.*, 1981). Several reports (Seth & Fung, 1993) have suggested that a cytochrome P450 related enzyme could be responsible for the production of NO from organic nitrates, in cells not derived from the vascular smooth muscle. More recently a membrane-bound enzyme responsible for generating NO from GTN in these latter cells had been characterized (Seth & Fung, 1993). The enzyme activity was enhanced by several thiols and was suppressed by several reagents known to bind free sulphydryl groups.

These findings have prompted the development of compounds acting as NO-donating pro-drugs, devoid of nitrate tolerance (Needleman & Johnson, 1973) and with a longer duration of action than classic organic nitrate esters (Yamada *et al.*, 1991; Ånggård, 1991).

Recently, we described a new class of compounds, with a furoxan structure, which have been shown to possess NO-mimetic pharmacological activities (Calvino *et al.*, 1992; Ghigo *et al.*, 1992). The mechanism of action of furoxancarboxamides at the molecular level has been investigated by Feelisch *et al.* (1992) who found that these compounds react with sulphydryl groups of low molecular weight thiols and proteins. Furoxans are thus pro-drugs, which increase the level of guanosine 3':5'-cyclic monophosphate (cyclic GMP) via liberation of NO, as found for classic nitrovasodilators.

In structure-activity relationship studies, we have also investigated the influence of different substitutions on the furoxan ring, i.e. bis-(phenylsulphonyl) and 3- or 4-(1,1-dinitroethyl), in relation to their vasodilator and platelet antiaggregatory profile (Ferioli *et al.*, 1993; Gasco *et al.*, 1993a,b).

In the present paper, the *in vitro* biological activity of twenty R-substituted phenylfuroxans (derivatives **2-11 a, b**), as well as of three di-R-substituted furoxans (derivatives **1, 12, 13**), is described; their proposed mechanism of action as NO donors makes them interesting models in the design of new drugs.

Methods

Synthesis of the compounds

All the derivatives were synthetized according to the methods previously reported (Gasco & Boulton, 1981; Gasco *et al.*,

[1] Author for correspondence.

1993a,b). The purity (>98%) of the samples used for the pharmacological tests was checked by h.p.l.c. (RP-C18 column, 5 μm, 250 × 4.6 mm; eluent; methanol/water).

Study of the vasodilator effects

Male New Zealand white rabbits weighing 1.8–2.3 kg were anaesthetized with sodium pentobarbitone (40–50 mg kg^{-1}) via an ear vein, the carotid artery was cannulated and the animals bled. The thorax was rapidly opened and the aorta was removed carefully, to protect the endothelial lining, cleared of fat and connective tissue and cut in 2–3 mm wide transverse rings. Four rings were joined together by surgical silk to form a chain and placed in a glass organ bath containing Krebs Henseleit bicarbonate solution at 37°C, constantly oxygenated with a mixture of 95% O_2/5% CO_2. After an equilibration period of 1 h under a basal tension of 2 g, changes in isometric contraction were monitored by a force transducer (Basile, mod. 7004) connected to a Gemini 7070 Basile pen recorder.

Responses to vasodilator agents were studied following the protocol described previously (Ferioli *et al.*, 1993). Glyceryl trinitrate (GTN, 0.01-3.0 μM) was used as a reference standard.

The composition (mM) of the Krebs buffer was: NaCl 118.90, KCl 4.66, KH_2PO_4 1.18, $MgSO_4$ 1.10, $CaCl_2$ 2.52, glucose 5.55, $NaHCO_3$ 25 (Merck; Darmstadt, Germany); pH was 7.4.

The following compounds were used: acetylcholine HCl (Sigma Chemical Company; St. Louis, Missouri, U.S.A.), glyceryl trinitrate (Perlinganit Lösung; Schwarz Pharma; Monheim, Germany), dimethylsulphoxide (Sigma), noradrenaline bitartrate (Sigma), ascorbic acid (Merck), methylene blue (Sigma).

Preparation of haemoglobin

Bovine haemoglobin Type 1 (H-2500 Sigma Chemical Co.) contains a mixture of oxyhaemoglobin and the oxidized derivative methaemoglobin. Pure oxyhaemoglobin was prepared, protected from the light, by adding to a solution of commercially available haemoglobin in phosphate saline buffer, pH 7.4, a 10 fold molar excess of the reducing agent sodium dithionite ($Na_2S_2O_4$), at 4°C. Within a few minutes, the mixture was centrifuged at 1000 *g* for 10 min, at 4°C, and the supernatant loaded onto a chromatographic column (Sephadex G-25 coarse, Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer.

The purity of the oxyhaemoglobin solution was determined spectrophotometrically (λ_{max} = 576 nm, 541 nm; ε = 14600, 13800) and the solutions were frozen in aliquots at −20°C and stored for 15 days.

Platelet aggregation studies

Human platelet aggregation was determined by the Born turbidimetric technique (Born, 1962), according to the procedure previously described (Ferioli *et al.*, 1993). The activity of each compound was tested 5 times with platelet rich plasma (PRP) obtained from different donors. IC_{50} values were generated from regression analysis of concentration-response curves.

Preparation of guanylate cyclase from rat lungs

Male, Sprague Dawley rats (Charles River, Calco (CO), Italy), weighing 200–220 g were anaesthetized with urethane (1 g kg^{-1}, i.p.) and killed by decapitation. The procedure was carried out at +4°C; pieces (100 g) of rat lung were chopped, suspended in 300 ml buffer [(Tris/HCl 10 mM, containing sucrose (0.25 M), ethylendiamino-tetraacetic acid (EDTA, 1 mM) and dithiothreitol (DTT, 2 mM), pH 7.4] and homogenized in a Polytron homogenizer. The homogenate was centrifuged at 500 *g* for 10 min, the supernatant collected and further centrifuged at 105,000 *g* for 1 h. The high speed supernatant (cytosol fraction) was applied to a column (4 × 15 cm) of DEAE cellulose (DE-52, Whatman) which was pre-washed (2.0 l) with buffer A [Tris/HCl (10 mM) containing DTT (2 mM), at pH 7.4]. The column was further washed with buffer A (1.0 l) followed by washing (1.0 l) with buffer B [Tris/HCl (10 mM), DTT (2 mM), NaCl (0.1 mM), at pH 7.4]. For the elution step, the column was finally washed with buffer C [Tris/HCl (10 mM), DTT (2 mM), NaCl (0.5 mM), at pH 7.4] and 10 ml fractions were collected. Fractions containing enzymic activity were collected and dialysed overnight against buffer A. The dialysate was applied to a column (1.5 × 19 cm) of Blue Sepharose CL-6B (Pharmacia) previously equilibrated with 1.0 l of buffer B. The enzyme was eluted with buffer C and 3 ml fractions were collected. The main active fractions (absorbance at 280 nm above 0.1) were pooled and dialysed overnight against buffer A. This procedure removed most of the haemoglobin from the eluate of the DE-52 column. The dialysate was frozen in aliquots at −70°C, thawed before use and not refrozen. Using SDS-polyacrylamide gel electrophoresis, about 50% of the protein migrated at 72 kD, the molecular weight reported for soluble GC subunit of bovine lung (Nakane *et al.*, 1988; 1990).

Assay of guanylate cyclase activity

The guanylate cyclase activity was measured by preincubating the enzyme (10 μg) for 5 min at 37°C in 50 mM Tris/HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM ethylenglycol-*bis*-(α-aminoethyl ether)-*N,N,N,N*, tetraacetic acid (EGTA), 5 mM L-cysteine and 0.5 mM of all the tested furoxans. The reaction was started by addition of 0.2 mM guanosine-5'-triphosphate (GTP) in a final volume of 300 μl and terminated after 20 min at 37°C, by heating for 3 min at 90°C. Cyclic GMP was measured by a radioimmunoassay kit using [^{3}H]-cyclic GMP (Amersham). The generation of cyclic GMP was linear with time (10–30 min) and cyclic GMP-phosphodiesterase inhibitors did not affect enzymatic activity, so they were not added to reaction mixture. The maximal enzymatic activity (E) was expressed in pmol cyclic GMP min^{-1} mg^{-1} protein. Values are given as means ± s.e.mean (n = 6–11). It refers to the activity displayed by each furoxan compound at 0.5 mM.

Furoxan compounds did not show cross-reactivity in the cyclic GMP radioimmunoassay. Protein was calculated according to Lowry *et al.* (1951).

Measurement of NO_2^-

Solutions (0.1 mM) of all the tested furoxans were prepared at 37°C, using 50 mM phosphate buffer, pH 7.4, (diluting 10 mM DMSO stock solutions) in presence or absence of 5 mM L-cysteine; after 1 h under gentle stirring, 1 ml aliquots of drug solutions were mixed with 0.25 ml aliquots of Griess reagent (4% sulphanilamide; 0.4% N-naphtylethylendiamine dihydrochloride; 10% H_3PO_4, w/v) and incubated at room temperature for 10 min to form an azo-dye. Absorbance was monitored at 550 nm using a UV-1204 Shimadzu spectrophotometer and NO_2^- was quantified with $NaNO_2$ as a standard (linearity of the standard curve was from 0.01 to 0.1 mM). The capacity to release NO was expressed as NO_2^-%, i.e. the ratio between the number of NO_2^- mol detected and the number of furoxan mol, multiplied by 100.

Statistical analysis

EC_{50} values represent the concentration required to produce 50% relaxation in a noradrenaline-contracted strip (n = 4–6). R_M% represents the peak relaxation observed at the maximal drug concentration employed.

Analysis of concentration-response curves was performed

using ALLFIT computer programme; comparisons were made by Student's t test. A probability of 0.05 or less was considered significant.

Linear regression analyses were performed according to Coburn (1987) on a IBM AT personal computer. The figures in parentheses for the regression lines are 95% confidence intervals; r = correlation coefficient ($r^2 \times 100$: variance in the data 'explained' by the regression equation); s = standard deviation from regression; P = level of significance; n = number of data.

Results

Vasodilator effects

The vasodilator activity of the compounds under investigation, expressed as their EC_{50} values ± 95% confidence limits (CL), as well as percentage of maximal relaxation (R_M% values), is reported in Table 1. Compounds **2a**, **2b**, **3a** and **4a** were inactive, while the EC_{50} values of the other derivatives ranged between 0.005–100 μM. Maximal potency was shown by compounds **11b**, **11a** and **13**, characterized by a potency ratio 3–10 fold higher than GTN. The majority of the furoxan derivatives (with the exclusion of compounds **1**, **3a**, **3b**, **4a**, **5b**), caused complete relaxation of the vascular tone, induced by noradrenaline. Furoxans showing the highest potency as vasodilators were also tested on aortic rings that had been pretreated (15 min) with methylene blue or oxyhaemoglobin (10 μM) (Figure 1). The relative EC_{50} values (see legend to Figure 1) reflect the expected rightward shift.

Effects on platelet aggregation

Compounds **7a**, **7b**, **11a**, **11b**, **12** and **13** were also tested for their ability to inhibit collagen-induced platelet aggregation. They showed IC_{50} values in the sub-micromolar range (Table 2).

Activation of soluble guanylate cyclase

The ability of the furoxan derivatives to activate the partially purified, soluble guanylate cyclase, obtained from rat lung, expressed as maximal enzymatic activity (E), is shown in Table 1.

Diphenylfuroxan **1** is characterized by low activity; substitution of the CH_3, NH_2, C_6H_5S, $COOCH_3$ groups for 3-phenyl or 4-phenyl moiety affords derivatives with similar (**2a**, **2b**, **3b**, **4a**) or slightly enhanced (**3a**, **4b**, **5a**, **5b**) biological activity, when compared to **1**.

A different situation occurs with the remaining pair of isomers (**6a**, **6b**; **7a**, **7b**: **8a**, **8b**; **9a**, **9b**; **10a**, **10b**; **11a**, **11b**), in which the 3-R-substituted isomers always display a level of stimulatory effect higher than that displayed by the 4-R analogues. The 3-R derivatives of this group can be listed in increasing order of efficacy as follows: **11b>10b>9b>**sodium nitroprusside (SNP)$\geq$**8b>7b>6b**.

Finally the high degree of efficacy as activators of guanylate cyclase of compounds **12** and **13** is noteworthy.

Formation of nitrite from furoxans

The capacity of furoxans to release NO (measured as NO_2^-) upon coincubation with 5 mM L-cysteine, is reported in Table 1. There was no spontaneous formation of NO; NO releasing-capacity followed the series: **10b = 10a$\leq$9b>12>**

Table 1 Chemical structure and biological activity of NO-releasing furoxans

Compound	*R*	*R'*	*Vasodilator activity* EC_{50} (μM) ± 95% CL	*Vasodilator activity* R_M %	*Stimulation of guanylate cyclase activity* E	*Release of NO* NO_2^- %
GTN			0.06 ± 0.03	111 ± 3	Not tested	45 ± 1
SNP			Not tested	Not tested	2182 ± 106	58 ± 6
1	Ph	Ph	100 ± 3	50 ± 1	179 ± 5	3 ± 2
2a	CH_3	Ph	Inactive	Inactive	309 ± 40	7 ± 3
2b	Ph	CH_3	Inactive	Inactive	270 ± 13	1 ± 1
3a	NH_2	Ph	–	46 ± 2	391 ± 46	2 ± 2
3b	Ph	NH_2	70.5 ± 0.3	71 ± 7	104 ± 15	0 ± 0
4a	Ph-S	Ph	–	48 ± 4	175 ± 46	8 ± 1
4b	Ph	Ph-S	3.5 ± 0.3	102 ± 2	464 ± 102	3 ± 2
5a	$COOCH_3$	Ph	3.6 ± 1.2	116 ± 10	482 ± 36	5 ± 3
5b	Ph	$COOCH_3$	67.6 ± 1.2	66 ± 9	348 ± 75	2 ± 2
6a	Cl	Ph	0.5 ± 0.1	116 ± 4	230 ± 20	2 ± 2
6b	Ph	Cl	1.7 ± 0.3	123 ± 2	714 ± 26	14 ± 1
7a	Ph-SO_2	Ph	1.7 ± 0.3	98 ± 5	575 ± 38	5 ± 2
7b	Ph	Ph-SO_2	0.63 ± 0.06	98 ± 5	1008 ± 68	22 ± 1
8a	$CONH_2$	Ph	6.1 ± 6.4	128 ± 4	517 ± 113	2 ± 0
8b	Ph	$CONH_2$	6.0 ± 1.2	127 ± 6	2062 ± 130	7 ± 4
9a	OCH_3	Ph	5.1 ± 2.6	128 ± 4	484 ± 36	5 ± 2
9b	Ph	OCH_3	0.5 ± 0.3	124 ± 6	2656 ± 55	76 ± 2
10a	NO_2	Ph	7.2 ± 2.9	98 ± 2	374 ± 27	76 ± 3
10b	Ph	NO_2	1.0 ± 0.6	110 ± 4	2847 ± 180	80 ± 1
11a	CN	Ph	0.007 ± 0.002	122 ± 3	1212 ± 126	6 ± 2
11b	Ph	CN	0.005 ± 0.008	124 ± 4	3744 ± 192	40 ± 2
12	Ph-SO_2	Ph-SO_2	0.049 ± 0.015	125 ± 4	2615 ± 73	59 ± 4
13	CN	CN	0.016 ± 0.006	113 ± 6	2087 ± 417	38 ± 7

R_M %: percentage of the maximum vascular relaxation. E: maximal enzymatic activity. NO_2^-%: ratio between the number of NO_2^- mol detected and the number of furoxan mol, multiplied by 100. The EC_{50} value has not been reported when R_M % was less than 50%. Basal enzymatic activity was 0.077 ± 0.004 nmol min^{-1} mg^{-1} prot. Values are given as means ± s.e.mean for 6 to 11 replicates.

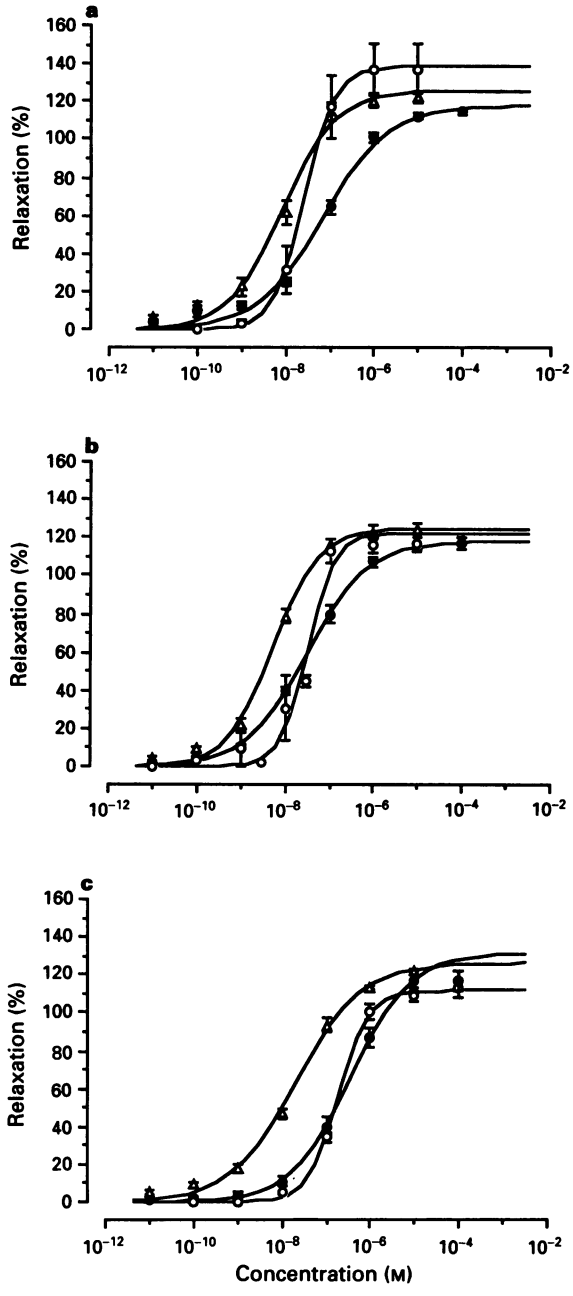


Figure 1 Concentration-response curves of the vasodilator activity of selected furoxan compounds, in the absence (Δ) or presence of methylene blue (10 μM) (●) or oxyhaemoglobin (10 μM) (○). EC_{50} (μM) ± 95% CL: (a) compound **11a**, control 0.007 ± 0.002, +MB 0.07 ± 0.02 ($P<0.05$), +HbO_2 0.030 ± 0.005 ($P<0.05$); (b) compound **11b**, control 0.005 ± 0.001, + MB 0.031 ± 0.009 ($P<0.05$), +HbO_2 0.032 ± 0.005 ($P<0.05$); (c) compound **13**, control 0.016 ± 0.004, +MB 0.29 ± 0.05 ($P<0.05$), +HbO_2 0.32 ± 0.02 ($P<0.05$). Each value is the mean ± 95% CL of 4–6 different experiments.

Table 2 Inhibitory effect of selected furoxan compounds on collagen-induced, human platelet aggregation

Compound	*IC_{50} ± 95% confidence limits* (μM)
SNP	2.2 ± 0.5
7a	3.4 ± 1.4
7b	0.38 ± 0.02
12	0.31 ± 0.05
11a	0.84 ± 0.12
11b	0.20 ± 0.02
13	0.24 ± 0.02

11b = 13>7b>6b>11a and remaining compounds. No release of NO was observed in the absence of L-cysteine.

Comparison of the data reported above with those of stimulation efficacy of the guanylate cyclase activity (E), shows a statistically significant degree of correlation, able to explain only 46.3% of variance (Equation 1; Coburn, 1987).

Equation 1: E = 26.7 (±6.3) %NO_2^- + 500.9 (±208.7)
$r = 0.68$ $s = 797.9$ $P<0.01$ $n = 23$.

The furoxan derivatives **11b**, **10a** and **8b** show 'residuals' (observed *minus* estimate) larger than twice the standard deviation; if these compounds are removed, the 'cleaned' *equation 2* is obtained.

Equation 2: E = 34.1 (±2.5) %NO_2^- + 305.9 (±73.8)
$r = 0.96$ $s = 272.5$ $P<0.01$ $n = 20$

This equation is very satisfactory from a statistical point of view and explains 91.4% of variance. Only derivative **11a** is now an outlier and its removal affords Equation 3.

Equation 3: E = 34.8 (±1.9) %NO_2^- + 253.8 (±59.1)
$r = 0.98$ $s = 211.5$ $P<0.01$ $n = 19$

This equation is highly satisfactory and explains 95.1% of variance.

Discussion

The results described in the present study indicate that the furoxan compounds represent interesting prototypes of NO-donating pro-drugs characterized by significant biological activity.

The majority of the furoxan compounds studied possess vasodilator properties, with adequate efficacy to cause complete functional antagonism of the noradrenaline-induced vascular tone. Their vasodilator action cannot be ascribed to antagonism of the α_1-adrenoceptor, since a similar vasodilator profile was also evident in aortic ring depolarized by K^+ (data not shown). In addition, in a limited number of experiments, the vasodilator effect of furoxans was tested in vascular preparations in which the endothelium had been completely removed through rubbing of the intima, and found to be fully independent of endothelial integrity, as already reported for organic nitrates.

The mechanism of action of the furoxan compounds seems to be due to a thiol-dependent formation of NO, since no or minimal spontaneous formation of NO was observed after 1 h in absence of cysteine. Moreover, a high, statistically significant degree of correlation has been observed between the stimulation efficacy of the guanylate cyclase activity (E) and ability to release NO, for nineteen derivatives of the series. This is in line with what has been reported for the widely used nitrovasodilators, glyceryl trinitrate and sodium nitroprusside, which release nitric oxide on redox activation (Ignarro *et al.*, 1981; Kruszyna *et al.*, 1987). The high degree of statistical significance observed, might have been facilitated by the experimental model employed, based on the simple interaction between a substrate (NO) and a purified enzyme (guanylate cyclase). At the moment we have no evidence to explain the behaviour of four 'outliers' (**11a**, **11b**, **10a**, **8b**). A convincing argument to explain the behaviour of 4-nitro-3-phenylfuroxan **10a**, which displays a negative residue, is that this compound quickly undergoes nucleophilic substitution by action of a thiol group affording nitrites and a derivative similar to **4a**, both weak activators at the concentration used in the experiment. In effect we found that **10a** easily reacts with thiol derivatives giving 4-SR substituted products (Gasco *et al.*, unpublished results). No clear correlation was found when $1/EC_{50}$ values of the vasodilator activity of the different furoxans were plotted vs E or %NO_2^-. The considerable shift of the EC_{50} values for the vasodilator activity of the furoxan compounds, observed in the presence of methylene blue or oxyhaemoglobin, provides further sup-

port in favour of a NO-mediated, cyclic GMP-dependent mechanism of action.

Some selected furoxan compounds showing the highest potency as vasodilators, also inhibit collagen-induced, human platelet aggregation. This is consistent with the hypothesis that furoxan compounds, by releasing NO, share a common action mechanism both as antiaggregating agents and as vasodilators (Feelish *et al.*, 1992; Ghigo *et al.*, 1992).

Generally speaking as far as the nitric oxide release and the ability to activate the guanylate cyclase of the investigated furoxans are concerned, in the most active compounds ($\%NO_2^- > 10\%$, E pmol cyclic GMP min^{-1} mg^{-1} protein > 500) the 4-R substitution is more efficient than the corresponding 3-R substitution. Important substituents are CN, NO_2, OCH_3, $PhSO_2$, Cl, $CONH_2$ (in part) groups. Worthy of note is the high activity level of both the di-cyano and di-phenylsulphonyl substituted furoxans which suggest that furoxans, disubstituted with electron withdrawing moieties, should be very active compounds.

As pointed out before, as far as the vasodilator properties of the series are concerned, the structure-activity relationships are complex. The results we recently obtained in the study of NO-release by **11b** and other furoxan derivatives, under the action of thiophenol, suggest that this process is a complex pathway, strongly dependent on the nature of the substituent at the heterocyclic ring (work in progress).

Taken together our results indicate that furoxans represent a new class of compounds with an interesting potential as vasodilators and antiaggregating agents. The extensive structure-activity and -reactivity studies now in progress will hopefully clarify whether furoxans may provide a means to tailor NO lifetime and transport properties, and to elicit specific biological responses.

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References

ÄNGGÅRD, E.E. (1991). Endogenous nitrates-implications for treatment and prevention. *Eur. Heart J.*, **12**, 5–8.

BORN, G.V.R. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351**, 714–718.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

CALVINO, R., FRUTTERO, R., GHIGO, D., BOSIA, A., PESCARMONA, G.P. & GASCO, A. (1992). 4-Methyl-3-arylsulfonylfuroxans: a new class of potent inhibitors of platelet aggregation. *J. Med. Chem.*, **35**, 3296–3300.

COBURN, R.A. (1987). Quantitative structure-activity relationship studies. Medicinal chemistry regression machine. In *QSAR-PC: PAR.* ed. Coburn, R.A.. Cambridge, U.K.:Biosoft.

FEELISCH, M., SCHONAFINGER, S. & NOACK, E. (1992). Thiol Mediated generation of nitric oxide accounts for the vasodilator action of furoxans. *Biochem. Pharmacol.*, **44**, 1149–1157.

FERIOLI, R., FAZZINI, A., FOLCO, G.C., FRUTTERO, R., CALVINO, R., GASCO, A., BONGRANI, S. & CIVELLI, M. (1993). NO-mimetic furoxans: arylsulfonyl-furoxans and related compounds. *Pharmacol. Res.*, **28**, 203–212.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GASCO, A. & BOULTON, A.J. (1981). Furoxans and benzofuroxans. *Adv. Heterocycl. Chem.*, **29**, 251–340.

GASCO, A.M., DI STILO, A., FRUTTERO, R., SORBA, G., GASCO, A. & SABATINO, P. (1993a). Synthesis and structure of a trimer of the furoxan system with high vasodilator and platelet antiaggregatory activity. *Liebigs Ann. Chem.*, 441–444 and the other papers of the series.

GASCO, A.M., DI STILO, A., SORBA, G., GASCO, A., FERIOLI, R., FOLCO, G.C., CIVELLI, M. & CARUSO, P. (1993b). 1,1-Dinitroethyl substituted furoxans: a new class of vasodilators and inhibitors of platelet aggregation. *Eur. J. Med. Chem.*, **28**, 433–438.

GHIGO, D., HELLER, R., CALVINO, R., ALESSIO, P., FRUTTERO, R., GASCO, A., BOSIA, A. & PESCARMONA, G.P. (1992). Characterization of a new compound, S35B as a guanylate cyclase activator in human platelets. *Biochem. Pharmacol.*, **43**, 1281–1288.

HIBBS, J.B. Jr., VAVRIN, Z. & TAINTOR, R.R. (1987). L-Arginine is required for expression of the active macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.

HOLTZ, J., FOSTERMANN, U., POHL, U., GIESLER, M. & BASENGE, E. (1984). Flow- dependent, endothelium-mediated dilatation of epicardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. *J. Cardiovasc. Pharmacol.*, **6**, 1161–1169.

IGNARRO, L.J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 535–560.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHANDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

KRUSZYNA, H., KRUSZYNA, R., SMITH, R.P. & WILCOX, D.E. (1987). Red blood cells generate nitric oxide from directly acting, nitrogenous vasodilators. *Toxicol. Appl. Pharmacol.*, **91**, 429–438.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

NAKANE, M., ARAI, K., SAHEKI, S., KUNO, T., BUECHLER, W. & MURAD, F. (1990). Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. *J. Biol. Chem.*, **265**, 16841–16845.

NAKANE, M., SAHEKI, S., KUNO, T., ISHII, K. & MURAD, F. (1988). Molecular cloning of a cDNA coding for 70 kilodalton subunit of soluble guanylate cyclase from rat lung. *Biochem. Biophys. Res. Commun.*, **157**, 1139–1147.

NEEDLEMAN, P., JAKSCHIK, B. & JOHNSON, E.M. (1973). Sulphydryl requirement for relaxation of vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **187**, 324–331.

NEEDLEMAN, P. & JOHNSON, E.M. (1973). Mechanism of tolerance development to organic nitrates. *J. Pharmacol. Exp. Ther.*, **184**, 709–715.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.*, **92**, 181–187.

RUBANYI, G.M. & VANHOUTTE, P.M. (1987). Nature of endothelium-derived relaxing factor: are there two relaxing mediators? *Circ. Res.*, **61**, II61–II67.

SETH, P. & FUNG, H.L. (1993). Biochemical characterization of a membrane-bound enzyme responsible for generating nitric oxide from nitroglycerin in vascular smooth muscle cells. *Biochem. Pharmacol.*, **46**, 1481–1486.

YAMADA, H., YONEYAMA, F., SATOH, K. & TAIRA, N. (1991). Comparison of the effects of the novel vasodilator FK409 with those of nitroglycerin in isolated coronary artery of the dog. *Br. J. Pharmacol.*, **103**, 1713–1718.

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Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions

[1]Armin Hatzelmann, *Hermann Tenor & Christian Schudt

Byk Gulden, Department of Biochemistry, Konstanz and *University of Konstanz, Faculty of Biology, Konstanz, Germany

1 The effect of non-selective (3-isobutyl-1-methylxanthine, IBMX; theophylline) and type IV- or type III/IV-selective (rolipram, RP 73401; zardaverine, tolafentrine) phosphodiesterase (PDE) inhibitors on human eosinophil functions was investigated.

2 For this purpose human eosinophils were purified from blood of healthy donors by a magnetic cell separation (MACS) technique to a purity $\geqslant 99\%$. From the stimuli investigated (complement C5a; N-formyl-methionyl-leucyl-phenylalanine, fMLP; platelet activating factor, PAF; opsonized zymosan) C5a was selected to test the influence of the above mentioned compounds on secretion of granule constituents (eosinophil cationic protein, ECP; eosinophil-derived neurotoxin, EDN) as well as on formation of reactive oxygen species measured by luminol-enhanced chemiluminescence in intact cells. For comparison, inhibition of PDE IV activity in the cytosol of disrupted cells, which contains about 75% of total PDE IV activity, was determined.

3 Both theophylline and IBMX inhibited the two cell responses with IC_{50} values which were in the range of their IC_{50} values obtained for inhibition of PDE IV activity in the cell-free system. The β_2-adrenoceptor agonist, salbutamol ($1\ \mu mol\ l^{-1}$), which by itself did not substantially influence the two cell responses, only marginally improved the potency of theophylline and IBMX in inhibiting ECP/EDN secretion. Only the IC_{50} value of IBMX for inhibition of chemiluminescence was lowered by about one order of magnitude in the presence of salbutamol.

4 In contrast, none of the selective PDE inhibitors tested substantially inhibited the two cell responses at concentrations up to $10\ \mu mol\ l^{-1}$. This was surprising because all of the compounds investigated inhibited PDE IV activity in the cell-free system with IC_{50} values which were at least 30 fold lower than the highest concentration of the compounds used with intact cells. In combination with salbutamol, however, both ECP/EDN secretion and chemiluminescence was inhibited by rolipram and zardaverine with IC_{50} values similar to the IC_{50} values for inhibition of PDE IV activity. Although RP 73401 and tolafentrine also inhibited both cell responses in the presence of salbutamol, the potency of these two compounds in inhibiting eosinophil function in intact cells was at least two orders of magnitude lower than would have been expected from the inhibition of PDE IV activity in the cell-free system.

5 These results indicate that (i) C5a-stimulated human eosinophils are sensitive to inhibition by the non-selective PDE inhibitors theophylline and IBMX, (ii) the inhibitory effect of these non-selective PDE inhibitors cannot be mimicked by selective PDE IV or PDE III/IV inhibitors although human eosinophils almost exclusively contain PDE IV; (iii) the selective PDE inhibitors need an additional cyclic AMP trigger like a β_2-adrenoceptor agonist to be effective; but (iv) under the latter conditions inhibition of cell responses in intact cells does not correspond to inhibition of PDE IV activity in the cell-free system.

6 We conclude that the non-selective PDE-inhibiting xanthines may inhibit C5a-stimulated human eosinophil responses by other action(s) in addition to PDE IV inhibition, and that inhibition of PDE IV activity in the cell-free system by the selective inhibitors may not generally represent the potency of the compounds in intact cells.

Keywords: Human eosinophils; complement C5a; eosinophil cationic protein; eosinophil-derived neurotoxin; chemiluminescence; phosphodiesterase IV; phosphodiesterase inhibitors; xanthines

Introduction

In contrast to the former assumption that the basic defect in asthma lies in an abnormal contractility of airway smooth muscle, the dogma that 'asthma is a chronic inflammatory condition of the airways' has been generally accepted in the last few years (Barnes, 1992). The well-known anti-inflammatory as well as immunosuppressive actions of corticosteroids together with the efficacy of this class of drugs in the treatment of asthma (Barnes & Pedersen, 1993) provide one major piece of evidence for the importance of underlying inflammatory processes in the pathophysiology of asthma. However, the use of corticosteroids is limited by some side effects (Barnes & Pedersen, 1993), underlining the need for new anti-inflammatory drugs.

In most of the inflammatory cells elevated adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels are found to inhibit cellular responses. One way to elevate cyclic AMP within cells is to attenuate the breakdown of cyclic AMP mediated by the action of phosphodiesterases (PDEs). PDEs comprise a family of isoenzymes which have been classified into PDEs I–VI according to the nomenclature proposed by Beavo & Reifsnyder (1990). Interestingly, the predominant PDE isoenzyme in inflammatory cells is PDE IV (Giembycz, 1992) which is the only cyclic AMP-specific PDE among the classical PDE isoenzymes. Recently, another cyclic AMP-specific PDE has been cloned (Michaeli *et al.*, 1993) as a representative of a previously unknown PDE family (PDE VII) the role of which, however, has yet to be clarified. In addition to the established presence of PDE IV in inflammatory cells, a considerable number of *in vitro* func-

[1] Author for correspondence.

tional studies indicate that inhibition of PDE IV by selective inhibitors indeed abrogates cellular responses of inflammatory cells implicated in asthma pathogenesis (Giembycz, 1992). Among these cells the eosinophilic granulocytes are accepted as playing a pivotal effector role in the generation of airway obstruction observed in asthmatics (Gleich & Adolphson, 1986). Eosinophils are not only capable of releasing a multitude of mediators (bioactive lipids, cytokines) and reactive oxygen species, but also of secreting several cytotoxic proteins (major basic protein, MBP; eosinophil cationic protein, ECP; eosinophil-derived neurotoxin, EDN) stored in the so-called specific granules (Bruijnzeel, 1989). Elevated levels of these proteins have been demonstrated in association with *in vivo* asthma symptomatology (Gleich, 1990). While the inhibition of EDN release in human eosinophils by the non-selective PDE inhibitors IBMX (3-isobutyl-1-methylxanthine) and theophylline has been reported (Kita *et al.*, 1991), little information of the effect of selective PDE inhibitors on human eosinophil responses is available. Preliminary work has been published on the effect of the mixed-type PDE III/IV inhibitor zardaverine on the respiratory burst activity (Dent *et al.*, 1990), on the effects of the PDE IV inhibitor WAY-PDA-641 on PAF-induced superoxide generation (Maruo *et al.*, 1993) and on PAF- or N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced migration (Tanimoto *et al.*, 1993), as well as on the effect of rolipram, zardaverine and the PDE III inhibitor motapizone on interleukin-5- and PAF-induced chemotactic responses (Shute *et al.*, 1993).

In the present study the effect of the selective PDE IV inhibitors rolipram (Schudt *et al.*, 1991b) and RP 73401 (Karlsson *et al.*, 1993) and the mixed-type PDE III/IV inhibitors zardaverine (Schudt *et al.*, 1991b) and tolafentrine (Schudt *et al.*, 1993) on ECP/EDN secretion as well as on formation of reactive oxygen species in human eosinophils was investigated in comparison with the non-selective PDE inhibitors, IBMX and theophylline. Apart from these functional studies in intact cells, the inhibition of PDE IV activity by these compounds in the cytosol of disrupted cells was assessed.

Methods

Isolation of human eosinophils

Fresh human blood, 5 × 50 ml (anticoagulated with 0.3% sodium citrate) from healthy donors was centrifuged at room temperature for 15 min at 200 g. After aspiration of the supernatant (platelet-rich plasma), the residue was diluted with HBSS/HEPES (composition, mmol l^{-1}: KCl 5.4, NaCl 137, Na_2HPO_4 0.3, KH_2PO_4 0.4, $NaHCO_3$ 4.2, glucose 5, HEPES 10, pH 7.4) to a total volume of 320 ml. Forty ml each was mixed with 20 ml of a 5% dextran solution (in HBSS/HEPES) and allowed to stand for about 30 min at room temperature; 25 ml of the supernatant was layered on top of 10 ml ficoll paque and centrifuged for 10 min at 800 *g*. The sediments were resuspended in HBSS/HEPES (4°C) and washed by centrifugation for 5 min at 315 *g*. The remaining red blood cells were lysed for 15 min at 4°C in a buffer containing, mmol l^{-1}: NH_4Cl 155, $KHCO_3$ 10 and EDTA 0.1 (pH 7.4). Afterwards the cells were washed first twice in HBSS/HEPES and then once in PBS/FCS (mmol l^{-1}: KCl 2.7, NaCl 137, Na_2HPO_4 8.1, KH_2PO_4 1.5, FCS 2%, pH 7.4) by centrifugation at 4°C for 5 min at 200 *g*. For the purification of eosinophils the magnetic cell separation (MACS) system from Miltenyi Biotec (Bergisch-Gladbach, Germany) was applied as described by Hansel *et al.* (1991) with some modifications. A two-step procedure was developed which reduces the consumption of anti-CD16 microbeads to about one third. Briefly, granulocytes were resuspended in PBS/FCS at a cell concentration of 10^7 cells/17.5 µl before addition of 2.5 µl anti-CD16 microbeads. After incubation for 30 min at 4°C, 10 ml PBS/FCS (4°C) was added and the cell suspension transferred to the top of a D-separation column in the magnetic field. The flow was adjusted to about 3.5 ml min^{-1} by a 3-way tap. The non-magnetically labelled cells (eosinophils) were eluted by rinsing the column with about 130 ml PBS/FCS. After this first step, the purity of the eosinophil cell suspension was 50–90%. After washing the cells in PBS/FCS (4°C) by centrifugation for 5 min at 200 *g*, the cells were resuspended in 50 µl PBS/FCS and the same volume of anti-CD16 microbeads was added. The cell suspension was again incubated for 30 min at 4°C, then 0.9 ml PBS/FCS was added and the cell suspension transferred to the top of a B_2-separation column. Eosinophils were eluted as described above with 12 ml of PBS/FCS and washed again in PBS/FCS. After this second purification step the purity of eosinophils was ≥ 99% as determined by combined May-Grünewald/Giemsa staining, and the viability was >97% as measured by Trypan blue exclusion. The yield of eosinophils was 2.13 ± 1.16 × 10^7 cells 250 ml^{-1} blood (n = 34). We did not determine eosinophil numbers of the blood donors in whole blood; however, assuming an average number of 0.17 × 10^6 eosinophils ml^{-1} blood of healthy subjects, the recovery of eosinophils by the isolation procedure employed can be estimated to be about 50%.

Secretion of ECP and EDN

All assays were performed in duplicate at a cell concentration of 10^6 cells ml^{-1} in Dulbecco's phosphate buffered saline (PBS) (pH 7.4) supplemented with 1 mmol l^{-1} Ca^{2+}/Mg^{2+}, 5 mmol l^{-1} glucose and 0.1% (w/v) BSA. The cell suspensions (1 ml) were preincubated for 10 min at 37°C before stimulation of the assays as indicated in the legends to the figures. Thereafter, the cells were spun down by centrifugation for 30 s in an Eppendorf centrifuge. Except for the inhibition experiments (see below) the supernatants were diluted 1:5 (v/v) in PBS containing 0.006% NP 40 and 1 mmol l^{-1} EGTA and stored at −20°C. The cell pellets were resuspended in an equivalent volume of PBS containing 0.5% NP 40 and 1 mmol l^{-1} EGTA and stored at −20°C. The cell pellets were diluted 1:50 (v/v) in PBS before radioimmunoassay (RIA) analysis. For the inhibition experiments the supernatants of the incubations (0.25 ml) were frozen directly and diluted 1:5 (v/v) in PBS prior to RIA analysis. Aliquots of the samples (50 µl) were taken for ECP- and EDN-measurements by a RIA method according to the manufacturers instructions.

Chemiluminescence assay

The formation of reactive oxygen species was assessed by luminol-enhanced chemiluminescence essentially as described (Schudt *et al.*, 1991a). Briefly, aliquots (0.5 ml) of the cell suspension (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C in a buffer consisting of mmol l^{-1}, NaCl 140, KCl 5, HEPES 10, $CaCl_2/MgCl_2$ 1, glucose 1 mg ml^{-1}, BSA 0.05% (w/v) luminol 10 µmol l^{-1} and microperoxidase 4 µmol l^{-1}. The assays were then transferred into a 'Multi-Bioluminat' LB 9505C from Berthold (Wildbad, Germany) before stimulation of the assays as indicated in the legends to the figures. Chemiluminescence was continuously recorded for the indicated times and the AUC's (area under the curve) calculated.

In order to assess unspecific quenching of chemiluminescence, formation of O_2^- was triggered in the absence of cells (but otherwise identical conditions) by xanthine oxidase (25 mu) in the presence of hypoxanthine (0.3 µmol l^{-1}). The chemiluminescence signal obtained under these conditions was of comparable magnitude.

PDE activity

For measuring of PDE activity under cell-free conditions, human eosinophils (10^7 cells ml^{-1}) were suspended in PBS

(pH 8.2) containing HEPES 10 mmol l^{-1} and 1 mmol l^{-1} EGTA, Mg^{2+} and β-mercaptoethanol before sonication of the cells for 1–1.5 min at 4°C using a Branson sonifier (microtip limit 1, duty cycle 10). Sonicates were centrifuged for 1 h at 100 000 *g*. Cytosolic and pellet fractions (resuspended in a corresponding volume of homogenization buffer) were stored at −20°C until measuring PDE activity.

PDE IV activity was determined as described by Thompson & Appleman (1979) with some modifications (Bauer & Schwabe, 1980). The assay mixture contained Tris-HCl 40 mmol l^{-1} (pH 7.4), $MgCl_2$ 5 mmol l^{-1}, cyclic AMP 0.5 μmol l^{-1} including [^{3}H]-cyclic AMP (about 50 000 c.p.m./assay), the indicated concentration of the inhibitor and an aliquot of eosinophil cytosol (15–25 μl) at a final assay volume of 200 μl. The assays were incubated for 30 min at 37°C and then stopped by the addition of 50 μl 0.2 N HCl. The assays were left on ice for about 10 min, incubated with 25 μg 5'-nucleotidase (*Crotalus atrox* snake venom) for 10 min at 37°C, and then loaded on QAE Sephadex A-25 columns (Econo colums, Bio-Rad, 1 ml bed volume). The columns were eluted with 2 ml ammonium formiate (pH 6.0) 30 mmol l^{-1} and the eluate was counted for radioactivity. Results were corrected for blank values (measured in the presence of denatured protein) which were below 2% of total radioactivity. The amount of cyclic AMP hydrolyzed did not exceed 20% of the original substrate concentration.

Chemicals and solutions

BSA (bovine serum albumin), complement C5a, dextran (mol.wt. 515,000), DMSO (dimethyl sulphoxide), EGTA (ethylene glycol-*bis*-[β-aminoethyl ether]*N,N,N',N'*-tetraacetic acid), fMLP (N-formyl-methionyl-leucyl-phenylalanine), glucose, hypoxanthine, IBMX, microperoxidase, nonidet P40 (NP 40), PAF (platelet activating factor), salbutamol, theophylline, xanthine oxidase and zymosan A were purchased from Sigma Chemie (Deisenhofen, Germany). HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) was obtained from Serva (Heidelberg, Germany), luminol from Boehringer (Mannheim, Germany), ficoll paque from Pharmacia LKB (Freiburg, Germany) and FCS (foetal calf serum) from Gibco (Paisley, Scotland). ^{125}I radioimmunoassays (RIAs) for ECP and EDN were purchased from Kabi Pharmacia (Freiburg, Germany). All other chemicals and solvents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Rolipram was a generous gift from Schering AG (Berlin, Germany), RP 73401 as well as zardaverine and tolafentrine have been synthesized at the chemistry facilities of Byk Gulden (Konstanz, Germany).

Opsonized zymosan was prepared by incubating zymosan A for 30 min at 37°C in human serum. The opsonized zymosan was washed three times in cold saline (0.9% NaCl in water, w/v) and stored at a concentration of 100 mg ml^{-1} at −20°C until use. Before opsonization, zymosan A had been boiled for 1.5–2 h in saline at a concentration of about 10 mg ml^{-1} and washed four times in boiling saline. A stock solution from fMLP (10 mmol l^{-1}) was prepared in DMSO and diluted 1:1000 (v/v) in the incubation buffer (see below). C5a was dissolved in water (100 μmol l^{-1}) and diluted 1:10 in buffer. A stock solution from PAF (1 mol l^{-1}) was prepared in ethanol and diluted 1:100 (v/v) in buffer. The solutions of all four stimuli were further diluted 1:100 (v/v) in the assays resulting in a final concentration of 100 nmol l^{-1} for fMLP, C5a and PAF and 1 mg ml^{-1} for opsonized zymosan, respectively. A stock solution from salbutamol (1 mmol l^{-1}) was prepared in water. Stock solutions from theophylline (25 mmol l^{-1}), IBMX (25 mmol l^{-1}) and zardaverine (10 mmol l^{-1}) were prepared in NaOH and from tolafentrine (10 mmol l^{-1}) in HCl so that the final assay concentrations of NaOH and HCl, respectively, were below 0.001 N at the highest inhibitor concentration tested. Stock solutions (10 mmol l^{-1}) of rolipram and RP 73401 were prepared in ethanol and DMSO, respectively, so that the final solvent concentration in the assays was below 0.01% at the highest inhibitor concentration tested. Appropriate dilutions of the inhibitors in water were further diluted 1:25 (v/v) in the assays to achieve the final concentrations indicated.

Statistics

Results are given as mean ± s.d. (standard deviation) from the number (*n*) of independent experiments (using cells from individual subjects) indicated. IC_{50} values were calculated from concentration-inhibition-curves by non-linear regression analysis using GraphPad InPlot (GraphPad Software Inc., Philadelphia, U.S.A.). Statistical significance (*P*) was determined by the paired *t* test (InStat, GraphPad Software Inc.).

Results

Secretion of ECP and EDN

In initial experiments the ability of fMLP, opsonized zymosan, C5a and PAF to trigger eosinophil granule release was compared. In a recent paper from Bach *et al.* (1991), possible pitfalls in the measurement of the secretion of granule proteins by human eosinophils have been described. A major problem seems to be the low recovery of granule proteins in the supernatant of stimulated cells compared to the loss of these proteins in the cell pellets. In order to address this problem under our experimental conditions, we measured EDN and ECP both in the supernatant and in the cell pellet of stimulated versus unstimulated cells (blank) after incubation of the cell suspensions for 30 min at 37°C.

We detected comparable amounts of EDN (2871 ± 833 ng/10^6 cells; $n = 6$) and ECP (2706 ± 1111 ng/10^6 cells; $n = 6$) in the cell pellet of unstimulated cells. These values are in agreement with those reported by others (Abu-Ghazaleh *et al.*, 1992). Under these conditions, the percentage of EDN and ECP measured in the supernatant of the cells was 3.7% and 2.9%, respectively. Figure 1a shows that all four stimuli tested lead to a comparable (2.5–3 fold compared to unstimulated cells) increase of both EDN- and ECP-levels in the cell supernatants. In general, EDN and ECP were released in parallel in agreement with the colocalization of these two granule proteins in the matrix of specific granules (Gleich & Adolphson, 1986). However, in the case of opsonized zymosan almost no ECP was detected in the supernatant, although a substantial portion of EDN was measured. Quantification of the loss of granule proteins in the cell pellets (expressed by the index: amount of the granule proteins in unstimulated divided by the amount in stimulated cells) revealed a similar pattern to that in the supernatants (Figure 1b). For opsonized zymosan a comparable loss of EDN and ECP was found in the cell pellet. The fact that no ECP could be detected in the cell supernatant with this stimulus remains unexplained.

Based on the amounts of the granule proteins lost in the cell pellets in stimulated versus unstimulated cells, the recovery of the proteins in the supernatants was calculated. For EDN the recovery was largely independent of the stimulus used and was found to be about 75% on average (data not shown). In contrast, for ECP the recovery was only about 20–25% on average (data not shown) with the exception of opsonized zymosan for which no ECP could be detected in the cell supernatant as already mentioned before. Despite the low recovery for ECP it is obvious from the data shown in Figure 1 that the stimulation pattern of granule proteins reflected either as increase of EDN and ECP in the cell supernatant (Figure 1a) or as decrease of the two proteins in the cell pellet (Figure 1b) is almost identical. These results suggest that the relative amounts of both proteins

measured in the supernatant of the cells are representative for the activation status of the cells concerning the secretion of EDN and ECP. Therefore, in further experiments assessing the influence of the compounds on EDN- and ECP-secretion, the supernatants of the cells were analysed.

For this purpose C5a was selected as a stimulus which showed the most reproducible and efficient response with respect to granule secretion (as shown above) and the chemiluminescence measurements (see below). As a prerequisite, the kinetics of EDN- and ECP-release were examined. Figure 2 demonstrates that the secretion of both proteins occurs very rapidly and plateaus at about 15 min Therefore, a stimulation time of 10 min was selected for the inhibition experiments.

Under these conditions both theophylline and IBMX concentration-dependently inhibited EDN- and ECP-release almost in parallel with IC_{50} values of 347/589 μmol l^{-1} and 50/51 μmol l^{-1}, respectively (Figure 3). The β_2-adrenoceptor agonist, salbutamol, did not show a significant concentration-dependent effect in the concentration-range tested (Figure 3). In contrast, none of the selective PDE IV (rolipram, RP 73401) or PDE III/IV (zardaverine, tolafentrine) inhibitors examined, substantially inhibited EDN- and ECP-release at concentrations up to 10 μmol l^{-1} (Figure 4). In combination with salbutamol (1 μmol l^{-1}), however, secretion of both proteins was inhibited by all four compounds tested in an obviously synergistic manner (Figure 4).

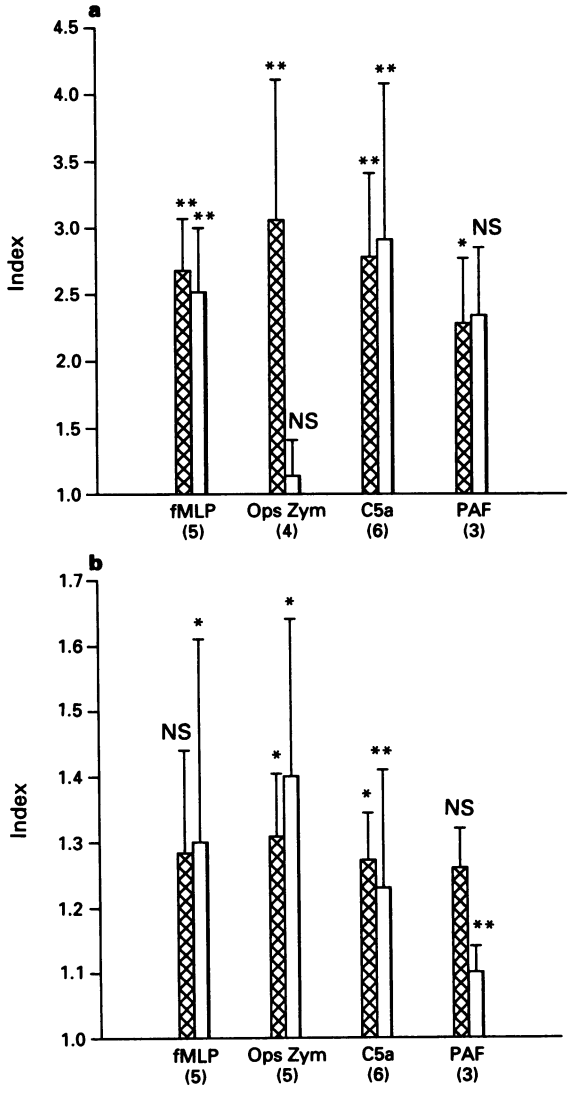


Figure 1 Stimulus-dependent secretion of eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP) by human eosinophils. Aliquots (1 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation with fMLP, C5a, PAF (100 nmol l^{-1}, each) or opsonized zymosan (1 mg ml^{-1}). Non-stimulated cells served as blank. After 30 min the incubations were stopped by centrifugation, the supernatants were removed and the cell pellets solubilized as described in Methods. Aliquots of appropriate dilutions of the supernatants (a) and pellets (b) were assayed for EDN (crosshatched columns) and ECP (open columns) by RIA. Amounts of EDN and ECP detected in the presence of the stimuli are expressed relative to the values measured in unstimulated cells by means of an index. In the supernatant the index is defined as the ratio of the amount of EDN or ECP in stimulated cells divided by that in unstimulated cells, in the pellet *vice versa*. Data are given as mean ± s.d. from the number of independent experiments given in parentheses. Statistical significance (**$P<0.01$; *$P<0.05$; NS, not significant) using the paired t test was calculated comparing the amounts of ECP and EDN measured in stimulated versus unstimulated cells.

Chemiluminescence measurements

The formation of reactive oxygen species in human eosinophils was assessed by luminol-enhanced chemiluminescence. In analogy to the secretion experiments, the ability of fMLP, PAF, C5a and opsonized zymosan to stimulate a chemiluminescence response was investigated in initial experiments. In contrast to secretion, the chemiluminescence response differed markedly between the four stimuli which is demonstrated by a representative experiment shown in Figure 5. Whereas the soluble agonists fMLP, PAF and C5a triggered a rapid chemiluminescence signal within seconds, which declines to baseline levels in about 1–3 min, opsonized zymosan stimulated chemiluminescence with a slow onset but a long duration up to 30 min. A quantitative evaluation of all experiments performed is shown in Table 1 which summarizes the AUC's for the corresponding time-periods of the stimuli in which the most pronounced chemiluminescence signal was detected. From these data it is evident that the C5a-stimulated chemiluminescence response was most reproducible and showed the highest relative efficacy compared to unstimulated cells (blank). Therefore, inhibition experiments were

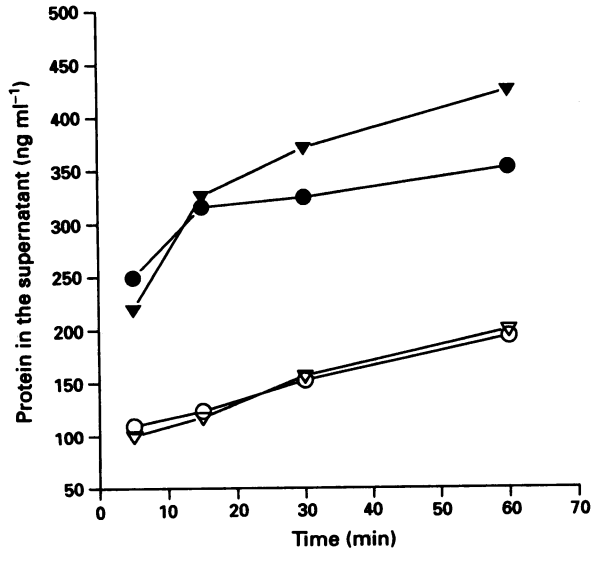


Figure 2 Kinetics of C5a-stimulated release of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. Aliquots (1 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation without (open symbols) or with 100 nmol l^{-1} C5a (solid symbols). At the indicated times the incubations were centrifuged and the amount of ECP (○,●) and EDN (△,▲) in the supernatant of the assays determined by RIA. Results are given as mean from two independent experiments.

performed with C5a using a stimulation time of 1 min. Similar to the secretion experiments both theophylline and IBMX concentration-dependently inhibited chemiluminescence with IC_{50} values of 525 μmol l^{-1} and 417 μmol l^{-1}, respectively (Figure 6). It was evident, however, that the potency of IBMX in inhibiting chemiluminescence was about one order of magnitude lower than for inhibition of secretion. Salbutamol also inhibited C5a-stimulated chemiluminescence in human eosinophils to some degree; however, almost the same inhibitory effect of salbutamol on chemiluminescence was obtained in a cell-free system consisting of hypoxanthine/xanthine oxidase (XOD) (Figure 6) which is known to produce a chemiluminescence signal by the production of superoxide anion radical (O_2^-). These results suggest that most, if not all, of the inhibitory effect of salbutamol on chemiluminescence in eosinophils is due to an unspecific quenching of O_2^- rather than to an inhibition of the production of reactive oxygen species themselves. A concentration of 1 μmol l^{-1} salbutamol had only a modest effect on chemiluminescence, therefore this concentration was used to study the combined effect of salbutamol and theophylline or IBMX, respectively, on C5a-stimulated chemiluminescence. While the effect of salbutamol on the inhibition of chemiluminescence by theophylline was simply additive (IC_{50} of 331 μmol l^{-1} versus 525 μmol l^{-1}), the IC_{50} value of IBMX for chemiluminescence inhibition was improved by more than one order of magnitude (31 μmol l^{-1} versus 417 μmol l^{-1}) in the presence of salbutamol (Figure 6).

Similar to the secretion experiments, none of the selective PDE inhibitors tested had a remarkable effect on C5a-

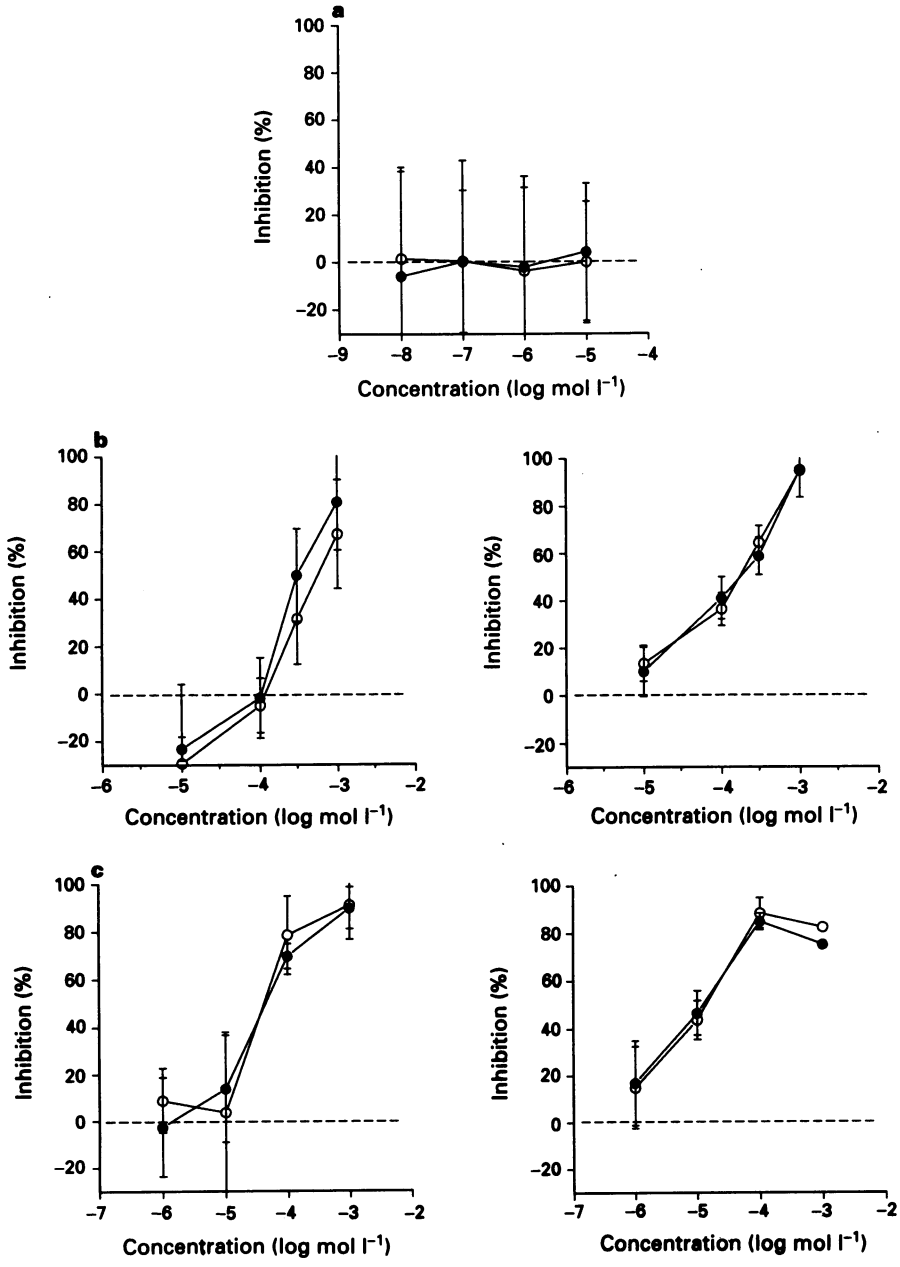


Figure 3 Influence of salbutamol (a), and theophylline (b) or 3-isobutyl-1-methylxanthine (c) (either alone left panel, or in combination with 1 μmol l^{-1} salbutamol right panel) on C5a-stimulated secretion of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. Aliquots (0.25 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated either in the absence or presence of the indicated concentrations of the compounds for 10 min at 37°C. After stimulation of the assays with C5a (100 nmol l^{-1}) for 10 min, the incubations were stopped by centrifugation. Aliquots of the supernatants were taken for measurement of ECP (○) and EDN (●) by RIA. Drug effect (% inhibition) was calculated based on the amount of ECP and EDN, respectively, measured in the absence of any compound (control). Results are given as mean ± s.d. from 3–5 independent experiments.

stimulated chemiluminescence (Figure 7). Again, potency was markedly improved on combination with 1 μmol l^{-1} salbutamol which facilitates a synergistic inhibition of chemiluminescence in the presence of all four selective PDE inhibitors tested (Figure 7).

Inhibition of PDE IV activity

In initial experiments the PDE isoenzyme activity pattern as well as the subcellular localization of PDE isoenzymes was investigated. For this purpose, cyclic nucleotide hydrolyzing activity was measured with either cyclic GMP or cyclic AMP at a concentration of 0.5 μmol l^{-1}. Compared to cyclic AMP-PDE activity (235 ± 26 pmol × min^{-1} × 10^{-8} cell equivalents; $n = 10$) only trace amounts (<3%) of cyclic GMP-PDE activity (6.5 ± 3.2 pmol × min^{-1} × 10^{-8} cell equivalents) were found, indicating the absence of both PDE I and V in human eosinophils. More than 95% of total cyclic AMP-PDE activity was blocked by rolipram (10 μmol l^{-1}). The residual cyclic AMP-PDE activity was neither affected by the PDE III-selective inhibitor motapizone (1 μmol l^{-1}) nor cyclic GMP (5 μmol l^{-1}) or Ca^{2+}/calmodulin which are the

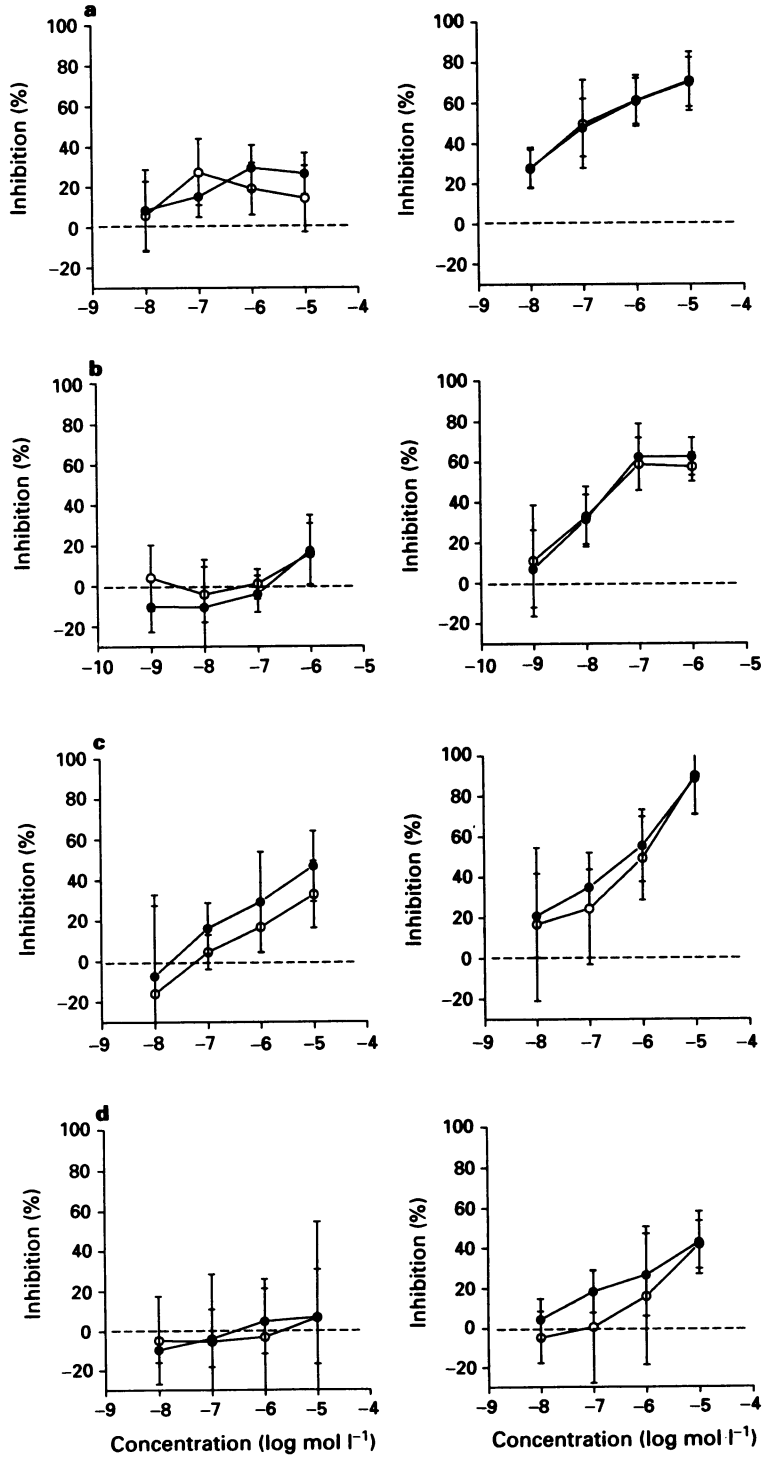


Figure 4 Influence of rolipram (a), RP 73401 (b), zardaverine (c) and tolafentrine (d) (either alone left panel, or in combination with 1 μmol l^{-1} salbutamol right panel) on C5a-stimulated secretion of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. For assay conditions see legend to Figure 3. Symbols refer to ECP (○) and EDN (●), respectively. Results are given as mean ± s.d. from 3–6 independent experiments.

Table 1 Quantitative analysis of the chemiluminescence response of human eosinophils stimulated by various agonists

	Blank (AUC, c.p.m. × 10^7)	*Stimulated* (AUC, c.p.m. × 10^7)	n	*AUC Stimulated/Blank* (fold)
fMLP (0–3 min)	2.6 ± 2.6	19.4 ± 13.5	8	7.5[a]
C5a (0–1 min)	0.8 ± 1.0	14.3 ± 2.9	9	17.9[b]
PAF (0–1 min)	0.5 ± 0.4	3.1 ± 3.5	8	6.2[c]
Ops Zym (0–30 min)	46.6 ± 25.7	191.6 ± 64.2	8	4.1[d]

For assay conditions see legend to Figure 5. Data shown correspond to the AUC's (c.p.m. × 10^7) calculated for the chemiluminescence signal at time-periods typical for each agonist: fMLP 0–3 min, C5a and PAF 0–1 min, and opsonized zymosan (Ops Zym) 0–30 min. These time-periods are derived from kinetic experiments (see Figure 5), and cover the times at which a stimulus-dependent chemiluminescence signal beyond the signal of unstimulated cells (blank) was measured. Results are given as mean ± s.d. from the number of experiments indicated. Statistical significance (P) using the paired t test was calculated comparing the AUC's of stimulated versus unstimulated cells. For abbreviations, see text.
[a]$P<0.01$; [b]$P<0.0001$; [c]$P<0.05$; [d]$P=0.0001$.

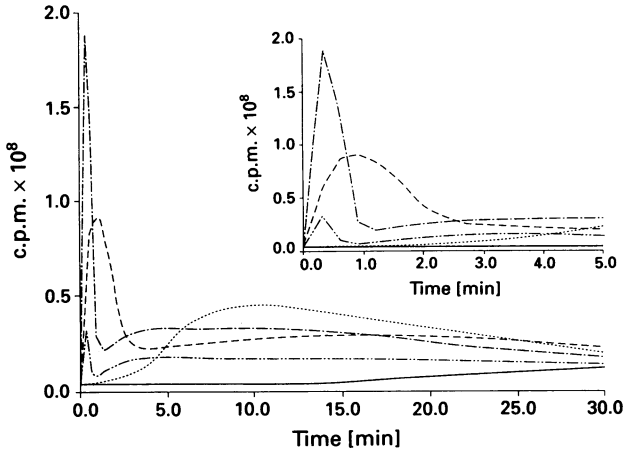


Figure 5 Stimulus-dependent chemiluminescence response of human eosinophils. Aliquots (0.5 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation with 100 nmol l^{-1} fMLP (------), 100 nmol l^{-1} C5a (-·-·-), 100 nmol l^{-1} PAF (-··-··-) or 1 mg ml^{-1} opsonized zymosan (·····). Non-stimulated cells served as blank (——). Luminol-enhanced chemiluminescence was continuously recorded. The figure shows the traces of a representative experiment.

activators of PDE I and PDE II, respectively. These results indicate that PDE IV is the predominant PDE isoenzyme in human eosinophils. In addition, about 75% of PDE IV activity is located in the cytosolic fraction.

This fraction, therefore, was taken to investigate the effect of the PDE inhibitors mentioned above on PDE IV activity of human eosinophils under cell-free conditions. For all six compounds monophasic inhibition curves were obtained, the calculated IC_{50} values of which are summarized in Table 2.

A comparison of the IC_{50} values for inhibition of PDE IV activity, secretion of ECP/EDN and chemiluminescence, respectively, reveals (Table 2) that theophylline inhibited all three parameters with about the same potency. On the whole this is also true for IBMX except for the inhibition of chemiluminescence, where IBMX required the presence of salbutamol to achieve an IC_{50} value similar to that obtained for inhibition of PDE IV activity in the cell-free system. In contrast, none of the selective PDE inhibitors substantially affected intact cell responses on their own, despite the fact that these compounds were much more potent inhibitors of PDE activity in the cell-free system compared to theophylline and IBMX. Interestingly, the selective PDE inhibitors were active in the presence of salbutamol in a synergistic manner. Under these conditions, zardaverine and rolipram inhibited both ECP/EDN secretion and chemiluminescence with IC_{50} values comparable to those obtained for inhibition of PDE IV activity, while RP 73401 and tolafentrine in intact cells were one to two or at least two orders of magnitude, respectively, less potent than would have been expected from the inhibition studies of PDE activity in the cell-free system.

Discussion

The present study addressed the question whether eosinophil responses like granule secretion or formation of reactive oxygen species, which are related to the inflammatory role of these cells in asthma pathogenesis, are inhibited by PDE inhibitors *in vitro*. This class of compounds has been postulated to be promising in asthma therapy and in particular PDE IV-selective inhibitors have attracted attention because of their general potential to suppress inflammatory cell functions (Giembycz, 1992).

Although the eosinophil is accepted to play a pivotal role as effector cell in the pathogenesis of asthma, very limited information on the effect of PDE inhibitors on human eosinophil functions is available. Kita *et al.* (1991) reported the inhibition of sIgA- or IgG-stimulated EDN secretion in human eosinophils by IBMX and theophylline. The IC_{50} values for IBMX (9 and 30 μmol l^{-1}) and theophylline (90 and 500 μmol l^{-1}) reported in that study are comparable to those we have found for both compounds in the present study for C5a-stimulated ECP/EDN secretion (Table 2). In addition, we have shown that both inhibitors are effective in inhibiting formation of reactive oxygen species as measured by luminol-enhanced chemiluminescence. From Kita's studies it is evident that stimulus-dependent differences in the potency of the compounds in inhibiting human eosinophil secretion may occur. Based on initial experiments comparing different physiological stimuli, we have selected complement C5a for the inhibition experiments because under our experimental conditions the cells responded to C5a most effectively and most reproducibly. At least in the guinea-pig the importance of complement activation for antigen-induced bronchoconstriction has been considered (Regal *et al.*, 1993a,b).

We have found that human eosinophils contain PDE IV as the predominant PDE isoenzyme. Under our experimental conditions most of the PDE IV activity (75%) was recovered from the cytosol. According to our experimental protocol we used freshly isolated cells for the preparation of subcellular fractions. We have made the observation, however, that freezing (storage at −20°C) of the cells before sonication or

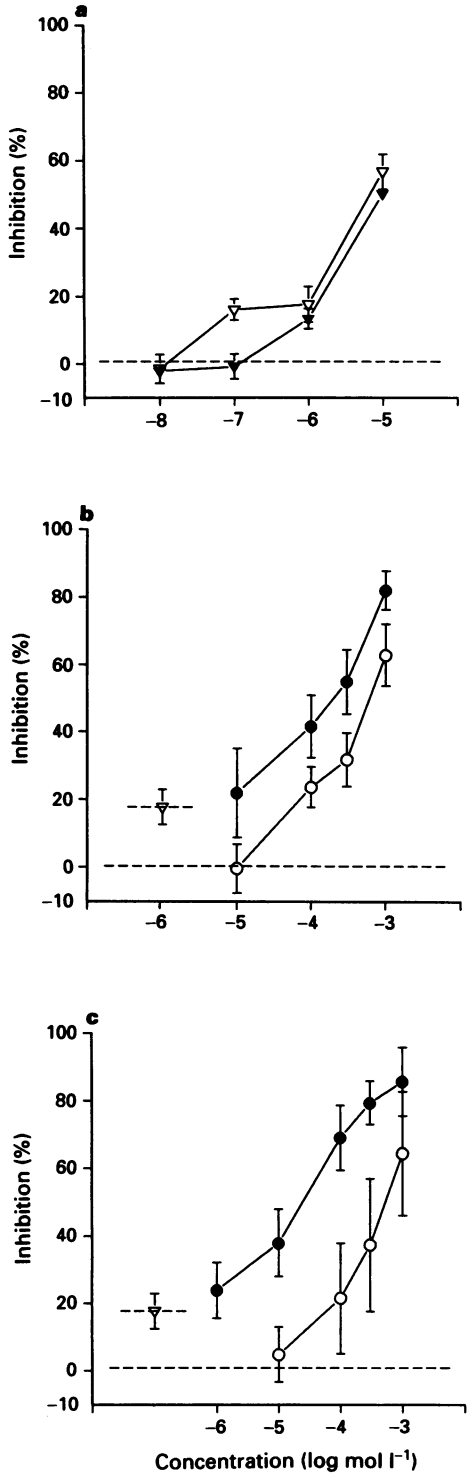


Figure 6 Influence of salbutamol (a) and theophylline (b) or 3-isobutyl-1-methylxanthine (c) (either alone or in combination with 1 μmol l^{-1} salbutamol) on C5a-stimulated chemiluminescence response of human eosinophils. Aliquots (0.5 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated either in the presence or absence of the indicated concentrations of the compounds for 10 min at 37°C. Then the assays were stimulated with C5a (100 nmol l^{-1}) for 1 min. Chemiluminescence was continuously recorded and the AUC's calculated. Drug effect (% inhibition) was calculated based on the chemiluminescence signal obtained in the absence of any compound (control). Symbols refer to the effect of PDE inhibitors alone (○) or in combination with 1 μmol l^{-1} salbutamol (●), and the effect of salbutamol itself (△), respectively. Unspecific quenching of chemiluminescence by salbutamol (a, ▲) was assessed by triggering superoxide anion formation in a cell-free system using hypoxanthine (0.3 μmol l^{-1}) and xanthine-oxidase (25 mu) under otherwise identical conditions. Results are given as mean ± s.d. from 3–5 independent experiments.

enhancement of the energy output during sonication renders total PDE IV activity to be membrane-bound (data not shown). These results indicate that the handling of the cells prior to and during disruption is a critical determinant of PDE IV localization at least in human eosinophils. Differences in the experimental procedure for the preparation of subcellular fractions may therefore account for the membrane localization of PDE IV reported by others in human (Giembycz & Barnes, 1993) as well as guinea-pig (Souness *et al.*, 1991; Dent *et al.*, 1991) eosinophils. In addition, the localization of PDE IV in eosinophils may be species-dependent.

Based on the PDE isoenzyme pattern, one would have expected that the inhibition of human eosinophil functions by the non-selective PDE inhibitors IBMX and theophylline would be mimicked by selective PDE IV inhibitors, provided that the action of IBMX and theophylline is a consequence of PDE inhibition. Surprisingly, none of the selective PDE inhibitors tested substantially inhibited ECP/EDN secretion or the chemiluminescence response, although all of these compounds were much more potent PDE IV inhibitors than theophylline or even IBMX in the cell-free system. These results are in contrast to guinea-pig peritoneal eosinophils, the O_2^--formation of which has been demonstrated to be sensitive to inhibition by selective PDE IV inhibitors both in resting cells (Souness *et al.*, 1991) and in opsonized zymosan-stimulated cells (Dent *et al.*, 1991). Whether this difference represents species-specific effects or is due to differences in the experimental conditions remains unexplained at the moment.

One explanation for the failure of the selective PDE inhibitors to inhibit human eosinophil responses could be the insufficient basal cyclic AMP concentration in these cells. Due to the requirement of large cell numbers we were not able to perform cyclic AMP measurements: however, we tried to assess the influence of an additional cyclic AMP trigger indirectly by testing the influence of a β_2-adrenoceptor agonist (salbutamol). We are not aware of any literature directly proving the existence of β_2-adrenoceptors on human peripheral eosinophils purified from healthy blood donors. However, the enhancement of IBMX-mediated cyclic AMP levels by β_2-adrenoceptor agonists like salbutamol and isoprenaline and its reversal by the β-adrenoceptor antagonist propanolol as reported in the study of Kita *et al.* (1991) provides circumstantial evidence for the presence of such receptors on human eosinophils. In addition, β-adrenoceptors of the β_2-subtype that are coupled to adenylate cyclase have been characterized in eosinophils from patients with eosinophilia (Yukawa *et al.*, 1990). In our own experiments we used salbutamol and found that neither ECP/EDN secretion nor chemiluminescence was substantially affected by this compound. One should mention that according to the experimental protocol used in our studies, cells were treated with salbutamol 10 min prior to the stimulation with C5a; we therefore cannot exclude the possibility that salbutamol would have been effective after a shorter preincubation time diminishing the extent of cyclic AMP degradation by the action of PDE IV. The lack of effect of salbutamol on eosinophil responses is in agreement with the study of Kita *et al.* (1991), who showed no or only marginal inhibitory effects of salbutamol and isoprenaline on sIgA- and IgG-stimulated EDN secretion, respectively, but is in contrast to a recent study of Munoz *et al.* (1994) who demonstrated an approximately 50% inhibition of fMLP-stimulated EPO- (eosinophil peroxidase) and leukotriene C_4-release by salbutamol. The latter group, however, used cytochalasin B as a non-physiological agent in their assays which might facilitate the inhibition of eosinophil responses by β_2-adrenoceptor agonists. In support of this assumption Yukawa *et al.* (1990) failed to find an inhibitory effect of salbutamol on both O_2^- generation and EPO release in eosinophils from patients with eosinophilia. Formoterol at high concentrations (1–100 μmol l^{-1}) was reported to inhibit modestly (<40%)

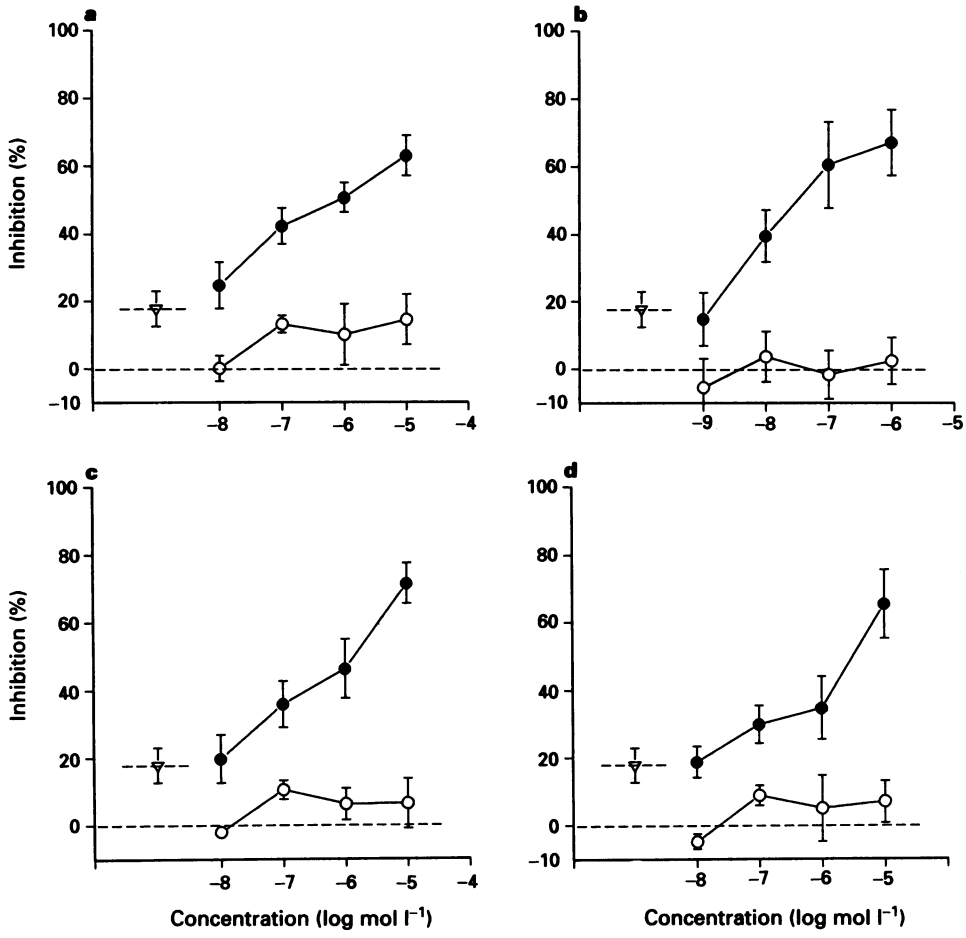


Figure 7 Influence of rolipram (a), RP 73401 (b), zardaverine (c) and tolafentrine (d) (either alone or in combination with 1 μmol l^{-1} salbutamol) on C5a-stimulated chemiluminescence response of human eosinophils. For assay conditions see legend to Figure 6. Symbols refer to the effect of PDE inhibitors alone (○) or in combination with 1 μmol l^{-1} salbutamol (●); the effect of salbutamol itself is also shown (△). Results are given as mean ± s.d. from 3–4 independent experiments.

Table 2 IC_{50} values (μmol l^{-1}) of various PDE inhibitors for inhibition of C5a-stimulated secretion of ECP/EDN and the chemiluminescence response in intact human eosinophils, as well as for inhibition of PDE IV activity in the cytosol of human eosinophils

	Secretion of ECP/EDN		*Chemiluminescence*		*PDE IV activity*
	− Salbut	+ Salbut	− Salbut	+ Salbut	
	IC_{50} (μM)		IC_{50} (μM)		IC_{50} (μM)
Theophylline	589/347	204/257	525	331	288
IBMX	51/50	12/11	524	45	14
Rolipram	NE	0.1/0.2	NE	0.5	0.3
RP 73401	NE	0.05/0.04	NE	0.01	0.0007
Zardaverine	>10	0.7/0.4	NE	0.9	0.3
Tolafentrine	NE	>10	NE	7.6	0.03

Studies of the effect of PDE inhibitors in intact cells were performed either in the absence or presence of salbutamol (Salbut) (1 μmol l^{-1}) which by itself was without appreciable effect. IC_{50} values were calculated from concentration-inhibition-curves by non-linear regression analysis from at least three independent experiments.
NE, no effect. For abbreviations, see text.

PAF-induced ECP release, however, this effect of formoterol was not abolished by propanolol, making a β_2-adrenoceptor-triggered response unlikely (Eda *et al.*, 1993).

Despite the lack of effect of salbutamol itself, this compound allowed the selective PDE inhibitors to inhibit both ECP/EDN secretion and chemiluminescence in a synergistic manner. Obviously, only the cyclic AMP synthesis triggered by the action of the β_2-adrenoceptor agonist and the simultaneous inhibition of cyclic AMP degradation by PDE inhibitors are sufficient to inhibit C5a-stimulated cell responses. Under these conditions the IC_{50} values of rolipram and zardaverine for inhibition of eosinophil responses were in the order of magnitude that would have been expected from the IC_{50} values for inhibition of PDE IV activity in the cell-free system. In contrast, such a conformity did not exist for tolafentrine and RP 73401. Such a poor correlation between the PDE inhibitory actions of certain compounds and their effectiveness in inhibiting O_2^- formation has also been observed in guinea-pig eosinophils and has been attributed to a barrier impeding access of the compounds to the enzyme (Souness *et al.*, 1991). We have tested the compounds used in the present study in other cells and have found no

differences in the ability of the compounds to penetrate into the cells. Alternatively, the aforementioned lack of correlation may be explained by the existence of two sites (a catalytic site and a second biologically important site) on PDE IV as proposed for the guinea-pig eosinophil enzyme (Souness *et al.*, 1992). The second site seems to be (partially) concealed in freshly prepared membranes but is exposed by solubilization or vanadate/glutathione treatment. The IC_{50} values of inhibitors against solubilized PDE IV and their potencies in intact cells (elevation of intracellular cyclic AMP) strongly correlate, implying that in most eosinophils, PDE IV exists in a form similar to the solubilized or vanadate/glutathione-treated enzyme. Although these results obtained with the membrane-bound guinea-pig PDE IV may not directly translate to the cytosolic (soluble) human enzyme, they raise the possibility that the state of human eosinophil PDE IV, including inhibitor-sensitivity in the cytosol, is different from the state of the enzyme in intact cells. We are at present trying to clarify this hypothesis.

Based on the results discussed so far, the question arises why the non-selective PDE inhibitors IBMX and theophylline are able to suppress eosinophil functions in contrast to the selective PDE IV and III/IV inhibitors. The fact that human eosinophils contain PDE IV as the exclusive PDE isoenzyme suggests that other mechanisms besides or in addition to PDE inhibition may contribute to the inhibitory action of IBMX and theophylline. Other modes of action have been described for these xanthine derivatives such as inhibitions of calcium currents (Simasko & Shaochun, 1993), blockade of the adenylate cyclase-inhibitory regulatory protein G_i (Parsons *et al.*, 1988), direct activation of cyclic AMP-dependent protein kinase (Tomes *et al.*, 1993) or, in particular, adenosine receptor antagonism (Ukena *et al.*, 1993). These or other aspects have to be considered in further experiments elucidating the precise mode of action of IBMX and theophylline on human eosinophil functions. At the moment we are able to exclude only adenosine receptor antagonism in the actions of IBMX and theophylline on human eosinophils, based on the fact that human eosinophils have been reported to be very poor producers of adenosine (Resnick *et al.*, 1993) and that the chemiluminescence response is not influenced by adenosine deaminase (data not shown). Nevertheless, the different effects of the PDE inhibitors described in this paper demonstrate that results obtained with non-specific PDE inhibitors may not always translate to specific PDE inhibitors even if the PDE isoenzyme pattern of a certain cell type has been considered as shown here for human eosinophils. Theophylline is a well-established drug in the therapy of airway diseases, although, its mode of action *in vivo* is still controversial (Milgrom & Bender, 1993). Although speculative, the direct inhibitory effect of theophylline on human eosinophil responses observed *in vitro* (Kita *et al.*, 1991; present study) may contribute to the recent clinical finding that after treatment with theophylline a significant reduction in the number of EG2-positive activated eosinophils beneath the lung epithelial basement membrane of mild asthmatics was found (Sullivan *et al.*, 1994). PDE inhibition is ascribed a major role in the action of theophylline on both bronchodilatation and inhibition of inflammation. The benefit arising from theophylline therapy has encouraged the pharmaceutical industry to develop more specific and more potent PDE inhibitors in order to enhance efficacy in parallel with fewer side effects. It will be interesting to see whether such compounds can mimic the action of theophylline in patients suffering from airway diseases. With the restriction that all conclusions are based on experiments using one particular stimulus (C5a), the results presented in this paper indicate that specific PDE IV inhibitors need an additional cyclic AMP-triggering signal to be effective towards eosinophils. We have used a β_2-adrenoceptor agonist (salbutamol) in our *in vitro* experiments; however, it can be assumed that the action of salbutamol is representative of other receptor agonists positively coupled to adenylate cyclase in human eosinophils. Similar to other leukocytes such agonists may include prostaglandins such as PGE_2, adenosine, histamine, endogenous adrenaline and others presumed to be present in inflammatory loci.

In addition, one might speculate whether certain factors (e.g. cytokines, colony-stimulating factors or other inflammatory mediators) present in the circulation and/or in the lung of atopic patients alter the sensitivity of eosinophils towards PDE inhibitors. The study of Maruo *et al.* (1993) may be interpreted in this direction. In that study, an inhibitory effect of the PDE IV inhibitor WAY-PDA-641 on PAF-induced superoxide generation was found in purified eosinophils from allergic subjects, which is in contrast to our own results demonstrating no effect of PDE IV and III/IV inhibitors on C5a-stimulated chemiluminescence in eosinophils from healthy donors. This general aspect is of particular importance for assessing *in vitro*-data on the effects of PDE inhibitors on pro-inflammatory cells for the *in vivo*-efficacy of these drugs in asthmatic patients.

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References

ABU-GHAZALEH, R.I., DUNNETTE, S.L., LOEGERING, D.A., CHECKEL, J.L., KITA, H., THOMAS, L.L. & GLEICH, G.J. (1992). Eosinophil granule proteins in peripheral blood granulocytes. *J. Leukoc. Biol.*, **52**, 611–618.

BACH, M.K., BRASHLER, J.R., SANDERS, M.E. & BIENKOWSKY, M.J. (1991). Pitfalls in the quantitative estimation of the secretion of granule proteins in eosinophils. *J. Immunol. Methods*, **142**, 243–250.

BARNES, P.J. (1992). New aspects of asthma. *J. Internal Med.*, **231**, 453–461.

BARNES, P.J. & PEDERSEN, S. (1993). Efficacy and safety of inhaled corticosteroids in asthma. *Am. Rev. Respir. Dis.*, **148**, S1–S26.

BAUER, A.C. & SCHWABE, U. (1980). An improved assay of cyclic 3',5'-nucleotide phosphodiesterase with QAE Sephadex A-25. *Naunyn-Schmied. Arch. Pharmacol.*, **311**, 193–198.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

BRUIJNZEEL, P.L.B. (1989). Contribution of eosinophil-derived mediators in asthma. *Int. Arch. Allergy Appl. Immunol.*, **90**, 57–63.

DENT, G., EVANS, P.M., CHUNG, K.F. & BARNES, P.J. (1990). Zardaverine inhibits respiratory burst activity in human eosinophils. *Am. Rev. Respir. Dis.*, **141**, A392.

DENT, G., GIEMBYCZ, M.A. RABE, K.F. & BARNES, P.J. (1991). Inhibition of eosinophil cyclic nucleotide PDE activity and opsonized zymosan-stimulated respiratory burst by type IV-selective PDE inhibitors. *Br. J. Pharmacol.*, **103**, 1339–1346.

EDA, R., SUGIYAMA, H., HOPP, R.J., OKADA, C., BEWTRA, A.K. & TOWNLEY, R.G. (1993). Inhibitory effects of formoterol on platelet-activating factor induced eosinophil chemotaxis and degranulation. *Int. Arch. Allergy Immunol.*, **102**, 391–398.

GIEMBYCZ, M.A. (1992). Could isoenzyme-selective phosphodiesterase inhibitors render bronchodilator therapy redundant in the treatment of bronchial asthma? *Biochem. Pharmacol.*, **43**, 2041–2051.

GIEMBYCZ, M.A. & BARNES, P.J. (1993). Stimulus-response coupling in eosinophils. In *Immunopharmacology of Eosinophils*. ed. Smith, H, & Cook, R.M. pp. 91–118. London, San Diego, New York, Boston, Sydney, Tokyo, Toronto: Academic Press.

GLEICH, G.J. (1990). The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.*, **85**, 422–436.

GLEICH, G.J. & ADOLPHSON, C.R. (1986). The eosinophilic leukocyte: structure and function. *Adv. Immunol.*, **39**, 177–253.

HANSEL, T.T., DE VRIES, I.J.M., IFF, T., RIHS, S., WANDZILAK, M., BETZ, S., BLASER, K. & WALKER, C. (1991). An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J. Immunol. Meth.*, **145**, 105–110.

KARLSSON, J.-A., SOUNESS, J., RAEBURN, D., PALFREYMAN, M. & ASHTON, M. (1993). Anti-inflammatory effects of novel selective cyclic nucleotide phosphodiesterase inhibitors. In *T-lymphocyte and Inflammatory Cell Research in Asthma*. ed. Jolles, G., Karlsson, J.-A. & Taylor, J. pp. 323–351. London, San Diego, New York, Boston, Sydney, Tokyo, Toronto; Academic Press.

KITA, H., ABU-GHAZALEH, R.I., GLEICH, G.J. & ABRAHAM, R.T. (1991). Regulation of Ig-induced eosinophil degranulation by adenosine 3',5'-cyclic monophosphate. *J. Immunol.*, **146**, 2712–2718.

MARUO, H., TANIMOTO, Y., BEWTRA, A.K. & TOWNLEY, R.G. (1993). Effect of phosphodiesterase IV inhibitor (WAY-PDA-641) on PAF-induced superoxide generation from human eosinophils. *J. Allergy Clin. Immunol.*, **93**, 569.

MICHAELI, T., BLOOM, T.J., MARTINS, T., LOUGHNEY, K., FERGUSON, K., RIGGS, M., RODGERS, L., BEAVO, J.A. & WIGLER, M. (1993). Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient Saccharomyces cerevisiae. *J. Biol. Chem.*, **268**, 12925–12932.

MILGROM, H. & BENDER, B. (1993). Current issues in the use of theophylline. *Am. Rev. Respir. Dis.*, **147**, S33-S39.

MUNOZ, N.M., VITA, A.J., NEELEY, S.P., MCALLISTER, K., SPAETHE, S.M., WHITE, S.R. & LEFF, A.R. (1994). Beta adrenergic modulation of formyl-methionine-leucine-phenylalanine-stimulated secretion of eosinophil peroxidase and leukotriene C_4. *J. Pharmacol. Exp. Ther.*, **268**, 139–143.

PARSONS, W.J., RAMKUMAR, V. & STILES, G.L. (1988). Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein G_i. *Mol. Pharmacol.*, **34**, 37–41.

REGAL, J.F., FRASER, D.G., ANDERSON, D.E. & SOLEM, L.E. (1993a). Enhancement of antigen-induced bronchoconstriction after intravascular complement activation with cobra venom factor. *J. Immunol.*, **150**, 3496–3505.

REGAL, J.F., FRASER, D.G. & TOTH, C.A. (1993b). Role of the complement system in antigen-induced bronchoconstriction and changes in blood pressure in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **267**, 979–988.

RESNICK, M.B., COLGAN, S.P. PATAPOFF, T.W., MRSNY, R.J., AWTREY, C.S., DELP-ARCHER, C., WELLER, P.F. & MADARA, J.L. (1993). Activated eosinophils evoke chloride secretion in model intestinal epithelia primarily via regulated release of 5'-AMP. *J. Immunol.*, **151**, 5716–5723.

SCHUDT, C., WINDER, S., FORDERKUNZ, S., HATZELMANN, A. & ULLRICH, V. (1991a). Influence of selective phosphodiesterase inhibitors on human neutrophil functions and levels of cAMP and Ca_i. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 682–690.

SCHUDT, C., WINDER, S., MÜLLER, B. & UKENA, D. (1991b). Zardaverine as a selective inhibitor of phosphodiesterase isozymes. *Biochem. Pharmacol.*, **42**, 153–162.

SCHUDT, C., TENOR, H., WNEDEL, A., ELTZE, M., MAGNUSSEN, H. & RABE, K.F. (1993). Influence of the PDE III/IV inhibitor B9004-070 in contraction and PDE activities in airway and vascular smooth muscle. *Am. Rev. Respir. Dis.*, **147**, A183.

SHUTE, J.K., SCHUDT, C. & CHURCH, M.K. (1993). Differential inhibition of phosphodiesterase inhibitors of Il-5 and PAF induced chemotactic responses of normal and atopic human eosinophils. *J. Allergy Clin. Immunol.*, **93**, 298.

SIMASKO, S.M. & SHAOCHUN, Y. (1993). 3-Isobutyl-l-methylxanthine inhibits sustained calcium current independently of cyclic AMP in neuronal and endocrine cells. *Mol. Pharmacol.*, **44**, 622–627.

SOUNESS, J.E., CARTER, C.M., DIOCEE, B.K., HASSALL, G.A., WOOD, L.J. & TURNER, N.C. (1991). Characterization of guinea-pig eosinophil phosphodiesterase activity. *Biochem. Pharmacol.*, **42**, 937–945.

SOUNESS, J.E., MASLEN, C. & SCOTT, L.C. (1992). Effects of solubilization and vanadate/glutathione complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase. *FEBS Letters*, **302**, 181–184.

SULLIVAN, P., BEKIR, S., JAFFAR, Z., PAGE, C., JEFFERY, P. & COSTELLO, J. (1994). Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. *Lancet*, **343**, 1006–1008.

TANIMOTO, Y., MARUO, H., BEWTRA, A.K. & TOWNLEY, R.G. (1993). Effects of phosphodiesterase IV inhibitor (WAY-PDA-641) on human eosinophil and neutrophil migration *in vitro*. *J. Allergy Clin. Immunol.*, **93**, 570.

THOMPSON, W.J. & APPLEMAN, M.M. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv. Cycl. Nucl. Res.*, **10**, 69–92.

TOMES, C., ROSSI, S. & MORENO, S. (1993). Isobutylmethylxanthine and other classical cyclic nucleotide phosphodiesterase inhibitors effect cAMP-dependent protein kinase activity. *Cell. Signal.*, **5**, 615–621.

UKENA, D., SCHUDT, C. & SYBRECHT, G.W. (1993). Adenosine receptor-blocking xanthines as inhibitors of phosphodiesterase isozymes. *Biochem. Pharmacol.*, **45**, 847–851.

YUKAWA, T., UKENA, D., KROEGEL, C., CHANEZ, P., DENT, G., CHUNG, K.F. & BARNES, P.J. (1990). $Beta_2$-adrenergic receptors on eosinophils. *Am. Rev. Respir. Dis.*, **141**, 1446–1452.

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The inhibition of CYP enzymes in mouse and human liver by pilocarpine

†Tanja Kimonen, †Risto O. Juvonen, *Esko Alhava & [1],†,**Markku Pasanen

†Department of Pharmacology and Toxicology, *Department of Surgery, University of Kuopio, P.O. Box 1627, FIN 70211 Kuopio and **Department of Pharmacology and Toxicology, University of Oulu, Kajaanitie 52 D, FIN 90220 Oulu, Finland

1 Pilocarpine is a cholinomimetic natural alkaloid. Its interactions with testosterone hydroxylations, coumarin 7-hydroxylase (COH), dimethylnitrosamine N-demethylase (DMNA), pentoxyresorufin O-dealkylase (PROD) and 7-ethoxyresorufin O-deethylase (EROD), which are indicative of the activities of cytochrome P4502A5 (CYP2A5) or 6, 2E1, 2B, 1A, were examined in mouse and human liver microsomes.

2 In mouse liver microsomes the IC_{50} values of pilocarpine were 6 μM for COH and testosterone 15α-hydroxylase (T15αOH) activities, 4 μM for PROD, ≈100 μM for DMNA and testosterone 6β-hydroxylase (T6βOH) activities and >1 mM for EROD activity.

3 In human liver microsomes, the IC_{50} value for COH was 6 μM and for DMNA 10 μM; T15αOH and PROD activities were not detectable but T6βOH and testosterone 16β/2β-hydroxylase activities were moderately inhibited (IC_{50} 70 μM).

4 These results suggest that pilocarpine has (i) a high affinity towards phenobarbitone-inducible CYP2A4/5 and CYP2B activities in mouse liver, (ii) a high affinity towards CYP2A6 in human liver microsomes and (iii) a moderate affinity towards CYP3A enzyme(s) in both microsomal preparations.

5 The low IC_{50} concentrations *in vitro* indicate potential metabolic interactions between pilocarpine and several P450 enzymes.

Keywords: Cytochrome P450; coumarin 7-hydroxylase; human liver; mouse liver; microsomal mono-oxygenases; pilocarpine

Introduction

Metabolism is the principle means by which lipophilic compounds are made more water soluble and suitable for excretion. Cytochromes P450 are key enzymes in phase I of the metabolism of many medicinal drugs. However, xenobiotics can also interfere with metabolizing enzymes and, therefore, 'diagnostic' substrates and inhibitors of xenobiotic metabolizing enzymes have been used both *in vivo* and/or *in vitro* in order to characterize the metabolic pathways of a chemical substance (see Guengerich & Shimada 1991; Wrighton *et al.*, 1993; Pasanen & Pelkonen 1994). Some inhibitors, such as methoxsalen (Mäenpää *et al.*, 1993b; 1994) or quinidine (Guengerich *et al.*, 1986; Otton *et al.*, 1988) bind to metabolizing enzymes without forming any metabolic end product.

The P450 superfamily consists of up to 36 families that participate in the metabolism of xenobiotics as well as the synthesis and catabolism of endogenous compounds such as steroids, fatty acids, prostaglandins and vitamins. Those belonging to families CYP1 to 4, are mainly associated with the metabolism and bioactivation of xenobiotics (Nelson *et al.*, 1993), and about 30 different P450 enzymes have been identified and characterized from human tissues (Guengerich 1994).

Pilocarpine is a natural alkaloid which is an agonist at muscarinic cholinoceptors. Pilocarpine eye drops are used throughout the world for the control of elevated intraocular pressure associated with glaucoma. Most of the locally applied drug is absorbed into the systemic circulation through the tear ducts resulting in measurable pilocarpine blood concentrations (Patton & Francoeur, 1978). These systemic levels could potentially interact with xenobiotic metabolizing enzymes. Pilocarpine is largely excreted unchanged by the kidneys but it is also metabolized to pilocarpic acid, isopilocarpic acid and isopilocarpine (Hill & Barcza, 1966; Anderson & Cowle, 1968) *in vivo* by tissue and plasma esterases (Ellis *et al.*, 1972). Here, we demonstrate potent interactions of pilocarpine with human and mouse liver microsomal CYP enzymes *in vitro*.

[1] Author for correspondence at Department of Pharmacology and Toxicology, University of Kuopio.

Methods

Treatment of animals

Female, 7-10 weeks old DBA/2/KUO (D2) mice (weighing 23.4 ± 2.5 g) were purchased from the National Laboratory Animal Centre of the University of Kuopio and were housed in macrolon cages (22 ± 1°C and 50-60% humidity). They were kept on 12 h light/12 h dark cycle (0.7 h 00 min-19 h 00 min/19 h 00 min-7 h 00 min) with food (Ewos, Sweden) and tap water *ad libitum*. To induce selectively certain cytochrome P450s, animals (ten mice in each group two pools per treatment) were injected intraperitoneally with either pyrazole (3 × 200 mg kg^{-1} daily, saline), phenobarbitone (3 × 80 mg kg^{-1} daily, saline), 3-methylcholanthrene (80 mg kg^{-1} in corn oil) or an equal volume of appropriate vehicle. Acetone was given in drinking water (2% v/v) for one week. After the treatment, microsomes were isolated in five volume of 150 mM KCl, 10 mM K_2-EDTA, pH 7.4 and suspended in 100 mM potassium phosphate, 1 mM K_2-EDTA in 20% glycerol at pH 7.4 according to Honkakoski & Lang (1989).

Human liver samples

Liver samples were obtained from patients undergoing surgery in connection with laparotomy under the approval of the ethics committee of the University of Kuopio. Liver

samples were frozen in liquid nitrogen and stored at −80°C for no more than one week before preparation of microsomes. Only tumour-free tissue was used for the preparation of microsomes, and the microsomal fraction was isolated as described above. HL16 was from a 48 year-old female who died of cerebral haemorrhage, with no known medication; HL17 was from 66-year male with no medication and whose liver exhibited normal histology; HL141 was from 59-year male who had an accidental splenic rupture and in laparotomy a liver tumour was found, no medication; HL 212 was from a 66-year old female, with metastatic adenocarcinoma. She had been treated with mianserin (10 mg per day) for several months. HL237 was from 66-year female with cholangiocarcinoma, with no medication. Xenobiotic metabolizing activities were detected in HL17, HL212 and HL237, and the testosterone metabolism was studied in HL141, HL212 and HL237 microsomes. Due to restricted amounts of human liver microsomes a narrower concentration-range was used for the inhibition assays than in mouse liver microsomes.

Biochemical assays

Microsomal protein concentrations were determined by the bicinchonic acid method (Smith *et al.*, 1985). Cytochrome P450 contents were determined according to Omura & Sato (1964); coumarin 7-hydroxylase activity was measured by the method of Aitio (1978) using 2 μM coumarin as a substrate; 7-pentoxyresorufin O-dealkylase (0.1 μM substrate) and 7-ethoxyresorufin O-deethylase (0.1 μM substrate) activities were determined according to Burke *et al.* (1985). Dimethylnitrosamine N-demethylase activity (60 μM and 0.2 mM substrate) was measured according to the method of Nash (1953) modified by Juvonen *et al.* (1985). The method of Waxman *et al.* (1983) was used for testosterine hydroxylase activity determinations with 20 μM ^{14}C-labelled substrate. Testosterone hydroxylations were carried out with both male and female control mouse liver microsomes. COH activity was examined in pyrazole (PYR)-induced, DMNA activity in acetone-induced, PROD activity in phenobarbitone (PB)-induced and EROD activity in 3-methylcholanthrene (MC)-induced female liver microsomes. All enzymatic assays were measured as duplicates and $n = 3$ for each of testosterone hydroxylations and xenobiotic metabolizing activities.

Materials

Coumarin, dimethylnitrosamine, Tween 80, bicinchonic acid, bovine serum albumin, nicotinamide adenine dinucleotide phosphate (reduced), 5-bromo-4-chloro-3-indolyl phosphate, testosterone, were from Sigma (St. Louis, MO, U.S.A.). Pilocarpine was from Leiras Ltd, Finland; resorufin was obtained from Aldrich-Europe (Steinhem, Germany); 7-ethoxy- and 7-pentoxyresorufin were purchased from Pierce (Rockford, OR, U.S.A.). [4-^{14}C]-testosterone and Hyperfilm MP autoradiography films were obtained from Amersham (Amersham, U.K.). All other chemicals were of at least analytical grade.

Results

The mouse and human liver microsomal testosterone hydroxylations and xenobiotic metabolizing mono-oxygenases are shown in Tables 1 and 2. In control mouse liver, testosterone 15α-hydroxylase (T15αOH), testosterone 7α-hydroxylase (T7αHO) and testosterone 6α-hydroxylase (T6αOH) activities were higher in females than males. Testosteron 6β-hydroxylase (T6βOH) and testosterone 16β/2β-hydroxylase (T16β/2βOH) activities were the only ones that could be determined reliably in the human microsomal samples. These results agree with those of (Mäenpää *et al.*, 1991) in that human liver microsomes do not exhibit significant T15αOH activity.

Changes of testosterone hydroxylations, as percentages of control in mouse and human liver microsomes are summarised in Figure 1. T15αOH activity, an activity marker of CYP2A4 in mouse liver, was the most sensitive to inhibition by pilocarpine in samples from either sex. The IC_{50} was 4 μM in female mouse and 20 μM in male microsomes. T6βOH activity was moderately inhibited ($IC_{50} \approx 100$ μM) by pilocarpine in the mouse microsomal preparations of either sex. In

Table 1 Testosterone hydroxylation activities in female and male mouse and human liver microsomes

	Testosterone hydroxylations (nmol mg^{-1} microsomal protein min^{-1})					
Microsomes	*15α*	*16α*	*7α*	*6β*	*6α*	*16β/2β*
Mouse						
female	0.62 ± 0.14	0.88 ± 0.26	1.16 ± 0.16	0.54 ± 0.07	0.30 ± 0.07	0.12 ± 0.02
male	0.32 ± 0.10	0.98 ± 0.38	0.85 ± 0.18	0.43 ± 0.02	0.17 ± 0.04	0.11 ± 0.01
Human	ND	ND	ND	0.48 ± 0.26	ND	0.06 ± 0.02

The activities were detected at 20 μM testosterone concentration in female and male DBA/2N mouse liver microsomes and human liver microsomes. 15α, 16α *etc* indicate the hydroxylation product of testosterone; ($n = 3$, ± s.d.). ND, no detectable.

Table 2 Xenobiotic metabolizing mono-oxygenases in female mouse and human liver microsomes

	Xenobiotic metabolizing mono-oxygenases (nmol mg^{-1} microsomal protein min^{-1})			
Microsomes	*COH*	*PROD*	*DMNA*	*EROD*
Mouse				
control†	0.250–0.500	0.02–0.06	0.8–1.2	0.03–0.06
induced	2.93 ± 0.27	2.6 ± 0.9	2.2 ± 0.41	1.2 ± 0.2
Human	0.72 ± 0.13	ND	0.41 (n = 1) 0.51 ± 0.352*	1.1 ± 0.17

Abbreviations: Coumarin 7-hydroxylase (COH), pentoxyresorufin O-dealkylase (PROD), 7-ethoxyresorufin O-deethylase (EROD) and dimethylnitrosamine *N*-demethylase (DMNA) activities ($n = 3$, ± s.d.), see text under Methods for the treatments of induced mouse live microsomes.
COH activity was determined at 2 μM, PROD and EROD at 0.1 μM and DMNA at 0.2 mM substrate concentration in mouse liver and at 0.2 mM and 60 mM* in human liver microsomes.
†Ranges for control values are taken from our earlier experiments; ND, not detectable.

human liver microsomes the IC_{50} for T6βOH was 70 μM. Other testosterone hydroxylases (T16β/2βOH or 16αOH activities), markers of CYP2B and 2D subfamilies, were only marginally affected in mouse liver microsomes. However, in human liver microsomes T16β/2βOH activity was moderately inhibited (IC_{50}, 70 μM).

The activities and the enzymes catalysing xenobiotic biotransformations are given in Table 2. COH (an activity marker of mouse CYP2A5 and human CYP2A6) and PROD (CYP2B) activities in mouse liver microsomes were the most sensitive to the inhibition by pilocarpine (Figure 2a,b.) PROD activity was below the limit of detection in all human liver microsome preparations. The IC_{50} for COH activity was 6 μM both in human and mouse liver microsomes and 4 μM for mouse liver PROD activity indicating a high affinity of pilocarpine for these enzymes. Pilocarpine effectively inhibited human liver DMNA activity (IC_{50} 10 μM, 60 mM substrate), traditionally classified as a marker for CYP2E1 activity, but only moderately ($IC_{50} \approx 100$ μM) inhibited the orthologous enzyme in mouse liver microsomes. DMNA activity was detectable only in one human liver preparation at 0.2 mM substrate concentration (Table 2). EROD, an activity marker of CYP1A enzymes, was not affected by pilocarpine in any of the samples.

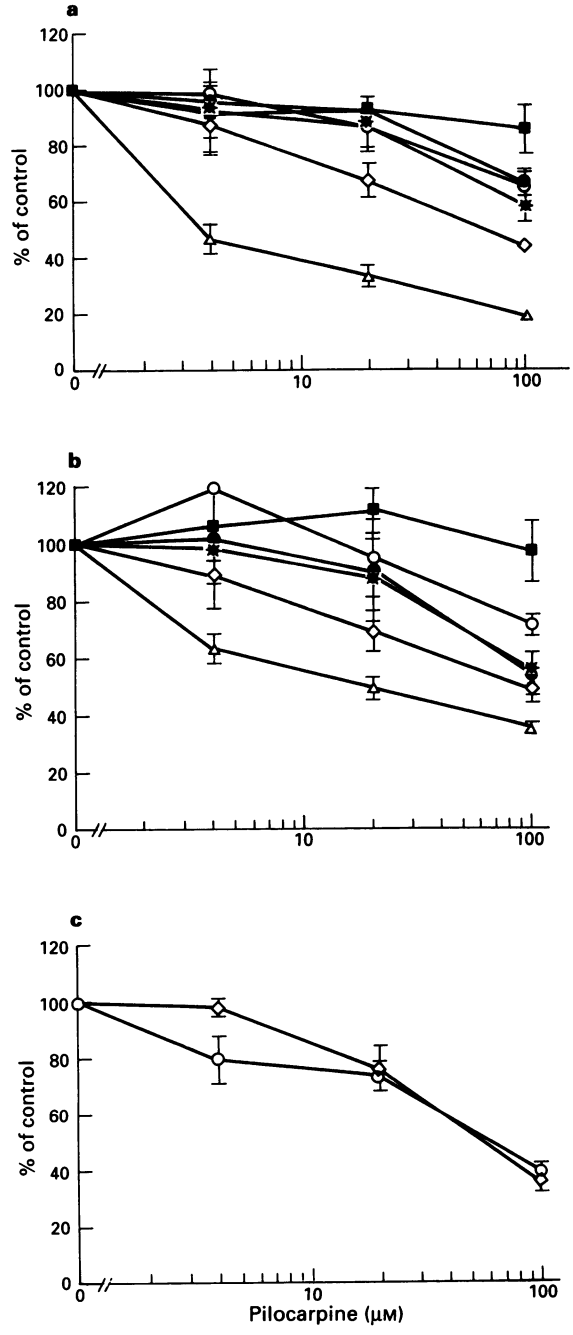


Figure 1 The inhibition of testosterone hydroxylation activities by pilocarpine in female (a) and male (b) mouse liver and (c) human liver microsomes. Symbols: (○) 16β, (◇) 6β, (●) 6α, (*) 7α, (■) 16α, (△) 15α. Results are expressed as a percentage of control mean ± s.d., $n = 3$.

Discussion

This study described potential interactions of a cholinomimetic drug, pilocarpine, with human and mouse mono-oxygenases. The enzymes most sensitive to inhibition by pilocarpine were activities of CYP2A and CYP2B subfamilies and moderate inhibition was observed for CYP3A enzyme(s).

In the present study, pilocarpine was equally potent in inhibiting CYP2A4 and CYP2A5 activities. This is not surprising because in mouse the CYP2A5 catalysing COH

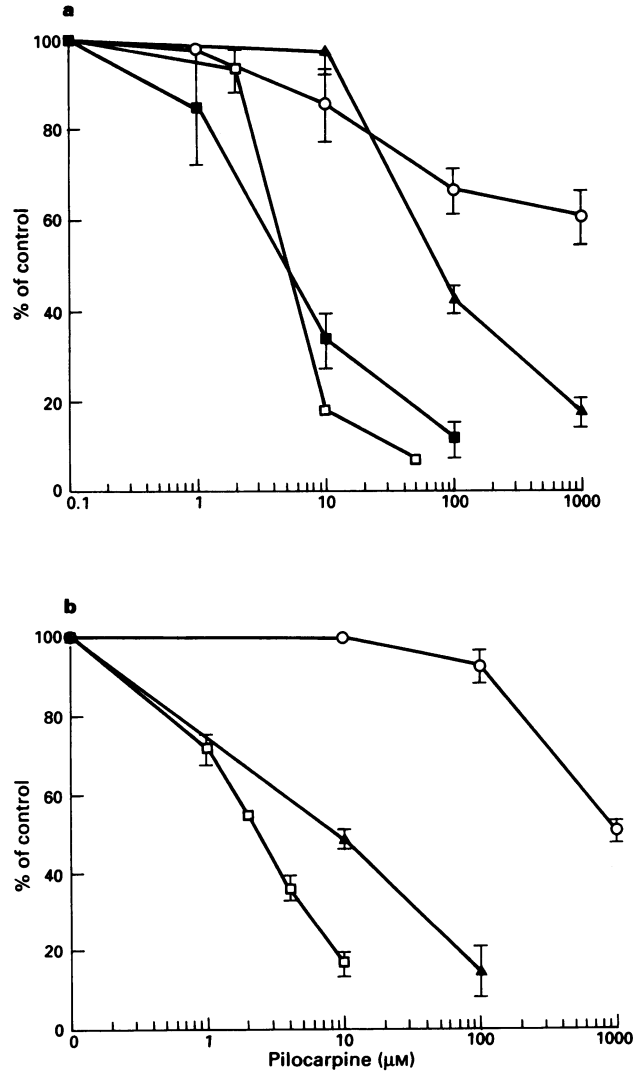


Figure 2 The inhibition of coumarin 7-hydroxylase (COH, □), dimethylnitrosamine N-demethylase (DMNA, ▲), pentoxyresorufin O-dealkylase (PROD, ■) and 7-ethoxyresorufin O-deethylase (EROD, ○) activities by pilocarpine in female mouse (a) and human (b) liver microsomes. The COH, DMNA, PROD and EROD activities are for animals treated with pyrazole, acetone, phenobarbitone and 3-methylacholanthrene, respectively. Results are expressed as percentage of control mean ± s.d, $n = 3$.

activity has a 98.3% homology with the 2A4 enzyme which is mainly responsible for T15αOH activity. The two enzymes differ from each other in 11 amino acid residues (Lindberg *et al.*, 1989; Negishi *et al.*, 1989) and, therefore, polyclonal antibodies against CYP2A4 or 5 (Negishi *et al.*, 1989) detect both of them. Interestingly, T7αOH, which is an activity marker of the third member of CYP2A activities (Yamano *et al.*, 1990; Iwasaki *et al.*, 1993), and which exhibited the highest specific activity of all the testosterone hydroxylations in mouse liver microsomes, was not inhibited in mouse liver microsomes by pilocarpine. This indicates remarkable differencies in the substrate binding pocket of CYP2A enzymes CYP2A4/5 *versus* T7αOH.

Coumarin 7-hydroxylase activity was inhibited to a similar extent by pilocarpine in mouse and human liver microsomes. This can be explained by the considerable homology (83%) of the enzymes, CYP2A5 in mouse and CYP2A6 in man, catalysing the COH activity (Raunio *et al.*, 1988). However, there exist certain species differencies between mouse 2A5 and human 2A6, since inhibitor studies demonstrate that linear furanocoumarins and metyrapone are potent inhibitors of mouse enzyme but have no effect on the human enzyme (Mäenpää *et al.*, 1993b). Our present results demonstrate that drug-drug interactions with COH activity may occur but because of the large inter-individual variations of COH activity in the human population (Pelkonen *et al.*, 1985; Miles *et al.*, 1990; Yamano *et al.*, 1990; Camus *et al.*, 1993) potential clinical impact of such interactions is hard to predict.

CYP2B catalyses PROD activity and is sensitive to pilocarpine inhibition at micromolar concentrations. Wahlström (1979) reported supersensitivity of rats to hexobarbitone if given in conjunction with pilocarpine and suggested that cholinergic mechanisms may mediate hexobarbitone recovery in rat. Our present data suggest that, at least partially, supersensitivity reactions to the higher hexobarbitone doses could be due to an interaction with the enzymes which terminate the action of this barbiturate. Tsujimoto *et al.* (1972) reported that pilocarpine inhibited the metabolism of hexobaritone nicotine and aniline in rabbit microsomes. Our results demonstrate that the COH and PROD activities are even more sensitive to inhibition by pilocarpine.

An earlier study showed that pilocarpine does not affect the aromatisation of testosterone (Verhoeven, 1979). However, in this study we have shown that pilocarpine effectively inhibited *in vitro* hydroxylation of testosterone to positions 15α and 6β. Pilocarpine moderately inhibited T6βOH activity which is an activity marker of the CYP3A subfamily in human or rodent liver (Bornheim & Correia, 1990; Yun *et al.*, 1992). The CYP3A subfamily proteins are quantitatively the most prominent CYP fraction in human liver. This family consists of several members and they are expressed at relatively high, although individually variable, levels in man (see Murray, 1992; Wrighton & Stevens, 1992; Kinirons *et al.*, 1993). They are also expressed during the foetal period (Kitada *et al.*, 1987; Mäenpää *et al.*, 1993a; Schuetz *et al.*, 1993). They are known to be regulated by sex hormones and some CYP3A enzymes are also inducible by phenobarbitone, glucocorticoids and macrolide antibiotics (see Murray, 1992; Wrighton & Stevens, 1992). The CYP3A enzymes appear to metabolize and activate a large number of drugs including lovastatin, nifedipine, cyclosporin, diazepam, ethinyloestradiol, imidazole antifungal agents and chemical carcinogens like aflatoxin B_1 (Murray, 1992; Wrighton & Stevens, 1992; Yun *et al.*, 1992). Therefore, the potential for an interaction between several important drugs and pilocarpine exists.

In the present study we examined the DMNA activity determinations using acetone-induced mouse liver microsomes when the specific content of CYP2E1 is maximally increased (see Gonzalez, 1989). The DMNA activity was moderately inhibited by pilocarpine in mouse and effectively inhibited in human liver microsomes. In human liver microsomes the DMNA inhibition at a high substrate concentration was at the same level as that of COH activity. At 0.2 mM substrate concentrations DMNA activity could be detected only in one human liver microsomal preparation. There is strong evidence that enzymes of the CYP2A family participate in nitrosamine metabolism especially at high substrate concentrations in mouse and man (Crespi *et al.*, 1990; Camus *et al.*, 1993). If this is the case, then the inhibition with DMNA activity by pilocarpine represents an interaction with CYP2A5 in mouse.

EROD activity was only marginally inhibited by pilocarpine (1 mM). Therefore, a potential interaction with pilocarpine with CYP1A substrates and inducers is unlikely.

The potent inhibition of phenobaritone-inducible CYP2A activities (COH and T15αOH activities) and CYP2B (PROD activity) indicates that pilocarpine is bound to these enzymes with a high affinity. Pilocarpine is largely excreted as such by the kidneys. It is also metabolized to pilocarpic acid, isopilocarpic acid and isopilocarpine (Hill & Barcza, 1966; Anderson & Cowle, 1968) *in vivo* by tissue and plasma esterases (Ellis *et al.*, 1972). However, the present results demonstrate that pilocarpine as with other potent inhibitors such as methoxsalen (Mäenpää *et al.*, 1993b) or quinidine (Guengerich *et al.*, 1986; Otton *et al.*, 1988) can interfere with several CYP activities that are not involved in its own metabolism.

In summary, these results *in vitro* suggest that pilocarpine reacts specifically with CYP2A activities; with COH which is an activity marker for CYP2A5 in mouse and CYP2A6 in man and T15αOH for CYP2A4 in mouse liver (which raises the potential for interaction in local steroid metabolism) but not with the third member CYP7αOH. CYP2B activity, PROD, was also inhibited to a similar extent. Interestingly, the activities most sensitive to inhibition by pilocarpine were phenobarbitone-inducible. Coumarin and pilocarpine both contain a lactone structure, and such substrate structure analogies may be used in characterizing the active centre of CYP2A proteins. Previous reports of unexplained adverse drug-drug or drug-P450 reactions may be due to such interactions.

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References

AITIO, A. (1978). A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal. Biochem.*, **85**, 488–491.

ANDERSON, R.A. & COWLE, J.B. (1968). Influence of pH on the effect of pilocarpine on aqueous dynamics. *Br. J. Ophthalmol.*, **52**, 607–611.

BORNHEIM, L.M. & CORREIA, M.A. (1990). Selective inactivation of mouse liver cytochrome P450IIIA by cannabidiol. *Mol. Pharmacol.*, **38**, 319–326.

BURKE, M.D., THOMPSON, S., ELCOMBE, C.R., HALPERT, J., HAAPARANTA, T. & MAYER, R.T. (1985). Ethoxy- pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.*, **34**, 3337–3345.

CAMUS, A., GENESTE, O., HONKAKOSKI, P., BEREZIAT, J., HENDERSON, C.J., WOLF, C.R., BARTSCH, H. & LANG, M.A. (1993). High variability of nitrosamine metabolism among individuals: role of cytochromes P450 2A6 and 2E1 in the dealkylation of N-nitrosodiethylamine in mice and humans. *Mol. Carcinogenesis*, **7**, 268–275.

CRESPI, C.L., PEMAN, B.W., LEAKEY, J.A.E., ARLOTTO, M.P., STARK, A., PARKINSON, A., TURNER, T., STEIMEL, D.T., RUDO, K., DAVIES, R.L. & LANGENBACH, R. (1990). Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis*, **11**, 1293–1300.

ELLIS, P.P., LITTLEJOHN, K. & DEITRICH, R.A. (1972). Enzymatic hydrolysis of pilocarpine. *Investig. Ophthalmol.*, **11**, 747–751.

GONZALEZ, F.J. (1989). The molecular biology of cytochrome P450s. *Pharmacol. Rev.*, **40**, 243–288.

GUENGERICH, F.P. (1994). Catalytic selectivity of human cytochrome P450 enzyme -Relevance to drug metabolism and toxicity. *Toxicol. Letters.*, **70**, 133–138.

GUENGERICH, F.P., MÜLLER-ENOCH, D. & BLAIR, I.A. (1986). Oxidation of quinidine by human liver cytochrome P-450. *Mol. Pharmacol.*, **30**, 287–295.

GUENGERICH, F. & SHIMADA, T. (1991). Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem. Res. Toxicol.*, **4**, 391–407.

HILL, R.K. & BARCZA, S. (1966). Stereochemistry of the jaborandi alkaloids. *Tetrahedron*, **22**, 2889–2893.

HONKAKOSKI, P. & LANG, M.A. (1989). Mouse liver phenobarbital-inducible P450 system: purification, characterization and differential inducibility of four cytochrome P450 isozymes from D2 mouse. *Arch. Biochem. Biophys.*, **273**, 42–57.

IWASAKI, M., LINDBERG, R.L.P., JUVONEN, R.O. & MEGISHI, M. (1993). Site directed mutagenesis of mouse steroid 7α-hydroxylase (cytochrome P-450): Role of residue-209 in determining steroid-cytochrome P-450 interaction. *Biochem. J.*, **291**, 569–573.

JUVONEN, R.O., KAIPAINEN, P.K. & LANG, M.A. (1985). Selective induction of coumarin 7-hydroxylase by pyrazole in D2 mice. *Eur. J. Biochem.*, **152**, 3–8.

KINIRONS, M.T., O'SHEA, D., DOWNING, T.E., FITZWILLIAM, A.T., JOELLENBECK, L., GROOPMAN, J.D., WILKINSON, G.R. & WOOD, A.J.J. (1993). Absence of correlations among three putative *in vivo* probes of human cytochrome P4503A activity in young healthy men. *Clin. Pharmacol. Ther.*, **54**, 621–629.

KITADA, M., KAMATAKI, T., ITAHASHI, K., RIKIHISA, T. & KANAKUBO, Y. (1987). Significance of cytochrome P-450 (P-450 HFLa) of human fetal livers in the steroid and drug oxidation. *Biochem. Pharmacol.*, **36**, 453–456.

LINDBERG, R., BURKHART, B., ICHIKAWA, T. & NEGISHI, M. (1989). The structure and characterization of type I P-450$_{15\alpha}$ gene as a major steroid 15α-hydroxylase and its comparison with type II P-450$_{15\alpha}$ gene. *J. Biol. Chem.*, **264**, 6465–6471.

MILES, J.S., MCLAREN, A.W., FORRESTER, L.M., GLANCEY, M.J. LANG, M.A. & WOLF, C.R. (1990). Identification of human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochem. J.*, **267**, 365–371.

MURRAY, M. (1992). P450-enzymes -inhibition mechanisms, genetic regulation and effects of liver disease. *Clin. Pharmacokin.*, **23**, 132–146.

MÄENPÄÄ, J., JUVONEN, R., RAUNIO, H., RAUTIO, A. & PELKONEN, O. (1994). Methoxsalen and coumarin metabolism interactions in man and mouse. *Biochem. Pharmacol.*, **48**, 1363–1369.

MÄENPÄÄ, J., PELKONEN, O., CRESTEIL, T. & RANE, A. (1993a). The role of cytochrome P450 3A (CYP3A) isoform(s) in oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *J. Steroid Biochem. Mol. Biol.*, **44**, 61–67.

MÄENPÄÄ, J., SIGUSCH, H., RAUNIO, H., SYNGELMÄ, T., VUORELA, P., VUORELA, H. & PELKONEN, O. (1993b). Differential inhibition of coumarin 7-hydroxylase activity in mouse and human liver microsomes. *Biochem. Pharmacol.*, **45**, 1035–1042.

MÄENPÄÄ, J., SYNGELMÄ, T., HONKAKOSKI, P., LANG, M.A. & PELKONEN, O. (1991). Comparative studies on coumarin and testosterone metabolism in mouse and human livers. Differential inhibition by the anti-P450Coh antibody and metyrapone. *Biochem. Pharmacol.*, **42**, 1229–1235.

NASH. T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.*, **55**, 416–421.

NEGISHI, M., LINDBERG, R., BURKHART, B., ICHIKAWA, T., HONKAKOSKI, P. & LANG, M.A. (1989). Mouse steroid 15α-hydroxylase gene family: Identification of type II P-45015α as coumarin 7-hydroxylase. *Biochemistry*, **28**, 4169–4172.

NELSON, D.R., KAMATAKI, T., WAXMAN, D.J., GUENGERICH, F.P., W, E.R., FEYEREISEN, R., GONZALEZ, F.J., COON, M.J., GUNSALUS, I.C., GOTOH, O., OKUDA, K. & NEBERT, D.W. (1993). The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomeclature. *DNA Cell Biol.*, **12**, 1–51.

OMURA, T. & SATO, R. (1964). The carbon monooxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.*, **239**, 2370–2378.

OTTON, S.V., BRINN, R.U. & GRAM, L.F. (1988). *In vitro* evidence against the oxidation of quinidine by the sparteine/deprisoquine monooxygenase of human liver. *Drug. Metab. Dispos.*, **16**, 15–17.

PASANEN, M. & PELKONEN, O. (1994). The expression and environmental regulation of P450 enzymes in human placenta. *Crit. Rev. Toxicol.*, **24**, 211–229.

PATTON, T.F. & FRANCOEUR, M. (1978). Ocular bioavailability and systemic loss of topically applied ophthalmic drugs. *Am. J. Ophthalmol.*, **85**, 225–229.

PELKONEN, O., SOTANIEMI, E.A. & AHOKAS, J.T. (1985). Coumarin 7-hydroxylase activity in human liver microsomes. Properties of the enzyme and interspecies comparisons. *Br. J. Clin. Pharmacol.*, **19**, 59–66.

RAUNIO, H., SYNGELMÄ, T., PASANEN, M., JUVONEN, R., HONKAKOSKI, P., KAIRALUOMA, M., SOTANIEMI, E.A., LANG, M.A. & PELKONEN, O. (1988). Immunochemical and catalytical studies on hepatic coumarin 7-alphahydroxylase in man, rat, and mouse. *Biochem. Pharmacol.*, **37**, 3889–3895.

SCHUETZ, J.D., KAUMA, S. & GUZELIAN, P.S. (1993). Identification of the fetal liver cytochrome-CYP3A7 in human endometrium and placenta. *J. Clin. Invest.*, **92**, 1018–1024.

SMITH, P.K., KROHN, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. (1985). Measurement of protein using bicinchronic acid. *Anal. Biochem.*, **150**, 76–85.

TSUJIMOTO, A., DOHI, T. & KOJIMA, S. (1972). Potent inhibitory action of pilocarpine on hepatic drug metabolism. *Jpn. J. Pharmacol.*, **22**, 736–739.

VERHOEVEN, G. (1979). Stimulation effect of neurotransmitters on the aromatization of testosterone by Sertoli cell-enriched cultures. *Mol. Cell. Endocrinol.*, **13**, 241–253.

WAHLSTRÖM, G. (1979). Some aspects of the changes induced by chronic barbital treatments in the male rat. *Drug Alcohol Depend.*, **4**, 221–233.

WAXMAN, D.J., KO, A. & WALSH, C. (1983). Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J. Biol. Chem.*, **258**, 11937–11947.

WRIGHTON, S.A. & STEVENS, J.C. (1992). The human hepatic cytochromes P450 involved in drug metabolism. *Crit. Rev. Toxicol.*, **22**, 1–22.

WRIGHTON, S.A. VANDENBRANDEN, M., STEVENS, J.C., SHIPLEY, L.A., RING, B.J., RETTIE, A.E. & CASHMAN, J.R. (1993). *In vitro* methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metab. Rev.*, **25**, 453–484.

YAMANO, S., TATSUNO, J. & GONZALEZ, F.J. (1990). The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry*, **29**, 1322–1329.

YUN, C.H.Y., SHIMADA, T. & GUENGERICH, P.F. (1992). Roles of human liver cytochrome P4502C and 3A enzymes in the 3-hydroxylation of benzo(a)pyrene. *Cancer Res.*, **52**, 1868–1874.

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The effects of phenelzine and other monoamine oxidase inhibitor antidepressants on brain and liver I_2 imidazoline-preferring receptors

Regina Alemany, Gabriel Olmos & [1]Jesús A. García-Sevilla

Laboratory of Neuropharmacology, Department of Fundamental Biology and Health Sciences, University of the Balearic Islands, E-07071 Palma de Mallorca, Spain

1 The binding of [^{3}H]-idazoxan in the presence of 10^{-6} M (−)-adrenaline was used to quantitate I_2 imidazoline-preferring receptors in the rat brain and liver after chronic treatment with various irreversible and reversible monoamine oxidase (MAO) inhibitors.

2 Chronic treatment (7–14 days) with the irreversible MAO inhibitors, phenelzine (1–20 mg kg^{-1}, i.p.), isocarboxazid (10 mg kg^{-1}, i.p.), clorgyline (3 mg kg^{-1}, i.p.) and tranylcypromine (10 mg kg^{-1}, i.p.) markedly decreased (21–71%) the density of I_2 imidazoline-preferring receptors in the rat brain and liver. In contrast, chronic treatment (7 days) with the reversible MAO-A inhibitors, moclobemide (1 and 10 mg kg^{-1}, i.p.) or chlordimeform (10 mg kg^{-1}, i.p.) or with the reversible MAO-B inhibitor Ro 16-6491 (1 and 10 mg kg^{-1}, i.p.) did not alter the density of I_2 imidazoline-preferring receptors in the rat brain and liver; except for the higher dose of Ro 16-6491 which only decreased the density of these putative receptors in the liver (38%).

3 *In vitro*, phenelzine, clorgyline, 3-phenylpropargylamine, tranylcypromine and chlordimeform displaced the binding of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors from two distinct binding sites. Phenelzine, 3-phenylpropargylamine and tranylcypromine displayed moderate affinity (K_{iH} = 0.3–6 μM) for brain and liver I_2 imidazoline-preferring receptors; whereas chlordimeform displayed high affinity (K_{iH} = 6 nM) for these receptors in the two tissues studied, Clorgyline displayed very high affinity for rat brain (K_{iH} = 40 pM) but not for rat liver I_2 imidazoline-preferring receptors (K_{iH} = 169 nM).

4 Preincubation of cortical or liver membranes with phenelzine (10^{-4} M for 30 min) did not alter the total density of I_2 imidazoline-preferring receptors, indicating that this irreversible MAO inhibitor does not irreversibly bind to I_2 imidazoline-preferring receptors. In contrast, preincubation with 10^{-6} M clorgyline reduced by 40% the B_{max} of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors.

5 Chronic treatment (7 days) with the inducers of cytochrome P-450 enzymes phenobarbitone (40 or 80 mg kg^{-1}, i.p.), 3-methylcholanthrene (20 mg kg^{-1}, i.p.) or 2-methylimidazole (40 mg kg^{-1}, i.p.) did not alter the binding parameters of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors. The compound SKF 525A, a potent inhibitor of cytochrome P-450 enzymes which forms a tight but reversible complex with the haemoprotein, completely displaced with moderate affinity (K_{iH} = 2–10 μM) the specific binding of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors. Preincubation of total liver homogenates with 3×10^{-4} M phenelzine in the presence of 10^{-3} M NADH, a treatment that irreversibly inactivates the haeme group of cytochrome P-450, did not reduce the density of liver I_2 imidazoline-preferring receptors. These results discounted a possible interaction of [^{3}H]-idazoxan with the haeme group of cytochrome P-450 enzymes.

6 Together the results indicate that the down-regulation of I_2 imidazoline-preferring receptors is associated with an irreversible inactivation of MAO (at least in the brain) that is not related either to the affinity of the MAO inhibitors for I_2 imidazoline-preferring receptors or to an irreversible binding to these putative receptors. These findings indicate a novel effect of irreversible MAO inhibitors in the brain and suggest a new target for these compounds that could be of relevance in the treatment of depression, a disease in which an increased density of brain I_2 imidazoline-preferring receptors has been reported.

Keywords: Imidazoline-preferring receptors; phenelzine; clorgyline; tranylcypromine; moclobemide; monoamine oxidase (MAO); [^{3}H]-idazoxan

Introduction

Several tissues and cell lines express specific and functional binding sites for imidazoli(di)ne/guanidine compounds which have been termed imidazoline-preferring receptors (Michel & Ernsberger, 1992). The structure of a possible endogenous ligand for these receptors (a component of the so-called clonidine-displacing substance; CDS) has been recently determined and identified as agmatine (Li *et al.*, 1994); this substance is bioactive, promoting release of catecholamines from adrenal chromaffin cells and its biosynthetic enzyme, arginine decarboxylase is expressed in the rat brain (Li *et al.*, 1994). The imidazoline-preferring receptors appear to comprise at least two subtypes, recently designated as I_1 and I_2 (Ernsberger, 1992), which differ in their pharmacological profiles and also in their tissue and subcellular distributions (for a review see Michel & Insel, 1989; Atlas, 1991; Hieble & Ruffolo, 1992; Kilpatrick *et al.*, 1992).

The I_1 imidazoline-preferring receptors have been found to

[1] Author for correspondence at: Lab Neurofarmacolgia, Dept. Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Cra, Valldemossa Km 7.5, E-07071 Palma de Mallorca, Spain.

be increased in platelet membranes from depressed patients and down-regulated after desipramine treatment (Piletz *et al.*, 1990; 1991). The I_2 imidazoline-preferring receptors might also be increased in the frontal cortex of depressed suicide victims (Meana *et al.*, 1993) and recently, down-regulation of these putative receptors after chronic treatment with the irreversible monoamine oxidase (MAO) inhibitors clorgyline and pargyline has been reported in the rat brain (Olmos *et al.*, 1993). The I_2 imidazoline-preferring receptors and the enzyme MAO show some similarities: (1) they are located on the mitochondrial outer membrane (Tesson & Parini, 1991; Tesson *et al.*, 1991); (2) in the rat and human brain the regional distribution of I_2 imidazoline-preferring receptors correlates well with that of the B but not A isoform of the MAO (Sastre & García-Sevilla, 1993); and (3) the density of I_2 imidazoline-preferring receptors and MAO-B increases with age in the human brain (Sastre & García-Sevilla, 1993). These findings suggest some relationship between these two proteins; however, a direct interaction of imidazoline ligands with the active centre of MAO-A and B isoenzymes has been previously discounted (Olmos *et al.*, 1993; Sastre & García-Sevilla, 1993).

The MAO inhibitors consist of a structurally diverse group of compounds whose antidepressant properties have been attributed to their common ability to increase synaptic availability of certain monoamine neurotransmitters. However, maximal inhibition of MAO activity usually precedes antidepressant effects, suggesting that the therapeutic effects of these compounds may be mediated by more than one target (for a review see Baker *et al.*, 1992). Thus, interactions of MAO inhibitors with cytochrome P-450 enzymes (Muakkassah & Yang, 1981; Bélanger & Atitsé-Gbeasson, 1982; Dupont *et al.*, 1987) have been described. The synthesis of different cytochrome P-450 forms can be induced by a variety of compounds in the mitochondria and endoplasmic reticulum of the liver and other tissues (Murrey & Reidy, 1990). Therefore, if the I_2 imidazoline-preferring receptors were a form of cytochrome P-450 enzymes, their density in the liver and brain might be modulated by chronic treatment with one of these inducing agents.

The present study was thus designed (1) to assess further and extend the effects of chronic treatments with irreversible and reversible MAO inhibitors on rat brain and liver I_2 imidazoline-preferring receptors; (2) to investigate *in vitro* the pharmacological properties of the interaction of these drugs with the imidazoline-preferring receptors and (3) to seek for a possible link between I_2 imidazoline-preferring receptors and cytochrome P-450 enzymes.

Methods

Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2°C with a 12 h light/dark cycle. In one series of experiments and for the chronic treatments the animals received i.p., every 12 h either 0.9% saline vehicle, the irreversible MAO inhibitors, phenelzine (0.3–20 mg kg^{-1} for 7 days), isocarboxazid (10 mg kg^{-1} for 7 days), clorgyline (3 mg kg^{-1} for 14 days) or tranylcypromine (10 mg kg^{-1} for 7 days). The time-course for the effect of phenelzine (3 mg kg^{-1}) was studied on days 1 (acute), 7, 14 and 21 after treatment. Chronic treatment with these MAO inhibitors has been shown to result in a profound and sustained inhibition of MAO-A/B (80–90%) in the rat brain and liver (Mousseau *et al.*, 1993).

In another series of experiments rats were treated i.p., every 12 h for 7 days with the reversible MAO-A inhibitors, moclobemide (1 and 10 mg kg^{-1}) or chlordimeform (10 mg kg^{-1}) or with the reversible MAO-B inhibitor, Ro 16-6491 (1 and 10 mg kg^{-1}). Rats were also treated chronically with the cytochrome P-450 inducing agents phenobarbitone (40 or 80 mg kg^{-1}, i.p., every 24 h for 7 days), 3-methylcholanthrene (20 mg kg^{-1}, i.p., every 12 h for 7 days) or 2-methylimidazole (40 mg kg^{-1}, i.p. every 24 h for 7 days). Both in the acute and chronic treatments the rats were killed 24 h after the last injection.

Rat cortical and liver membrane preparation

The animals were decapitated and the parieto-occipital cortex and portions of liver were rapidly removed into ice-cold Tris-sucrose buffer (5 mM Tris-HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4) and frozen at − 80°C until required. Cortical and liver membranes (P_2 fractions) were prepared by established methods with modifications (Giralt & García-Sevilla, 1989). Briefly, the tissue samples were homogenized in 5 ml of ice-cold Tris-sucrose buffer containing 2 mM of the protease inhibitor, phenylmethylsulphonyl fluoride (PMSF). The homogenates were centrifuged at 1,100 *g* for 10 min, and the supernatants were then recentrifuged at 40,000 *g* for 10 min. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5). The final pellet was resuspended in an appropriate volume of this buffer to a final protein content of 800–1000 μg ml^{-1} for the cortical membranes and 900–1600 μg ml^{-1} for the liver membranes. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

[^{3}H]-idazoxan binding assay

Total [^{3}H]-idazoxan binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the cortical or liver membranes which were incubated with shaking for 30 min at 25°C. Binding of [^{3}H]-idazoxan to brain I_2 imidazoline-preferring receptors was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors (Olmos *et al.*, 1992; Miralles *et al.*, 1993a). In the liver it was found that (−)-adrenaline (10^{-10} to 10^{-3} M) did not compete against the binding of [^{3}H]-idazoxan (10^{-8} M) (only 10% of inhibition at 10^{-4} M) suggesting that the radioligand does not label α_2-adrenoceptors in this tissue, as previously described (Zonnenschein *et al.*, 1990). Because of this, total binding was defined in the liver membranes in the absence of (−)-adrenaline. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, as previously described (Olmos *et al.*, 1992). In the saturation studies, cortical and liver membranes were incubated with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 4×10^{-8} M) as above. The specific binding was defined as the difference between total binding (liver membranes) or total non-adrenergic binding (cortical membranes) and nonspecific binding and was plotted as a function of increasing concentrations of the radioligand. Specific binding represented 70% to 40% of total binding in cortical membranes and 90% to 80% in liver membranes. In the drug competition studies, membranes were incubated as above with [^{3}H]-idazoxan (10^{-8} M) and in the absence or presence of various concentrations of the competing drugs (10^{-14} M or 10^{-12} M to 10^{-3} M; 15–23 concentrations). Total binding was determined as above and plotted as a function of the drug concentration.

In some experiments, the effect of *in vitro* preincubation with phenelzine, clorgyline or chlordimeform on [^{3}H]-idazoxan binding to brain and liver I_2 imidazoline-preferring receptors was assessed as described previously (Olmos *et al.*, 1993). The pellet resulting from the last centrifugation (see preparation of membranes) was resuspended in 10 ml of fresh incubation buffer and incubated for 30 min at 25°C in the presence of phenelzine (10^{-4} M), clorgyline (10^{-6} M) or chlordimeform (10^{-6} M). Then, the membranes were washed twice with 10 ml of fresh incubation buffer and the final pellet was

resuspended as above and used in saturation or competition binding experiments.

Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C). Membrane-bound [^{3}H]-idazoxan was measured by vacuum filtration through Whatman GF/C glass fibre filters which had been presoaked with 0.5% polyethylenimine (Bruns *et al.*, 1983), using a Brandel 48R cell harvester (Biomedical Research & Development Laboratories, U.S.A.). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 57% efficiency (Packard model 1900 TR).

[^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding assays

In some experiments, the effect of *in vitro* preincubation with phenelzine (10^{-4} M) as above was assessed on the binding of [^{3}H]-Ro 41-1049 to liver MAO-A and that of [^{3}H]-Ro 19-6327 to liver MAO-B. Binding assays of these radioligands were performed as described previously (Sastre & García-Sevilla, 1993).

Analyses of binding data and statistics

Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed by computer-assisted nonlinear regression using the EBDA-LIGAND (Munson & Rodbard, 1980; McPherson, 1985) programmes. All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically using the extra sum of squares principle (F test) as outlined by Munson & Rodbard (1980). The more complex model was accepted if the P value resulting from the F test was less than 0.05.

Results are expressed as mean ± s.e.mean. One-way analysis of variance (ANOVA), followed by Scheffé's test, was used for the statistical evaluations. The level of significance was $P = 0.05$.

Drugs

[^{3}H]-idazoxan (specific activity, 41–46 Ci mmol^{-1}; batches 42 to 47) was purchased from Amersham International plc (U.K.). [^{3}H]-Ro 41-1049 [*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide HCl] (specific activity, 30.8 Ci mmol^{-1}) and [^{3}H]-Ro 19-6327 [*N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide HCl] (specific activity, 20.2 Ci mmol^{-1}) were generous gifts from Dr J. Richards and Dr J. Saura (F. Hoffmann-La Roche Ltd., Switzerland). For the binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. Other drugs (and their sources) included: (−)-adrenaline bitartrate (Sigma Chemical Co., U.S.A.); chlordimeform HCl (Ciba-Geigy, Switzerland); clorgyline HCl (Sigma); iproniazid (RBI, U.S.A.); isocarboxazid HCl (F. Hoffmann-La Roche Ltd.); 3-methylcholanthrene (Sigma); 2-methylimidazole (Sigma); moclobemide base (F. Hoffmann-La Roche Ltd.); naphazoline HCl (Sigma); phenelzine sulphate (Sigma); phenobarbitone (Sigma); 3-phenylpropargylamine HCl (RBI); Ro 16-6491 [*N*-(2-aminoethyl-*p*-chlorobenzamide] HCl (F. Hoffmann-La Roche Ltd.); semicarbazide HCl (RBI); SKF 525A [2-diethylaminoethyl diphenylpropylacetate] HCl (proadifen) (RBI); tranylcypromine sulphate (Smith-Kline, France). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

In vivo *effects of irreversible and reversible MAO inhibitors on brain and liver I_2 imidazoline-preferring receptors*

Chronic treatment (7 days) with the irreversible non-selective MAO inhibitor, phenelzine (1–20 mg kg^{-1}) dose-dependently decreased the density of I_2 imidazoline-preferring receptors in

Table 1 Effects of chronic treatments with irreversible monoamine oxidase (MAO) inhibitors on I_2 imidazoline-preferring receptors in the rat cerebral cortex and liver

		Cerebral cortex [^{3}H]idazoxan			*Liver* [^{3}H]-idazoxan		
Treatment	*Dose* (mg kg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n
Saline	–	14.4 ± 0.7	68 ± 3	24	9.0 ± 1.0	335 ± 21	24
Hydrazines							
Phenelzine	0.3	13.7 ± 0.7	75 ± 4	5	4.9 ± 0.3	237 ± 17	3
	1	12.1 ± 2.2	49 ± 3	4	8.4 ± 0.1	264 ± 10	3
	3	12.8 ± 2.9	42 ± 5*	4	8.8 ± 0.8	147 ± 10*	4
	10	9.2 ± 1.6	36 ± 2**	3	7.7 ± 0.4	133 ± 9***	6
	20	7.5 ± 0.5	30 ± 2***	6	8.2 ± 0.5	172 ± 16*	5
Isocarboxazid	10	9.5 ± 1.5	20 ± 3***	3	16.1 ± 0.7	124 ± 9*	3
Propargylamines							
Clorgyline	3	13.3 ± 1.9	34 ± 3***	6	7.1 ± 0.7	156 ± 14*	4
Cyclopropylamines							
Tranylcypromine	10	13.7 ± 4.9	31 ± 6***	4	12.0 ± 0.9	134 ± 13*	3

Each drug was administered i.p., every 12 h for 7 days, except for the clorgyline treatment which was for 14 days. The rats were killed 24 h after the last injection. Cortical and liver membranes were incubated at 25°C for 30 min with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 4×10^{-8} M). Total binding of [^{3}H]-idazoxan to I_2 imidazoline-preferring receptors was defined in the presence of 10^{-6} M adrenaline for the cortical membranes or buffer only for the liver membranes; non-specific binding was defined in the presence of 10^{-4} M naphazoline. Binding parameters (K_d, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programmes. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. Data for clorgyline treatment in the brain were taken from Olmos *et al.* (1993). One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} after chronic treatment with the irreversible MAO inhibitors ($F[8,50] = 20.86$, $P = 0.0001$ and $F[8,46] = 10.33$; $P = 0.0001$ for the cortical and liver membranes, respectively). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with saline-treated group (ANOVA followed by Scheffé's test).

the rat cerebral cortex (B_{max} values reduced by 28%–56%) and liver (B_{max} values reduced by 21%–60%) (Table 1). This effect of phenelzine was similar in both tissues, in spite of the marked different densities of I_2 imidazoline-preferring receptors in brain and liver (five times greater density in the liver; Table 1). The effect of phenelzine (3 mg kg^{-1}) was rapid (1 day) in the liver but not in the brain (7 days) and the down-regulation of I_2 imidazoline-preferring receptors was maintained in both tissues after 14 or 21 days of repeated treatment (B_{max} values reduced by 35% in brain and 60% in liver) (Figure 1). Similar chronic treatment with isocarboxazid (10 mg kg^{-1}), a hydrazine structurally related to phenelzine, also significantly decreased the density of brain and liver I_2 imidazoline-preferring receptors (B_{max} values reduced by 71% and 63%, respectively) (Table 1).

In addition to hydrazines the study was extended to other irreversible MAO inhibitors including propargylamines and cyclopropylamines. Chronic treatment (14 days) with the propargylamine, clorgyline (3 mg kg^{-1}) decreased by 50% the density of I_2 imidazoline-preferring receptors in the rat brain (data taken from Olmos *et al.*, 1993) and liver (Table 1). This effect was also induced by chronic treatment (7 days) with the cyclopropylamine, tranylcypromine (10 mg kg^{-1}) which reduced by 55%–60% the density of brain and liver I_2 imidazoline-preferring receptors (Table 1). No significant alterations of the K_d values for the binding of [^{3}H]-idazoxan to cortical or liver membranes were observed after the different chronic treatments with the irreversible MAO inhibitors (Table 1). However, chronic treatment with phenelzine also tended to increase the affinity of the radioligand for the brain I_2 imidazoline-preferring receptors (Table 1) (ANOVA for K_d values: $F[5,40] = 4.07$, $P = 0.004$).

In order to determine further (Olmos *et al.*, 1993) if the down-regulation of I_2 imidazoline-preferring receptors found after chronic treatment with these MAO inhibitors was related to their ability to inactivate irreversibly the enzyme, the effects of chronic treatments with three reversible MAO inhibitors were studied. Chronic treatment (7 days) with the selective MAO-A inhibitors, moclobemide (1 and 10 mg kg^{-1}) or chlordimeform (10 mg kg^{-1}) or with the selective MAO-B inhibitor, Ro 16-6491 (1 and 10 mg kg^{-1}) did not induce any significant change in the binding parameters (K_d, B_{max}) of [^{3}H]-idazoxan to rat brain membranes (Table 2). In the liver only the higher dose of Ro 16-6491 reduced significantly the density of I_2 imidazoline-preferring receptors (38%) (Table 2).

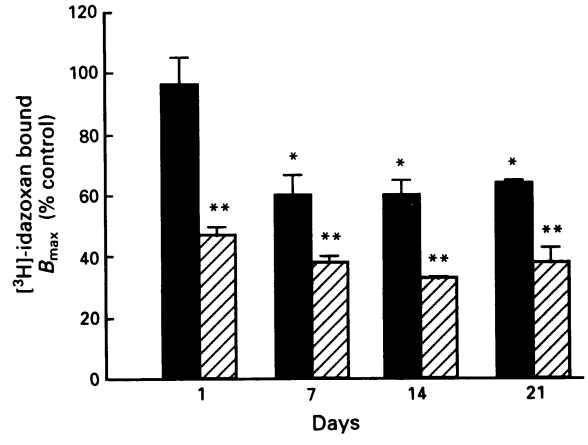


Figure 1 Time-course for the effect of phenelzine (3 mg kg^{-1}, i.p., every 12 h) on the density (B_{max}) of I_2 imidazoline-preferring receptors in the rat cerebral cortex (solid columns) and rat liver (hatched columns). Saturation curves of [^{3}H]-idazoxan binding (6×10^{-10} M to 4×10^{-8} M in the presence of 10^{-6} M adrenaline) to I_2 imidazoline-preferring receptors were determined in P_2 fractions of cortical or liver membranes as described in Table 1. B_{max} values are expressed as percentage of that from saline-treated rats and represent mean ± s.e.mean of 3 to 4 experiments with an animal per experiment. *$P < 0.05$ and **$P < 0.001$ as compared with saline-treated group (ANOVA followed by Scheffé's test).

In vitro *effects of irreversible and reversible MAO inhibitors on brain and liver I_2 imidazoline-preferring receptors*

In vitro competition curves for various irreversible and reversible MAO inhibitors against [^{3}H]-idazoxan binding to rat cerebral cortex and liver I_2 imidazoline-preferring receptors were biphasic (Table 3, Figures 2 and 3). Phenelzine, 3-phenylpropargylamine, tranylcypromine and the reversible MAO-B inhibitor, Ro 16-6491 displayed moderate affinity (low micromolar range) for I_2 imidazoline-preferring receptors with similar inhibition constants (K_i) in rat brain and liver (Table 3 and Figure 2). The hydrazine non-selective MAO inhibitors isocarboxazid, iproniazid and semicarbazide and the reversible MAO-A inhibitor moclobemide did not compete against [^{3}H]-idazoxan binding to I_2 imidazoline-preferring receptors in the two tissues studied (Table 3). Clorgyline competition curves against [^{3}H]-idazoxan binding to rat brain I_2 imidazoline-preferring receptors revealed the existence of a very high affinity ($K_{iH} = 40$ pM) site for this drug that represented about a 40% of total binding (Olmos *et al.*, 1993); in contrast, clorgyline only displayed moderate affinity ($K_{iH} = 169$ nM) for the same proportion of sites in the liver (Table 3 and Figure 3a). These differences between brain and liver I_2 imidazoline-preferring receptors were further

Table 2 Effects of chronic treatments with reversible monoamine oxidase (MAO) inhibitors on I_2 imidazoline-preferring receptors in the rat cerebral cortex and liver

		Cerebral cortex [^{3}H]-idazoxan			*Liver* [^{3}H]-idazoxan		
Treatment	*Dose* (mg kg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n
Saline	–	15.8 ± 1.8	75 ± 6	7	7.9 ± 0.5	272 ± 16	9
Moclobemide	1	16.3 ± 2.2	77 ± 7	3	8.3 ± 0.5	203 ± 7	4
	10	12.7 ± 1.6	69 ± 12	3	10.0 ± 1.1	197 ± 16	4
Chlordimeform	10	15.4 ± 1.6	76 ± 7	3	9.8 ± 0.6	307 ± 32	4
Ro 16-6491	1	16.5 ± 3.8	75 ± 9	3	6.4 ± 0.4	288 ± 31	4
	10	12.8 ± 3.0	70 ± 11	4	9.9 ± 0.2	170 ± 19*	4

Each drug was administered i.p., every 12 h for 7 days. The rats were killed 24 h after the last injection. Binding of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors and estimation of binding parameters (K_d, B_{max}) were done as described in Table 1. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} only after chronic treatment with Ro 16-6491 (10 mg kg^{-1}) in the liver ($F[5,23] = 6.31$; $P = 0.0008$). *$P < 0.05$ as compared with saline-treated group (ANOVA followed by Scheffé's test).

Table 3 Binding parameters of various monoamine oxidase (MAO) inhibitors on I_2 imidazoline-preferring receptors in the rat cerebral cortex and liver

	Cerebral cortex [^{3}H]-idazoxan				*Liver* [^{3}H]-idazoxan			
	K_{iH} (nM)	K_{iL} (μM)	$\%R_H$	n	K_{iH} (nM)	K_{iL} (μM)	$\%R_H$	n
MAO inhibitors								
Irreversibles								
Hydrazines								
Phenelzine	5,900	38.4	78	3	3,000	179	84	4
Isocarboxazid	–	>1000	–	2	–	>1000	–	2
Iproniazid	–	>1000	–	2	–	>1000	–	2
Semicarbazide	–	>1000	–	2	–	>1000	–	2
Propargylamines								
Clorgyline	0.04	10.6	38	10	169	15.0	45	4
3-Phenylpropargylamine	356	116	63	4	793	128	92	2
Cyclopropylamines								
Tranylcypromine	357	771	85	2	592	685	83	3
Reversibles								
Moclobemide	–	>1000	–	2	–	>1000	–	2
Chlordimeform	6.8	45.9	60	4	6.1	18.8	90	3
Ro 16-6491	1,900	–	100	2	–	–	–	–

Cortical or liver membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (10^{-8} M in the presence of 10^{-6} M adrenaline) and in the absence or the presence of the competing drugs (10^{-14} M or 10^{-12} M to 10^{-3} M). Binding parameters (K_{iH}, K_{iL} and $\%R_H$, defined as the percentage of high affinity sites for a given drug) were determined directly by simultaneous analysis of n independent experiments for each drug using the EBDA-LIGAND programmes. For the drugs tested (except Ro 16-6491) that completely inhibited radioligand binding computer-assisted curve fitting demonstrated that a two-site fit was significantly better than a one-site binding model ($P<0.001$, F test). The binding parameters for clorgyline and Ro 16-6491 competition curves in the brain are taken from Olmos *et al.* (1993).

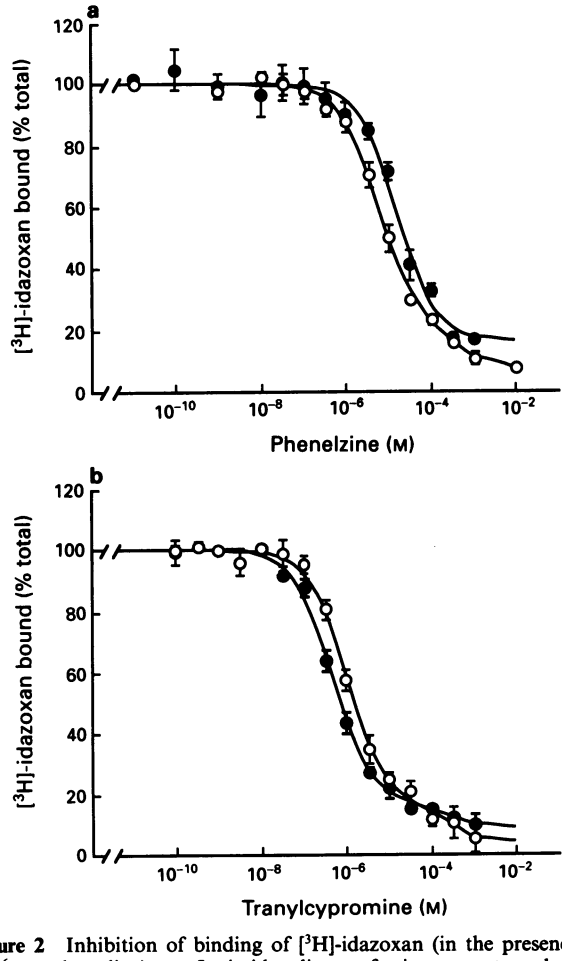


Figure 2 Inhibition of binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to I_2 imidazoline-preferring receptors by (a) phenelzine or (b) tranylcypromine in the rat cerebral cortex membranes (●) and in the rat liver membranes (○). Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that in all cases a two site fit was significantly better than a one site fit ($P<0.001$; F test). Data shown are mean ± s.d. or s.e.mean of 2 to 4 experiments. See Table 3 for K_i values and other details.

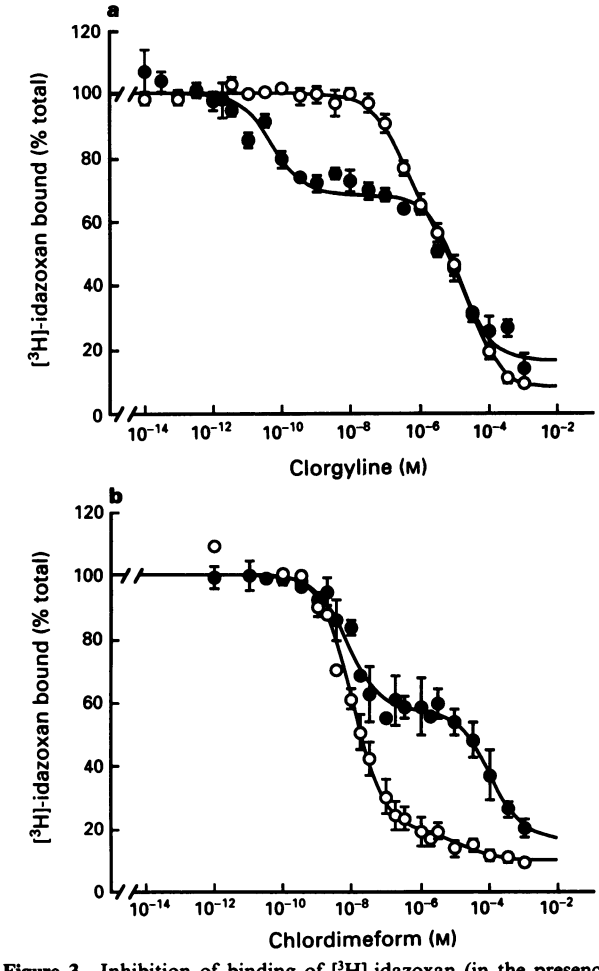


Figure 3 Inhibition of binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to I_2 imidazoline-preferring receptors by (a) clorgyline or (b) chlordimeform in the rat cerebral cortex membranes (●) and in the rat liver membranes (○). Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that in all cases a two site fit was significantly better than a one site fit ($P<0.001$; F test). Data shown are mean ± s.e.mean of 3 to 10 experiments. See Table 3 for K_i values and other details.

revealed by competition curves for chlordimeform against [^{3}H]-idazoxan binding. The pesticide and MAO inhibitor, chlordimeform (Hollingworth *et al.*, 1979) displayed high affinity (K_{iH}~6 nM) for the brain and liver I_2 imidazoline-preferring receptors. However, the proportion of high affinity sites for the drug represented 60% in the rat bain but 90% in the rat liver (Table 3 and Figure 3b).

In vitro *preincubation with phenelzine and chlordimeform on brain and liver I_2 imidazoline-preferring receptors*

To assess further the mechanisms of action involved in the *in vivo* down-regulation of brain and liver I_2 imidazoline-preferring receptors induced by phenelzine, incubation experiments were performed to determine if, consistent with its actions on MAO, this compound could also bind irreversibly to I_2 imidazoline-preferring receptors as described for clorgyline and pargyline (Olmos *et al.*, 1993). However, preincubation (30 min at 25°C) of brain or liver membranes with 10^{-4} M phenelzine did not alter the total density of I_2 imidazoline-preferring receptors (Figure 4). These results discounted an irreversible binding of phenelzine to I_2 imidazoline sites. Under the same experimental conditions (30 min at 25°C), however, preincubation with 10^{-4} M phenelzine decreased by 95% and 30% the binding of [^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 to liver MAO-A and B, respectively. In contrast, preincubation with 10^{-6} M clorgyline reduced by 40% ($P<0.01$) the B_{max} of [^{3}H]-idazoxan binding to brain and liver membranes (Figure 4).

Similar to the irreversible MAO-A inhibitor clorgyline, chlordimeform, a reversible MAO-A inhibitor (Hollingworth *et al.*, 1979), also displayed high affinity (low nanomolar range) for I_2 imidazoline-preferring receptors (Table 3). In order to determine whether this high affinity of the drug for I_2 sites could also result in irreversible binding, cortical membranes were preincubated with 10^{-6} M chlordimeform and reassessed for [^{3}H]-idazoxan binding. Preincubation with chlordimeform altered neither the density of sites nor the competition parameters of cirazoline ([^{3}H]-idazoxan binding, control membranes: $K_{iH}=4$ nM; $K_{iL}=3.3$ μM; $\%R_H=63$; $n=3$; preincubated membranes: $K_{iH}=8$ nM; $K_{iL}=2.3$ μM; $\%R_H=69$; $n=3$).

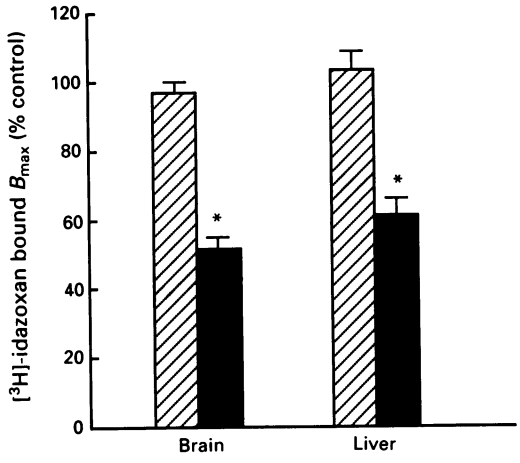


Figure 4 Effects of preincubation (30 min at 25°C) of cortical or liver membranes in buffer containing 10^{-4} M phenelzine (hatched columns) or 10^{-6} M clorgyline (solid columns) on the total density (B_{max}) of I_2 imidazoline-preferring receptors. Saturation curves of [^{3}H]-idazoxan binding (6×10^{-10} M to 4×10^{-8} M in the presence of 10^{-6} M adrenaline) to I_2 imidazoline-preferring receptors were determined in rat cerebral cortex or liver membranes after preincubation and washing as described in methods. B_{max} values are expressed as the percentage of that preincubated in buffer only (control) and represent mean ± s.e.mean of 3 to 6 experiments. *$P<0.05$ as compared with control group (ANOVA followed by Scheffé's test).

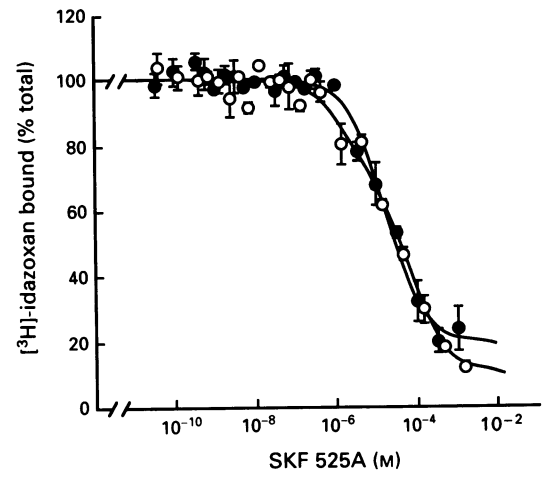


Figure 5 Inhibition of binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to I_2 imidazoline-preferring receptors by SKF 525A in the rat cerebral cortex membranes (●) and in the rat liver membranes (○). Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that for the competition curves in the rat liver a two site fit was significantly better than a one site binding model ($P<0.001$; F test). The binding parameters obtained were $K_i=10$ μM in the rat cerebral cortex; and $K_{iH}=1.6$ μM, $K_{iL}=36$ μM; $\%R_H=43$ in the rat liver. Data shown are mean ± s.d. of 2 experiments.

Table 4 Effects of chronic treatments with various inducers of cytochrome P-450 enzymes on I_2 imidazoline-preferring receptors in the rat cerebral cortex and liver

		Cerebral cortex [^{3}H]-idazoxan			*Liver* [^{3}H]-idazoxan		
Treatment	*Dose* (mg kg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n
Saline	–	13.2 ± 0.8	62 ± 3	17	12.8 ± 2.1	440 ± 24	9
Phenobarbitone	40	10.8 ± 0.7	62 ± 1	3	15.1 ± 3.2	370 ± 41	3
	80	11.3 ± 1.6	62 ± 6	4	–	–	–
3-Methylcholanthrene	20	16.9 ± 1.8	52 ± 8	3	15.2 ± 1.1	474 ± 34	3
2-Methylimidazole	40	10.8 ± 0.2	60 ± 3	2	–	–	–

Each drug was administered i.p., every 24 h (phenobarbitone and 2-methylimidazole) or every 12 h (3-methylcholanthrene) for 7 days. One-way ANOVA did not detect any significant change in the binding parameters of [^{3}H]-idazoxan to brain or liver I_2 imidazoline-preferring receptors after the various treatments. Other details as for Table 1.

Effects of manipulation of cytochrome P-450 enzymes on brain and liver I_2 imidazoline-preferring receptors

Chronic treatments (7 days) with the non-selective inducers of cytochrome P-450 forms phenobarbitone (40 or 80 mg kg^{-1}, i.p., every 24 h) and 3-methylcholanthrene (20 mg kg^{-1}, i.p., every 12 h) or with 2-methylimidazole (40 mg kg^{-1}, i.p., every 24 h), a selective inducer of the IIE form (Koop *et al.*, 1985), with doses enough to increase hepatic cytochrome P-450 activity by several fold (Cresteil *et al.*, 1986) did not significantly alter the binding parameters of [^{3}H]-idazoxan binding to rat liver and brain I_2 imidazoline-preferring receptors (Table 4). *In vitro*, however, the compound SKF 525A, a potent inhibitor of cytochrome P-450 enzymes which forms a tight but reversible complex with the haemoprotein, completely displaced with moderate affinity (low micromolar range) the specific binding of [^{3}H]-idazoxan to I_2 imidazoline-preferring receptors in brain and liver (Figure 5).

Phenelzine and probably other hydrazine MAO inhibitors (isocarboxazid and iproniazid) have been shown to directly bind to the ferrous haeme of cytochrome P-450 and to induce its irreversible inactivation in the presence of NADPH or NADH (Muakkassah & Yang, 1981; Jonen *et al.*, 1982). In order to assess if this mechanism could also underline the interaction of phenelzine with I_2 imidazoline-preferring receptors, total homogenates of rat liver were preincubated for 30 min at 37°C in the presence of 3×10^{-4} M phenelzine alone or with 10^{-3} M NADH; therafter P_2 fractions were obtained and assessed for [^{3}H]-idazoxan binding. Preincubation with phenelzine and NADH did not reduce the density of liver I_2 imidazoline-preferring receptors (phenelzine: $K_d = 18.7 \pm 0.9$ nM; $B_{max} = 444 \pm 18$ fmol mg^{-1} protein; $n = 3$; phenelzine plus NADH; $K_d = 19.0 \pm 1.5$ nM; $B_{max} = 501 \pm 34$ fmol mg^{-1} protein; $n = 3$), suggesting that [^{3}H]-idazoxan does not bind to the haeme group of cytochrome P-450.

Discussion

The present study demonstrates that chronic treatments with the MAO inhibitor antidepressant, phenelzine and other clinically effective irreversible MAO inhibitors including isocarboxazid, clorgyline and tranylcypromine are associated with down-regulation of brain and liver I_2 imidazoline-preferring receptors. However, the clinically effective antidepressant and reversible MAO inhibitor, moclobemide did not interact with brain and liver I_2 sites. These observations indicate a novel effect of irreversible MAO inhibitors in the brain and suggest a new target for these compounds that could be of relevance in the pathophysiology and treatment of depression, a disease in which an increased density of brain I_2 imidazoline-preferring receptors has been reported (Meana *et al.*, 1993).

Several conclusions can be deduced from the present data. Firstly, the reported down-regulation of brain I_2 imidazoline-preferring receptors is related to an irreversible inactivation of the enzyme MAO. Thus, chronic treatment with irreversible (phenelzine, isocarboxazid and tranylcypromine, present study; or clorgyline and pargyline, Olmos *et al.*, 1993) but not with reversible MAO-A (moclobemide, chlordimeform, present study; or Ro 41-1049, Olmos *et al.*, 1993) and MAO-B inhibitors (Ro 16-6491, present study) resulted in down-regulation of I_2 imidazoline-preferring receptors. This conclusion also applies to these putative receptors in the liver, except for the chronic effects of a high dose of the compound Ro 16-6491 (Table 2). Secondly, among the irreversible MAO inhibitors there is no relationship between the affinity displayed *in vitro* against [^{3}H]-idazoxan binding to brain and liver I_2 imidazoline-preferring receptors and the ability to down-regulate these receptors *in vivo*. Thus, clorgyline, tranylcypromine and phenelzine markedly differ in their affinities for I_2 imidazoline-preferring receptors (Table 3) but chronic treatment resulted in similar down-regulation of I_2 imidazoline-preferring receptors (Table 1); isocarboxazid did not compete for [^{3}H]-idazoxan binding to these receptors (Table 3) but it was more effective than phenelzine and tranylcypromine to down-regulate *in vivo* I_2 imidazoline-preferring receptors (Table 1). Thirdly, the down-regulation observed after chronic treatment with MAO inhibitors is not related to an irreversible binding of these compounds to I_2 imidazoline-preferring receptors (see also Olmos *et al.*, 1993). Thus, phenelzine, in contrast to clorgyline, does not irreversibly bind *in vitro* to brain or liver I_2 imidazoline-preferring receptors (Figure 4) but was able to down-regulate brain and liver I_2 imidazoline-preferring receptors after chronic treatment. Fourthly, the binding of [^{3}H]-idazoxan to brain and liver I_2 imidazoline-preferring receptors is heterogeneous in nature and competition curves with most MAO inhibitors were biphasic as previously described for other drugs (Miralles *et al.*, 1993a); the differences reported here for the *in vitro* interaction of clorgyline and chlordimeform with brain and liver I_2 imidazoline-preferring receptors indeed suggest that they may represent different molecular-pharmacological entities (see also Escribá *et al.*, 1994).

The possibility that [^{3}H]-idazoxan binds to the active site of MAO-A and/or B isoenzymes has been previously discounted (Olmos *et al.*, 1993; Sastre & García-Sevilla, 1993); however, it has been suggested that this radioligand could bind to another site on the MAO protein (Renouard *et al.*, 1993). This possibility is very unlikely, mainly because idazoxan, contrary to other imidazoline derivatives (e.g. cirazoline), did not inhibit up to 10^{-3} M rat liver basal MAO activity (Carpéné *et al.*, 1994). Moreover, the proposed molecular mass for the MAO-B (59 kDa) (Bach *et al.*, 1988; Chen *et al.*, 1993) does not correspond with those proposed for the main I_2 imidazoline-preferring receptors (29/30 and 70 kDa proteins) (Wang *et al.*, 1992; Escribá *et al.*, 1994). Recently, polyclonal antibodies have been raised against the 70 kDa imidazoline-receptor protein (Wang *et al.*, 1993) which in the present laboratory failed to recognize a purified fraction (up to 0.7 μg) of MAO from bovine plasma (Sigma Chemical Co.). Moreover, I_2 imidazoline-preferring receptors and the two isoforms of MAO display different isoelectric points (Limon *et al.*, 1992). On the other hand, the I_2 imidazoline-preferring receptors and the MAO-B are not subjected to the same regulation. Thus, chronic treatments with cirazoline and idazoxan that up-regulate the density of brain I_2 imidazoline-preferring receptors did not modify that of brain MAO-B (Olmos *et al.*, 1992; 1994). Moreover, the quinoline derivative EEDQ alkylates *in vivo* a significant proportion (50%) of I_2 imidazoline-preferring receptors without altering the density of MAO-B sites (Miralles *et al.*, 1993b). Together these findings indicate that although I_2 imidazoline-preferring receptors and MAO-B are co-expressed in the same brain areas (Sastre & García-Sevilla, 1993) they represent different proteins.

Irreversible MAO inhibitors including clorgyline, phenelzine and tranylcypromine have been shown also to interact with hepatic cytochrome P-450 enzymes (Muakkassah & Yang, 1981; Bélanger & Atitsé-Gbeasson, 1982; Dupont *et al.*, 1987). The rat brain also expresses these enzymes (Warner *et al.*, 1988; Bergh *et al.*, 1992) and a significant proportion are located on mitochondria (Walther *et al.*, 1986). Thus, the possibility that I_2 imidazoline-preferring receptors could be a form of these enzymes was investigated. Chronic treatment with the enzyme inducers phenobarbitone, 3-methylcholanthrene or 2-methylimidazole did not modulate the density of I_2 sites in brain and liver. On the other hand, phenelzine, SKF 525A and imidazole compounds have been shown to interact with the haeme group of cytochrome P-450 (Muakkassah & Yang, 1981; Yoshida & Aoyama, 1987). For instance, SKF 525A inhibited with moderate affinity ($K_i = 1-38$ μM) the binding of [^{3}H]-GBR-12935 to cytochrome P-450 in human brain (Allard *et al.*, 1994). Although

SKF 525A completely displaced with similar moderate affinity the specific binding of [^{3}H]-idazoxan to I_2 imidazoline-preferring receptors in brain and liver (Figure 5), preincubation with phenelzine in the presence of NADH did not reduce the density of liver I_2 imidazoline-preferring receptors, indicating that [^{3}H]-idazoxan does not bind to the haeme group of cytochrome P-450 enzymes. Moreover, there is no correlation between the densities of cytochrome P-450 (Allard *et al.*, 1994) and those of $I_{1/2}$ imidazoline-preferring receptors (De Vos *et al.*, 1994) in the various regions of the human brain ($r = 0.20$ and 0.03 for the I_1- and I_2 imidazoline-preferring receptors; $n = 10$).

Together these results are compatible with the view that an indirect mechanism is responsible for the *in vivo* down-regulation of brain I_2 imidazoline-preferring receptors found after chronic treatment with irreversible MAO inhibitors. In this sense, there is the possibility that some metabolite(s) of the irreversible MAO inhibitors could be active on I_2 imidazoline-preferring receptors. The endogenous amine 2-phenylethylamine is also a metabolite of phenelzine and has been proposed as one of the mediators of its antidepressant effects (McManus *et al.*, 1991; Paetsch *et al.*, 1993). However, 2-phenylethylamine displayed low affinity for the I_2 imidazoline-preferring receptors in the rat brain ($K_i = 2\ \mu M$). Further work is needed to unravel the mechanisms by which irreversible MAO inhibitors down-regulate I_2 imidazoline-preferring receptors in the brain.

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References

ALLARD, P., MARCUSON, J.O. & ROSS, S.B. (1994). [^{3}H]GBR-12935 binding to cytochrome P-450 in the human brain. *J. Neurochem.*, **62**, 342–348.

ATLAS, D. (1991). Clonidine-displacing substance (CDS) and its putative imidazoline receptor. New leads for further divergence of α_2-adrenergic receptor activity. *Biochem. Pharmacol.*, **41**, 1541–1549.

BACH, A.W., LAN, N.C., JOHNSON, D.L., ABELL, C.W., BEMBENEK, M.E., KWAN, S.W., SEEBURG, P.H. & SHIH, J.C. (1988). cDNA cloning of human liver monoamine oxidase A and B. Molecular basis of differences in enzymatic properties. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4934–4938.

BAKER, G.B., COUTTS, R.T., MCKENNA, K.F. & SHERRY-MCKENNA, R.L. (1992). Insights into the mechanisms of action of the MAO inhibitors phenelzine and tranylcypromine: a review. *J. Psychiatr. Neurosci.*, **17**, 206–214.

BÉLANGER, P.M. & ATITSÉ-GBEASSON, A. (1982). Inhibitory effect of tranylcypromine on hepatic drug metabolism in the rat. *Biochem. Pharmacol.*, **31**, 2679–2683.

BERGH, A.F. & STROBEL, H.W. (1992). Reconstitution of the brain mixed function oxidase system: purification of NADPH-cytochrome P450 reductase and partial purification of cytochrome P450 from whole brain. *J. Neurochem.*, **59**, 575–581.

BRUNS, R.F., LAWSON-WENDLING, K. & PUGSLEY, T.A. (1983). A rapid filtration assay for soluble receptors using polyethylenimine-treated filtres. *Anal. Biochem.*, **132**, 74–81.

CARPÉNÉ, C., COLLON, P., REMAURY, A., CORDI, A., HUDSON, A., NUTT, D. & LAFONTAN, M. (1994). Inhibition of amine oxidase activity by derivatives recognizing I_2-imidazoline sites. *J. Pharmacol. Exp. Ther.* (in press).

CHEN, K., WU, H.-F. & SHIH, J. (1993). The deduced amino acid sequences of human platelet and frontal cortex monoamine oxidase B are identical. *J. Neurochem.*, **61**, 187–190.

CRESTEIL, T., BEAUNE, P., CELIER, C., LEROUX, J.P. & GUENGERICH, F.P. (1986). Cytochrome P-450 isoenzyme content and monooxygenase activities in rat liver: effect of ontogenesis and pretreatment by phenobarbital and 3-methylcholanthrene. *J. Pharmacol. Exp. Ther.*, **236**, 269–276.

DE VOS H., BRICCA, G., DE KEYSER, J., DE BACKER, J.P., BOUSQUET, P. & VAUQUELIN, G. (1994). Imidazoline receptors, non-adrenergic idazoxan binding sites and α_2-adrenoceptors in the human central nervous system. *Neuroscience*, **59**, 589–598.

DUPONT, H., DAVIES, D.S. & STROLIN-BENEDETTI, M. (1987). Inhibition of cytochrome P-450-dependent oxidation reactions by MAO inhibitors in rat liver microsomes. *Biochem. Pharmacol.*, **36**, 1651–1657.

ERNSBERGER, P. (1992). Heterogeneity of imidazoline binding sites: proposed I_1 and I_2 subtypes. *Fundam. Clin. Pharmacol.*, **6** (Suppl 1), 55s, P18.

ESCRIBÁ, P.V., SASTRE, M., WANG, H., REGUNATHAN, S., REIS, D.J. & GARCÍA-SEVILLA, J.A. (1994). Immunodetection of putative imidazoline receptors proteins in the human and rat brain and other tissues. *Neurosci. Lett.*, **178**, 81–84.

GIRALT, M.T. & GARCÍA-SEVILLA, J.A. (1989). Acute and long-term regulation of brain α_2-adrenoceptors after manipulation of noradrenergic transmission in the rat. *Eur. J. Pharmacol.*, **164**, 455–466.

HIEBLE, J.P. & RUFFOLO, R.R. Jr. (1992). Imidazoline receptors: historical perspective. *Fundam. Clin. Pharmacol.*, **6** (Suppl 1), 7s–13s.

HOLLINGWORTH, R.M., LEISTER, J. & GHALI, G. (1979). Mode of action of formamidine pesticides: an evaluation of monoamine oxidase as the target. *Chem. Biol. Interactions*, **24**, 35–49.

JONEN, H.G., WERRINGLOER, J., PROUGH, R.A. & ESTABROOK, R.W. (1982). The reaction of phenylhydrazine with microsomal cytochrome P-450. *J. Biol. Chem.*, **257**, 4404–4411.

KILPATRICK, A.T., BROWN, C.C. & MACKINNON, A.C. (1992). Non-α_2-adrenoceptor idazoxan binding sites: a new target for drug development. *Biochem. Soc. Trans.*, **20**, 113–118.

KOOP, D.R., CRUMP, B.L., NORDBLOM, G.D. & COON, M.J. (1985). Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P-450 of rabbit liver microsomes by diverse agents: ethanol, imidazole, trichloroethylene, acetone, pyrazole, and isoniazid. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4065–4069.

LI, G., REGUNATHAN, S., BARROW, C.J., ESHRAGHI, J., COOPER, R. & REIS, D.J. (1994). Agmatine – an endogenous clonidine-displacing substance in the brain. *Science*, **263**, 966–969.

LIMON, I., COUPRY, I., LANIER, S.M. & PARINI, A. (1992). Purification and characterization of mitochondrial imidazoline-guanidinium receptive site from rabbit kidney. *J. Biol. Chem.*, **267**, 21645–21649.

LOWRY, O.H., ROSEBROUGH, N.F., FARR, A.G. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MCMANUS, D.J., MOUSSEAU, D.D., PAETSCH, P.R., WISHART, T.B. & GREENSHAW, A.J. (1991). β-adrenoceptors and antidepressants: possible 2-phenylethylamine mediation of chronic phenelzine effects. *Biol. Psychiatry*, **30**, 1122–1130.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for IBM PC. *J. Pharmacol. Methods*, **14**, 213–228.

MEANA, J.J., BARTUREN, F., MARTIN, I. & GARCÍA-SEVILLA, J.A. (1993). Evidence of increased non-adrenoceptor [^{3}H]idazoxan binding sites in the frontal cortex of depressed suicide victims. *Biol. Psychiatry*, **34**, 498–501.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the I site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13**, 369–370.

MICHEL, M.C. & INSEL, P.A. (1989). Are there multiple imidazoline binding sites? *Trends Pharmcol. Sci.*, **10**, 342–344.

MIRALLES, A., OLMOS, G., SASTRE, M., BARTUREN, F., MARTIN, I. & GARCÍA-SEVILLA, J.A. (1993a). Discrimination and pharmacological characterization of I_2-imidazoline sites with [^{3}H]idazoxan and alpha-2 adrenoceptors with [^{3}H]RX821002 (2-methoxy idazoxan) in the human and rat brains. *J. Pharmacol. Exp. Ther.*, **264**, 1187–1197.

MIRALLES, A., RIBAS, C., OLMOS, G. & GARCÍA-SEVILLA, J.A. (1993b). Differential effects of the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline on brain α_2-adrenoceptors and I_2-imidazoline sites in vitro and in vivo. *J. Neurochem.*, **61**, 1602–1610.

MOUSSEAU, D.D, MCMANUS, D.J., BAKER, G.B., JUORIO, A.V., DEWHURST, W.G. & GREENSHAW, A.J. (1993). Effects of age and of chronic antidepressant treatment on [^{3}H]tryptamine and [^{3}H]dihydroalprenolol binding to rat cortical membranes. *Cell. Mol. Neurobiol.*, **13**, 3–13.

MUAKKASSAH, S.F. & YANG, W.C.T. (1981). Mechanism of the inhibitory action of phenelzine on microsomal drug metabolism. *J. Pharmacol. Exp. Ther.*, **219**, 147–155.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for the characterization of ligand binding system. *Anal. Biochem.*, **107**, 220–239.

MURRAY, M. & REIDY, G.F. (1990). Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. *Pharmacol. Rev.*, **42**, 85–101.

OLMOS, G., ALEMANY, R., ESCRIBA, P.V. & GARCÍA-SEVILLA, J.A. (1994). The effects of chronic imidazoline drug treatment on glial fibrillary acidic protein concentrations in rat brain. *Br. J. Pharmacol.*, **111**, 997–1002.

OLMOS, G., GABILONDO, A.M., MIRALLES, A., ESCRIBA, P.V. & GARCÍA-SEVILLA, J.A. (1993). Chronic treatment with the monoamine oxidase inhibitors clorgyline and pargyline down-regulates non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain. *Br. J. Pharmacol.*, **108**, 597–603.

OLMOS, G., MIRALLES, A., BARTUREN, F. & GARCÍA-SEVILLA, J.A. (1992). Characterization of brain imidazoline receptors in normotensive and hypertensive rats: differential regulation by chronic imidazoline drug treatment. *J. Pharmacol. Exp. Ther.*, **260**, 1000–1007.

PAETSCH, P.R., BAKER, G.B. & GREENSHAW, A.J. (1993). Induction of functional down-regulation of β-adrenoceptors in rats by 2-phenylethylamine. *J. Pharmacol. Sci.*, **82**, 22–24.

PILETZ, J.E., HALARIS, A., SARAN, A. & MARLER, M.R. (1990). Elevated ^{3}H-para-aminoclonidine binding to platelet purified plasma membranes from depressed patients. *Neuropsychopharmacology*, **3**, 201–210.

PILETZ, J.E., HALARIS, A., SARAN, A. & MARLER, M.R. (1991). Desipramine lowers tritiated para-aminoclonidine binding in platelets of depressed patients. *Arch. Gen. Psychiatry*, **48**, 813–820.

RENOUARD, A., WIDDOWSON, P.S. & CORDI, A. (1993). [^{3}H]-idazoxan binding to rabbit cerebral cortex recognises multiple imidazoline I_2-type receptors: pharmacological characterization and relationship to monoamine oxidase. *Br. J. Pharmacol.*, **109**, 625–631.

SASTRE, M. & GARCÍA-SEVILLA, J.A. (1993). Opposite age-dependent changes of α_{2A}-adrenoceptors and non-adrenoceptor [^{3}H]idazoxan binding sites (I_2-imidazoline sites) in the human brain: strong correlation of I_2 with MAO-B sites. *J. Neurochem.*, **61**, 881–889.

TESSON, F. & PARINI, A. (1991). Identification of an imidazoline-guanidinium receptive site in mitochondria from rabbit cerebral cortex. *Eur. J. Pharmacol.*, **208**, 81–83.

TESSON, F., PRIP-BUUS, C., LEMOINE, A., PEGORIER J.-P. & PARINI, A. (1991). Subcellular distribution of imidazoline-guanidinium-receptive sites in human and rabbit liver. *J. Biol. Chem.*, **266**, 155–160.

WALTHER, B., GHERSI-EGEA, J.F., MINN, A. & SIEST, G. (1986). Subcellular distribution of cytochrome P-450 in the brain. *Brain Res.*, **375**, 338–344.

WANG, H., REGUNATHAN, S., MEELEY, M.P. & REIS, D.J. (1992). Isolation and characterization of imidazoline receptor protein from bovine adrenal chromaffin cells. *Mol. Pharmacol.*, **42**, 792–801.

WANG, H., REGUNATHAN, S., RUGGIERO, D.A. & REIS, D.J. (1993). Production and characterization of antibodies specific for the imidazoline receptor protein. *Mol. Pharmacol.*, **43**, 509–515.

WARNER, M., KÖHLER, C., HANSSON, T. & GUSTAFSSON, J.-A. (1988). Regional distribution of cytochrome P-450 in the rat brain: spectral quantitation and contribution of P-450b,e and P-450c,d. *J. Neurochem.*, **50**, 1057–1065.

YOSHIDA, Y. & AOYAMA, Y. (1987). Interaction of azole antifungal agents with cytochrome $P\text{-}450_{14DM}$ purified from *Saccahromyces cerevisiae* microsomes. *Biochem. Pharmacol.*, **36**, 229–235.

ZONNENSCHEIN, R., DIAMANT, S. & ATLAS, D. (1990). Imidazoline receptors in rat liver cells: a novel receptor or a subtype of α_2-adrenoceptor? *Eur. J. Pharmacol.*, **190**, 203–215.

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Interacting roles of nitric oxide and ATP in the pulmonary circulation of the rat

[1]Haroutioun Hasséssian & [2]Geoffrey Burnstock

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT

1 The potentiating effect of N^G-nitro-L-arginine methyl ester, (L-NAME) a nitric oxide synthesis inhibitor, on responses of the rat pulmonary vascular pressure (P_{VP}) to purinoceptor agonists was examined.

2 At a constant flow of 23 ml min^{-1} the P_{VP} was 22.4 ± 2.5 mmHg ($n = 15$), and treatment with 100 μM L-NAME for 15 min was without effect on the P_{VP}. After the tone was raised with 28 nmol 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U-46619), the P_{VP} was 29.4 ± 3.3 mmHg and treatment with 100 μM L-NAME was still without effect on the P_{VP}. It appears that there is a graded release of nitric oxide in response to different levels of steady shear stress and in our experimental model the threshold for detection was not reached under basal conditions.

3 In contrast, when the circulation was challenged with 30 s step, additive increases in flow between 11 and 50 ml min^{-1} ($n = 8$), treatment with 100 μM L-NAME produced a significant ($P < 0.05$) increase in P_{VP} suggesting that changes in flow-derived forces evoke the release of nitric oxide. This was evident for flow rates above 30 ml min^{-1}.

4 In preparations in which tone was raised with U-46619, a dose of 1×10^{-8} mol ATP or 2-meSATP evoked a drop in P_{VP} while α,β-meATP produced an increase in P_{VP} under constant flow of 23 ml min^{-1}. After treatment with 100 μM L-NAME, all three purinoceptor agonists evoked an increase in P_{VP}. The increase in P_{VP} evoked by α,β-meATP was not affected by L-NAME. These results suggest that P_{2Y}-purinoceptor stimulation evokes the release of nitric oxide to produce vasodilatation.

5 Under conditions of constant flow and basal pressure, 100 μM L-NAME significantly ($P < 0.05$) potentiated the increase in P_{VP} evoked by 1×10^{-6} mol ATP, although the increase evoked by 1×10^{-8} mol α,β-meATP, which was of similar magnitude, was not affected. These results indicate that a blockade of evoked nitric oxide release is responsible for the potentiation of the increase in P_{VP} evoked by ATP.

6 This study shows that, while nitric oxide does not appear to be released in the pulmonary circulation of the rat under constant flow conditions, nitric oxide release evoked by purinoceptor agonists attenuates increases in pulmonary vascular pressure.

Keywords: Purinoceptor; nitric oxide; pulmonary circulation; endothelium

Introduction

In the rat, single pulse stimulation of perivascular nerves which supply small intrapulmonary arteries produces excitatory junctional potentials that are mediated by a nonadrenergic, noncholinergic neurotransmitter which is probably adenosine-5'-triphosphate (ATP) (Inoue & Kannan, 1988). Furthermore, ATP is localized in endothelial cells, mast cells as well as erythrocytes, and in response to various stimuli, ATP can be liberated from these sources into the pulmonary circulation (Bergfeld & Forrester, 1992; Ralevic *et al.*, 1992). Hence, the role of ATP in the pulmonary circulation is essentially two fold. ATP released from non-sympathetic neurones evokes excitatory junctional potentials leading to vasoconstriction of pulmonary vessels (Inoue & Kannan, 1988), and purinoceptors mediating vasoconstriction have been demonstrated on pulmonary vascular smooth muscle (Liu *et al.*, 1989; Neely *et al.*, 1991), while activation of purinoceptors on endothelial cells produces vasodilatation (Liu *et al.*, 1989; Neely *et al.*, 1989).

Although the mechanism is not clear, a potentiation of vasoconstriction has been observed in a number of blood vessels following removal of endothelium (Hynes *et al.*, 1988), or after inhibition of nitric oxide synthesis (Vo *et al.*, 1991). In the rat pulmonary vascular bed, we examined whether enhancement of vasoconstriction to purinoceptor agonists occurs as a result of loss of agonist-evoked release of nitric oxide or alternatively, whether nitric oxide is constantly modulating reactivity of pulmonary vascular smooth muscle.

Methods

Isolation and perfusion of pulmonary vascular beds

The experiments were performed on male Wistar rats of 200–300 g weight. The rats were stunned and exsanguinated before the trachea was catheterized, and the lungs removed 'en block', together with the heart, and placed in a Petri dish containing Krebs solution (composition in mM: NaCl 118.0, KCl 5.0, $NaH_2PO_4 \cdot 2H_2O$ 1.2, $NaHCO_3$ 25.5, $MgSO_4 \cdot 7H_2O$ 1.2, D-glucose 5.6, $CaCl_2 \cdot 2H_2O$ 2.5 and 4% bovine serum albumin). The left atrium was removed and a Krebs-filled catheter was fixed inside the main pulmonary artery.

The preparation was transferred to a chamber which was open to room temperature (24.1 ± 0.3°C, $n = 16$), and the preparation was kept moist by gently superfusing with Krebs

[1] Present address: Department of Molecular Physiology and Biophysics, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030, U.S.A.

[2] Author for correspondence.

solution from a Pasteur pipette. The tracheal catheter was connected to a custom-made respirator (Hammersmith Hospital Engineering Unit), and ventilated with air at a frequency of 1 Hz. The mean tidal volume was 7.6 ± 1.0 ml min^{-1} ($n = 32$) and a mean positive end expiratory pressure of 8.5 ± 0.7 mmHg ($n = 32$) was maintained throughout all experiments. The preparation was perfused with Krebs solution (temperature: 23.4 ± 0.4°C, $n = 16$) which was bubbled with 95% O_2: 5% CO_2 to maintain pH (7.3 ± 0.0, $n = 16$).

Flow-pressure relationship

The tracheal and pulmonary artery catheters were attached to individual pressure transducers (Grass model T4) to monitor airway as well as pulmonary vascular pressure (P_{VP}). The perfusion pressure of the apparatus was measured daily to subtract later from the apparent pressure measured after connecting the preparation and thus determine the real perfusion pressure of the pulmonary circulation. The Masterflex (Cole Parmer Instruments Co., Chicago, Illinois 60648) pump delivered a constant flow.

After the preparation had stabilized, the flow rate was increased to preset levels in a step-wise manner, and the perfusion pressure steps were recorded before reducing the flow by the same steps. The pressure-flow relationship was the same whether the flow was being increased or decreased in a step-wise manner. At this point 700 µl of a N^G-nitro-L-arginine methyl ester (L-NAME) solution was introduced into the 700 ml Krebs reservoir to produce a 100 µM L-NAME solution. After perfusing with L-NAME for 15 min, the flow rate was once again increased to the preset levels as before. Control experiments were conducted in a series of preparations ($n = 3$) where the flow rate was increased to preset levels in a step-wise manner before reducing the flow by the same steps, and repeating this protocol four times. It was found that, in the absence of drug, the pressure-flow relationship did not change with repetition (results not shown).

All measures of P_{VP} were performed 30 s into the step-augmented flow. Flow steps of duration longer than 30 s caused oedema when the flow rate was increased above about 30 ml min^{-1}, therefore we chose 30 s steps in order to examine flow rates that produce P_{VP} substantially higher than 20 mmHg prior to treatment with L-NAME. During constant flow, the changes in vascular perfusion pressure reflect changes in vascular resistance. For a fixed volume perfusion with air, a sustained change in the pulse pressure reflects a change in the compliance of the airways. A rise in the compliance of the airways indicates oedema, and over the duration of the experiments in our study the pulse pressure was constant.

Bolus administration of drugs

With a Hamilton microsyringe, all purinoceptor agonists were injected in a 50 µl volume, through stethoscope tubing, into the Krebs perfusate proximal to the preparation. The drugs were prepared as 1 × 10^{-2} M stock solutions in distilled water and diluted as required. When the response to a drug had attenuated and the P_{VP} had regained its steady level, a further dose was administered. Doses of drugs were given in increasing strength. To avoid any possible desensitization by α,β-meATP, during dose-response analysis, a complete series of ATP doses was injected first, followed by the complete series of 2 meSATP and then by α,β-meATP. When required, the vascular tone was raised with a single bolus injection of 28 nmol 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U-46619) in a 10 µl volume. Such a dose of U-46619 was sufficient to keep the tone raised for the duration of the experiment.

Materials

The salts used to prepare the Krebs solution were purchased from BDH, while adenosine-5'-triphosphate (sodium salt, ATP) and α,β-meATP were purchased from Sigma. The 2-meSATP was obtained from Research Biochemicals Inc. (Natick, MA 01760, U.S.A.). The vasoconstrictor U-46619 was a kind gift from UpJohn (Kalamazoo, Michigan, U.S.A.).

Statistical analysis

Student's paired t test was used when the effect of L-NAME was being examined on a given response. The curves shown in Figure 2 were compared by testing for statistical significance between the difference of regression line slopes in the t statistic. Furthermore, individual points were compared with Student's paired t test and thus both point differences as well as the overall trend were analysed. All values are expressed as means ± s.e.mean, where n represents the number of animals. A probability of $P < 0.05$ was considered significant.

Results

Release of nitric oxide during increasing flow

To determine whether nitric oxide is released in the pulmonary circulation under basal pressure and constant flow conditions, the P_{VP} was measured in the absence and presence of L-NAME. At a constant flow of 23 ml min^{-1}, the P_{VP} was 22.4 ± 2.5 mmHg ($n = 15$). After perfusing with 100 µM L-NAME for 15 min, the P_{VP} was 23.8 ± 3.9 mmHg and not significantly different from before the L-NAME treatment (Figure 1). Similar results were found when treatment with L-NAME was investigated at a higher P_{VP} and constant flow. With 28 nmol U-46619 the perfusion pressure was significantly ($P < 0.05$, $n = 5$) raised to 29.4 ± 3.3 mmHg. Furthermore, 15 min after perfusing with 100 µM L-NAME, the perfusion pressure was 31.8 ± 2.4 mmHg and not significantly different from before the L-NAME treatment.

When the vascular bed was challenged with additive 30 s step increases in flow between 11 and 50 ml min^{-1} ($n = 8$),

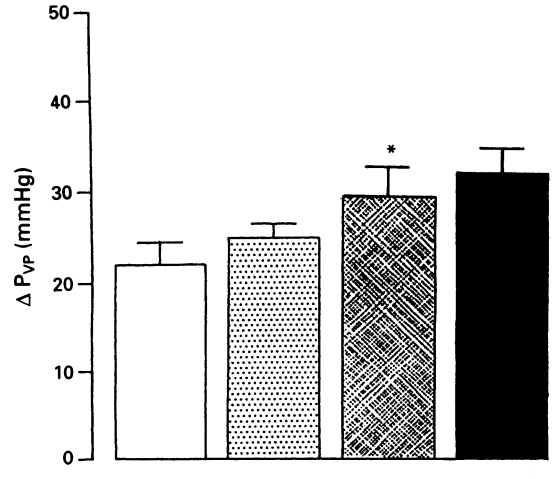


Figure 1 The pulmonary vascular pressure (P_{VP}) measured at a constant flow of 23 ml min^{-1} ($n = 15$), before treatment with any drug (open column), or after treatment with 100 µM N^G-nitro-L-arginine methyl ester (L-NAME) (stippled column), 28 nmol U-46619 alone (shaded column) and 28 nmol U-46619 with 100 µM L-NAME (solid column). The asterisk indicates that the P_{VP} after treatment with U-46619 was significantly ($P < 0.05$, $n = 5$) higher than before treatment with any drug.

the P_{VP} increased proportionately with increasing flow (Figure 2). After perfusion for 15 min with 100 μM L-NAME, similar step increases in flow produced greater increases of P_{VP} (Figure 2). The significantly ($P<0.001$) greater slope of the flow-perfusion pressure curve after L-NAME was introduced represents an increase in vascular resistance.

The vasoconstrictor response to ATP is potentiated by L-NAME

At a constant flow of 23 ml min^{-1} the P_{VP} was 22.2 ± 3.3 mmHg and raised to 28.8 ± 3.8 mmHg ($P<0.05$, $n=5$) by 28 nmol U-46619. In a raised tone preparation, injection of 1×10^{-8} mol ATP or 2-meSATP evoked a vasodilator response while injection of 1×10^{-8} mol α,β-meATP evoked a vasoconstriction (Figure 3). Following treatment with 100 μM L-NAME for 15 min the P_{VP} was 30.8 ± 3.2 mmHg and not significantly changed. Furthermore, injection of ATP or 2-meSATP evoked a vasoconstriction and the vasoconstriction to α,β-meATP was not significantly changed.

In a sample of preparations with basal P_{VP} (24.9 ± 3.2 mmHg, $n=7$) and constant flow (11.5 ± 0.0 ml min^{-1}) injection of 1×10^{-6} mol ATP raised P_{VP} by 4.5 ± 1.0 mmHg which was significantly ($P<0.05$) potentiated 2 fold after treatment with 100 μM L-NAME (Figure 4). Injection of 1×10^{-8} mol α,β-meATP evoked a rise in P_{VP} of 5.6 ± 2.0 mmHg which was not significantly changed in the presence of 100 μM L-NAME (Figure 4).

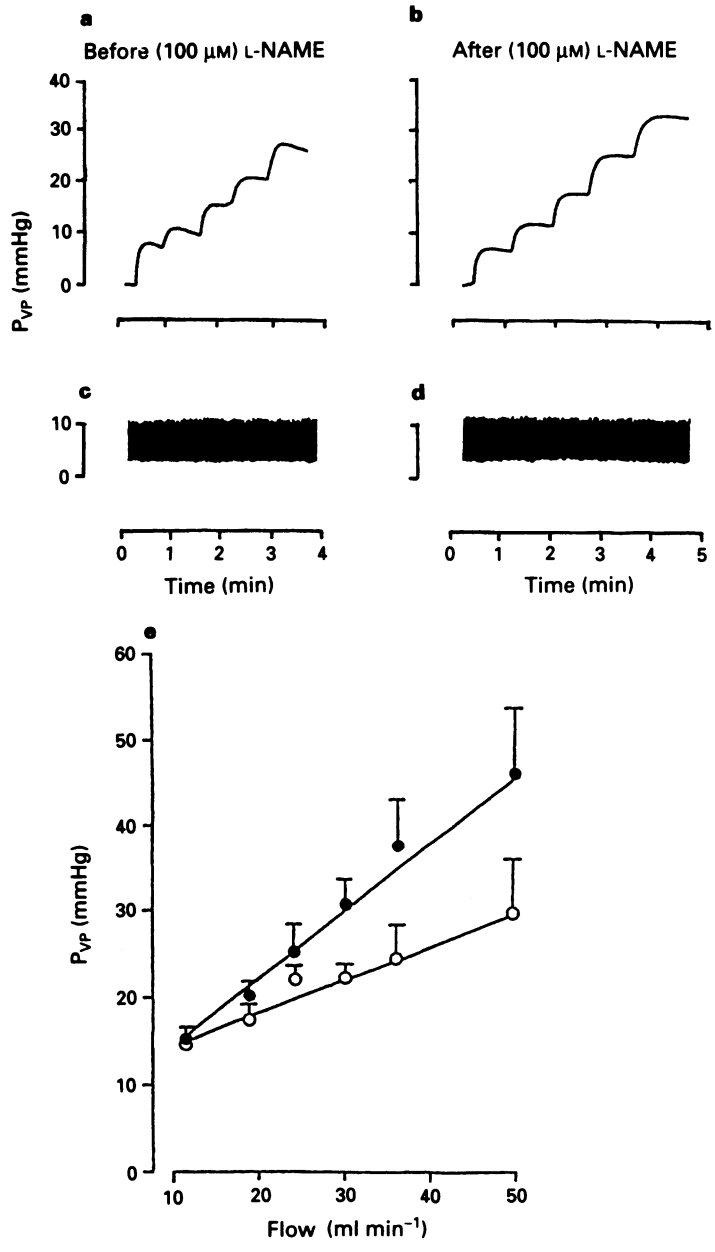


Figure 2 Polygraph traces showing the change in vascular perfusion pressure in (a) and (b) and the airway perfusion pressure in (c) and (d). Step augmentation of the Krebs flow rate (11-20-25-30-35-50 ml min^{-1}) produced corresponding step increases in vascular perfusion pressure without affecting the airways. In the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) the drop in perfusion pressure after the initial jump at each step has been blocked (b). Furthermore, the pressure jumps are to higher levels with each augmentation of flow rate. (e) The pulmonary vascular pressure (P_{VP}) measured at different flow rates before (○) and after (●) treatment with 100 μM L-NAME for 15 min. The regression line slope of the curve obtained after treatment with L-NAME is significantly ($P<0.001$, $n=8$) greater. Comparison of individual points with Student's paired t test, showed that the perfusion pressure for flow rates of 30 ml min^{-1} and higher is significantly ($P<0.05$ in each case) increased by treatment with 100 μM L-NAME.

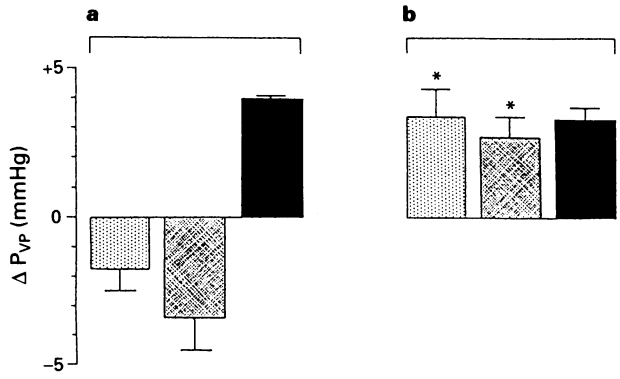


Figure 3 Changes in pulmonary vascular pressure (P_{VP}) evoked by 1×10^{-8} mol of ATP (stippled columns), 2-meSATP (shaded column) or α,β-meATP (solid column), before (a) and after (b) treatment with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) in preparations in which P_{VP} was raised with U-46619. The asterisks indicate that the response to ATP and 2-meSATP was significantly ($P<0.05$, $n=5$) different after treatment with L-NAME.

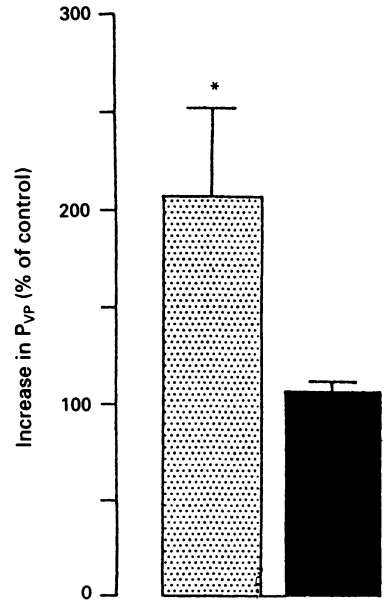


Figure 4 The increase in P_{VP} in response to ATP (stippled column) and α,β-meATP (solid column) at basal P_{VP} with constant flow, compared before and after treatment with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). The asterisk indicates that the increase in P_{VP} evoked by ATP was significantly ($P<0.05$, $n=7$) greater after treatment with L-NAME.

Discussion

To determine if constant release of nitric oxide could be detected we measured the effect of an active dose of L-NAME on the P_{VP} of low tone and raised tone preparations. Under constant flow conditions the P_{VP} was not changed following inhibition of nitric oxide synthesis, indicating that in our experimental model under constant basal flow conditions there does not appear to be a significant release of nitric oxide. It was found by Barer and colleagues (1993) that L-NAME occasionally induced constriction in normal rats under conditions of constant 50 ml min^{-1} flow which is beyond the range of flow that we studied. In other pulmonary circulation studies various inhibitors of nitric oxide synthesis (Gold *et al.*, 1990; Wiklund *et al.*, 1990; Dinh-Xuan *et al.*, 1991), produced an increase in basal vascular resistance. Inhibitors of G protein function have also produced similar results (Hyman *et al.*, 1989; Fineman *et al.*, 1991). In contrast, some investigators have not found an effect on perfusion pressure following disruption of nitric oxide-mediated vasodilatation by various means (Cherry & Gillis, 1987; Mazmanian *et al.*, 1989; Archer *et al.*, 1990; Nishiwaki *et al.*, 1992). Species differences aside, these studies have not addressed the effect of flow-derived changes in shear stress or the effect of reflexes which can be activated *in vivo* to counteract vascular changes. In our study, under conditions of step augmentation of flow-derived shear stress, treatment with L-NAME produced a significant increase in the resistance of the pulmonary circulation. Similar results were reported by Isaacson and colleagues (1994), where although L-NAME did not significantly change P_{VP} under constant flow conditions, there was an increase in P_{VP} when examined with step augmentations of flow. Although isolated vessel preparations and cultured endothelial cells have been found to release nitric oxide in the absence of flow (Moncada *et al.*, 1991), the contribution to vascular pressure of nitric oxide leaked from endothelial cells in the absence of agonist or flow-derived shear stress does not appear to be significant under the conditions that we studied. Furthermore, although nitric oxide was not detected in the perfusate from control rats with P_{VP} of 17.0 ± 1.4 mmHg, 20–25 nM nitric oxide was found in the perfusate of chronically hypoxic rats with mean pulmonary arterial pressure of 32.5 ± 3.3 mmHg which corroborate our study. Hence, it appears that increased shear stress due to augmented pulmonary vasoconstriction in chronically hypoxic rats causes release of nitric oxide (Barer *et al.*, 1993; Isaacson *et al.*, 1994). This suggests that *in vivo*, pulsatile changes in flow evoke nitric oxide release and that vascular changes evoked by such nitric oxide release can be blocked with L-NAME.

In a preparation in which P_{VP} was raised with U-46619, following blockade of nitric oxide synthesis, the vasodilatation in response to ATP as well as 2-meSATP was abolished and the response changed to vasoconstriction, although, the vasoconstrictor response to α,β-meATP was not changed. In contrast, at basal P_{VP} the vasoconstriction in response to ATP was increased by two fold, unlike the vasoconstriction produced by α,β-meATP which was not changed. The P_{2X}-purinoceptor subtype mediates vasoconstriction of large pulmonary vessels (Liu *et al.*, 1989; Neely *et al.*, 1991), and α,β-meATP is a highly selective agonist for this receptor (Burnstock & Kennedy, 1985), whereas 2-meSATP is selective for P_{2Y}-purinoceptors which mediate endothelium-dependent vasodilatation of pulmonary vessels (Liu *et al.*, 1989; Neely *et al.*, 1989). The natural agonist, ATP, is not selective for P_2-purinoceptor subtypes; therefore, exogenous ATP will stimulate both vasodilator and vasoconstrictor purinoceptors to produce a composite response. Blockade of nitric oxide synthesis with L-NAME removes the vasodilator component of the dual effect to reveal the vasoconstriction.

There is evidence which shows that ATP is released from vascular endothelial cells under conditions of increasing flow (Bodin *et al.*, 1991; 1992; Hasséssian *et al.*, 1993) or following hypoxia (Paddle & Burnstock, 1974; Forrester & Williams, 1977; Clemens & Forrester, 1981; Hopwood *et al.*, 1989). Furthermore, ATP has also been shown to be released from erythrocytes in response to hypoxia (Bergfeld & Forrester, 1992). It is possible that ATP released by flow-derived shear stress, hypoxia or other stimuli, is carried short distances in the blood to act on endothelial cells downstream, or ATP can act on the endothelial cell it is released from, to produce P_{2Y}-purinoceptor-mediated nitric oxide release in addition to possible autoregulation of ATP release. Hence ATP-evoked nitric oxide release from endothelial cells will constantly modulate vasoconstriction evoked by purinergic nerves. Therefore, under conditions where any component of endothelial purinergic mechanism is not fully functional,

vasoconstriction evoked by purinergic nerves will be potentiated.

In forms of pulmonary hypertension where there is loss of endothelial cells or trauma to the endothelium, infusion of ATP may aggravate the situation. In the pathological state, each component in isolation, as well as the interaction between vasoconstrictor and vasodilator mechanisms must be considered for a comprehensive understanding of pulmonary blood vessel reactivity.

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References

ARCHER, S.L., RIST, K., NELSON, D.P., DEMASTER, E.G., COWAN, N. & WEIR, E.K. (1990). Comparison of the hemodynamic effects of nitric oxide and endothelium dependent vasodilators in intact lungs. *J. Appl. Physiol.*, **68**, 735–747.

BARER, G., EMERY, C., STEWART, A., BEE, D. & HOWARD, P. (1993). Endothelial control of the pulmonary circulation in normal and chronically hypoxic rats. *J. Physiol.*, **463**, 1–16.

BERGFELD, G.R. & FORRESTER, T. (1992). Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovasc. Res.*, **26**, 40–47.

BODIN, P., BAILEY, D. & BURNSTOCK, G. (1991). Increased flow-induced ATP release from isolated vascular endothelial cells but not smooth muscle cells. *Br. J. Pharmacol.*, **103**, 1203–1205.

BODIN, P., MILNER, P., WINTER, R. & BURNSTOCK, G. (1992). Chronic hypoxia changes the ratio of endothelin to ATP release from rat aortic endothelial cells exposed to high flow. *Proc. R. Soc. B*, **247**, 131–135.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CHERRY, P.D. & GILLIS, C.N. (1987). Evidence for the role of endothelium derived relaxing factor in acetylcholine-induced vasodilation in the intact lung. *J. Pharmacol. Exp. Ther.*, **241**, 516–520.

CLEMENS, M.G. & FORRESTER, T. (1981). Appearance of adenosine triphosphate in the coronary sinus effluent from isolated working rat heart in response to hypoxia. *J. Physiol.*, **312**, 143–158.

DINH-XUAN, A.T., HIGENBOTTAM, T.W., CLELLAND, C.A., PEPKE-ZABA, J., CREMONA, G., BUTT, A.Y., LARGE, S.R., WELLS, F.C. & WALLWORK, J. (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N. Engl. J. Med.*, **324**, 1539–1547.

FINEMAN, J.R., HEYMANN, M.A. & SOIFER, S.J. (1991). N^W-nitro-L-arginine attenuates endothelium-dependent pulmonary vasodilation in lambs. *Am. J. Physiol.*, **260**, H1299–H1306.

FORRESTER, T. & WILLIAMS, C.A. (1977). Release of adenosine triphosphate from isolated adult heart cells in response to hypoxia. *J. Physiol.*, **268**, 371–390.

GOLD, M.E., WOOD, K.S., BYRNS, R.E., FUKUTO, J. & IGNARRO, L.J. (1990). N^G-methyl-L-arginine causes endothelium-dependent contraction and inhibition of cyclic GMP formation in artery and vein. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 4430–4434.

HASSÉSSIAN, H., BODIN, P. & BURNSTOCK, G. (1993). Blockade by glibenclamide of the flow-evoked endothelial release of ATP that contributes to vasodilation in the pulmonary vascular bed of the rat. *Br. J. Pharmacol.*, **109**, 466–472.

HOPWOOD, A.M., LINCOLN, J., KIRKPATRICK, K.A. & BURNSTOCK, G. (1989). Adenosine 5′-triphosphate, adenosine and endothelium-derived relaxing factor in hypoxic vasodilation of the heart. *Eur. J. Pharmacol.*, **165**, 323–326.

HYMAN, A.L., KADOWITZ, P.J. & LIPPTON, H.L. (1989). Methylene blue selectively inhibits pulmonary vasodilator responses in cats. *J. Appl. Physiol.*, **66**, 1513–1517.

HYNES, M.R., DANG, H. & DUCKLES, S.P. (1988). Contractile responses to adrenergic nerve stimulation are enhanced with removal of endothelium in rat caudal artery. *Life Sci.*, **42**, 357–365.

INOUE, T. & KANNAN, M.S. (1988). Nonadrenergic and noncholinergic excitatory neurotransmission in rat intrapulmonary artery. *Am. J. Physiol.*, **254**, H1142–H1148.

ISAACSON, T.C., HAMPL, V., WEIR, E.K., NELSON, D.P. & ARCHER, S.L. (1994). Increased endothelium-derived NO in hypertensive pulmonary circulation of chronically hypoxic rats. *J. Appl. Physiol.*, **76**, 933–940.

LIU, S.F., McCORMACK, D.G., EVANS, T.W. & BARNES, P.J. (1989). Characterization and distribution of P_2-purinoceptor subtypes in rat pulmonary vessels. *J. Pharmacol. Exp. Ther.*, **251**, 1204–1210.

MAZMANIAN, G., BAUDET, B., BRINK, C., CERRINA, J., KIRKIACHARIAN, S. & WEISS, M. (1989). Methylene blue potentiates vascular reactivity in isolated rat lungs. *J. Appl. Physiol.*, **66**, 1040–1045.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

NISHIWAKI, K., NYHAN, D.P., ROCK, P., DESAI, P.M., PETERSON, W.P., PRIBBLE, C.G. & MURRAY, P.A. (1992). N^W-nitro-L-arginine and pulmonary vascular pressure-flow relationship in conscious dogs. *Am. J. Physiol.*, **262**, H1331–H1337.

NEELY, C.F., KADOWITZ, P.J., LIPPTON, H., NEIMAN, M. & HYMAN, A.L. (1989). Adenosine does not mediate the pulmonary vasodilator response of adenosine 5′-triphosphate in the feline pulmonary vascular bed. *J. Pharmacol. Exp. Ther.*, **250**, 170–176.

NEELY, C.F., HAILE, D.M., CAHIL, B.E. & KADOWITZ, P.J. (1991). Adenosine and ATP produce vasoconstriction in the feline pulmonary vascular bed by different mechanisms. *J. Pharmacol. Exp. Ther.*, **258**, 753–761.

PADDLE, B.M. & BURNSTOCK, G. (1974). Release of ATP from perfused heart during coronary vasodilation. *Blood Vessels*, **11**, 110–119.

RALEVIC, V., LINCOLN, J. & BURNSTOCK, G. (1992). Release of vasoactive substances from endothelial cells. In *Endothelial Regulation of Vascular Tone*. ed. Ryan, U.S. & Rubanyi, G.M. pp. 297–328. New York: Marcel Dekker Inc.

VO, P.A., REID, J.J. & RAND, M.J. (1991). Endothelial nitric oxide attenuates vasoconstrictor responses to nerve stimulation and noradrenaline in the rat tail artery. *Eur. J. Pharmacol.*, **199**, 123–125.

WIKLUND, N.P., PERSSON, M.G., GUSTAFSSON, L.E., MONCADA, S. & HEDQVIST, P. (1990). Modulatory role of endogenous nitric oxide in pulmonary circulation *in vivo*. *Eur. J. Pharmacol.*, **185**, 123–124.

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The interaction of RS 25259-197, a potent and selective antagonist, with 5-HT_3 receptors, *in vitro*

E.H.F. Wong, *R. Clark, E. Leung, D. Loury, D.W. Bonhaus, L. Jakeman, *H. Parnes, [1]R.L. Whiting & R.M. Eglen

Institutes of Pharmacology and *Organic Chemistry, Syntex Discovery Research, 3401, Hillview Avenue, Palo Alto, California 94303, U.S.A.

1 A series of isoquinolines have been identified as 5-HT_3 receptor antagonists. One of these, RS 25259-197 [(3aS)-2-[(S)-1-azabicyclo[2.2.2]oct-3-yl]-2,3,3a,4,5,6-hexahydro-1-oxo-1*H*-benzo[de]isoquinoline-hydrochloride], has two chiral centres. The remaining three enantiomers are denoted as RS 25259-198 (**R,R**), RS 25233-197 (**S,R**) and RS 25233-198 (**R,S**).

2 At 5-HT_3 receptors mediating contraction of guinea-pig isolated ileum, RS 25259-197 antagonized contractile responses to 5-HT in an unsurmountable fashion and the apparent affinity (pK_B), estimated at 10 nM, was 8.8 ± 0.2. In this tissue, the $-\log K_B$ values for the other three enantiomers were 6.7 ± 0.3 (**R,R**), 6.7 ± 0.1 (**S,R**) and 7.4 ± 0.1 (**R,S**), respectively. The apparent affinities of RS 25259-197 and RS 25259-198, RS 25233-197 and RS 25233-198 at 5-HT_3 receptors in membranes from NG-108-15 cells were evaluated by a [^{3}H]-quipazine binding assay. The $-\log K_i$ values were 10.5 ± 0.2, 8.4 ± 0.1, 8.6 ± 0.1 and 9.5 ± 0.1, respectively, with Hill coefficients not significantly different from unity. Thus, at these 5-HT_3 receptors, the rank order of apparent affinities was (**S,S**) > (**R,S**) > (**S,R**) = (**R,R**).

3 RS 25259-197 displaced the binding of the selective 5-HT_3 receptor ligand, [^{3}H]-RS 42358-197, in membranes from NG-108-15 cells, rat cerebral cortex, rabbit ileal myenteric plexus and guinea-pig ileal myenteric plexus, with affinity (pK_i) values of 10.1 ± 0.1, 10.2 ± 0.1, 10.1 ± 0.1 and 8.3 ± 0.2, respectively. In contrast, it exhibited low affinity ($pK_i < 6.0$) at 28 other receptors in binding assays, including adrenoceptors (α_{1A}, α_{1B}, α_{2A}, α_{2B}, β_1, β_2), muscarinic (M_1-M_4), dopamine (D_1, D_2), opioid and other 5-HT (5-HT_{1A}, 5-HT_{1D}, 5-HT_{2C}, 5-HT_4) receptors.

4 RS 25259-197 was tritium labelled (specific activity: 70 Ci mmol^{-1}) and evaluated in pharmacological studies. Saturation studies with [^{3}H]-RS 25259-197 in membranes from NG-108-15 and cloned homomeric α subunits of the 5-HT_3 receptor from N1E-115 cells expressed in human kidney 293E1 cells, revealed an equilibrium dissociation constant (K_d) of 0.05 ± 0.02 and 0.07 ± 0.01 nM, and B_{max} of 610 ± 60 and 1068 ± 88 fmol mg^{-1}, respectively. Competition studies in NG-108-15 cells indicated a pharmacological specificity entirely consistent with labelling a 5-HT_3 receptor, i.e. RS 25259-197 > granisetron > (S)-zacopride > tropisetron > (R)-zacopride > ondansetron > MDL 72222.

5 In contrast to the majority of radioligands available to label 5-HT_3 receptors, [^{3}H]-RS 25259-197 labelled a high affinity site in hippocampus from human post-mortem tissue with an equilibrium dissociation constant (K_d) of 0.15 ± 0.07 nM and density (B_{max}) of 6.8 ± 2.4 fmol mg^{-1} protein. Competition studies in this tissue indicated a pharmacological specificity consistent with labelling of a 5-HT_3 receptor.

6 Quantitative autoradiographic studies in rat brain indicated a differential distribution of 5-HT_3 receptor sites by [^{3}H]-RS 25259-197. High densities of sites were seen in nuclear tractus solitaris and area postrema, a medium density in spinal trigeminal tract, ventral dentate gyrus and basal medial amygdala, and a low density of sites in hippocampal CA1, parietal cortex, medium raphe and cerebellum.

7 In conclusion, the functional, binding and distribution studies undertaken with the radiolabelled and non-radiolabelled RS 25259-197 (S,S enantiomer) established the profile of a highly potent and selective 5-HT_3 receptor antagonist.

Keywords: 5-HT_3 receptors; radioligand binding; autoradiography; guinea-pig ileum; antagonist; allosteric interaction; autoradiographic localization

Introduction

5-Hydroxytryptamine (5-HT) receptors are classified on operational, transductional and structural criteria (see Martin & Humphrey, 1994; Boess & Martin, 1994; Hoyer *et al.*, 1994 for reviews) into 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-ht_5, 5-ht_6 and 5-ht_7 subtypes. The 5-HT_3 receptor is thought to form a ligand gated ion channel (Derkach *et al.*, 1989; Peters *et al.*, 1991; Yakel, 1992). The distribution and pharmacology of 5-HT_3 receptors have been characterized by several radiolabelled ligands including [^{3}H]-GR 65630 (Kilpatrick *et al.*, 1988), [^{3}H]-ICS 205,930 (Hoyer & Neijt, 1988), [^{3}H]-quaternary ICS 205,930 (Watling *et al.*, 1988; McKernan *et al.*, 1990), [^{3}H]-quipazine (Milburn & Peroutka, 1989; Sharif *et al.*, 1991), [^{3}H]-zacopride (Barnes *et al.*, 1989a; Pinkus *et al.*, 1989), [^{3}H]-BRL 43694 (Nelson & Thomas, 1989), [^{3}H]-LY 278,584 (Wong *et al.*, 1989), [^{125}I]-iodozacopride (Laporte *et al.*, 1992) and, most recently, [^{3}H]-RS 42358-197 (Wong *et al.*, 1993a). Consistently, these studies and those using *in situ* hybridization (see Boess & Martin, 1994, for review) suggest that central 5-HT_3 receptors are discretely localized on cortical, limbic and brain stem structures. Peripherally, 5-HT_3 receptors appear to be exclusively located on neurones, activation of which causes contractions of gastrointestinal smooth muscle (Fozard, 1984; Butler *et al.*, 1990; Eglen *et al.*, 1990) and the von Bezold-Jarisch reflex (Fozard, 1984).

[1] Author for correspondence.

5-HT$_3$ receptors appear to exist in species variants, in that the guinea-pig 5-HT$_3$ receptor differs pharmacologically from the 5-HT$_3$ receptor in mouse, rat, ferret or man (Butler *et al.*, 1990; Newberry *et al.*, 1991; Kilpatick & Tyers, 1992; Wong *et al.*, 1993a). Recent binding studies at 5-HT$_3$ receptors in murine brain and ileum have provided some evidence for the existence of intra-species heterogeneity (Bonhaus *et al.*, 1993). Measurement of single ion conductances also support the existence of more than one receptor (see Boess & Martin, 1994, for review).

The identification of selective 5-HT$_3$ receptor antagonists has helped to establish the pharmacological relevance of these receptors. Anti-psychotic actions (Tricklebank, 1989), cognitive enhancing actions, and facilitation of withdrawal from drugs of abuse (Costall & Naylor, 1991) have all been ascribed to 5-HT$_3$ receptor antagonism (Costall *et al.*, 1990) although the neurochemical basis (Barnes *et al.*, 1989b; Johnson *et al.*, 1993), and, indeed, the behavioural actions of these antagonists *per se* remains controversial (Tricklebank, 1991; Greenshaw, 1993). In contrast, the efficacy of 5-HT$_3$ receptor antagonists as inhibitors of nausea and vomiting associated with neoplastic agents has clearly been demonstrated in dog or ferret and clinically (Oxford *et al.*, 1992; Andrews & Bhandari, 1993).

A recent investigation into the structure activity relationship of several novel 5-HT$_3$ receptor antagonists (Clark *et al.*, 1993) has identified one, RS 25259-197 [(3a**S**)-2-[(**S**)-1-azabicyclo-[2.2.2]oct-3-yl]-2,3,3a,4,5,6-hexahydro-1-oxo-1*H*-benzo [de]isoquinoline-hydrochloride] (Figure 1) as an antagonist of very high affinity. This compound is a structural analogue of RS 42358-197 (Wong *et al.*, 1993a; Eglen *et al.*, 1993), an antagonist that also exhibits high affinity and selectivity for the 5-HT$_3$ receptor (Clark *et al.*, 1993; Figure 1). In this study we have further characterized the functional, binding and autoradiographic characteristics of both radiolabelled and non-radiolabelled RS 25259-197 *in vitro*.

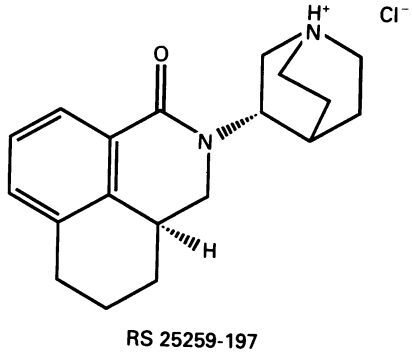
Methods

Contractility studies

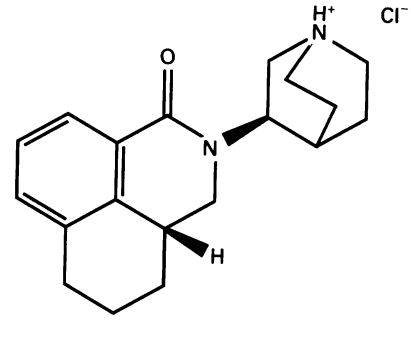
In guinea-pig isolated ileum, contractile responses to activation of 5-HT$_3$ receptors were measured according to the method of Eglen *et al.* (1990). All experiments were carried out at 37°C. Briefly, 2 cm segments of proximal ileum were suspended in Tyrode solution (composition mM: NaCl 137, KCl 2.7, $MgCl_2.H_2O$ 1.1, $NaHPO_4.2H_2O$ 0.4, glucose 5.6, $NaHCO_3$ 11.9, $CaCl_2.6H_2O$ 1.8) under 1 g resting tension. The Tyrode solution contained methysergide (1 μM) to antagonize 5-HT$_1$ and 5-HT$_2$ receptors. 5-Methoxytryptamine (10 μM) was included to desensitize 5-HT$_4$, receptors (Craig *et al.*, 1990). Concentration-response curves to 5-HT were constructed, non-cumulatively (10 nM – 10 μM) at incremental half-log concentrations, both in the absence and in the presence of antagonists, following equilibration for 60 min between consecutive concentration-response curves.

Binding studies

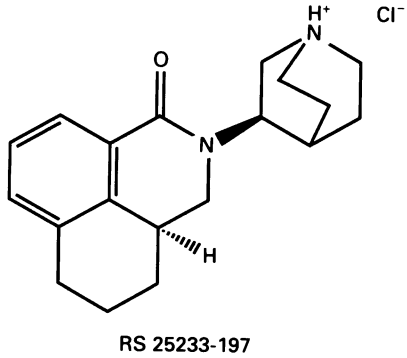
Radioligand binding assays at 5-HT$_3$ receptors were conducted with four 5-HT$_3$ receptor ligands, [^{3}H]-quipazine, [^{3}H]-granisetron, [^{3}H]-RS 42348-197 and [^{3}H]-RS 25259-197. Membranes were prepared according to the methods of Wong *et al.* (1993a) in a Tris-Krebs buffer (composition mM: NaCl 154, KCl 5.4, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.0, D-glucose 11, Tris 25, pH 7.4 at 25°C) and incubations were performed in 0.5 ml total volume at 25°C for 60 min. Saturation studies were conducted with eight concentrations of radioligand, ranging from 4 pM to 4 nM. Competition studies were conducted with 0.1 to 0.4 nM of radioligand. Non-specific binding was defined with 0.1 μM (S)-zacopride. Reactions were terminated by vacuum filtration over GF/B filters pretreated with 0.3% polyethyleneimine. The filters were then



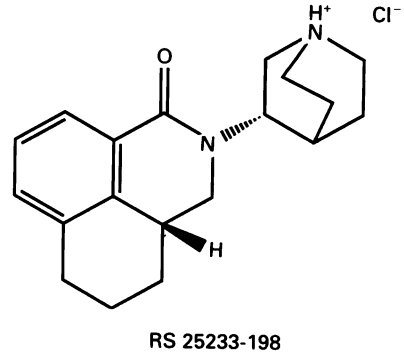
RS 25259-197



RS 25259-198



RS 25233-197



RS 25233-198

Figure 1 Structures of RS 25259-197 and enantiomers.

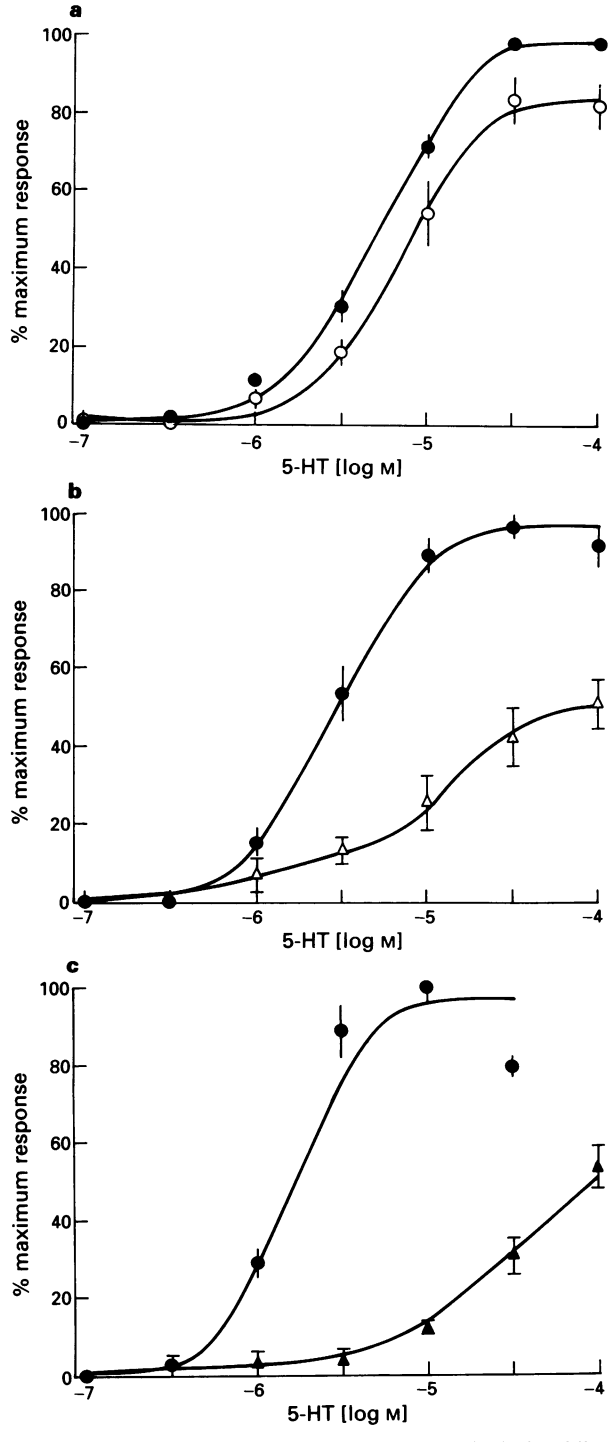


Figure 2 Antagonism of 5-HT response in guinea-pig, isolated ileum by RS 25259-197. Concentration-response curves for 5-HT were constructed in the absence (●), and presence of (a) 1 (○), (b) 10 (△) and (c) 100 (▲) nM of RS 25259-197.

washed for 10 s with ice-cold 0.1 M NaCl, dried and radioactivity retained on the filters was determined by liquid scintillation spectrometry. In all studies, the protein concentration was determined by the Biorad colormetric method with bovine gamma-globulin as the standard (Bradford, 1976). Competition curves were generated by use of 10 concentrations of non-radiolabelled compound. Association and dissociation rates for [^{3}H]-RS 25259-197 and [^{3}H]-granisetron were also determined.

Autoradiographical studies

Coronal sections of rat and mouse brains were cut at 20 μm thickness. Sections were dried and pre-incubated in Tris-HCl buffer (50 mM Tris, 120 mM NaCl, pH 7.4, 22°C) for 30 min. The sections were then covered with the same buffer containing 1.0 nM [^{3}H]-RS 42358-197 or [^{3}H]-RS 25259-197 for 60 min at 22°C. Non-specific binding was defined in the presence of 1.0 μM (S)-zacopride. The incubations were terminated by rinsing the slides for two washes of 5 min in ice cold buffer. The sections were dried and apposed, together with ^{3}H polymer standards (Amersham, Inc.) to tritium-sensitive X-ray film for 24 weeks. The autoradiograms were

Table 2 Affinity of RS 25259-197 at 5-HT$_3$ and other receptor binding assays

Assay radioligand	*p*K$_i$	
5-HT$_{1A}$	8-OH-DPAT	4.4 ± 0.1
5-HT$_{1D}$	5-HT	4.2 ± 0.3
5-HT$_{2A}$	Ketanserin	4.8 ± 0.2
5-HT$_{2C}$	Mesulergine	4.6 ± 0.3
5-HT$_3$	Quipazine	10.4 ± 0.2
5-HT upake	Paroxetine	5.3 ± 0.1
Dopamine D$_1$	SCH23390	<4.0
Dopamine D$_2$	Spiperone	<4.0
Alpha$_{1A}$	Prazosin	5.6 ± 0.3
Alpha$_{1B}$	Prazosin	5.4 ± 0.1
Alpha$_{2A}$	Rauwolscine	5.4 ± 0.2
Alpha$_{2B}$	Rauwolscine	5.4 ± 0.1
Beta$_1$	CGP-12177	<4.0
Beta$_2$	CGP-12177	<4.0
Angiotensin AT$_1$	Sar^{1}Ile^{8}Angiotensin II	<4.0
Angiotensin AT$_2$	Sar^{1}Ile^{8}Angiotensin II	<4.0
Muscarinic M$_1$	Pirenzepine	5.9 ± 0.1
Muscarinic M$_2$	NMS	4.9 ± 0.1
Muscarinic M$_3$	NMS	5.3 ± 0.1
Kappa Opioid	U69593	4.2 ± 0.4
Mu Opioid	RX783006	3.7 ± 0.2
Delta Opioid	DPDPE	4.0 ± 0.4
GABA$_A$	GABA	<4.0
GABA$_A$/BDZ	Diazepam	<4.0
GABA$_A$/Picrotoxin	TBPS	<4.0
NMDA channel	MK-801	<4.0
Ca^{2+} channel	PN200-10	<4.0
Na$^+$ channel	Saxitoxin	<4.0
NK$_1$ Substance P	<4.0	

Values are means ± s.e.mean of at least three separate determinations.

Table 1 Binding affinity and antagonist potency of RS 25259-197 and enantiomers

Compound	*p*K$_i$	n_H	*p*K$_B$
RS 25259-198 (**R,R**)	8.40 ± 0.07	0.89 ± 0.08	6.7 ± 0.1
RS 25233-197 (**S,R**)	8.63 ± 0.13	1.40 ± 0.60	6.7 ± 0.3
RS 25233-198 (**R,S**)	9.48 ± 0.05	0.91 ± 0.07	7.4 ± 0.1
RS 25259-197 (**S,S**)	10.45 ± 0.18	1.03 ± 0.10	8.8 ± 0.2
RS 25259-197 (**S,S**)[1]	10.10 ± 0.09	0.95 ± 0.05	–
RS 25259-197 (**S,S**)[2]	10.19 ± 0.06	0.91 ± 0.07	–
RS 25259-197 (**S,S**)[3]	10.14 ± 0.04	0.91 ± 0.04	–
RS 25259-197 (**S,S**)[4]	8.30 ± 0.18	0.85 ± 0.12	–

p*K*$_i$ and n$_H$ values (means ± s.e.mean; *n* = 3 animals) were generated from competition experiments using [^{3}H]-quipazine in NG-108-15 cells and [^{3}H]-RS 42358-197 in [1]NG-108-15, [2]rat cortex, [3]rabbit ileum and [4]guinea-pig ileum.
All p*K*$_B$ values were derived from guinea-pig isolated ileum using 10 nM RS 25259-197.

then analysed by digital image analysis with the MCID imaging system (Imaging Research, Inc.). Brain areas were verified on cresyl violet stained sections after autoradiography, using the areas described in the rat brain atlas of Paxinos & Watson (1985).

Data analysis

In functional studies, potencies (EC_{50}) were calculated for each tissue using the relationship of Parker & Waud (1971) by non-linear iterative curve fitting procedures (Leung *et al.*, 1992). The apparent affinity (pK_B) was derived by the method of Furchgott (1972), i.e. K_B = Antagonist concentration/Agonist concentration-ratio − 1. Statistically significant differences were assessed by Student's t test, with $P < 0.05$ being considered significant. Competition binding data were analysed by fitting the data to a four parameter logistic equation, followed by the Cheng-Prusoff correction (Cheng & Prusoff, 1973). Saturation binding data were analysed with the programme LIGAND (Munson & Rodbard, 1980).

Compounds used

[^{3}H]-quipazine (60 Ci mmol^{-1}) and [^{3}H]-ganisetron (84 Ci mmol^{-1}) were purchased from Dupont-NEN Corp. (Boston, MA, U.S.A.). [^{3}H]-RS 42358-197 (55 Ci mmol^{-1}) and [^{3}H]-RS 25259-197 (70 Ci mmol^{-1}) were synthesized in the Radiochemistry Group, Syntex Discovery Research. (**S**) and (**R**)-zacopride (4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide HCl), pancopride (*N*-(1-azabicycle-[2.2.2]-octane-3-yl)-2-cyclopropyl-methoxy-4-amino-5-chlorobenzamide), granisetron (BRL 43694, endo-1-methyl-*N*-(9-methyl-9-azabicyclo[3.3.1]-non-3-yl]-1*H*-indazole-3-carboxamide), tropisetron (ICS 205,930, endo-e-methyl-8-azabicyclo[3.2.1]oct-3-yl-1*H*-indole-3-carboxylate), ondansetron (racemic GR 38032, 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazole-1-yl) methyl]-4*H*-carbazole-4-one HCl.2H$_2$O)) and MDL 72222 (endo-8-methyl-8-azabicyclo [3.2.1.] oct-3-yl 3,5-dichlorobenzoate HCl) were synthesized at the Institute of Organic Chemistry, Syntex Discovery Research, Palo Alto, CA, U.S.A. (1-(*meta*-chlorophenyl)-biguanide hydrochloride was purchased from Cookson Chemicals Ltd. (Southampton, U.K.). Other chemicals and reagents were purchased from Sigma Chemical Company or Research Biochemicals Incorporated (Boston, MA, U.S.A.). NG 108-15 cells were obtained from the Institute of Biochemistry and Cell Biology, Syntex Discovery Research, Palo Alto, CA, U.S.A. The human kidney 293 cell-line, permanently transfected with a homomeric alpha subunit of murine 5-HT$_3$ receptor, was a gift from Dr David Julius, Department of Pharmacology, University of California, San Francisco, U.S.A. Post-mortem human brain hippocampal tissue was obtained from Dr Gavin Reynolds, University of Sheffield, U.K.

Results

Functional studies

RS 25259-197 (**S,S**) constitutes one of four enantiomers, the other three being denoted as RS 25259-198 (**R,R**), RS 25233-197 (**S,R**) and RS 25233-198 (**R,S**) (Figure 1). These four enantiomers were tested for their apparent affinity at 5-HT$_3$ receptors in guinea-pig, isolated ileum. Non-parallel, dextral shifts in the concentration-response curves to 5-HT were seen in the presence of all four enantiomers (Table 1). The rank order of pK_B values was (**S,S**) > (**R,S**) > (**S,R**) = (**R,R**). RS 25259-197, only, unsurmountably antagonized responses to 5-HT (Figures 2a–c).

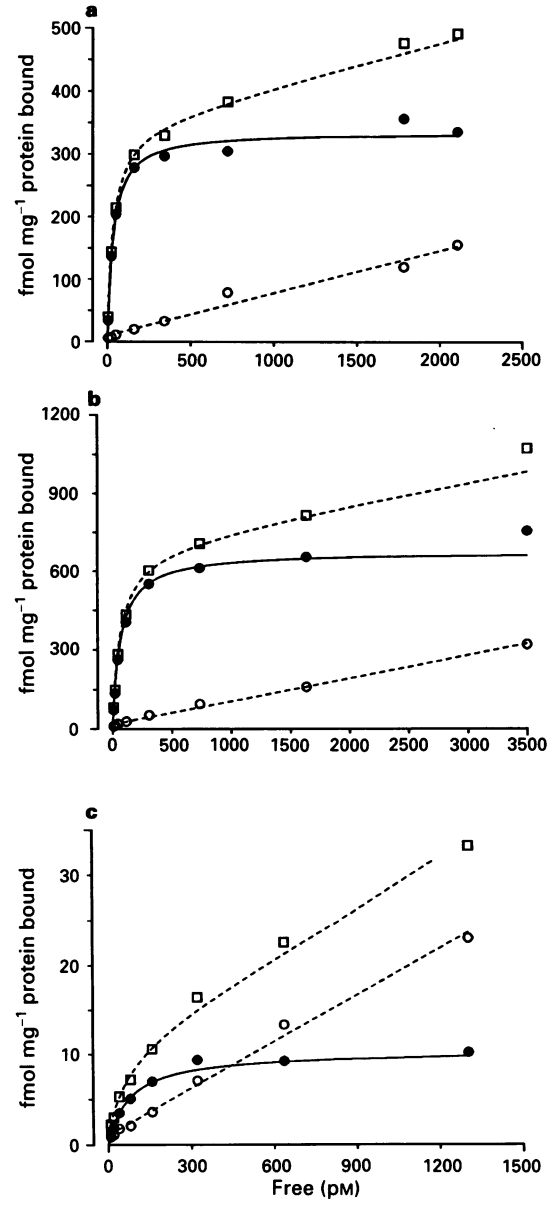


Figure 3 Saturation studies of [^{3}H]-RS 25259-197 (0.004–3.5 nM) binding in membranes from (a) NG-108-15 cells, (b) human kidney 293 cells transfected with the cloned homomeric α-subunit of 5-HT$_3$ receptor, and (c) human hippocampus. Non-specific binding was defined by 0.1 μM (S)-zacopride. (□) Total, (○) non-specific and (●) specific binding are shown.

Table 3 Saturation studies of [^{3}H]-RS 25259-197 and [^{3}H]-RS 42358-197 in membranes from NG-108-15 cells, human kidney 293E1 cells transfected with cloned 5-HT$_3$ receptor homomeric subunit and human hippocampus

Preparation	*[^{3}H]-RS 25259-197*		*[^{3}H]-RS 42358-197*	
	K_d (nM)	B_{max} (fmol mg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1})
NG 108-15 cells	0.05 ± 0.02	610 ± 60	0.20 ± 0.01	660 ± 70
293E1 cells	0.07 ± 0.01	1068 ± 88	0.20 ± 0.03	888 ± 79
Human hippocampus	0.15 ± 0.07	6.9 ± 2.4	–	–

Values are means ± s.e.mean; $n = 3$ animals.

Radioligand binding studies with [^{3}H]-quipazine and [^{3}H]-RS 42358-197

In radioligand binding studies, using [^{3}H]-quipazine to label sites in membranes from NG 108-15 cells, a similar rank order of pK_i values was seen. These estimates of affinities were higher than those derived in the functional studies (Table 1). Due to the high affinity of RS 25259-197 at these 5-HT$_3$ receptors, the interaction of this enantiomer was further evaluated at other 5-HT$_3$ receptors. At 5-HT$_3$ receptors labelled by [^{3}H]-RS 42358-197, in membranes from NG 108-15 cells, rat cortex, rabbit ileum or guinea-pig ileum, RS 25259-107 exhibited apparent affinity values of 10.1 ± 0.1, 10.2 ± 0.1, 10.1 ± 0.1 and 8.3 ± 0.2, respectively. In all these studies, the Hill coefficients were not significantly different from unity (Table 1).

Pharmacological selectivity

The affinity of RS 25259-197 at other neurotransmitter receptors and ion channels was evaluated. RS 25259-197 was highly selective for the 5-HT$_3$ receptor, since low affinities (pK_i < 6.0) were seen at twenty eight neurotransmitter receptor binding assays, including other 5-HT receptors (Table 2). The lack of affinity for several ion channels was suggested by the low affinity (pK_i < 4.0) for the *N*-methyl-D-aspartate (NMDA) receptor-coupled cation channel, the voltage-sensitive Ca^{2+} channel and the Na^+ channel, as labelled by [^{3}H]-MK-801, [^{3}H]-PN200-100 and [^{3}H]-saxitoxin, respectively.

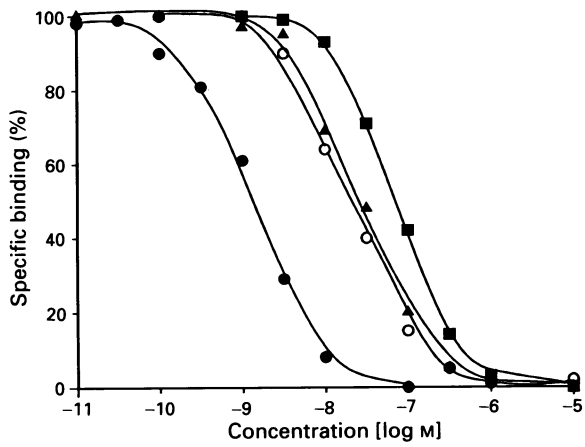


Figure 4 Displacement of [^{3}H]-RS 25259-197 binding in membranes from NG-108-15 cells by 5-HY$_3$ receptor ligands: (●) **S**-zacopride; (○) **R**-zacopride; (▲)-ondansetron; (■)*m*-chlorophenyl-biguanide.

Radioligand binding studies with [^{3}H]-RS 25259-197

The affinity of [^{3}H]-RS 25259-197 was determined in saturation binding studies, using membranes from NG 108-15 cells and 293 E1 cells expressing the alpha subunit of a murine 5-HT$_3$ receptor (Figure 3a and b; Table 3). In human hippocampal membranes, [^{3}H]-RS-25259-197 specifically labelled a site with an affinity (K_d) of 0.15 ± 0.07 nM and a density (B_{max}) of 6.8 ± 2.4 fmol.mg^{-1} protein (Figure 3c).

A stoichiometric comparison of these sites, labelled by [^{3}H]-RS 25259-197, was also made with the dehydro analogue of RS 252529-197, [^{3}H]-RS 42358-197 (Wong *et al.*, 1993a).

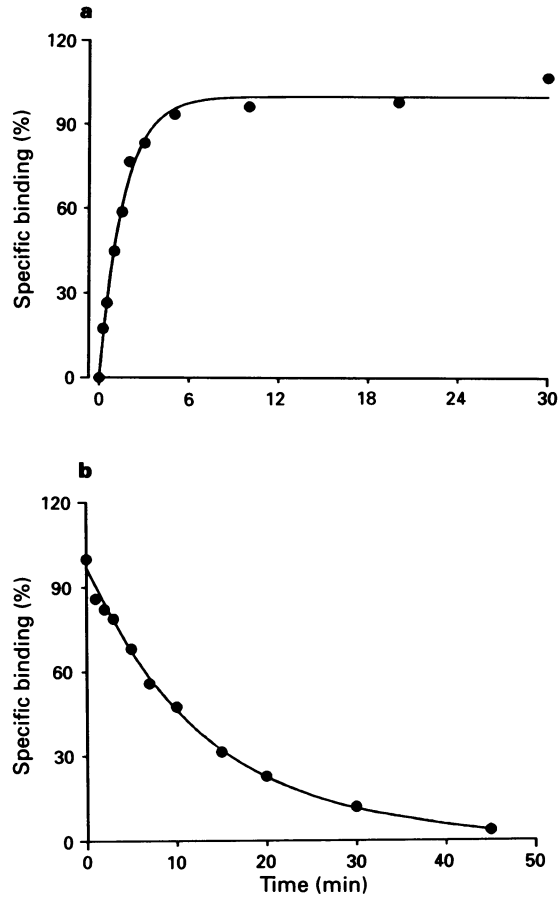


Figure 5 Kinetic analysis of [^{3}H]-RS 25259-197 (0.2 nM) binding in membranes from NG-108-15 cells: (a) association, and (b) dissociation by 1 μM RS 25259-197. Non-specific binding was defined with 0.1 μM (S)-zacopride.

Table 4 Affinities of 5-HT$_3$ compounds for [^{3}H]-RS 25259-197 at binding sites in NG 108-15 cells and human hippocampus

	[^{3}H]-RS 25259-197				*[^{3}H]-quipazine*	
	NG 108-15		*Human hippocampus*		*NG 108-15*	
	pK_i	n_H	pK_i	n_H	pK_i	n_H
RS 25259-197	10.06 ± 0.05	1.03 ± 0.02	10.01	1.69	10.45 ± 0.18	1.03 ± 0.10
(S)-zacopride	9.57 ± 0.09	1.09 ± 0.06	9.81	1.19	9.53 ± 0.08	1.12 ± 0.19
Pancopride	–	–	–	–	9.20 ± 0.30	1.10 ± 0.11
Granisetron	8.91 ± 0.03	0.87 ± 0.03	–	–	–	–
Tropisetron	8.79 ± 0.33	1.11 ± 0.12	–	–	8.68 ± 0.39	0.70 ± 0.20
(R)-zacopride	8.46 ± 0.08	0.93 ± 0.17	8.56	1.14	8.54 ± 0.08	1.26 ± 0.04
Ondansetron	8.19 ± 0.05	1.08 ± 0.10	–	–	8.39 ± 0.10	1.00 ± 0.15
mCPBG	7.75 ± 0.02	1.17 ± 0.04	–	–	–	–
MDL 72222	7.59 ± 0.02	1.12 ± 0.09	–	–	7.66 ± 0.02	1.07 ± 0.04
2-methyl-5-HT	–	–	6.76	1.69	6.30 ± 0.10	0.92 ± 0.09

Values are means ± s.e.mean; n = 3 animals in studies with NG-108-15 cell membranes.
[1]Due to a lack of tissue available, human data are obtained from a single determination.

In NG 108-15 and 293 E1 cell membranes, [^{3}H]-RS 25259-197 exhibited a 3–4 fold higher affinity than [^{3}H]-RS 42358-197, although the same density (B_{max}) of sites was labelled (Table 3).

The pharmacological specificity of [^{3}H]-RS 25259-197 was characterized in both NG 108-15 cells (Figure 4) and human hippocampus. A series of 5-HT$_3$ receptor ligands displaced [^{3}H]-RS 25259-197 binding in these tissues with a similar

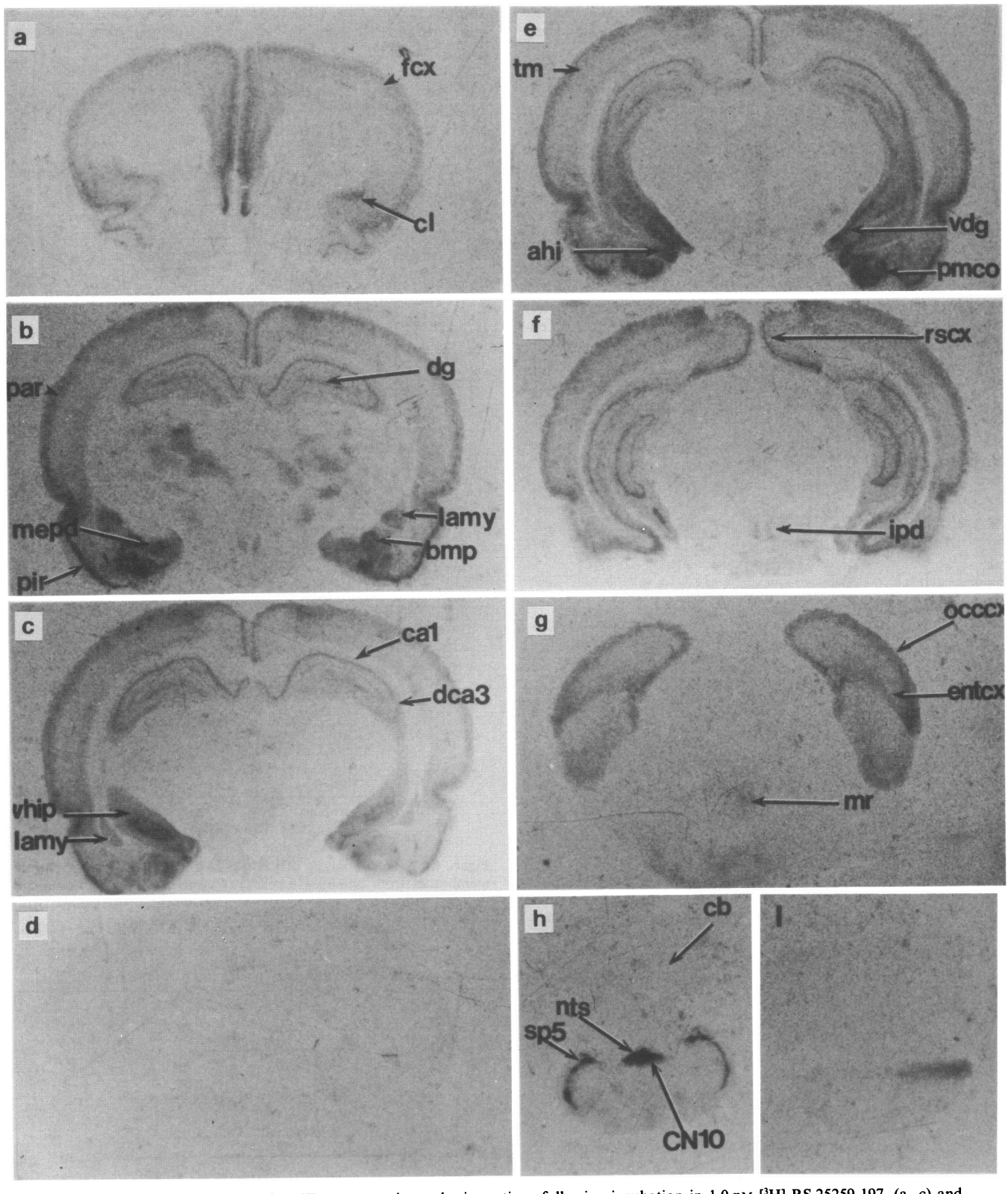


Figure 6 Autoradiography of 5-HT$_3$ receptors in rat brain sections following incubation in 1.0 nM [^{3}H]-RS 25259-197. (a–c) and (e–h) Total binding; (d) (i) non-specific binding of sections adjacent to (c) and (f), respectively, defined in the presence of 1.0 μM (S)-zacopride. Abbreviations correspond to regions in Table 5: claustrum, (cl), frontal cortex (fcx), parietal cortex (par), retrosplenial cortex (rscx), basomedial posterior amygdala (bmp), lateral amygdala (lamy), amygdalo-hippocampal region (ahi), piriform cortex (pir), ventral hippocampus (vhip), medial amygdala (mepd), posterior medial cortical amygdala (pmco), ventral dentate gyrus (vdg), temporal cortex (tm), interpeduncular nucleus (ipd), occipital cortex (occcx), entorhinal cortex (entcx), median raphe nucleus (mr), cerebellum (cb), nucleus tractus solitarius (nts), vagal motor nucleus (CN10), spinal trigeminal tract (sp5).

rank order of affinity (Table 3). A similar rank order of affinity was also observed in a binding assay using [^{3}H]-quipazine to label 5-HT$_3$ receptors in NG 108-15 cell membranes (Table 4).

Kinetics of [^{3}H]-RS 25259-197 binding

[^{3}H]-RS 25259-197 rapidly bound to a high affinity binding site in NG-108-15 cell membranes with an association constant (K_{+1}) of 2.15 nM^{-1} min^{-1} (Figure 5a). Association of the ligand/receptor complex was completed within 10 min and no further change in binding was observed for a further 80 min. Following incubation for 60 min, dissociation was induced by 1.0 μM unlabelled RS 25259-197 (Figure 5b). This followed a monophasic profile, indicating dissociation from a single population of sites, with a half-life ($t_{\frac{1}{2}}$) of 9.6 min (K_{-1} of 0.072 ± 0.004 min^{-1}). This equilibrium constant, calculated from these kinetic parameters was 0.033 nM, closely matching the K_d determined by saturation studies (Table 3). In parallel studies, the association and dissociation constants for [^{3}H]-granisetron were also measured. The corresponding K_{+1} and K_{-1} values were 0.166 nM^{-1} min^{-1} and 0.166 ± 0.009 min^{-1}, respectively, with a calculated K_d of 1.0 nM. This is comparable to the K_d value, obtained from saturation studies, of 1.8 nM (Wong *et al.*, 1993b).

Autoradiographic studies

The distribution of high affinity binding sites for [^{3}H]-RS 25259-197 in sections from rat brain (Figure 6) was characterized by quantitative ligand autoradiography *in vitro*. Parallel studies were carried out with a related 5-HT$_3$ receptor antagonist, [^{3}H]-RS 42358-197 (Wong *et al.*, 1993a). A high percentage of specific binding was measured in most of the regions studied (Table 5). Both of these ligands exhibited similar patterns of distribution, in that the regions with the highest density of binding sites were found in the hind brain vagal complex (nucleus tractus solitarius, are postrema, dorsal motor nucleus of the vagus). A moderate density of binding sites was found in the spinal trigeminal tract, amygdala, hippocampus, and cerebral cortex. Low numbers of sites were detected in median raphe, inferior colliculus and cerebellum (Table 5).

Discussion

Clark *et al.* (1993) have identified several conformationally constrained 5-HT$_3$ receptor antagonists, leading to the identification of a series of isoquinolin-1-ones. Of these, RS 25259-197 (S,S) appeared to exhibit the highest affinity (Clark *et al.*, 1993). The aim of the present study was to characterize the interaction of this enantiomer at several 5-HT$_3$ receptors.

RS 25259-197, forms one of the four enantiomers (Figure 1). It was observed from both functional and radioligand binding studies that RS 25259-197 was a 5-HT$_3$ receptor antagonist of high affinity (Figures 2 and 4, Table 3). Potent and unsurmountable antagonism was observed with RS 25259-

Table 5 Autoradiographic distribution of [^{3}H]-RS 25259-197 and [^{3}H]-RS 42358-197 binding in rats

Region	*[^{3}H]-RS 25259-197* *Specific bound* (fmol mg^{-1}) mean	(s.e.mean)	*% Specific* (specific/total) mean	(s.e.mean)	*[^{3}H]-RS 42358-197* *Specific bound* (fmol mg^{-1}) mean	(s.e.mean)
NTS (nts)	75.1	7.1	95.5	1.0	57.1	2.7
Area postrema	46.0	24.3	85.9	2.8	20.0	6.9
CN10 nucleus (cn10)	33.0	4.4	80.9	2.0	14.7	3.8
Spinal trigeminal n. (sp5)	21.0	3.3	84.2	1.6	8.9	0.9
Biriform cortex (pir)	19.9	1.4	89.0	0.9	ND	
bmp amygdala (bmp)	19.4	9.3	94.7	7.3	ND	
Ventral dentate gyrus (vdg)	18.2	1.6	84.0	6.2	14.9	1.4
Amygdala-hippocampal (ahi)	17.3	1.4	87.5	1.1	12.5	0.9
PMCO amygdala (pmco)	14.9	0.8	88.8	1.4	14.2	1.0
Perirhinal cortex	14.3	2.3	84.6	1.7	8.2	0.6
Ventral hippocampus (vhip)	11.4	0.9	84.4	0.9	11.2	1.3
Pre-subiculum	9.4	1.6	81.7	3.6	3.8	0.7
Temporal cortex (tm)	9.2	0.8	78.3	3.1	5.9	0.5
Para-subiculum	9.0	1.3	78.2	4.2	6.1	1.6
Entorhinal cortex (entcx)	8.2	0.5	81.6	1.6	7.9	1.6
Lateral amyg nucleus (lamy)	7.2	0.8	77.6	2.7	5.7	0.4
Claustrum (cl)	7.1	0.7	70.8	5.1	12.2	4.4
Occipital cortex (occcx)	7.0	0.7	77.2	3.2	4.3	0.5
CA2 hippocampus	6.0	0.5	70.8	2.2	4.8	0.5
Dorsal CA3 hippocampus (dca3)	6.0	0.4	72.1	3.5	5.3	0.6
Retrosplenial cortex (rscx)	5.7	0.3	76.6	1.0	4.0	0.4
CA1 hippocampus (ca1)	5.3	1.0	66.4	4.9	3.8	0.4
Dorsal denate gyrus (dg)	5.0	0.2	72.8	2.6	3.9	0.8
Parietal cortex L.2,3 (par)	4.6	0.4	69.0	4.4	3.6	0.3
Interpedunclar n. (ipd)	4.5	0.2	64.8	5.1	3.5	0.7
Mepd amygdala (mepd)	4.0	2.0	57.8	10.9	ND	
CN12 nucleus	2.4	0.5	37.7	5.8	1.0	0.2
Parietal cx. L.1-6	2.3	0.2	54.3	2.6	2.0	0.3
Central grey	0.8	0.2	26.7	5.8	0.6	0.7
Median raphe (mr)	0.8	0.3	19.6	7.5	1.1	0.6
Inferior colliculus	0.4	0.1	17.0	5.8	0.6	0.2
Cerebellum (cb)	0.3	0.1	12.1	5.2	0.1	0.1

Values are mean ± s.e.mean of measurements from 3–6 brains in two separate experiments. Binding density was measured following incubation in 1.0 nM [^{3}H]-RS 25259-197 or [^{3}H]-RS 42358-197. Non-specific binding was defined in the presence of 1 μM (S)-zacopride. Values are corrected for 100% receptor occupancy (K_d = 0.15 and 0.25 nM, respectively). ND not determined.
RS 25259-197 displaced [^{3}H]-RS 42358-197 binding in all brain areas to non-specific levels. Representative pK_i values were determined by autoradiography in nts (10.4 ± 0.2), bma (10.3 ± 0.2) and vhip (10.3 ± 0.2), n = 3.

197 at 5-HT$_3$ receptors in guinea-pig ileum, in that a clear depression of maximal response was observed by 10 nM of RS 25259-197. Increasing the concentration of 5-HT up to 100 μM did not overcome the blockade (Figure 2). Similar antagonism has been reported with pancopride, which also has a high affinity for 5-HT$_3$ receptors ($pK_i = 9.3$, Fernandez *et al.*, 1990). No evidence was seen for deviations from competitive antagonism in binding studies at any of the tissues, including guinea-pig ileum. The reason for such non-competitive antagonism is, therefore, likely to be related to the formation of a hemi-equilibrium state between the agonist and antagonist at the ileal 5-HT$_3$ receptor (Rang, 1966).

Several lines of pharmacological evidence support the existence of a guinea-pig variant of the 5-HT$_3$ receptor (Butler *et al.*, 1991; Newberry *et al.*, 1991; Kilpatrick & Tyers, 1992; Wong *et al.*, 1993a; Richardson *et al.*, 1985) in that most antagonists exhibited a significantly lower affinity (10 to 1000 fold) for 5-HT$_3$ receptors in this species. In support of these data, the affinities for RS 25259-197 and enantiomers at this 5-HT$_3$ receptor were less than the affinities observed at 5-HT$_3$ receptors in rat cortex, NG-108-15 cells and rabbit ileum (Table 1). RS 25259-197 did not discriminate between 5-HT$_3$ receptors in membrane preparations from the remaining species, including man (Tables 1 and 3). These competition binding studies, regardless of the species, revealed that RS 25259-197 exhibited affinities for 5-HT$_3$ receptors higher than many antagonists thus far reported (Table 4). Moreover, this high affinity was accompanied by a high degree of selectivity in that relatively low affinities at twenty eight other receptors were seen (Table 2). Indeed, up to 1 μM of RS 25259-197 did not exhibit agonist or antagonist action in the guinea-pig ileum contractile assay for 5-HT$_4$ receptor (Leung *et al.*, unpublished data).

The interaction of RS 25259-197 with 5-HT$_3$ receptors was further defined by the use of [^{3}H]-RS 25259-197. In saturation binding studies, the ligand exhibited a high affinity of 5-HT$_3$ receptors in NG-108-15 cells, human kidney 293E1 cells transfected with an α subunit of a murine 5-HT$_3$ receptor (Maricq *et al.*, 1991) and human hippocampus (Table 2). These sites appeared to be pharmacologically similar as judged by the rank orders of competing ligands, although large differences were observed in terms of receptor density. The similarity in B_{max} values between [^{3}H]-RS 42358-197 and [^{3}H]-RS 25259-197 in a given preparation (Table 3) also supports the specificity of [^{3}H]-RS 25259-197 for the 5–HT$_3$ receptor. This differed from data obtained with other radioligands, whereby potential heterogeneity was indicated when [^{3}H]-quipazine, [^{3}H]-GR 65630 and [^{3}H]-granisetron labelled a different number of sites (Wong *et al.*, 1993b). The reasons for this discrepancy remain unclear.

The high density of 5-HT$_3$ receptors in NG 108-15, NCB-20 or N1E-115 neuroblastoma cells has facilitated purification and electrophysiological studies of 5-HT$_3$ receptors (e.g. McKernan *et al.*, 1990; Peters *et al.*, 1991). In contrast, the low density of 5-HT$_3$ receptors in human brain tissue has hindered characterization and, consequently, only a limited number of studies have been reported (Barnes *et al.*, 1989a,b; Waeber *et al.*, 1989; Abi-Dargham *et al.*, 1993). The ability of [^{3}H]-RS 25259-197 to detect low but measurable levels of 5-HT$_3$ receptors, particularly in human tissue, endorses the usefulness of this radioligand in studies of this nature. Indeed, the pharmacological specificity of [^{3}H]-RS 25259-197 obtained from human hippocampus was consistent with previous studies of human brain tissues using other radioligands (Barnes *et al.*, 1989a,b; Waeber *et al.*, 1989; Abi-Dargham *et al.*, 1993). Furthermore, a comparison of this specificity to that observed in NG-108-15 cells argues against a species difference between human and murine 5-HT$_3$ receptors.

Given the fact that RS 25259-197 has the same high affinity for rat brain regions (Table 5) and in NG-108-15 cells, and the similarity in pharmacological specificity of the related radioligand [^{3}H]-RS 42358-197 at the same two preparations, the selectivity of [^{3}H]-RS 25259-197 for the 5-HT$_3$ receptor in the rat brain seems assured. Autoradiographic studies in rat brain with [^{3}H]-RS 25259-197 indicated a regional distribution of sites similar to the distribution reported with [^{3}H]-RS 42358-197 (Table 5), [^{3}H]-GR 65630 (Kilpatrick *et al.*, 1988; 1989), [^{3}H]-quipazine (Perry, 1990), [^{3}H]-tropisetron (Waeber *et al.*, 1989) or [^{3}H]-(S)-zacopride (Waeber *et al.*, 1990). The highest density of receptors was found in the hind-brain vagal complex, while medium levels were found in the spinal trigeminal tract, amygdala, hippocampus and cerebral cortex (Table 5). The higher density of binding sites in emetic centres including nucleus tractus solitarius, dorsal nucleus of the vagus or area postrema, are consistent with a central anti-emetic action of 5-HT$_3$ receptor antagonists. Distribution studies in human brain with [^{3}H]-tropisetron support this observation (Waeber *et al.*, 1989).

In conclusion, RS 25259-197 is a novel, high affinity, selective 5-HT$_3$ receptor antagonist. These properties are consistent with the potent anti-emetic action *in vivo* (Eglen *et al.*, 1995). Finally, the ability of [^{3}H]-RS 25259-197 to label selectively 5-HT$_3$ receptors in human brain or guinea-pig ileum highlight the advantage of the ligand for characterizing receptors in preparations expressing either a low density of sites or low affinities toward 5-HT$_3$ receptor ligands.

References

ABI-DARGHAM, A., LARUELLE, M., WONG, D.T., ROBERTSON, D.W., WEINBERGER, D.R. & KLEINMAN, J.E. (1993). Pharmacological and regional characterization of [^{3}H]LY278584 binding sites in human brain. *J. Neurochem.*, **60**, 730–737.

ANDREWS, P.L. & BHANDARI, P. (1993). The 5-hydroxytryptamine receptor antagonists as antiemetics: preclinical evaluation and mechanism of action. *Eur. J. Cancer*, **29A** (Suppl.), S11–S16.

BARNES, J.M., BARNES, N.M., COSTALL, B., IRONSIDE, J.W. & NAYLOR, R.J. (1989a). Identification and characterization of 5-hydroxytryptamine$_3$ recognition sites in human brain tissue. *J. Neurochem.*, **53**, 1787–1793.

BARNES, J.M., BARNES, N.M., COSTALL, B., NAYLOR, R.J. & TYERS, M.B. (1989b). 5-HT$_3$ receptors mediate inhibition of acetylcholine release in cortical tissue. *Nature*, **338**, 762–764.

BOESS, F.G. & MARTIN, I.L. (1994). Molecular biology of 5-HT receptors. *Neuropharmacol.*, **33**, 275–317.

BONHAUS, D.W., WONG, E.H.F., STEFANICH, E., KUNYSZ, E.A. & EGLEN, R.M. (1993). Pharmacological characterization of 5-hydroxytryptamine$_3$ receptor in murine brain and ileum using the novel radiologand [^{3}H]-RS-42358-197: evidence for receptor heterogeneity. *J. Neurochem.*, **61**, 1927–1932.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BUTLER, A., ELSWOOD, C.J., BURRIDGE, J., IRELAND, S.J., BUNCE, K.T., KILPATRICK, G.J. & TYERS, M.B. (1990). The pharmacological characterization of 5-HT$_3$ receptors in three isolated preparations derived from guinea-pig tissues. *Br. J. Pharmacol.*, **101**, 591–598.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **92**, 881–894.

CLARK, R.D., MILLER, A.B., BERGER, J., REPKE, D.B., WEINHARDT, K.K., KOWALCZYK, B.A., EGLEN, R.M., BONHAUS, D.W., LEE, C.-H., MICHEL, A.D., SMITH, W.L. & WONG, E.H.F. (1993). 2-(quinuclidin-3-yl)pyrido[4,3-b]indol-1-ones and isoquinolin-1-ones. Potent conformationally restricted 5-HT$_3$ receptor antagonists. *J. Med. Chem.*, **36**, 2645–2657.

COSTALL, B. & NAYLOR, R.J. (1991). Pharmacological properties and functions of central 5-HT$_3$ receptors. *Therapie (Paris).*, **46**, 437–444.

COSTALL, B., NAYLOR, R.J. & TYERS, M.B. (1990). The psychopharmacology of 5-HT$_3$ receptors. *Pharmacol. Ther.*, **47**, 181–202.

CRAIG, D.A., EGLEN, R.M., WALSH, L.K., PERKINS, L.A., WHITING, R.L. & CLARKE, D.E. (1990). 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a discriminative tool for the 5-HT$_3$ and putative 5-HT$_4$ receptors in guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 9–16.

DERKACH, V., SUPRENANT, A. & NORTH, R.A. (1989). 5-HT$_3$ receptors are membrane ion channels. *Nature*, **339**, 706–709.

EGLEN, R.M., LEE, C., SMITH, W.L., JOHNSON, L.G., WHITING, R.L. & HEGDE, S.S. (1993). RS-42358-197, a novel and potent 5-HT$_3$ receptor antagonist, *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.*, **266**, 535–543.

EGLEN, R.M., LEE, C.H., SMITH, W.L., JOHNSON, L.G., WHITING, R.L. & HEGDE, S.S. (1995). Pharmacological characterization of RS-25259-197, a novel and selective 5-HT$_3$ receptor antagonist, *in vivo*. *Br. J. Pharmacol.*, **114**, 860–866.

EGLEN, R.M., SWANK, S.R., WALSH, L.K.M. & WHITING, R.L. (1990). Characterization of 5-HT$_3$ and 'atypical' 5-HT receptors mediating guinea-pig ileal contractions. *in vitro*. *Br. J. Pharmacol.*, **101**, 513–520.

FERNANDEZ, A.G., PUIG, J., BOU, J., FRISTWOOD, R.W. & BERGA, P. (1990). Pancopride: a novel 5-HT$_3$ antagonist with potent antiemetic action. *Eur. J. Pharmacol.*, **183**, P.we.046.

FOZARD, J.R. (1984). Neuronal 5-HT$_3$ receptors in the periphery. *Neuropharmacol.*, **23**, 1473–1486.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory In *Handbook of Experimental Pharmacology*, Vol. 33, pp. 283–335. Berlin: Springer Verlag.

GREENSHAW, A.J. (1993). Behavioral pharmacology of 5-HT$_3$ receptor antagonists: a critical update on therapeutic potential. *Trends Pharmacol. Sci.*, **14**, 265–270.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHERY, P.P.A. (1994). VII. International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.*, **46**, 157–203.

HOYER, D. & NEIJT, H.C. (1988). Identification of serotonin 5-HT$_3$ recognition sites in membranes of N1E-115 neuroblastoma cells by radioligand binding. *Mol. Pharmacol.*, **33**, 303–309.

JOHNSON, R.M., INOUYE, G.T., EGLEN, R.M. & WONG, E.H.F. (1993). 5-HT$_3$ receptor ligands lack modulatory influence on acetylcholine release in rat entorhinal cortex. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 241–247.

KILPATRICK, G.J., JONES, B.J. & TYERS, M.B. (1988). The distribution of specific binding of the 5-HT$_3$ receptor ligand [^{3}H] GR65630 in rat brain using quantitative autoradiography. *Neurosci. Lett.*, **94**, 156–160.

KILPATRICK, G.J., JONES, B.J. & TYERS, M.B. (1989). Binding of the 5-HT$_3$ ligand, [^{3}H] GR65630, rat area postrema, vagus nerve and the brains of several species. *Eur. J. Pharmacol.*, **159**, 157–164.

KILPATRICK, G.J. & TYERS, M.B. (1992). Inter-species variants of the 5-HT$_3$ receptor. *Biochem. Soc. Trans.*, **20**, 118–121.

LAPORTE, A.M., KOSCIELNIAK, T., PONCHANT, M., VERGE, D., HAMON, M. & GOZLAN, H. (1992). Quantitative autoradiography mapping of 5-HT$_3$ receptors in the rat CNS using [^{125}I]iodozacopride and [^{3}H]zacopride as radioligands. *Synapse*, **10**, 271–281.

LEUNG, E., MICHELSON, S., VILLARUBIA, C., PERKINS, L.A. & EGLEN, R.M. (1992). Analysis of concentration-response relationships by seemingly unrelated nonlinear regression (SUNR) technique. *J. Pharmacol. Toxicol. Methods*, **28**, 209–216.

MARICQ, A.V., PETERSON, A.S., BRAKE, A.J., MYERS, R.M. & JULIUS, D. (1991). Primary structure and functional expression of the 5-HT$_3$ receptor, a serotonin-gated ion channel. *Science*, **254**, 432–436.

MARTIN, G.R. & HUMPHREY, P.P.A. (1994). Receptors for 5-hydroxytryptamine: current perspective on classification and nomenclature. *Neuropharmacol.*, **33**, 261–273.

McKERNAN, R.M., GILLARD, N.P., QUIRK, K., KNEEN, C.O., SYEVENSON, G.I., SQAIN, C.J. & RAGAN, C.I. (1990). Purification of the 5-hydroxtryptamine 5-HT$_3$ receptors from NCB-20 cells. *J. Biol. Chem.*, **265**, 13572–13577.

MILBURN, D.M. & PEROUTKA, S.J. (1989). Characterization of [^{3}H]quipazine binding to 5-hydroxytryptamine$_3$ receptors in rat brain membranes. *J. Neurochem.*, **52**, 1787–1792.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerized approach for characterization of ligand systems. *Anal. Biochem.*, **107**, 220–239.

NELSON, D.R. & THOMAS, D.R. (1989). [^{3}H]-BRL43694 (Granisetron), a specific ligand for 5-HT$_3$ binding sites in rat brain cortical membranes. *Biochem. Pharmacol.*, **38**, 1693.

NEWBERRY, N.R., CHESHIRE, S.H. & GILBERT, M.J. (1991). Evidence that 5-HT$_3$ receptors of the rat, mouse and guinea-pig superior cervical ganglion may be different. *Br. J. Pharmacol.*, **102**, 615–620.

OXFORD, A.W., BELL, J.A., KILPATRICK, G.J., IRELAND, S.J. & TYERS, M.B. (1992). Ondansetron and related 5-HT$_3$ antagonists: recent advances. *Prog. Med. Chem.*, **29**, 239–270.

PARKER, P.B. & WAUD, D.R. (1971). Pharmacological estimates of drug-receptor dissociation constants. Statistical evaluation. I. Agonist. *J. Pharmacol. Exp. Ther.*, **177**, 1–12.

PAXINOS, G. & WATSON, C. (1985). *The Rat Brain in Stereotaxic Coordinates*, 2nd edition. New York: Academic Press.

PERRY, D.C. (1990). Autoradiography of [^{3}H]quipazine in rodent brain. *Eur. J. Pharmacol.*, **187**, 75–85.

PETERS, J.A., MALONE, H.M. & LAMBERT, J.J. (1991). Characterization of 5-HT$_3$ receptor mediated electrical response in nodose ganglion neurones and clonal neuroblastoma cells maintained in culture. In *Serotonin: Molecular Biology, Receptors and Functional Effects* ed. Fozard, J.R. & Saxena, P.R. pp. 84–94. Basel: Birkhauser.

PINKUS, L., SARBIN, N.S., BAREFOOT, D.S. & GORDON, J.C. (1989). Association of [^{3}H]zacopride with 5-HT$_3$ binding sites. *Eur. J. Pharmacol.*, **168**, 355–362.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc. B.*, **164**, 488–510.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

SHARIF, N.A., WONG, E.H.F., LOURY, D.N., STEFANICH, E., MICHEL, A.D., EGLEN, R.M. & WHITING, R.L. (1991). Characterization of 5-HT$_3$ binding sites in NG108-15, NCB-20 neuroblastoma cells and rat cerebral cortex using [^{3}H]-quipazine and [^{3}H]-GR 65630 binding. *Br. J. Pharmacol.*, **102**, 919–925.

TRICKLEBANK, M.D. (1989). Interactions between dopamine and 5-HT$_3$ receptors suggest new treatments for psychosis and drug addiction. *Trends Pharmacol. Sci.*, **10**, 127–129.

WAEBER, C., HOYER, D. & PALACIOS, J.M. (1989). 5-Hydroxytryptamine$_3$ receptors in the human brain: autoradiographic visualization using [^{3}H]ICS 205,930. *Neurosci.*, **31**, 393–400.

WAEBER, C., DIXON, K., HOYER, D. & PALACIOS, J.M. (1990). The (S)-isomer of [^{3}H]zacopride labels 5-HT$_3$ receptors with high affinity in rat brain. *Eur. J. Pharmacol.*, **181**, 283–297.

WATLING, K.J., ASPLEY, S., SWAIN, C.J. & SAUNDERS, J. (1988). [^{3}H] Quaternised ICS 205-930 labels 5-HT$_3$ receptor binding sites in rat brain. *Eur. J. Pharmacol.*, **149**, 397–398.

WONG, D.T., ROBERTSON, D.W. & REID, L.R. (1989). Specific [^{3}H]LY 278584 binding to 5-HT$_3$ recognition sites in rat cerebral cortex. *Eur. J. Pharmacol.*, **166**, 107–110.

WONG, E.H.F., BONHAUS, D.W., STEFANICH, E. & EGLEN, R.M. (1993a). Labelling of 5-hydroxytryptamine$_3$ receptors with a novel 5-HT$_3$ receptor ligand, [^{3}H]RS-42358-197. *J. Neurochem.*, **60**, 921–930.

WONG, E.H.F., BONHAUS, D.W., LEE, J.A., WU, I., LOURY, D.N. & EGLEN, R.M. (1993b). Different densities of 5-HT$_3$ receptors are labelled by [^{3}H]quipazine, [^{3}H]GR 65630 and [^{3}H]granisetron. *Neuropharmacol.*, **32**, 869–875.

YAKEL, J.L. (1992). 5-HT$_3$ receptors as cation channels. In *Central and Peripheral 5-HT$_3$ Receptors*, ed. Hamon, M. pp. 103–128, London, England: Academic Press.

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Pharmacological characterization of RS 25259-197, a novel and selective 5-HT_3 receptor antagonist, *in vivo*

[1]R.M. Eglen, C.-H. Lee, W.L. Smith, L.G. Johnson, *R. Clark, R.L. Whiting & S.S. Hegde

Institute of Pharmacology, *Institute of Organic Chemistry, Syntex Discovery Research, 3401 Hillview Avenue, Palo Alto, CA-94304, U.S.A.

1 The pharmacological effects *in vivo*, of RS 25259-197, a selective 5-HT_3 receptor antagonist, have been investigated.

2 In anaesthetized rats, RS 25259-197, administered by the intravenous, intraduodenal or transdermal route, dose-dependently inhibited the von Bezold-Jarisch reflex induced by 2-methyl 5-HT (ID_{50} = 0.04 μg kg^{-1}, i.v., 3.2 μg kg^{-1}, i.d. and 32.8 μg per chamber, respectively). In this regard, when administered intraduodenally, RS 25259-197 was more potent and exhibited a longer duration of action than either ondansetron or granisetron.

3 In conscious ferrets, RS 25259-197, administered intravenously or orally, dose-dependently inhibited emesis induced by cisplatin. The ID_{50} estimates of RS 25259-197 were 1.1 μg kg^{-1}, i.v. and 3.2 μg kg^{-1}, p.o. In this respect, RS 25259-197 was more potent than ondansetron and equipotent with granisetron.

4 In conscious dogs, RS 25259-197, administered intravenously or orally, dose-dependently inhibited emesis induced by cisplatin (ID_{50} = 1.9 μg kg^{-1}, i.v. and 8.5 μg kg^{-1}, p.o.), dacarbazine (ID_{50} = 4.1 μg kg^{-1}, i.v. and 9.7 μg kg^{-1}, p.o.), actinomycin D (ID_{50} = 4.9 μg kg^{-1}, i.v. and 2.5 μg kg^{-1}, p.o.) and mechlorethamine (ID_{50} = 4.4 μg kg^{-1}, i.v. and 3.0 μg kg^{-1}, p.o.). Against each of the emetogenic agents, RS 25259-197 was very much more potent than ondansetron. When tested at equi-effective intravenous doses against cisplatin-induced emesis in dogs, RS 25259-197 had a longer duration of anti-emetic activity (7 h) than ondansetron (4 h). At doses up to and including 1000 μg kg^{-1}, p.o., neither RS 25259-197 nor ondansetron was capable of inhibiting apomorphine-induced emesis.

5 At doses up to 1000 μg kg^{-1}, i.v., RS 25259-197 produced no meaningful haemodynamic changes in anaesthetized dogs.

6 In summary, RS 25259-197 is a novel, highly potent and orally active 5-HT_3 receptor antagonist *in vivo*. With respect to its anti-emetic activity, RS 25259-197 appears to be a significant improvement over ondansetron in terms of potency and duration of action.

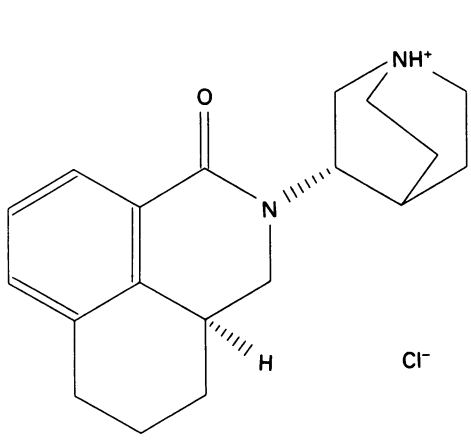
Keywords: 5-HT_3 antagonist; RS 25259-197; RS 25259; ondansetron; emesis; cisplatin; cancer chemotherapy; anti-emetic; von Bezold-Jarisch reflex

Introduction

It is now well established that the side-effects of nausea and emesis associated with anti-cancer therapy are due to activation of 5-HT_3 receptors at a peripheral and/or central locus (Kilpatrick *et al.*, 1990; Tyers, 1991). The vast majority of evidence favours a peripheral mechanism involving activation of 5-HT_3 receptors, localized on vagal afferents supplying the upper gastrointestinal tract, by 5-HT released from enterochromaffin cells as a consequence of exposure to cytotoxic drugs or X-ray irradiation (Costall & Naylor, 1992). The advent of selective 5-HT_3 receptor antagonists has shown that, in cancer patients, these drugs are highly effective in preventing chemotherapy-induced emesis and therefore improving the quality of life.

Numerous 5-HT_3 receptor antagonists have been synthesized to date. The most widely characterized compounds include ondansetron, granisetron, zacopride and tropisetron (Oxford *et al.*, 1992). Emerging evidence indicates that significant differences in potency, efficacy and duration of action exist between the compounds which may have an impact on their clinical usefulness (Marr *et al.*, 1991; Andrews *et al.*, 1992). For example, the anti-emetic effects have been reported to be dose-dependent for granisetron but not for ondansetron (Andrews *et al.*, 1992). Also, in the ferret, granisetron was reported to be more potent as an anti-emetic when administered by the oral route than the intravenous route, whereas the reverse was true for ondansetron (Fitzpatrick *et al.*, 1990). Ondansetron, the most extensively studied 5-HT_3 receptor antagonist, is poorly effective in controlling mild and delayed emesis (Kris *et al.*, 1992; Levitt *et al.*, 1993). Furthermore, none of the currently available 5-HT_3 receptor antagonists is capable of completely blocking the incidence of nausea and emesis in cancer patients. Reports of side-effects, such as chest pain and extrapyramidal symptoms with ondansetron (Ballard *et al.*, 1992; Halpern & Murphy, 1992), and animal toxicity such as granisetron-induced hepatic carcinoma (Joss & Dott, 1993) may also limit the clinical utility of these compounds. Hence, there is clearly a need for the development of selective 5-HT_3 receptor antagonists which are more safe, potent and efficacious. RS 25259-197((3aS)-2-[(S)-1-azabicyclo[2.2.2]oct-3-yl]2,3,3a,4,5,6-hexahydro-1-oxo-1-1*H*-benz[*de*] isoquinoline hydrochloride) is a novel selective 5-HT_3 receptor antagonist (Wong *et al.*, 1995) (Figure 1). Homogenate radioligand binding and functional pharmacological studies have established that RS 25259-197 exhibits a high affinity at 5-HT_3 receptors (pK_i = 10.4 at 5-HT_3 receptors in rat cortex and $pA_2 \sim 8.8$ at 5-HT_3 receptors in guinea-pig ileum). In the present paper, we present our findings on the *in vivo* pharmacological effects of this compound using ondansetron and/or granisetron as reference compounds.

[1] Author for correspondence.



RS-25259-197

Figure 1 Chemical structure of RS 25259-197 ((3aS)-2-[(S)-1-azabicyclo[2.2.2]oct-3-yl]-2,3,3a,4,5,6-hexahydro-1-oxo-1-1H-benz[de] isoquinoline hydrochloride).

Methods

Studies on the von Bezold-Jarisch reflex in rats

Intravenously administered 5-HT or 2-methyl 5-HT elicits an immediate and short-lasting vagally mediated reflex bradycardia (referred to as the Von Bezold-Jarisch reflex) which is specifically mediated by 5-HT$_3$ receptors and is antagonized by selective 5-HT$_3$ receptor antagonists (Fozard, 1984). RS 25259-197 was evaluated for its ability to inhibit this reflex, using ondansetron and granisetron as reference compounds.

Male Sprague Dawley rats (Charles River, 250–380 g) were given food and water *ad libitum*, except those used for intraduodenal drug administration; these rats were deprived of food overnight. Rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and placed on an aquatic K module heating pad to maintain body temperature at 37°C. The left jugular vein or duodenum, trachea and left femoral vein were cannulated for drug administration (i.v. or i.d.), facilitation of respiration and injection of 2-methyl 5-HT, respectively. Heart rate was derived from a limb lead II ECG monitored via subdermal platinum electrodes and was recorded with ECG/Biotach amplifiers connected to a Gould recorder (RS 3800). A dose-response curve to 2-methyl 5-HT (5–100 μg kg^{-1}, i.v.) was constructed in each rat to establish a submaximal dose (usually 10 or 20 μg kg^{-1}, i.v.) which would elicit a reproducible bradycardic response. Each rat then received a single dose of RS 25259-197, ondansetron or granisetron and was then challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 420 and 480 min post dosing. A separate group of rats receiving vehicle (saline for i.v., deionized water for i.d.) was similarly tested in each study. Preliminary experiments showed that there was no evidence of tachyphylaxis in the responses to 2-methyl 5-HT. Duration of action of the compounds was assessed by determining the period of time for which the inhibitory effects remained significantly different from vehicle controls.

In a separate series of experiments, RS 25259-197, when applied topically on the abdominal skin of rats, was evaluated for its ability to inhibit the reflex. An aqueous solution of the drug was absorbed onto a Hill Top chamber (25 mm in diameter) in a volume of 0.4 ml. A submaximal dose of 2-methyl 5-HT that could elicit a reproducible bradycardic response was first determined in each rat. RS 25259-197 (0.01–1000 μg per chamber) was then applied topically to the depilitated abdominal area and the rat was challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240 and 300 min post dose.

Anti-emetic studies in ferrets and dogs

The ferret (Florczyk *et al.*, 1982) and the dog (Gylys *et al.*, 1979) are well established animal models of emesis which respond to cancer chemotherapeutic agents in a manner similar to that observed in man.

Adult male ferrets (1–1.4 kg, Marshall farms) were randomly assigned to different treatment groups. Each animal was anaesthetized with metofane inhalant. A jugular vein was cannulated and exteriorized from the outside of the neck. Following recovery from anaesthesia, each animal was dosed with either RS 25259-197 (1–100 μg kg^{-1}, i.v. or 0.3–100 μg kg^{-1}, p.o.), ondansetron (30–1000 μg kg^{-1}, p.o.) or vehicle (1 ml kg^{-1}, i.v. or p.o.) 30 min prior to administration of cisplatin (10 mg kg^{-1}, i.v.). The compounds were given orally in hard gelatin capsules. Each animal was counted for the number of emetic episodes for 5 h following cisplatin administration.

Adult male dogs (8–20 kg) (Hazleton Research Products) were randomly assigned to different treatment groups. Each animal was dosed with either RS 25259-197 (0.3–300 μg kg^{-1}, i.v. or p.o.), vehicle control (0.1 ml kg^{-1}, i.v. or p.o.), ondansetron (1–1000 μg kg^{-1}, i.v. or p.o.) or haloperidol (5 mg kg^{-1}, p.o.) at 30 min prior to the administration of apomorphine (0.1 mg kg^{-1}, s.c.), or 120 min prior to the administration of either dacarbazine (30 mg kg^{-1}, i.v.) or mechlorethamine (0.4 mg kg^{-1}, i.v.) and actinomycin D (0.15 mg kg^{-1}, i.v.) or at 60 min after the administration of cisplatin (3 mg kg^{-1}, i.v.). The compounds were given intravenously via the cephalic vein or orally in hard gelatin capsules. Each animal was counted for the number of emetic episodes for 5 h following the administration of each emetic. An emetic episode was defined as the successful evacuation of stomach contents. In separate experiments designed to examine the duration of anti-emetic activity, dogs were pretreated with vehicle (0.1 ml kg^{-1}, i.v.), RS 25259-197 (30 μg kg^{-1}, i.v.) or ondansetron (300 μg kg^{-1}, i.v.) at 24, 12, 8, 7, 6, 5, 4, 3, 2 or 1 h prior to the administration of cisplatin, after which each animal was observed for an additional 5 h.

Haemodynamic studies in anaesthetized dogs

Mongrel dogs (4 male, 4 female, 11–16 kg, Hazelton Laboratories) were anaesthetized with pentobarbitone sodium (36 mg kg^{-1}, i.v.). The dogs were then artificially ventilated with Harvard respirators. A catheter inserted into a carotid artery was used for measurement of arterial pressure and for blood sampling (pH/blood gas analysis with a Corning Model 168 analyser). The ipsilateral femoral vein was cannulated for the administration of test compound or vehicle and for the administration of a maintenance infusion of pentobarbitone sodium (3–8 mg kg^{-1} h^{-1}). A Millar 7F Mikro-Tip Catheter pressure transducer was inserted into the left ventricle for the measurement of left ventricular systolic pressure (LVP) and left ventricular end-diastolic pressure (LVEDP), and dp/dt_{max} was obtained by electronic differentiation of the LVP signal. The right external jugular vein was isolated and a 5F Swan-Ganz catheter (Spectramed SP5105H) placed in the pulmonary artery to permit determination of cardiac output (CO) by thermodilution using a Gould Cardiac Index Computer (Model 435). Heart rate (HR) was measured with a cardiotachometer triggered by the R-wave of a limb lead II ECG recorded from needle electrodes inserted subcutaneously. Mean arterial pressure (MAP) was calculated as 1/3 (systolic arterial pressure-diastolic arterial pressure) + diastolic arterial pressure. Directly monitored parameters as well as electronically derived dp/dt_{max} and HR were recorded on a polygraph (Gould RS 3800).

Following completion of instrumentation, each dog was allowed to equilibrate for 30–60 min before beginning an experiment. Each dog received vehicle (0.02 ml kg^{-1}, i.v.) or

a single dose of RS 25259-197 (10, 100 and 1000 μg kg^{-1}, i.v.). Recorded parameters were measured immediately prior to the first treatment dose and at 2, 5, 15, 30, 45 and 60 min after each dose.

Statistical analysis

Statistical analysis of the data was performed by a repeated measure analysis of variance (ANOVA) and, in some cases, was followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test. Statistical significance was defined as $P<0.05$. In emetic studies, an ID_{50} (dose required to produce 50% of the maximal inhibition) was calculated wherever appropriate. ID_{50} was calculated with NONLIN84 software, a nonlinear modelling programme.

Materials

RS 25259-197, granisetron and ondansetron were synthesized at the Institute of Organic Chemistry, Syntex Research. 5-HT, 2-methyl 5-HT, actinomycin D, cisplatin, dacarbazine, mechlorethamine and apomorphine were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.).

Results

Studies on the von Bezold-Jarisch reflex

When administered intravenously, RS 25259-197 (0.01–10 μg kg^{-1}), ondansetron (1–300 μg kg^{-1}) and granisetron (0.03–300 μg kg^{-1}) produced dose-dependent inhibition of the 2-methyl 5-HT induced bradycardic response, yielding ID_{50} estimates (95% confidence interval) of 0.04(0.02–0.06), 2.2 (0.5–3.96) and 0.1(0.01–0.19) μg kg^{-1}, respectively (Figure 2a). When administered intraduodenally, RS 25259-197, ondansetron and granisetron produced dose-dependent inhibition of the bradycardic response (Figure 2b). By the i.d. route, RS 25259-197 was much more potent (ID_{50} (95% confidence interval)): [3.2(2.7–3.7 μg kg^{-1}] than ondansetron [144(53.2–234.2) μg kg^{-1}] and granisetron [49.1(21.2–76.9) μg kg^{-1}]. The duration of inhibitory effects was dose-dependent for all three compounds (Figure 3a–c). At equi-effective doses (lowest dose required to produce 100% inhibition) the duration of the inhibitory effects for RS 25259-197 (10 μg kg^{-1}, i.d., 420 min) was greater than that of ondansetron (1000 μg kg^{-1}, i.d., 300 min) and granisetron (300 μg kg^{-1}, i.d., 300 min).

Transdermal administration of RS 25259-197 (0.01–1000 μg/chamber) produced a dose-dependent inhibition of the bradycardia induced by 2-methyl 5-HT (Figure 4a) yielding an ID_{50} (95% confidence interval) of 32.8 (12.8–52.7) μg per chamber. The onset of the inhibitory effects was inversely proportional to the concentration of the drug in the chamber (Figure 4b). At doses of 1–100 μg per chamber, the inhibitory effects of the drug lasted for greater than 300 min.

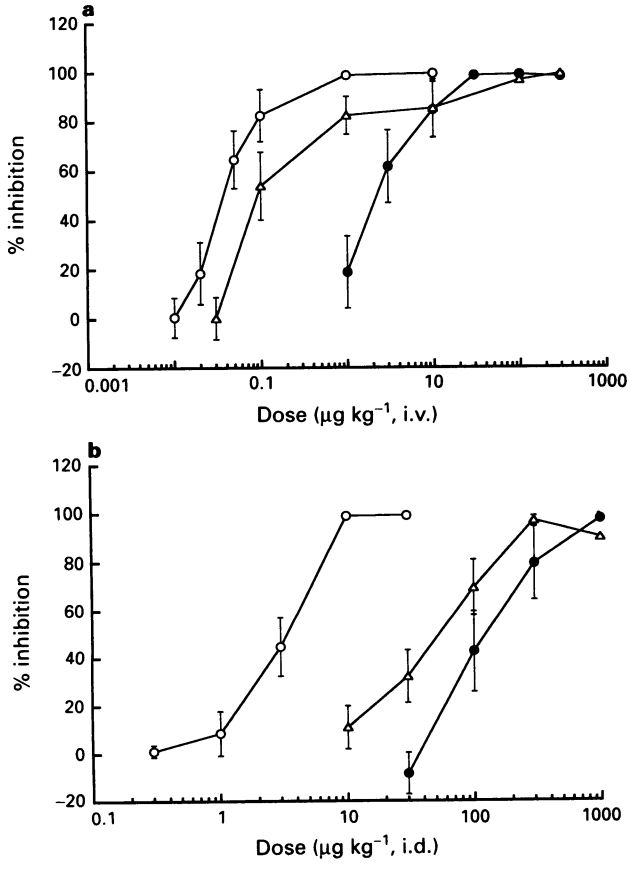


Figure 2 Inhibitory effects of RS 25259-197 (○), ondansetron (●) and granisetron (△), administered intravenously (a) and intraduodenally (b), on the 2-methyl 5-HT-induced bradycardia. Each point represents the mean ± s.e.mean, $n = 8–10$.

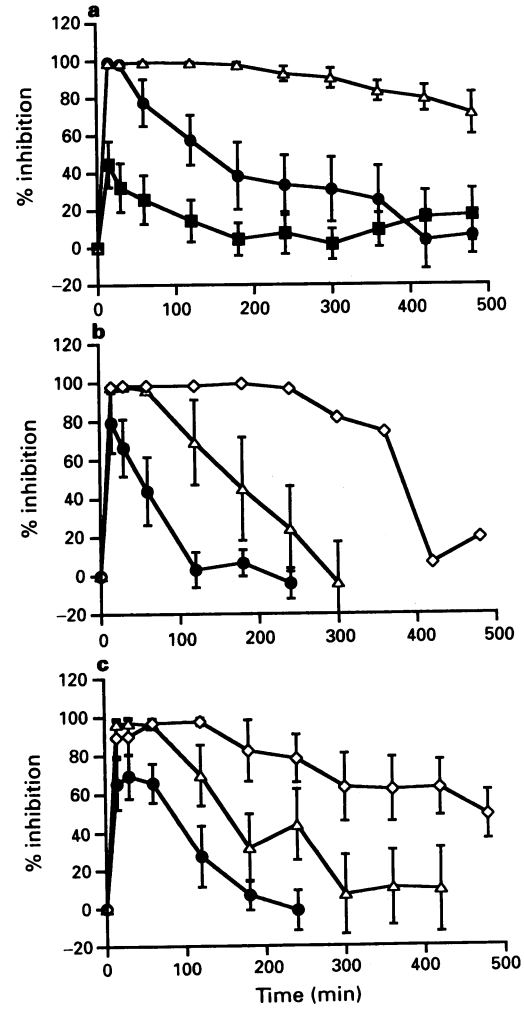


Figure 3 Duration of the inhibitory effects of RS 25259-197 (a), ondansetron (b) and granisetron (c) on the 2-methyl-5-HT-induced bradycardia. (a) RS 25259-197, (■) 3 μg kg^{-1}, i.d., (●) 10 μg kg^{-1}, i.d., (△) 30 μg kg^{-1}, i.d., (b) Ondansetron, (●) 300 μg kg^{-1}, i.d., (△) 1000 μg kg^{-1}, i.d., (◇) 3000 μg kg^{-1}, i.d. (c) Granisetron, (●) 100 μg kg^{-1}, i.d., (△) 300 μg kg^{-1}, i.d., (◇) 1000 μg kg^{-1}, i.d. Each point represents the mean ± s.e.mean, $n = 8–10$.

Anti-emetic activity in ferrets and dogs

Compared with vehicle control, RS 25259-197 (1–30 μg kg^{-1}, i.v. and 1–30 μg kg^{-1}, p.o.), ondansetron (30–1000 μg kg^{-1}, p.o.) and granisetron (3–300 μg kg^{-1}, p.o.) given 30 min prior to cisplatin produced significant and dose-dependent reduction in the number of emetic episodes in ferrets (Figure 5, Table 1). When administered orally, RS 25259-197 was 2 and 13 fold more potent than granisetron and ondansetron, respectively.

As shown in Figure 6 and Table 2, RS 25259-197 (0.3–100 μg kg^{-1}, i.v. and 1–100 μg kg^{-1}, p.o.) and ondansetron (3–300 μg kg^{-1}, i.v. and 10–100 μg kg^{-1}, p.o.) produced dose-dependent inhibition of the emesis induced by cisplatin, actinomycin D, dacarbazine and mechlorethamine in dogs. ID_{50} estimates are shown in Table 3. When administered orally, RS 25259-197 was about 30 fold more potent than ondansetron as an anti-emetic against each of the emetogenic agents. In studies designed to examine the duration of anti-emetic activity, RS 25259-197 (30 μg kg^{-1}, i.v.) was effective for 7 h in inhibiting cisplatin-induced emesis, whereas the anti-emetic activity of ondansetron (300 μg kg^{-1}, i.v.) lasted for 4 h (Figure 7).

Neither RS 25259-197 (1–1000 μg kg^{-1}, p.o.) nor ondansetron (1000 μg kg^{-1}, p.o.) were effective in inhibiting apomorphine induced emesis in dogs (data not shown). In contrast, haloperidol (5 mg kg^{-1}, p.o.) produced significant inhibition of apomorphine induced emesis (data not shown).

Haemodynamic effects in anaesthetized dogs

Baseline values for the measured parameters did not differ significantly between the vehicle and RS 25259-197 treatment groups. Administration of vehicle or RS 25259-197 had no significant effects on MAP, CO, *dP/dT* and SVR (data not

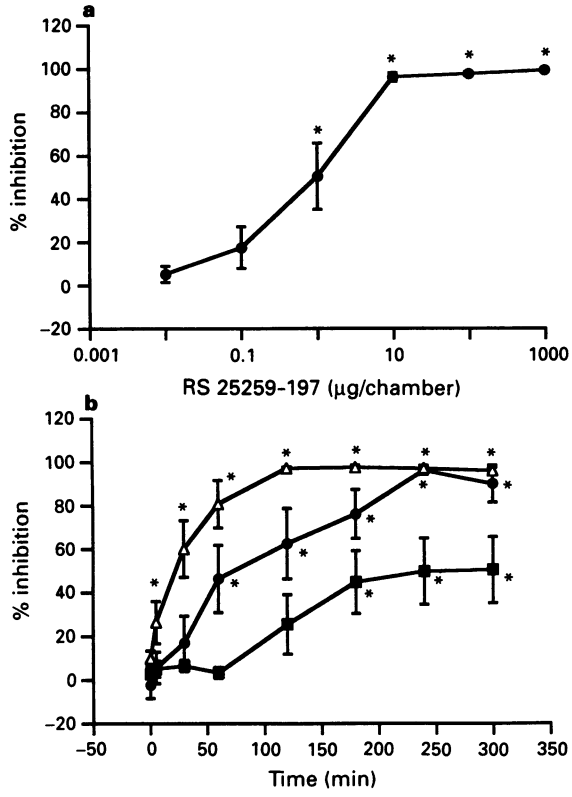


Figure 4 Inhibitory effects of RS 25259-197 (administered transdermally) on the 2-methyl 5-HT-induced bradycardia. (a) Dose-response curve to RS 25259-197. (b) Duration of the inhibitory effects of RS 25259-197, (■) 1 μg per chamber, (●) 10 μg per chamber, (Δ) 100 μg per chamber. Each point represents the mean ± s.e.mean, $n = 10$. *Significantly different ($P < 0.05$) from vehicle control.

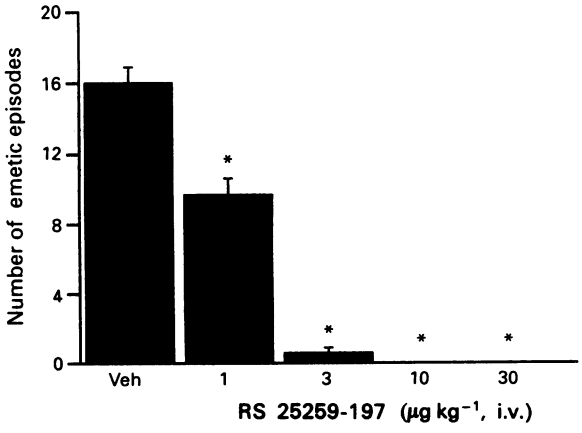


Figure 5 Inhibitory effects of intravenously administered RS 25259-197 on cisplatin-induced emesis in ferrets. Each column represents the mean ± s.e.mean, $n = 6$. *Significantly different ($P < 0.05$) from vehicle control (Veh).

Table 1 ID_{50} estimates of RS 25259-197, ondansetron and granisetron against cisplatin-induced emesis in ferrets

	ID_{50} (μg kg^{-1} (95% confidence intervals))	
	i.v.	*p.o.*
RS 25259-197	1.1 (1.0–1.2)	3.2 (1.6–4.8)
Ondansetron	ND	43 (18.0–100)
Granisetron	ND	6.9 (1.0–40)

ND = not determined.

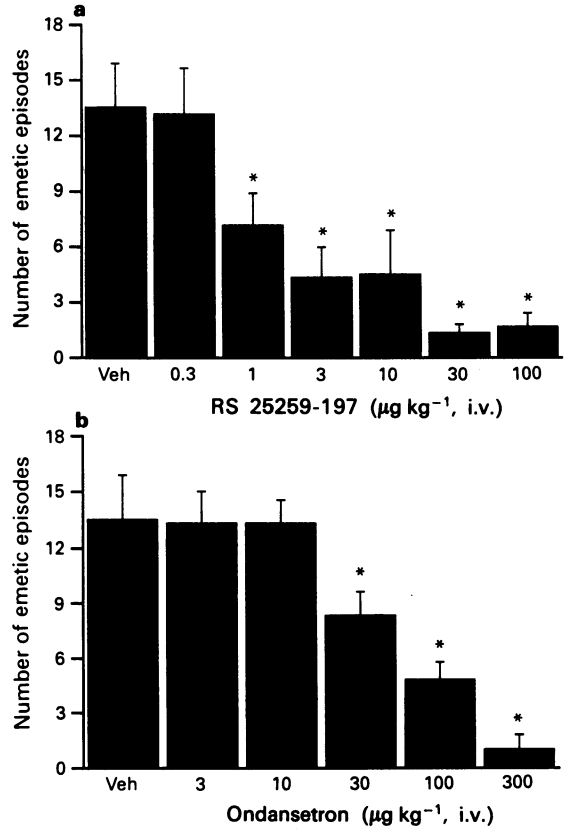


Figure 6 Inhibitory effects of intravenously administered RS 25259-197 (a) and ondansetron (b) on cisplatin-induced emesis in dogs. Each point represents the mean ± s.e.mean, $n = 6$. *Significantly different ($P < 0.05$) from vehicle control (Veh).

shown). RS 25259-197 produced a significant decrease in HR at 1000 μg kg^{-1}, i.v. but only at 45 min post-drug (Figure 8).

Discussion

The data obtained in the present study show that RS 25259-197, a novel 5-HT$_3$ receptor antagonist, displays several pharmacodynamic and pharmacokinetic differences from the 5-HT$_3$ receptor antagonists, ondansetron and granisetron.

Studies on the Von Bezold-Jarisch reflex

Inhibition of the 2-methyl 5-HT induced bradycardia (Von Bezold-Jarisch reflex) is a useful test for the evaluation of 5-HT$_3$ receptor antagonists *in vivo*. In this assay, RS 25259-197 administered either intravenously, intraduodenally or transdermally, was effective in inhibiting the responses to 2-methyl 5-HT. By the intravenous route, RS 25259-197 was approximately 3 and 55 fold more potent than granisetron and ondansetron, respectively. The affinity of RS 25259-197

Table 2 Anti-emetic activity of RS 25259-197 in dogs

		Number of emetic episodes			
Drug	*Dose*	*Dacarbazine* (30 mg kg^{-1}, i.v.)	*Actinomycin D* (0.15 mg kg^{-1}, i.v.)	*Mechlorethamine* (0.4 mg kg^{-1}, i.v.)	*Cisplatin* (3 mg kg^{-1}, i.v.)
Vehicle (ml kg^{-1}, p.o.)	0.1	14.2 ± 1.6	14.5 ± 2.3	9.5 ± 2.9	14.2 ± 3.3
RS 25259-197	1	NT	NT	NT	12.8 ± 1.9
(μg kg^{-1}, p.o.)	3.2	12.8 ± 2.7	5.8 ± 0.6*	4.5 ± 1.4	16.2 ± 2.1
	10	6.8 ± 0.2*	6.7 ± 1.6*	2.8 ± 1.9*	4.0 ± 1.6*
	31.6	0.3 ± 0.2*	3.3 ± 1.9*	0.3 ± 0.3*	2.7 ± 0.7*
	100	0 ± 0*	1.0 ± 0.7*	0 ± 0*	0 ± 0*
Ondansetron	10	NT	NT	NT	12.7 ± 1.2
(μg kg^{-1}, p.o.)	31.6	10.8 ± 1.1	9.8 ± 3.1	5.7 ± 1.0	18.3 ± 3.4
	100	6.2 ± 2.2*	10.3 ± 1.9	8.5 ± 1.3	10.5 ± 1.3
	316	3.0 ± 1.2*	4.2 ± 0.8*	2.8 ± 1.5*	3.2 ± 0.5*
	1000	0.2 ± 0.2*	1.5 ± 0.6*	0 ± 0*	2.0 ± 0.9*

All values are expressed as mean ± s.e.mean ($n = 6$ per group).
NT = not tested.
*Statistically significant from vehicle control ($P < 0.05$).

Table 3 ID_{50} (μg kg^{-1}) estimates of RS 25259-197 and ondansetron against various emetogenic agents in dogs

		Emetogenic agent			
Drug	*Route of administration*	*Cisplatin*	*Dacarbazine*	*Actinomycin D*	*Mechlorethamine*
RS 25259-197	i.v.	1.9 (0.28–13.0)	4.1 (2.1–6.1)	4.9 (0.45–54)	4.4 (2.1–9.1)
	p.o.	8.5 (3.6–20)	9.7 (7.3–12.0)	2.5[$]	3.0 (0.85–10.0)
Ondansetron	i.v.	46.0 (17.0–74.0)	80.0[$]	200.0 (47.0–900)	36.0[$]
	p.o.	160.0 (48.0–270)	83.0[$]	160.0[$]	280.0[$]

[$]Meaningful confidence intervals could not be obtained because of lack of dose-dependency.

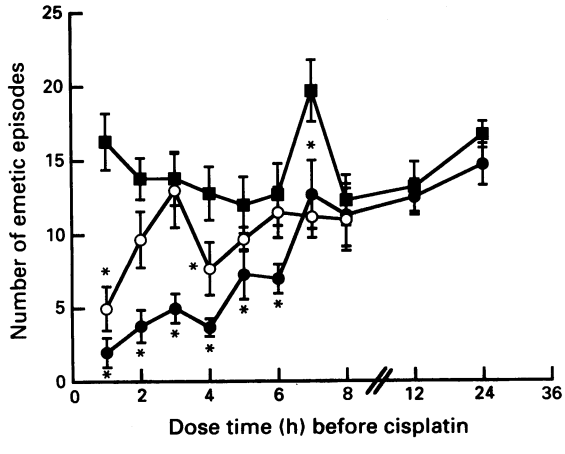


Figure 7 Duration of the inhibitory effects of vehicle (0.1 ml kg^{-1}) (■), RS 25259-197 (30 μg kg^{-1}, i.v.) (●) and ondansetron (300 μg kg^{-1}, i.v.) (○) on cisplatin-induced emesis in dogs. Dogs were pre-treated with the drugs at 24, 12, 8, 7, 6, 5, 4, 3, 2 and 1 h prior to the administration of cisplatin. Each point represents the mean ± s.e.mean, $n = 6$ *Significantly different ($P < 0.05$) from vehicle control.

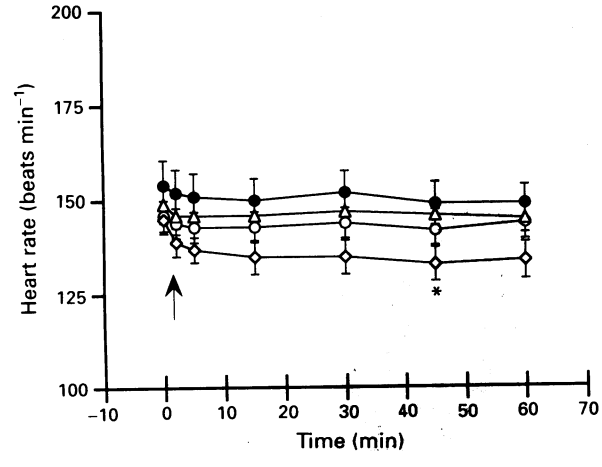


Figure 8 Effects of intravenously administered RS 25259-197 on heart rate (HR) in anaesthetized dogs. (○) Vehicle, (●) RS 25259-197 (10 μg kg^{-1}, i.v.), (△) RS 25259-197 (10 μg kg^{-1}, i.v.), (◇) RS 25259-197 (1000 μg kg^{-1}, i.v.). Each point represents the mean ± s.e.mean, $n = 6$. *Significantly different ($P < 0.05$) from vehicle control.

for displacing [^{3}H]-quipazine in the rat cerebral cortex is 10.4 (Wong *et al.*, 1994) whereas the reported pK_i values for ondansetron and granisetron in rat tissues range from 8.3–9.0 (for ondansetron) and from 9.2–9.8 (for granisetron) (Kilpatrick *et al.*, 1990). Thus the order of potency in inhibiting the Von Bezold-Jarisch reflex parallels the relative affinities of the three compounds at 5-HT$_3$ receptors. By the intraduodenal route, RS 25259-197 was about 15 and 45 fold more potent than ondansetron and granisetron. The i.d./i.v. potency ratio for RS 25259-197, ondansetron and granisetron was calculated to be 80, 65 and 490, respectively. These data suggest that RS 25259-197 and ondansetron possess better bioavailability characteristics than granisetron. Consequently, by the intraduodenal route, the better bioavailability and higher affinity of RS 25259-197 for 5-HT$_3$ receptors make it the most potent of the three compounds. Besides being more potent, RS 25259-197 also appears to have a longer duration of action; its inhibitory effects persisted longer than those of granisetron and ondansetron when all were tested at equi-effective doses.

Oral administration of drugs to cancer patients undergoing chemotherapy is problematic owing to the high incidence of emesis in these patients. Thus, availability of a 5-HT$_3$ receptor antagonist in the form of a transdermal formulation would greatly facilitate the management of emesis in these patients. The present study has shown that RS 25259-197, when administered by the transdermal route, causes potent and sustained inhibition of the 2-methyl-5-HT-induced bradycardia. These data suggest that transdermal formulations of RS 25259-197 may prove to be convenient drug-delivery systems in patients experiencing emesis due to cancer chemotherapy.

Anti-emetic studies

The emetogenic effects of anti-cancer drugs is most pronounced with platinum drugs such as cisplatin. Cisplatin, when administered to dogs and ferrets, produces an emetic and behavioural profile which most closely resembles the clinical symptoms (Gylys *et al.*, 1979; Florczyk *et al.*, 1982). The present study has shown that, RS 25259-197, administered intravenously or orally, potently inhibits cisplatin induced emesis in both the ferret and dog. Comparison of the oral anti-emetic potency in the ferret showed that RS 25259-197 was approximately 2 and 13 fold more potent than granisetron and ondansetron, respectively. In the dog, RS 25259-197 was about 30 fold more potent than ondansetron in inhibiting cisplatin-induced emesis. The lower potency of ondansetron relative to RS 25259-197 is again consistent with the relative affinity of these compounds for 5-HT$_3$ receptors. When tested at equi-effective doses in dogs, RS 25259-197 appeared to have a longer duration of anti-emetic action (7 h) than ondansetron (4 h).

RS 25259-197 produced complete inhibition of emesis induced by cisplatin in ferrets (i.v. but not p.o. route) and dogs (p.o. but not i.v. route). This is a pertinent finding because, in the clinical setting, it is critical to produce complete block rather than partial attenuation in the number of emetic episodes, as even mild nausea and emesis can severely compromise the success of chemotherapy. The maximal inhibitory effects of RS 25259-197 appear to depend on the route of administration. The precise reason for this paradoxical finding is unclear at present.

Besides cisplatin, other cancer chemotherapeutic agents such as dacarbazine, mechlorethamine and actinomycin D also cause emesis although of a lesser severity. These agents also induced emesis in dogs which was dose-dependently inhibited by RS 25259-197 indicating that the anti-emetic effects of this compound are not restricted to cisplatin alone. In the case of dacarbazine and mechlorethamine, complete inhibition of the emetic episodes was achieved with 100 μg kg^{-1}, p.o. of RS 25259-197. Ondansetron was also effective against the above emetogenic agents although it was about 100 fold less potent than RS 25259-197. Also, at the doses tested, ondansetron could produce complete blockade of the emetic episodes only against mechlorethamine but not against cisplatin, dacarbazine and actinomycin D. RS 25259-197 was ineffective against apomorphine-induced emesis suggesting that the anti-emetic effects are not a result of dopamine receptor antagonism. Such an interpretation is consistent with ligand binding studies which have shown that RS 25259-197 does not interact with D_1 and D_2 receptors (Wong *et al.*, 1995).

Haemodynamic studies

The effects of RS 25259-197 on haemodynamics in dogs were studied to determine whether the compound evoked cardiovascular effects at doses that showed anti-emetic activity. This study was undertaken in view of the reported clinical cardiovascular adverse effects reported with ondansetron (Ballard *et al.*, 1992). RS 25259-197 produced a modest but significant decrease in HR at 1000 μg kg^{-1}. However, in dogs, anti-emetic effects of RS 25259-197 were observed at doses as low as 1 μg kg^{-1}. Thus, RS 25259-197 was essentially devoid of any adverse cardiovascular side-effects at doses that produced anti-emetic effects.

In summary, RS 25259-197 appears to be a novel, orally active 5-HT$_3$ receptor antagonist that is more potent and has a longer duration of action than either ondansetron or granisetron. As discussed in the Introduction, currently available 5-HT$_3$ receptor antagonists such as ondansetron suffer from drawbacks most notable of which is their poor effectiveness in controlling mild and delayed emesis. It is unclear whether this is due to the intrinsic property of these drugs or is a commonality for all 5-HT$_3$ receptor antagonists. The present study has shown that, at least in animal models, RS 25259-197 represents a significant improvement over ondansetron as an anti-emetic agent with respect to potency and duration of action. It should be noted that granisetron has also been reported to possess the same advantages over ondansetron (Andrews *et al.*, 1992). As the present study lacks extensive anti-emetic efficacy data with granisetron, it is unclear as to how RS 25259-197 would compare with granisetron. Future studies should, therefore, be aimed at a direct comparison of the two drugs. Studies are also in progress to evaluate the clinical effectiveness of RS 25259-197.

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References

ANDREWS, P.L.R., BHANDARI, P., DARVEY, P.T., BINGHAM, S., MARR, H.E. & BLOWER, P.R. (1992). Are all 5-HT$_3$ receptor antagonists the same? *Eur. J. Cancer*, **28A** (Suppl. 1), S2–S6.

BALLARD, H.S., BOTTINO, G. & BOTTINO, J. (1992). Ondansetron and chest pain. *Lancet*, **340**, 1107.

COSTALL, B. & NAYLOR, R.J. (1992). Neuropharmacology of emesis in relation to clinical response. *Br. J. Cancer*, **19** (Suppl), S12–S13.

FITZPATRICK, L.R., LAMBERT, R.M., PENDLEY, C.E., MARTIN, G.E., BOSTWICK, J.S., GESSNER, G.W., AIREY, J.E., YOUSSEFYEH, R.D., PENDELTON, R.G. & DECKTOR, D.L. (1990). RG 12915: a potent 5-hydroxytryptamine-3 antagonist that is an orally effective inhibitor of cytotoxic drug-induced emesis in the ferret and dog. *J. Pharmacol. Exp. Ther.*, **254**, 450–455.

FLORCZYK, A.P., SCHURIG, J.E. & BRADNER, W.T. (1982). Cisplatin-induced emesis in the ferret: a new animal model. *Cancer Treat. Rep.*, **66**, 187–189.

FOZARD, J.R. (1984). MDL 72222: a potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **326**, 36–44.

GYLYS, J.A., DORAN, K.M. & BUYNISKI, J.P. (1979). Antagonism of cisplatin-induced emesis in the dog. *Commun. Chem. Pathol. Pharmacol.*, **23**, 61–68.

HALPERN, J.R. & MURPHY, B. (1992). Extrapyramidal reaction to ondansetron. *Cancer*, **69**, 1275.

JOSS, R.A. & DOTT, C.S. (1993). Clinical studies with Granisetron, a new 5-HT3 receptor antagonist for the treatment of cancer chemotherapy-induced emesis. *Eur. J. Cancer*, **29A**, S22–S29.

KILPATRICK, G.J., BUNCE, K.T. & TYERS, M.B. (1990). 5-HT$_3$ receptors. *Med. Res. Rev.*, **10**, 441–475.

KRIS, M.G., TYSON, L.B., CLARK, R.A. & GRALLA, R.J. (1992). Oral ondansetron for the control of delayed emesis after cisplatin. *Cancer*, **70** (Suppl 4), 1012–1016.

LEVITT, M., WARR, D., YELLE, L., RAYER, H.L., LOFTERS, W.S., PERAULT, D.J., WILSON, K.S., LATREILLE, J., POTVIN, M., WARNER, E., PRITCHARD, K.I., PALMER, M., LEE, B. & PATER, J.L. (1993). Ondansetron compared with dexamethasone and metoclopramide as antiemetics in the chemotherapy of breast cancer with cyclophosphamide, methotraxate and fluorouracil. *N. Engl. J. Med.*, **328**, 1081–1084.

MARR, H.E., DARVEY, P.T. & BARTLETT, A.J. (1991). Emerging differences between 5-HT3 receptor antagonists. *Anticancer Drugs*, **2**, 513–518.

OXFORD, A.W., BELL, J.A., KILPATRICK, G.J., IRELAND, S.J. & TYERS, M.B. (1992). Ondansetron and related 5-HT$_3$ receptor antagonists: recent advances. *Prog. Med. Chem.*, **29**, 239–270.

TYERS, M.B. (1991). 5-HT$_3$ receptors and the therapeutic potential of 5-HT$_3$ receptor antagonists. *Therapie (Paris)*, **46**, 431–435.

WONG, E.H.F., CLARK, R., LEUNG, E., LOURY, D., BONHAUS, D., JAKEMAN, L., PARNES, H., WHITING, R.L. & EGLEN, R.M. (1995). The interaction of RS 25259-197, a potent and selective 5-HT$_3$ receptor antagonist at 5-HT$_3$ receptors *in vitro*. *Br. J. Pharmacol.*, **114**, 851–859.

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Rapid degradation of endothelin-1 by an enzyme released by the rat isolated perfused mesentery

[1]Francisco Pérez-Vizcaíno, Andrea C. Cooper, Roger Corder, *Alain Fournier & [2]Timothy D. Warner

The William Harvey Research Institute, The Medical College of Saint Bartholomews' Hospital, Charterhouse Square, London EC1M 6BQ and *Institut National de la Recherche Scientifique (INRS)-Santé, Montréal, P.Q., Canada, H9R 1G6

1 *In vivo* the effects of endothelin-1 (ET-1) are limited by its rapid removal from the circulation and possibly by its metabolism by enzymes such as neutral endopeptidase 24.11, deamidase or carboxypeptidase A. Here, using as a model the isolated perfused mesenteric arterial bed of the rat, we have examined the involvements of these enzymatic activities in the vascular responses to ET-1.

2 Samples of Krebs buffer which had been recirculated through the mesenteric arterial bed for 30 min rapidly destroyed the activity of ET-1 as assessed either by bioassay on rings of rat thoracic aorta or by high performance liquid chromatography (h.p.l.c.). For instance, after 15 min incubation with the recirculated-Krebs solution (recirc-K) the contraction induced by 3×10^{-9} M ET-1 was reduced by more than 90%. Contractions induced by sarafotoxin 6b (3×10^{-9} M) were similarly suppressed by pre-incubation with recirc-K whereas those to Arg-vasopressin (3×10^{-9} M) were unaffected.

3 The degradation of ET-1 by recirc-K was prevented by 1,10-phenanthroline (10^{-3} M), abolished by heating the recirc-K solution to 90°C for 15 min, and reduced by EGTA (5×10^{-3} M) or ET-$1_{(16-21)}$ (10^{-5} M). For instance, in the presence of ET-$1_{(16-21)}$ ($n = 6$) the contraction induced by ET-1 was reduced by only 40% after 15 min incubation with recirc-K buffer. Leupeptin (3×10^{-4} M), dichloroisocoumarin (5×10^{-5} M), phenylmethyl-sulphonyl fluoride (10^{-3} M), a combination of bacitracin (300 mg ml^{-1}), bestatin (10^{-5} M), captopril (10^{-5} M), phosphoramidon (10^{-4} M) and thiorphan (10^{-4} M) or Polypep (a proprietary protein digest) did not inhibit the degradation of ET-1 by recirc-K.

4 In experiments examining directly the vascular responses of the isolated perfused mesentery of the rat, the addition of cumulative concentrations of ET-1 to the recirculating Krebs solution caused small concentration-dependent increases in perfusion pressure. The inclusion of ET-$1_{(16-21)}$, ET-$1_{(17-21)}$, or ET-$1_{(18-21)}$ (10^{-5} M) greatly potentiated these responses, but not those to Arg-vasopressin or methoxamine. The effects of 1,10-phenanthroline or EGTA could not be examined in this system because these agents both depressed non-specifically the vasoconstrictor responses of the mesenteric vascular bed.

5 Thus, the rat mesentery releases an enzyme that very rapidly destroys ET-1 or the very closely related peptide, sarafotoxin 6b but not Arg-vasopressin. This enzyme is most probably a metallopeptidase because of its sensitivity to inhibition by 1,10-phenanthroline or EGTA. It is particularly interesting that a simple vascular bed such as the mesentery produces such a powerful endothelin metabolising enzyme. It is tempting, therefore, to speculate that the endothelin degrading enzyme active at neutral pH that we have found is important in the metabolism of ET-1 throughout the vasculature.

Keywords: Endothelin-1; endothelin converting enzyme; endothelin degradation; rat isolated perfused mesentery

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide of 21 amino acids, originally found in the supernatant of porcine endothelial cells in culture (Yanagisawa *et al.*, 1988). ET-1 produces very prolonged responses both *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988; de Nucci *et al.*, 1988) suggesting that its activity must be very tightly controlled. Indeed, ET-1 is rapidly removed from the circulation with a circulating half-life of 30–60 s (Änggård *et al.*, 1989; Corder & Vane, 1994). Furthermore, ET-1 is readily metabolised by enzymes within the circulation such as neutral endopeptidase 24.11 (Vijayaraghavan *et al.*, 1990; Sokolovsky *et al.*, 1990), deamidase (Jackman *et al.*, 1993), or carboxypeptidase-like activities (Deng *et al.*, 1992; Jeng & Deng, 1993). Here, using as a model the isolated perfused mesentery of the rat, we have investigated the role of ET-1-metabolising enzymes in governing the vascular responses to ET-1 in a simple vascular bed. Some of these data have already been presented (Perez-Vizcaíno *et al.*, 1994).

Methods

Isolated perfused mesentery of the rat

The isolated perfused mesentery of the rat was prepared by the method of McGregor (1965). Male albino Wistar rats were anaesthetized with pentobarbitone sodium (120 mg kg^{-1}, i.p.), the abdomen opened and the ileocolic and colic branches of the superior mesenteric artery ligated. The superior mesenteric artery was cannulated and the superior mesenteric vascular bed perfused via the artery for 5 min (2 ml min^{-1}) with Krebs buffer containing heparin (100 u ml^{-1}). The animal was then killed by cutting the diaphragm. The intestine was separated from the mesentery and the preparation supported on a Petri-dish and the arteries perfused at a constant flow of 2 ml min^{-1} with warmed (37°C) and gassed (95% O_2:5% CO_2) Krebs buffer. Changes in perfusion pressure were measured with a transducer approximately 15 cm from the tip of the arterial cannula (Warner, 1989). The preparation was allowed to equilibrate for 45 min and its viability was then checked by a bolus injection of 6×10^{-5} mol KCl. After a further 15 min the effluent from the mesentery was collected and reperfused through the preparation. In this way the mesentery was perfused in a closed system with a total volume of 10 ml of Krebs buffer.

[1] Present address: Dept. Pharmacology, School of Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain.
[2]Author for correspondence.

After 30 min this recirculated-Krebs buffer (recirc-K) was removed for assay of ET-1 degrading activity (see below).

In a further series of experiments increasing concentrations of ET-1 (10^{-10} to 3×10^{-8} M) were added at 45 min intervals into the reservoir of the recirculating system. In some experiments ET-$1_{(16-21)}$, ET-$1_{(17-21)}$, or ET-$1_{(18-21)}$ (10^{-5} M), which are C-terminal fragments of ET-1, were added to the recirculating system 5 min before each addition of ET-1.

Rat thoracic aortic rings: assay of ET-1 degrading activity

Male Wistar rats (250–400 g) were killed with thiopentone sodium (Sagatal, 120 mg kg^{-1}, i.p.). Rings of rat aorta with the endothelium removed (2–3 mm, resting tension 2 g) were then set up in isolated organ baths containing 9 ml of warmed (37°C) and gassed (95% O_2:5% CO_2) Krebs buffer. Responses of the tissues were detected by isometric transducers (Hugo Sachs Electronik, Germany) and displayed on a chart recorder (Graphtec WR3101). After an equilibration period of 1 h the tissues were maximally contracted by 8×10^{-2} M KCl.

For the assay of ET-1 degrading activity, 1 ml of recirc-K from the mesentery (see above), or fresh Krebs buffer was incubated at 37°C with 3×10^{-8} M ET-1, sarafotoxin 6b or Arg-vasopressin for 15 min in the presence of vehicle or (i) a cocktail of peptidase inhibitors (bacitracin 300 mg ml^{-1}, bestatin 10^{-5} M, captopril 10^{-5} M, phosphoramidon 10^{-4} M and thiorphan 10^{-5} M, Maggi *et al.*, 1989); (ii) dichloroisocoumarin (5×10^{-4} M); (iii) EGTA (5×10^{-3} M); (iv) leupeptin (3×10^{-4} M); (v) N-ethylmaleimide (10^{-3} M); (vi) 1,10-phenanthroline (10^{-3} M); (vii) phenylmethylsulphonyl fluoride (10^{-3} M); (viii) Polypep (50 mg l^{-1}); (ix) ET-$1_{(16-21)}$ (10^{-5} M). In some experiments both the fresh and recirc-K were heated to 90°C for 15 min before ET-1 was added. At the end of the incubation period with ET-1 the entire volume of Krebs (1 ml) was added to the bath containing the aortic ring to give a final apparent concentration for ET-1, sarafotoxin 6b or Arg-vasopressin of 3×10^{-9} M. The subsequent contraction was recorded and standardized by comparison with the KCl contraction.

High performance liquid chromatography analysis

H.p.l.c. identification of ET-1 was performed with a column of 5 μm TSK gel ODS-120T, (4.6 × 250 mm, TOSOH Corporation, Japan from Anachem, Luton, Beds.). Samples were first acidified and extracted with TechElut SPE C_{18} columns (HPLC Technology, Macclesfield, Cheshire), eluted with 1 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid, concentrated under a stream of N_2, and injected onto the h.p.l.c. system. The h.p.l.c. column was eluted at a flow rate of 1 ml min^{-1} with a gradient of acetonitrile in 0.1% trifluoroacetic acid using a Pharmacia LKB model 2249 pump. The gradient used was 0–20% over 3 min, 20–32% over 12 min, 32–40% over 15 min, and 40–48% over 8 min. The column effluent was monitored at 280 nm with a Pharmacia LKB model 2141 variable wavelength monitor. In this system the elution time for ET-1 was 30.1 ± 0.1 min ($n = 7$).

Materials

The Krebs buffer had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$. $7H_20$ 1.17, $CaCl_2.6H_20$ 2.5, $NaHCO_3$ 25, glucose 5.6. ET-1 and sarafotoxin 6b were purchased from Peptide Institute (Osaka, Japan). Arg-vasopressin, bacitracin, bestatin, captopril, dichloroisocoumarin, EGTA, leupeptin, methoxamine, N-ethylmaleimide, 1,10-phenanthroline, phenylmethylsulfonyl fluoride, Polypep and thiorphan were obtained from Sigma Chemical Co. (Poole, Dorset). ET-$1_{(16-21)}$, ET-$1_{(17-21)}$, and ET-$1_{(18-21)}$ were synthesized at the INRS-Sante (Montreal, P.Q., Canada). All agents were dissolved in either distilled water or saline, except for phenylmethylsulphonyl fluoride which was dissolved in acetone. The salts for the Krebs solution were obtained from BDH (Lutterworth, Leics.).

Statistics

Statistical differences between points were determined by Student's unpaired two tailed t test, Mann-Whitney unpaired one tail two sample test, or one-way ANOVA with *post hoc* Bonferroni t test, as appropriate. A $P < 0.05$ was taken as significant.

Results

Detection of ET-1 degrading activity in Krebs buffer

ET-1 incubated with fresh Krebs buffer for 30 min and then added to the organ baths at a final concentration of 3×10^{-9} M caused a contraction of the rings of rat thoracic aorta equivalent to 72 ± 12% of that to KCl ($n = 12$). When incubated with recirc-K the ET-1 contractile activity was rapidly lost (Figure 1). For instance, after 15 min the incubate only caused a contraction equivalent to 7 ± 3% KCl ($n = 10$; $P < 0.001$ from control, ANOVA). This reduction in contractile activity was associated with destruction of ET-1 as assessed by h.p.l.c. (Figure 2).

The loss of ET-1 contractile activity was prevented by heating the recirc-K ($n = 3$; $P > 0.05$ from fresh Krebs buffer control, Student's t test) or by including 1,10-phenanthroline (10^{-3} M; $n = 6$; $P > 0.05$, Student's t test) in the incubate. However, control responses to ET-1 were reduced by 38% ($n = 3$) when 1,10-phenanthroline (10^{-4} M) was added directly to the organ baths. Co-incubation with EGTA or ET-$1_{(16-21)}$ greatly reduced ($P < 0.0001$ and $P < 0.007$ from recirc-K, respectively, Student's t test), but did not prevent the loss of ET-1 contractile activity (Table 1). N-ethylmaleimide reduced control responses to ET-1 by 62% ($n = 3$) and its effects on the metabolism of ET-1 was therefore not assessed. All the other agents were without protective effects (Table 1).

Sarafotoxin 6b (3×10^{-9} M) incubated with 1 ml of fresh Krebs buffer for 20 min caused a contraction of the rat thoracic aorta of 70 ± 21% KCl ($n = 3$). However, after incubation with recirc-K it produced a contraction of only

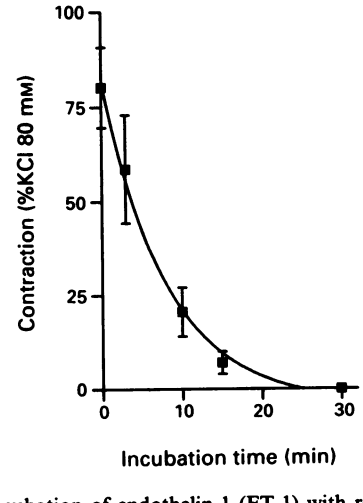


Figure 1 Incubation of endothelin-1 (ET-1) with recirculated-Krebs buffer for 0–30 min resulted in the rapid loss of contractile activity when assayed on isolated rings of rat thoracic aorta (apparent final concentration of ET-1, 3×10^{-9} M). Each point represents the mean (± s.e.mean) ($n = 3$–10).

4 ± 2% KCl ($n = 3$). Contractions induced by Arg-vasopressin (3×10^{-9} M, $n = 3-4$) were unaffected by 20 min incubation with recirc-K ($P > 0.05$, $n = 4$, Student's t test).

Vasoconstrictions induced by ET-1 in the rat isolated perfused mesentery

When added to the recirculating system ET-1 (3×10^{-10} to 3×10^{-8} M, $n = 7$) produced very little increase in perfusion pressure (Figure 3). However, in the presence of either ET-$1_{(16-21)}$, ET-$1_{(17-21)}$ or ET-$1_{(18-21)}$ the concentration-dependent responses to ET-1 were very much potentiated (Figure 3). Conversely, the increase in perfusion pressure caused by ET-1 (3×10^{-8} M) added immediately after commencing recirculation was unaffected by ET-$1_{(17-21)}$ ($n = 4-5$; Figure 4). Responses to Arg-vasopressin (10^{-10} to 10^{-8} M) or methoxamine (10^{-7} to 10^{-5} M) were unaffected by ET-$1_{(17-21)}$ ($n = 3-5$ data not shown).

The effects of 1,10-phenanthroline (10^{-3} M) or EGTA (5×10^{-3} M) on the responses of the mesentery to ET-1 could not be tested because these two agents strongly decreased the responsiveness of the mesenteric vessels to bolus injections of KCl or ET-1.

Discussion

As assayed by either bioassay or h.p.l.c., ET-1 was rapidly destroyed by Krebs buffer recirculated through the mesentery. This destruction was most probably due to the action of an endothelin-degrading enzyme (EDE) for it was prevented by heating the recirc-K, which will denature proteins. Interestingly, the EDE released from the mesentery was substrate-selective for it degraded ET-1 or sarafotoxin 6b but not Arg-vasopressin. This may well be because it acts upon the C-terminal portion of the ET/sarafotoxin peptides, a suggestion that is supported by two of our observations. Firstly, that inclusion of excess ET-$1_{(16-21)}$, in the incubates led largely to the preservation of the biological activity of ET-1, which may be explained by ET-$1_{(16-21)}$ providing an alternative substrate for the EDE. This was not a general effect of peptide fragments for Polypep, a proprietary protein digest that readily inhibits non-specific peptide or protein degradation, did not inhibit the degradation of ET-1. Secondly, addition of ET-$1_{(16-21)}$, ET-$1_{(17-21)}$ or ET-$1_{(18-21)}$ to the recirculating Krebs-buffer passing through the mesentery potentiated vasoconstrictions induced by ET-1. As before, this potentiation can be explained by the ability of these agents to inhibit by substrate competition the degradation of ET-1 by EDE. This was not a non-specific effect, for ET-$1_{(16-21)}$ did not

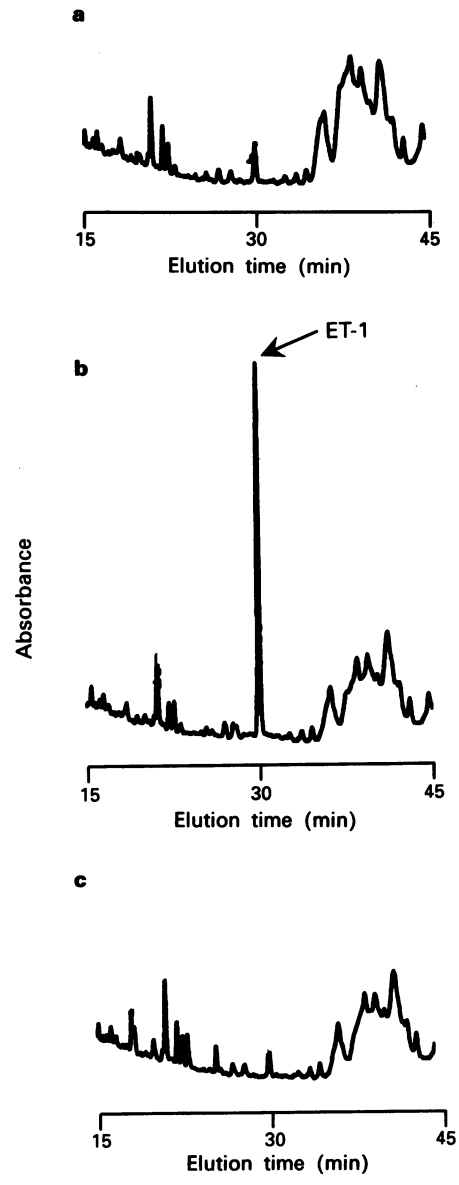


Figure 2 High performance liquid chromatography (h.p.l.c.) analysis of recirculated-Krebs solution resulted in the trace displayed in (a). The lower traces represent h.p.l.c. analysis of recirculated-Krebs buffer incubated with endothelin-1 (ET-1, 3×10^{-7} M) for 0 (b) and 15 min (c).

Table 1 Stability of endothelin-1 (ET-1) in recirculated Krebs buffer (recirc-K) assayed by the contraction of rings of rat thoracic aorta

Inhibitor	n	*Concentration*	*Remaining ET-1 response* (% control)
None	10		10
1,10-phenanthroline	6	(10^{-3} M)	100
EGTA	4	(5×10^{-3} M)	71
$ET_{(16-21)}$	6	(10^{-5} M)	63
Leupeptin	3	(3×10^{-4} M)	16
Dichloroisocoumarin	5	(5×10^{-5} M)	6
Phenylmethylsulphonyl fluoride	3	(10^{-3} M)	0
†Polypep	4	(50 mg l^{-1})	0
‡'Cocktail'	5	(as below)	0
Heating	3		100

ET-1 was incubated with recirc-K for 15 min in the absence or presence of the above agents (total incubate volume 1 ml). The ET-1-contractile activity retained within the incubate was then assayed by its ability to contract a ring of rat thoracic aorta (total final bath volume, 10 ml). The contractions induced by ET-1 (apparent final concentration, 3×10^{-9} M) are expressed as the % of the contraction induced by ET-1 incubated with fresh Krebs buffer for 20 min before addition to the rat aortic rings. †Polypep is a proprietary protein digest; ‡cocktail = bacitracin (300 mg ml^{-1}), bestatin (10^{-5} M), captopril (10^{-5} M), phosphoramidon (10^{-4} M), and thiorphan (10^{-4} M). (Effective concentrations of peptidase inhibitors from Benyon & Bond, 1990).

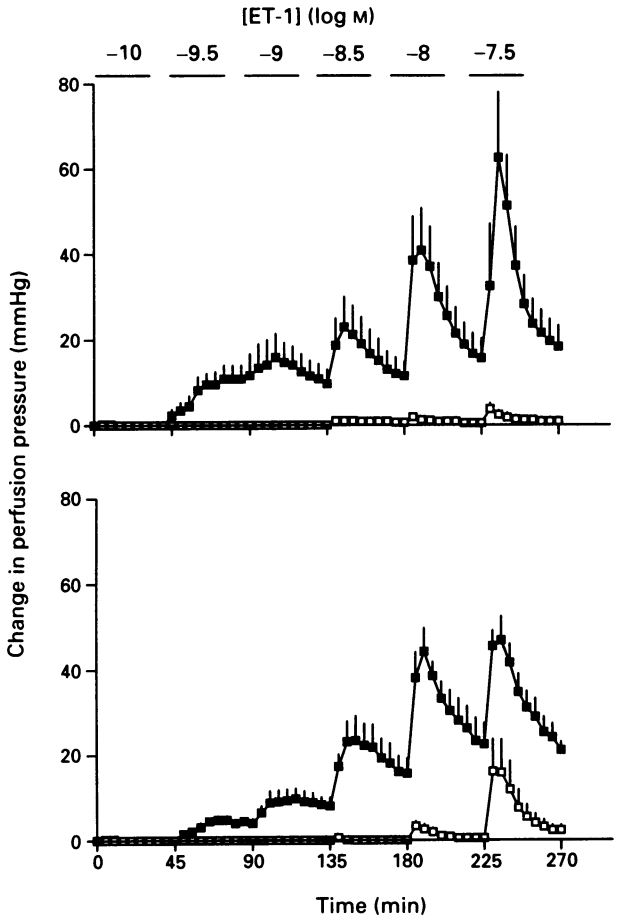


Figure 3 Increases in perfusion pressure in the isolated perfused mesentery of the rat induced by ET-1 (10^{-10} to 3×10^{-8} M) either in control conditions (□) or in the presence of ET-$1_{(16-21)}$ (10^{-5} M, ■, upper panel) or ET-$1_{(17-21)}$ (10^{-5} M, ■, lower panel). Each point with a vertical bar represents the mean (± s.e.mean) ($n = 4-5$).

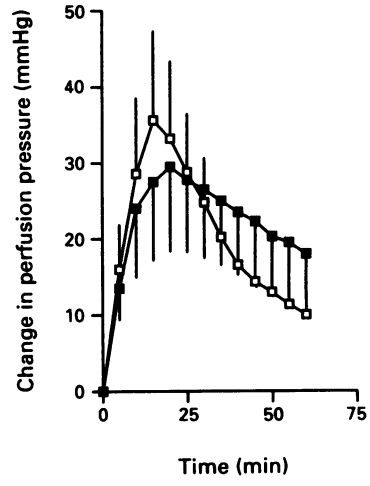


Figure 4 Increase in perfusion pressure in the isolated perfused mesentery of the rat induced by endothelin-1 (ET-1, 3×10^{-8} M) either in control conditions (□) or in the presence of ET-$1_{(17-21)}$ (10^{-5} M, ■). ET-1 was added to the Krebs buffer immediately after starting recirculation. Each point represents the mean (± s.e.mean) ($n = 4-5$).

potentiate responses to ET-1 when it was added to the Krebs buffer immediately reperfusion was started, i.e. before the EDE was present. In addition, ET-$1_{(17-21)}$ did not affect vasoconstrictions induced by either Arg-vasopressin or methoxamine.

It is interesting to examine the characteristics of this enzyme originating from such a simple vascular bed and compare it with other proposed EDEs. Firstly, we may conclude that it is a metalloprotease, for it was inhibited by 1,10-phenanthroline or EGTA. It is unlikely to be a serine or cysteine protease for it was not inhibited by dichloroisocoumarin, phenylmethylsulphonyl fluoride or leupeptin. Similarly, it does not appear to be neutral endopeptidase 24.11, angiotension converting enzyme or aminopeptidase for it was insensitive to inhibition by the 'cocktail' of inhibitors (Maggi *et al.*, 1989) that included phosphoramidon, captopril and bestatin (see Beynon & Bond, 1990). There are, however, a number of other endothelin-degrading enzymes, commonly derived from homogenates of renal tissue, to which we may compare this activity. The first of these is an enkephalinase-like activity, which actively degrades ET-1, but which we may discount as being the EDE we describe here, for this former enzyme is inhibited by phosphoramidon (Edano *et al.*, 1994). Our EDE may be more like another kidney-derived ET-1-inactivating peptidase that is inhibited by both 1,10-phenanthroline and by EDTA. However, the EDE derived from the mesentery is very active at pH 7.4, whereas this other enzyme is active mainly between pH 5 and 7 (Janas *et al.*, 1994). An appealing possibility is that the mesenteric-EDE is similar to a kidney enzyme that cleaves ET-1 between residues Ile^{20} and Trp^{21}, forming ET-$1_{(1-20)}$ (Deng *et al.*, 1992; Jeng & Deng, 1993). The attraction of this proposal is that this enzymatic step would very effectively limit the effects of ET-1 because ET-$1_{(1-20)}$ is 1000 fold less potent as a vasoconstrictor (Kimura *et al.*, 1988). However, this enzyme, unlike our EDE, has little activity at pH 7.4 and is inhibited by phenylmethylsulphonyl fluoride and not by metalloprotease inhibitors (Deng *et al.*, 1994). Another possible identity for the mesentery-derived EDE is deamidase, for bovine cultured aortic endothelial cells (Jackman *et al.*, 1993) and platelets (Jackman *et al.*, 1992) contain an enzyme of this type that degrades ET-1 to ET-$1_{(1-20)}$. However, this enzyme differs from the EDE described here in that it is not sensitive to inhibition by 1,10-phenanthroline. Possibly the activity of our EDE is similar to the membrane-bound metallo-endopeptidase found in rat kidney that cleaves ET-1 predominantly between Leu^{17} and Asp^{18} (Yamaguchi *et al.*, 1992). Finally, it may be that the mesentery releases/contains multiple EDEs, for with h.p.l.c. analysis we did not detect the formation of one major product from ET-1. This contrasts, for instance, with Jeng & Deng (1993) who clearly found that the EDE they isolated from rat kidney produced a single peptide from ET-1, identified as ET-$1_{(1-20)}$ by FAB mass spectrometry.

The role of EDE in regulating the responses to ET-1 *in vivo* is not clear. However, as vasoconstrictions induced by ET-1 are prolonged and slow to reverse both *in vitro* and *in vivo* (Marsault *et al.*, 1993; Warner *et al.*, 1994), and as ET-1 binds almost irreversibly to its receptors, with a dissociation half-life in excess of 30 h (Waggoner *et al.*, 1992) it is clearly important that its survival within tissues is tightly controlled. With this in mind, it is particularly interesting that a simple vascular bed such as the mesentery produces such a powerful endothelin metabolising enzyme. This might not be expected, while the presence of EDEs in the kidney (Yamaguchi *et al.*, 1992; Deng *et al.*, 1992; 1994; Jeng & Deng, 1993; Edano *et al.*, 1994; Janas *et al.*, 1994), with its well known metabolic activities is much less surprising. The EDE active at neutral pH that we have found in the mesentery may, therefore, be representative of a similar enzyme that is important in the metabolism of ET-1 throughout the vasculature.

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References

ÄNGGÅRD, E., GALTON, S., RAE, G., THOMAS, R., MCLOUGHLIN, L., DE NUCCI, G. & VANE, J.R. (1989). The fate of radioiodinated endothelin-1 and endothelin-3 in the rat. *J. Cardiovasc. Pharmacol.*, **13** (suppl.5), S46–S49.

BENYON, R.J. & BOND, J.S. (1990). *Proteolytic Enzymes: a Practical Approach*. Oxford: IRL Press at Oxford University Press.

CORDER, R. & VANE, J.R. (1994). Radioimmunoassay evidence that the pressor effect of big endothelin-1 in anaesthetised rats is due to local conversion to endothelin-1. *Br. J. Pharmacol.*, **111,** 225P.

DENG, Y., MARTIN, L.L., BALWIERCZAK, J.L. & JENG, A.Y. (1994). Purification and characterization of an endothelin degradation enzyme from rat kidney. *J. Biochem.*, **115,** 120–125.

DENG, Y., MARTIN, L.L., DELGRANDE, D. & JENG, A.Y. (1992). A soluble protease identified from rat kidney degrades endothelin-1 but not proendothelin-1. *J. Biochem.*, **112,** 168–172.

DE NUCCI, G., THOMAS, G., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 9797–9800.

EDANO, T., ARAI, K., OHSHIMA, T., KOSHI, T., HIRATA, M., OHKUCHI, M. & OKABE, T. (1994). Purification and characterization of endothelin-1 degradation activity from porcine kidney. *Biol. Pharmacol. Bull.*, **17,** 379–382.

JACKMAN, H.L., MORRIS, P.W., DEDDISH, P.A., SKIDGEL, R.A. & ERDÖS, E.G. (1992). Inactivation of endothelin 1 by deamidase (lysosomal protective protein). *J. Biol. Chem.*, **267,** 2872–2875.

JACKMAN, H.L., MORRIS, P.W., RABITO, S.F., JOHANSSON, G.B., SKIDGEL, R.A. & ERDÖS, E.G. (1993). Inactivation of endothelin-1 by an enzyme of the vascular endothelial cells. *Hypertens.*, **21,** 925–928.

JANAS, J., SITKIEWICZ, D., PULAWSKA, M.F., WARNAWIN, K. & JANAS, R.M. (1994). Purification of endothelin-1-inactivating peptidase from the rat kidney. *J. Hypertens.*, **12,** 375–382.

JENG, A.Y. & DENG, Y. (1993). Rapid inactivation of endothelin-1 by a carboxypeptidase-like enzyme purified from rat kidney. *J. Cardiovasc. Pharmacol.*, **22** (Suppl.8), S69–S72.

KIMURA, S., KASUYA, Y., SAWAMURA, T., SHINMI, O., SUGITA, Y., YANAGISAWA, M., GOTO, K. & MASAKI, T. (1988). Structure-activity relationships of endothelin: importance of the C-terminal moiety. *Biochem. Biophys. Res. Commun.*, **156,** 1182–1186.

MAGGI, C.A., GIULIANI, S., PATACCHINI, R., ROVERO, P., GIACHETTI, A. & MELI, A. (1989). The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur. J. Pharmacol.*, **174,** 23–31.

MARSAULT, R., FEOLDE, E. & FRELIN, C. (1993). Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. *Am. J. Physiol.*, **264,** C687–C693.

MCGREGOR, D.D. (1965). The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric blood vessels of the rat. *J. Physiol.*, **177,** 21–30.

PEREZ-VIZCAÍNO, F., WARNER, T.D., COOPER, A.C., CORDER, R. & VANE, J.R. (1994). Vasoconstrictions induced by big endothelin-1 in the isolated perfused mesentery of the rat are limited by the rapid degradation of endothelin-1. *Can. J. Physiol. Pharmacol.*, **72** (Suppl. 1), 109.

SOKOLOVSKY, M., GALRON, R., KLOOG, Y., BDOLAH, A., INDIG, F.E., BLUMBERG, S. & FLEMINGER, G. (1990). Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: Possible physiological significance. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 4702–4706.

VIJAYARAGHAVAN, J., SCICLI, G., CARRETERO, O.A., SLAUGHTER, C., MOOMAW, C. & HERSH, L.B. (1990). The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J. Biol. Chem.*, **265,** 14150–14155.

WAGGONER, W.G., GENOVA, S.L. & RASH, V.A. (1992). Kinetic analyses demonstrate that the equilibrium assumption does not apply to [125I]endothelin-1 binding data. *Life Sci.*, **51,** 1869–1876.

WARNER, T.D. (1989). Simultaneous perfusion of rat isolated superior mesenteric arterial and venous beds: comparison of their vasoconstrictor and vasodilator responses to agonists. *Br. J. Pharmacol.*, **99,** 427–433.

WARNER, T.D., ALLCOCK, G.H. & VANE, J.R. (1994). Reversal of established responses to endothelin-1 *in vivo* and *in vitro* by the endothelin receptor antagonists BQ-123 and PD 145065. *Br. J. Pharmacol.*, **112,** 207–213.

YAMAGUCHI, I., KIDO, H. & KATUNUMA, N. (1992). A membrane-bound metallo-endopeptidase from rat kidneys. Characteristics of its hydrolysis of peptide hormones and neuropeptides. *Eur. J. Biochem.*, **204,** 547–552.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYISHI, Y., MISTUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332,** 411–415.

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Multiple effects and stimulation of insulin secretion by the tyrosine kinase inhibitor genistein in normal mouse islets

J.C. Jonas, *T.D. Plant, P. Gilon, P. Detimary, M. Nenquin & [1]J.C. Henquin

Unité d'Endocrinologie et Métabolisme, Faculty of Medicine, University of Louvain, B-1200 Brussels, Belgium and *I. Physiologisches Institut, University of Saarland, D-66421 Homburg/Saar, Germany

1 Islets from normal mice were used to test the acute effects of genistein, a potent tyrosine kinase inhibitor, on stimulus-secretion coupling in pancreatic β-cells.

2 Genistein produced a concentration-dependent (10-100 μM), reversible, increase of insulin release. This effect was marginal on basal release or in the presence of non-metabolized secretagogues, and much larger in the presence of glucose or other nutrients. The increase in insulin release caused by 100 μM genistein was abolished by adrenaline or omission of extracellular Ca^{2+}. It was not accompanied by any rise of cyclic AMP, inositol phosphate or adenine nucleotide levels.

3 Although genistein slightly inhibited ATP-sensitive K^+ channels, as shown by ^{86}Rb efflux and patch-clamp experiments, this effect could not explain the action of the drug on insulin release because the latter persisted when ATP-sensitive K^+ channels were all blocked by maximally effective concentrations of glucose and tolbutamide. Genistein was also effective when ATP-sensitive K^+ channels were opened by diazoxide and the β-cell membrane depolarized by 30 mM K, but ineffective in the presence of diazoxide and normal extracellular K.

4 Genistein paradoxically decreased Ca^{2+} influx in β-cells, as shown by the inhibition of glucose-induced electrical activity, by the inhibition of Ca^{2+} currents (perforated patches) and by the lowering of cytosolic $[Ca^{2+}]_i$ (fura-2 technique). Genistein thus increases insulin release in spite of a lowering of $[Ca^{2+}]_i$ in β-cells.

5 Daidzein, an analogue of genistein reported not to affect tyrosine kinases, was slightly less potent than genistein on $K^+$and Ca^{2+} channels, but increased insulin secretion in a similar way. Three other tyrosine kinase inhibitors, tyrphostin A47, herbimycin A and an analogue of erbstatin variably affected insulin secretion.

6 Genistein exerts a number of heretofore unrecognized effects. The unusual mechanisms, by which genistein increases insulin release in spite of a decrease in β-cell $[Ca^{2+}]_i$ and without activating known signalling pathways, do not seem to result from an inhibition of tyrosine kinases.

Keywords: Genistein; daidzein; tyrosine kinase inhibitors; pancreatic β-cells; insulin release; K^+ channels; Ca^{2+} channels; stimulus-secretion coupling

Introduction

Protein phosphorylation by protein kinases is a widespread mechanism of signal transduction in eukaryotic cells. Among serine/threonine kinases, the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A and the diacylglycerol-dependent protein kinase C are involved in the regulation of insulin release by hormones and neurotransmitters, while Ca^{2+}-calmodulin dependent kinases play a central role in the coupling between the increase in cytoplasmic $[Ca^{2+}]_i$ and exocytosis (Prentki & Matschinsky, 1987; Jones *et al.*, 1991; Hughes & Ashcroft, 1992; Wenham *et al.*, 1994). On the other hand, it is not known whether tyrosine kinases are involved in stimulus-secretion coupling in pancreatic β-cells.

IGF-1 receptors, which possess an intrinsic tyrosine kinase activity, are expressed by rat pancreatic A- and B-cells (Van Schravendijk *et al.*, 1987), and their activation by physiological concentrations of IGF-1 has been reported to inhibit (Leahy & Vandekerkhove, 1990; Van Schravendijk *et al.*, 1990) or increase (Clark *et al.*, 1990) glucose-induced insulin release *in vitro*. Vanadate, a phosphotyrosine phosphatase inhibitor (Swarup *et al.*, 1982) that increases the number of phosphotyrosine residues in many cell types, potentiates insulin release from mouse islets in a dose- and glucose-dependent manner (Zhang *et al.*, 1991). This effect was mainly attributed to a complex interplay between changes in β-cell membrane potential, Ca^{2+} handling and an increase in phosphoinositide metabolism (Zhang *et al.*, 1991). All these data indirectly suggest that modulation of islet phosphotyrosine content could be implicated in the regulation of insulin release.

This hypothesis was also recently supported by two brief reports showing that genistein, a putatively selective inhibitor of tyrosine kinases, increased insulin release from the MIN 6 cell line (Ohno *et al.*, 1993) and from neonatal rat islets (Sorenson *et al.*, 1994). However, circumstantial evidence indicates that, in other systems, the effects of genistein might not always be related to tyrosine kinase inhibition (Abler *et al.*, 1992; Ozaki *et al.*, 1993; Young *et al.*, 1993). In this study, normal mouse islets were used to characterize the effects of genistein on stimulus-secretion coupling.

Methods

Preparation

Most experiments were performed at 37°C, with islets isolated by collagenase digestion of the pancreas of fed female NMRI mice (25-30 g). Patch-clamp experiments were carried out at room temperature, with β-cells obtained by dispersion of the isolated islets.

[1] Author for correspondence at Unité d'Endocrinologie et Métabolisme, UCL 55.30, Avenue Hippocrate 55, B-1200 Brussels, Belgium.

Solutions

Except for patch-clamp experiments (see below), the medium used was a bicarbonate-buffered solution containing (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and $NaHCO_3$ 24. It was gassed with O_2/CO_2 (94/6) to maintain pH 7.4 and was supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany). Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$. When the concentration of KCl was increased to 30 mM, that of NaCl was reduced to 94.8 mM to keep the osmolarity of the medium unchanged.

Insulin release measurements

After isolation, the islets were preincubated for 1 h in a medium containing 15 mM glucose, a concentration that produces half-maximum stimulation of mouse β-cells. In one type of experiments, they were then incubated for 60 min, in batches of 3, in 1 ml of medium containing appropriate concentrations of glucose and test substances. At the end of the incubation, a portion of the medium was withdrawn and diluted before insulin assay. In another type of experiment, preincubated islets were placed, in batches of 30, in parallel perifusion chambers. Effluent fractions were collected at 2 min intervals for insulin assay. Insulin was measured by a double-antibody radioimmunoassay with rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark).

Measurements of ^{86}Rb efflux

The islets were first loaded with ^{86}Rb (used as tracer for K) during 90 min of preincubation in 1 ml of medium containing 15 mM glucose and supplemented with $^{86}RbCl$. The concentration of Rb never exceeded 0.4 mM. After being washed, the islets were then placed in perifusion chambers permitting ^{86}Rb efflux to be monitored in the effluent fractions (Garrino & Henquin, 1988).

Measurements of cytosolic $[Ca^{2+}]_i$

In the experiments aimed at measuring cytosolic Ca^{2+}, the islets were loaded with fura-2 during a preincubation of 40 min in the presence of 10 mM glucose. They were then washed and transferred to a Ca^{2+} measuring system in which the perifused tissue was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science Ltd., Tunbridge Wells, United Kingdom). The images were analysed by the system MagiCal (Applied Imaging, Sunderland, United Kingdom). The technique has been described in detail previously (Gilon & Henquin, 1992).

Electrical recordings

The membrane potential of β-cells was measured with a high resistance microelectrode (Meissner & Schmelz, 1974). A piece of mouse pancreas was fixed in a perifusion chamber, and a few islets were partially microdissected by hand. After impalement, β-cells were identified by the typical electrical activity that they display in the presence of 15 mM glucose. The experiments were performed at 37°C and the perifusion medium was as described above, except for the absence of albumin.

Patch-clamp experiments were performed at room temperature on single β-cells maintained in culture for 1–2 days. ATP-sensitive and voltage-sensitive K^+currents were studied using the normal whole-cell configuration. Ca^{2+} currents were recorded by the perforated patch technique. Experimental details and composition of the solutions used have been described previously (Plant *et al.*, 1991). In some experiments, the extracellular solution contained 1 mg ml^{-1} BSA. Because BSA was without effect alone and did not influence the action of the tested substances, all results have been pooled.

Measurements of cyclic AMP, inositol phosphates and adenine nucleotides

In the experiments aimed at measuring cyclic AMP, preincubated islets were incubated, in batches of 12, in 1 ml of medium containing 15 mM glucose, with or without genistein. The incubation was stopped, and the cyclic AMP concentration of the islets measured by radioimmunoassay as described previously (Gembal *et al.*, 1993). In the experiments aimed at measuring inositol phosphates, the islets were loaded with *myo*-[2-^{3}H]-inositol during a preincubation of 120 min in the presence of 15 mM glucose. After washing, batches of 50 islets were incubated for 60 min in 1 ml of medium containing 15 mM glucose, 10 mM LiCl, 1 mM unlabelled inositol, with or without genistein. The incubation was stopped by addition of 3 ml of $CHCl_3/CH_3OH$/concentrated HCl (200:100:1) and 100 µl of EDTA (100 mM). Free inositol and inositol phosphates were then separated by anion exchange chromatography, as described previously (Gembal *et al.*, 1993). In the experiments aimed at measuring adenine nucleotides, preincubated islets were incubated, in batches of 5, in 0.4 ml of medium containing 15 mM glucose and 4.8 mM K or 10 mM glucose and 30 mM K, with or without genistein. The incubation was then stopped, ATP and ADP were extracted and assayed by a luminometric method, as described previously (Gembal *et al.*, 1993).

Drugs

Genistein, herbimycin A and the erbstatin analogue methyl 2,5-dihydroxycinnamate were obtained from Calbiochem-Behring (San Diego, CA, USA), daidzein from either Carl Roth GmbH (Karlsruhe, Germany) or LC Laboratories (Woburn, MA, U.S.A.), tyrphostin A47 (RG 50864) from Calbiochem-Behring or LC Laboratories, and genistin from Sigma (St. Louis, MO, U.S.A.). Tolbutamide was from Hoechst AG (Frankfurt/Main, Germany) and diazoxide from Schering-Plough Avondale (Rathdrum, Ireland). Stock solutions of genistein, daidzein and genistin (50 mM), of tyrphostin and erbstatin (100 mM), and of herbimycin A (2 mM) were prepared in dimethyl sulphoxide. Radiochemicals were purchased from the Radiochemical Centre (Amersham, Bucks, United Kingdom).

Statistical analysis

Measurements of intracellular Ca^{2+}, membrane potential and ion channel currents are illustrated by recordings which are representative of the indicated number of experiments. Otherwise, results are expressed as means ± s.e.mean for the indicated number of experiments (different animals or islet preparations) or batches of islets. The statistical significance of differences between means was assessed by unpaired *t* test, or by analysis of variance followed by a Dunnett's test when more than two groups were compared. Differences were considered significant at $P<0.05$.

Results

Characteristics of the effects of genistein on insulin release

The glucose- and concentration-dependence of the effects of genistein on insulin release were studied with incubated islets (Figure 1). In the presence of a non-stimulatory concentration of glucose (3 mM), genistein increased basal release only slightly at 100 µM. On the other hand, genistein potentiated the releasing effect of higher glucose concentrations, including a maximally effective one (30 mM) (Figure 1a). This effect

was statistically significant ($P<0.05$ or less) at 10 μM of the drug. As shown in Figure 1b, the sigmoidal relationship between glucose concentration and insulin release was not markedly altered by 100 μM genistein. The glucose concentrations causing half-maximal stimulation of release were 17 and 14 mM without and with genistein, respectively. Adrenaline (1 μM) inhibited insulin release induced by 15 mM glucose alone (1.25 ± 0.10 ng per islet h^{-1}) or with 100 μM genistein (1.04 ± 0.08 ng per islet h^{-1}).

The dynamics of the potentiation of glucose-induced insulin release by genistein was tested with perifused islets (Figure 2a). In the presence of 15 mM glucose, the effect of genistein was rapid in onset, reached a plateau within about 15 min, and was completely reversible upon withdrawal of the drug. Figure 2a also shows that omission of Ca^{2+} from the perifusion medium prevented both glucose-induced insulin release and its potentiation by genistein.

The effects of genistein were then tested in the presence of nutrient and non-nutrient insulin secretagogues (Table 1). A mixture of 10 mM leucine and glutamine stimulated insulin release as did 10 mM glucose. Genistein similarly potentiated both responses. On the other hand, genistein had little or no significant effect on insulin release induced by barium substitution for Ca^{2+} or by 30 mM K (in the presence of diazoxide which opens K^+_{ATP} channels) when the medium did not contain a nutrient. However, when high K was combined with glucose or with a mixture of leucine and glutamine, a

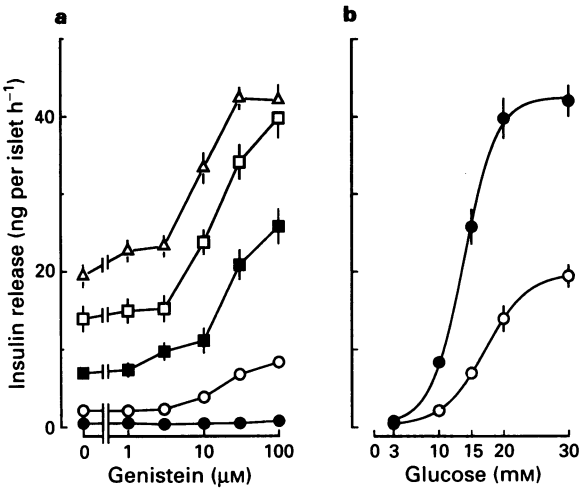


Figure 1 Concentration- and glucose-dependence of genistein effects on insulin release from incubated mouse islets. Batches of 3 islets were incubated for 1 h in 1 ml of medium. (a) Effects of increasing concentrations of genistein in the presence of different glucose concentrations: (△) 30; (□) 20; (■) 15; (○) 10 and (●) 3 mM. (b) Effects of increasing concentrations of glucose in the absence (○) or presence of 100 μM genistein (●). Values are means ± s.e.mean for 17–18 batches of islets from three separate experiments.

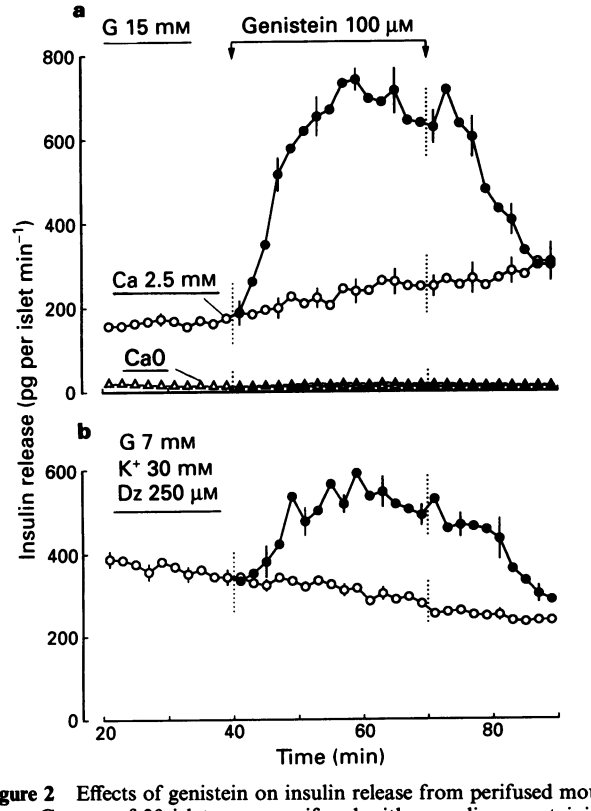


Figure 2 Effects of genistein on insulin release from perifused mouse islets. Groups of 30 islets were perifused with a medium containing: (a) 15 mM glucose (G) and 2.5 mM Ca^{2+} or 0 Ca, or (b) 7 mM glucose, 30 mM K^+ and 250 μM diazoxide (Dz). In each series, genistein (100 μM) was added from 40 to 70 min to one group of islets (●, ▲), while the other group, perifused in parallel, served as control. Values are means ± s.e. mean for five experiments.

Table 1 Effects of genistein on insulin release by mouse islets incubated under various conditions

Experimental conditions (mM)	*Insulin release* (ng per islet h^{-1}) *Control*	*+ Genistein*
Glucose 0	0.43 ± 0.04	0.72 ± 0.09[b]
Glucose 10	2.14 ± 0.21	8.33 ± 0.63[b]
Glucose 15	9.15 ± 0.57	26.4 ± 2.11[b]
Leu 10 + Gln 10	2.13 ± 0.41	7.20 ± 0.70[b]
G 0 + Ba^+ 2.5	7.57 ± 0.38	8.95 ± 0.58[a]
G 0 + K 30	12.5 ± 0.69	15.0 ± 1.00
G 10 + K 30	16.9 ± 0.92	32.4 ± 1.30[b]
G 0 + K 30 + Leu 10 + Gln 10	26.1 ± 1.34	44.6 ± 1.74[b]

Batches of 3 islets were incubated for 1 h in 1 ml of medium containing the indicated concentration of glucose (G), leucine (Leu) and glutamine (Gln), in the absence (control) or presence of 100 μM genistein. $CaCl_2$ was omitted from the medium containing $BaCl_2$. Diazoxide (0.25 mM) was present in the solutions containing 30 mM K. Values are means ± s.e.mean for 14–25 batches of islets from three to five separate experiments.
[a]$P<0.05$; [b]$P<0.01$ versus controls, by unpaired t test.

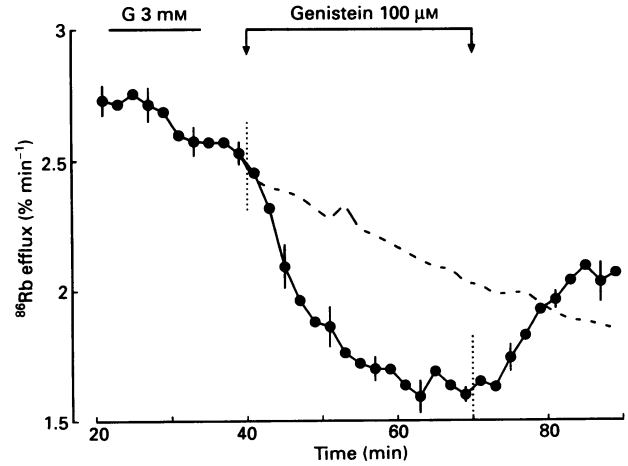


Figure 3 Effects of genistein on ^{86}Rb efflux from perifused mouse islets. After loading with the tracer, groups of 30 islets were perifused with a medium containing 3 mM glucose (G). Genistein (100 μM) was added from 40 to 70 min. Control experiments without genistein are shown by the broken line. Values are means ± s.e.mean for five experiments.

strong potentiating effect of genistein on insulin release became apparent (Table 1). The dynamics of the potentiation of insulin release produced by genistein in the presence of 7 mM glucose, high K and diazoxide is shown in Figure 2b. The increase in the secretory rate reached a plateau after about 15 min and returned to control values 20 min after withdrawal of genistein.

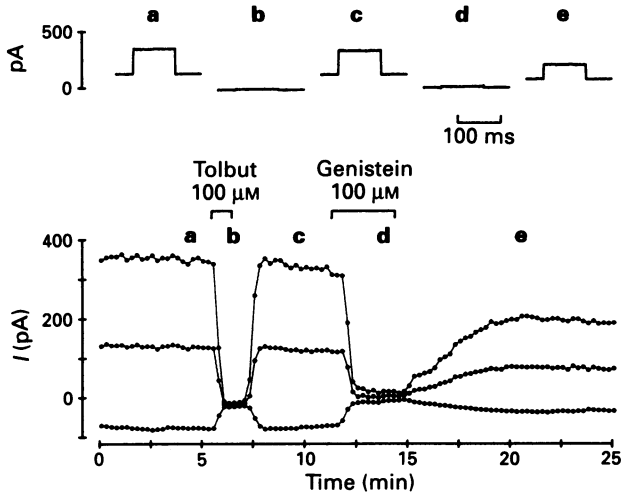


Figure 4 Effects of genistein on whole-cell ATP-sensitive K^+ currents in mouse β-cells. These experiments were performed at room temperature on cultured single β-cells. Current values were recorded at 15 s intervals, at −60, −70 and −80 mV (upper, middle and lower traces of the lower panel, respectively). Tolbutamide (100 μM) and genistein (100 μM) were applied as indicated by the bars. In the upper panel are shown currents recorded during 100 ms pulses from −70 to −60 mV at the times indicated by the letters in the lower panel.

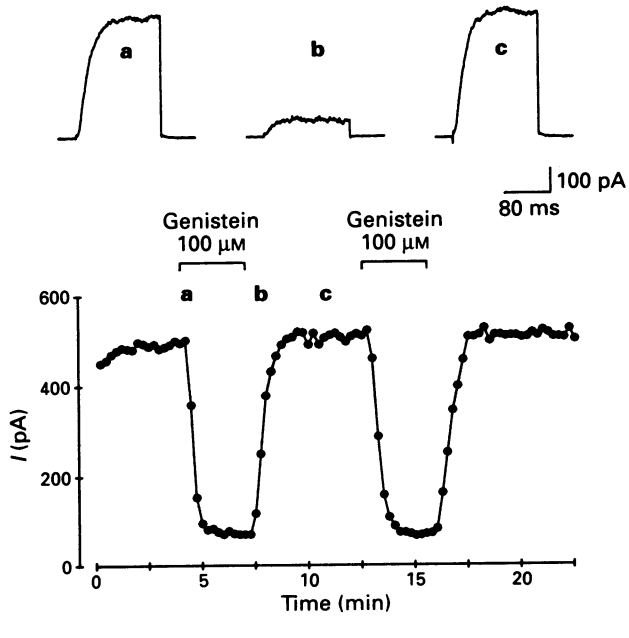


Figure 5 Effects of genistein on voltage-dependent K^+ currents in mouse β-cells. These experiments were performed at room temperature on cultured single β-cells. The currents (a,b,c) shown in the upper panel were recorded during 150 ms voltage steps from −70 to 0 mV, at the times shown in the lower panel. This panel shows values of the maximum K^+ current measured during pulses to 0 mV, applied at 15 s intervals. Genistein 100 μM was applied as indicated by the bars. The bath solution contained 100 μM tolbutamide to inhibit ATP-sensitive K^+ currents and 100 μM Cd^{2+} to block voltage-dependent Ca^{2+} currents and Ca^{2+}-activated K^+ currents.

Effects of genistein on K^+ channels in β-cells

Hypoglycaemic sulphonylureas and many other drugs increase insulin release by closing ATP-sensitive K^+channels (K^+_{ATP} channels); this closure depolarizes the β cell membrane and stimulates Ca^{2+} influx (Ashford, 1990; Henquin, 1990). Closure of K^+_{ATP} channels is readily detected by a decrease in the rate of ^{86}Rb efflux from islets perifused with a low concentration (3 mM) of glucose (Henquin *et al.*, 1992). As shown in Figure 3, genistein inhibited ^{86}Rb efflux under these conditions. The inhibition reached a maximum after about 15 min, and was completely reversible upon withdrawal of the drug.

The whole cell mode of the patch clamp technique was then used to determine whether the drug directly affects the K^+_{ATP} channels (Figure 4). At the beginning of the trace, ATP-sensitive K^+ currents are large because the cell interior has equilibrated with the pipette solution containing a low concentration of ATP (Plant *et al.*, 1991). Tolbutamide (100 μM) rapidly and reversibly inhibited the ATP-sensitive K^+ current. Addition of 100 μM genistein to the bath produced a slightly smaller and less rapidly reversible inhibition (Figure 4). The steady-state inhibition was 82 ± 2% ($n = 9$) for genistein. Under similar conditions, 100 μM tolbutamide inhibited the current by 93 ± 1% (Plant *et al.*, 1991). At the lower concentration of 25 μM, genistein had inconsistent effects; it slightly and transiently increased the ATP-sensitive K^+ current in some cells, and decreased it in others.

Blockade of K^+_{ATP} channels is expected to depolarize the β-cell membrane. The resting membrane potential of β-cells, measured with an intracellular microelectrode, during perifusion of the islets with a medium containing 3 mM glucose averaged −63 ± 2 mV. Genistein (100 μM) depolarized it by 7.8 ± 1.3 mV ($n = 5$), but did not induce the appearance of electrical activity.

Voltage-dependent K^+ channels were studied in the whole cell mode, by depolarizing the β cell membrane from −70 to 0 mV. As shown in Figure 5, 100 μM genistein also reversibly inhibited these channels. The inhibition amounted to 82 ± 1% ($n = 5$).

Role of the blockade of K^+_{ATP} channels in the potentiation of insulin release by genistein

The increase in insulin release brought about by tolbutamide is entirely attributable to a blockade of K^+_{ATP} channels

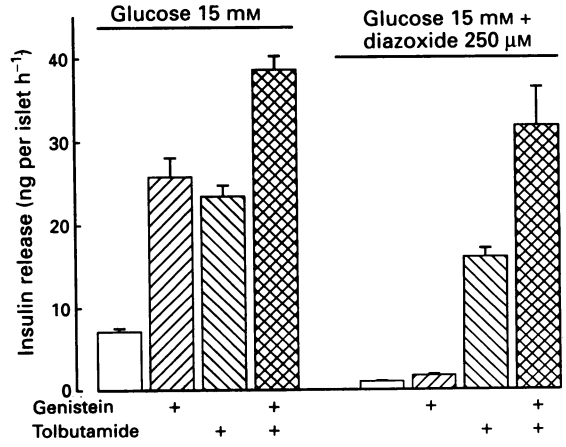


Figure 6 Comparison of the effects of genistein and tolbutamide on insulin release from incubated mouse islets. Batches of 3 islets were incubated for 1 h in 1 ml of medium containing 15 mM glucose and supplemented with 100 μM genistein and/or 250 μM tolbutamide as indicated. In the experiments shown by the 4 right-hand columns, 250 μM diazoxide was also present in the medium. Values are means ± s.e.mean for 18–20 batches of islets from four separate experiments.

(Ashford, 1990; Henquin, 1990). At a maximally effective concentration of 250 μM, tolbutamide potentiated glucose-induced insulin release to the same extent as did 100 μM genistein (Figure 6). The combination of both agents produced a much larger effect than either agent alone ($P<0.01$).

When K^+_{ATP} channels were opened by 250 μM diazoxide, glucose-induced insulin release was inhibited by 87 ± 1% (Figure 6). This inhibition was not significantly reversed by 100 μM genistein, whereas 250 μM tolbutamide increased insulin release even more than did glucose in the absence of diazoxide. Again, the combination of both agents produced a larger effect than either agent alone.

These results, therefore, indicate that a mechanism other than the inhibition of K^+_{ATP} channels by genistein plays a role in the increase in insulin release that the drug produces.

Effects of genistein on β-cell cytoplasmic $[Ca^{2+}]_i$ and Ca^{2+} channels

In the presence of 15 mM glucose, β-cells display a rhythmic electrical activity that reflects the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Figure 7). Agents that close K^+_{ATP} channels increase this electrical activity and stimulate Ca^{2+} influx.

Unexpectedly, genistein inhibited glucose-induced electrical activity to a variable degree from cell to cell. Even when the inhibition was strong, small oscillations of the membrane potential persisted and the membrane never repolarized to the resting level (Figure 7a and b). Figure 7c shows one cell in which the inhibition was less pronounced.

The intermittent influx of Ca^{2+} (during the phases of electrical activity) causes oscillations of $[Ca^{2+}]_i$ in β-cells (Gilon & Henquin, 1992; Santos *et al.*, 1991). Addition of genistein to a medium containing 15 mM glucose was followed by a progressive disappearance of these oscillations (Figure 8a) and a stabilization of $[Ca^{2+}]_i$ at a level that remained about 80 nM higher than basal $[Ca^{2+}]_i$.

When K^+_{ATP} channels are open by diazoxide, an increase of extracellular K to 30 mM causes sustained depolarization

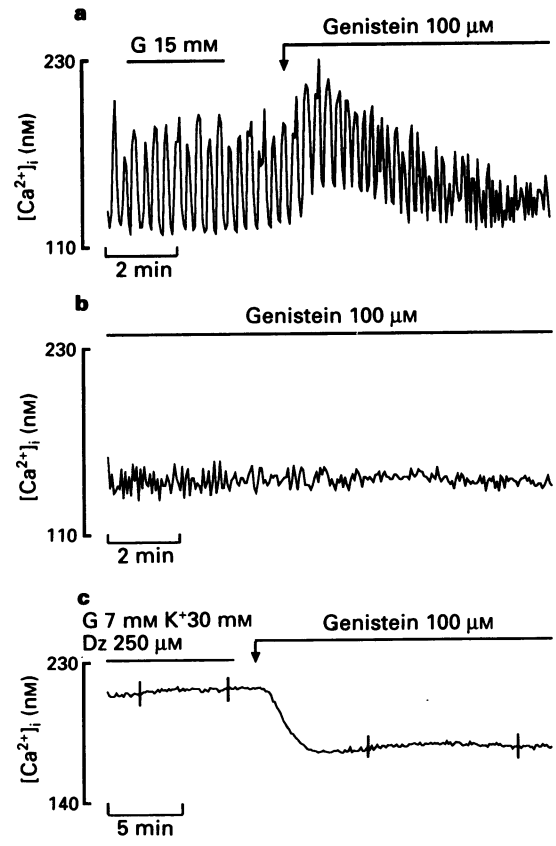


Figure 8 Effects of genistein on the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in islet cells. After loading with fura-2, islets were perifused with a medium containing 15 mM glucose (a,b) or 7 mM glucose, 30 mM K^+ and 250 μM diazoxide (c). Genistein 100 μM was added as indicated by the arrows. Panel (b) is the continuation of (a); they show an experiment that is representative of results obtained with 15 islets. Panel (c) shows means ± s.e.mean for 9 islets.

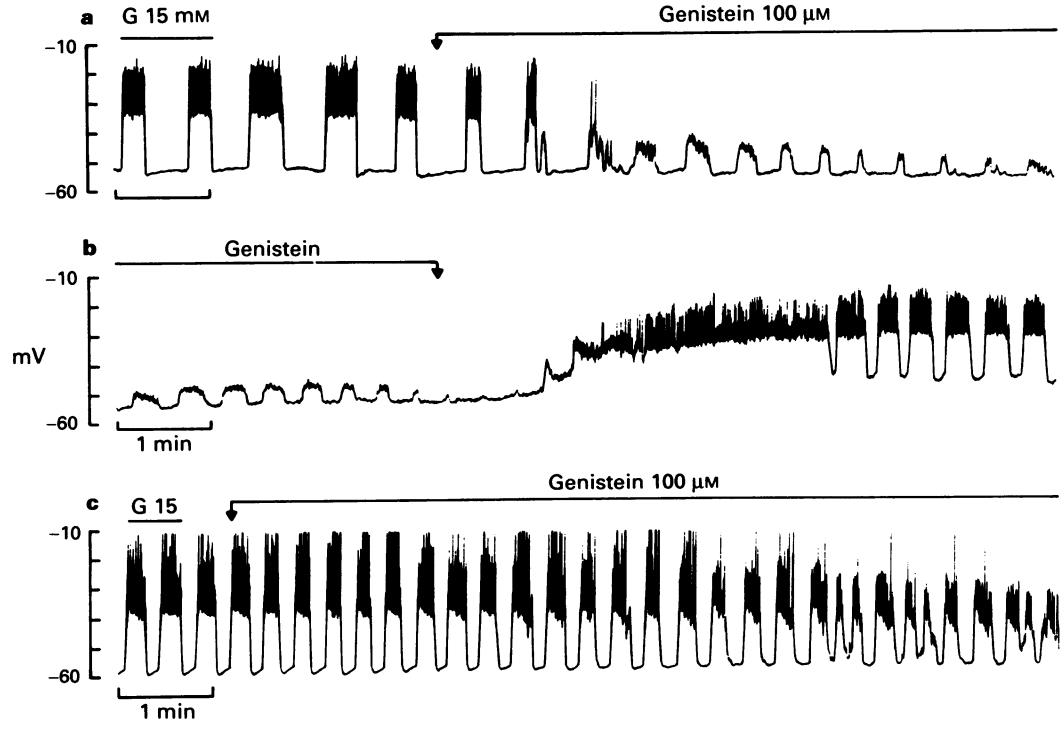


Figure 7 Effects of genistein on the membrane potential of a mouse β-cell perifused with 15 mM glucose (G). Genistein (100 μM) was added and removed as indicated by the arrows. Panels (a) and (b) show a single experiment without interruption. Panel (c) illustrates results obtained in another cell. These recordings are representative of results obtained in five experiments with different mice.

of the β-cell membrane and rise in β-cell $[Ca^{2+}]_i$ (Gembal *et al.*, 1993). Under these conditions, genistein was without effect on the membrane potential (not shown), but decreased $[Ca^{2+}]_i$ (Figure 8c).

These paradoxical effects of genistein led us to investigate a possible action of the drug on voltage-dependent Ca^{2+} channels. These were studied by the perforated patch technique (Plant *et al.*, 1991) during depolarization steps from −70 to 0 mV. As shown in Figure 9, genistein reversibly inhibited Ca^{2+} currents. The effect of the drug on peak current averaged 65 ± 4%, and was slowly reversible upon withdrawal.

Effects of genistein on cyclic AMP, inositol phosphate and adenine nucleotide levels

Genistein did not affect cyclic AMP levels in islets incubated in the presence of 15 mM glucose (Table 2). No effect could be detected either after blockade of the cyclic AMP phosphodiesterase by 0.5 mM isobutylmethylxanthine (26.6 ± 2.5 and 26.9 ± 2.6 fmol cyclic AMP per islet without and with genistein, $n = 9$). Genistein did not affect IP_3 and IP_2 levels, and slightly decreased IP_1 levels in islets incubated in the presence of 15 mM glucose (Table 2). Neither ATP nor ADP levels were affected by genistein in the presence of 15 mM glucose or of 10 mM glucose with high K and diazoxide. The ratio ATP/ADP was slightly decreased only in the latter condition (Table 2).

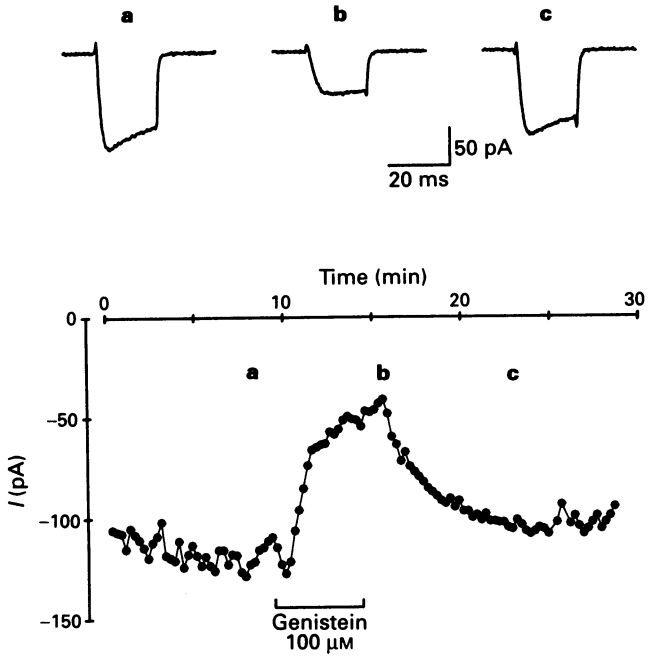


Figure 9 Effects of genistein on Ca^{2+} currents in mouse β-cells. Ca^{2+} currents were recorded by the perforated patch technique. The currents (a,b,c) shown in the upper panel were recorded during 20 ms voltage steps from −70 to 0 mV, at the times shown in the lower panel. This panel shows peak Ca^{2+} currents recorded at 15 s intervals. Genistein 100 μM was applied as indicated by the bar. To block K^+ currents, the bath solution contained tolbutamide (100 μM) and tetraethylammonium (20 mM), and the pipette solution contained 140 mM Cs^+. To block Na^+ currents, the bath solution contained 0.5 μM tetrodotoxin. The concentration of Ca^{2+} in the bath was 10 mM.

Effects of daidzein and genistin

Daidzein is an analogue of genistein reported to be without effect on tyrosine kinases (Akiyama & Ogawara, 1991). At a concentration of 50 μM, daidzein increased insulin release from islets perifused with a medium containing 15 mM glucose. This effect was similar to that produced by 50 μM genistein (10.7 ± 0.7 and 11.5 ± 1.1 ng per islet 30 min^{-1}, $n = 4$). Insulin release induced by 7 mM glucose, 30 mM K and 0.25 mM diazoxide (6.8 ± 0.2 ng per islet 30 min^{-1}, $n = 4$) was also similarly potentiated by 100 μM daidzein and genistein (13.8 ± 1.1 and 15.6 ± 2.0 ng per islet 30 min^{-1}, respectively). The effects of 100 μM daidzein on K^+_{ATP} channels were variable as were those of 25 μM genistein. Both slight activation and inhibition were observed. The effects of daidzein on cytoplasmic $[Ca^{2+}]_i$ could not be tested because the fluorescence of the drug interfered with the measurements. Ca^{2+} currents were inhibited by daidzein, but this inhibition was smaller than that produced by genistein (41 ± 11%, $n = 4$). In contrast to daidzein, genistin, another analogue of genistein without effect on tyrosine kinases (Akiyama & Ogawara, 1991), did not potentiate glucose-induced insulin release (data not shown).

Effects of tyrphostin A47, herbimycin A and an erbstatin analogue

Three tyrosine kinase inhibitors other than genistein were tested on insulin release at concentrations selected according to their potency on the kinase (Levitzki *et al.*, 1991; Uehara & Fukazawa, 1991; Umezawa & Imoto, 1991). In these experiments, the islets were incubated for 60 min in a medium containing 15 mM glucose. As compared to controls insulin release was decreased to 48 ± 6% ($n = 15$, $P < 0.01$) by 100 μM tyrphostin A47, increased to 130 ± 10% ($n = 15$, $P < 0.05$) by 2 μM herbimycin A and not affected (93 ± 9%, $n = 10$) by 100 μM of the erbstatin analogue.

Discussion

In the present study we show that the tyrosine kinase inhibitor, genistein, produces a number of sometimes paradoxical effects in mouse islets, which result in a marked potentiation of insulin release. This potentiation occurred over a range of concentrations (3–100 μM) which encompasses the IC_{50} value (~25 μM) for the inhibition by genistein of tyrosine kinases (Akiyama & Ogawara, 1991). Although

Table 2 Effects of genistein on cyclic AMP, inositol phosphate and adenine nucleotide levels in mouse incubated islets

Experimental conditions (mM)	*Cyclic AMP* (fmol per islet)	*IP_1*	*IP_2*	*IP_3* (d.p.m. per islet)	*ATP*	*ADP* (pmol per islet)	*ATP/ADP*
Glucose 15	6.7 ± 0.7	98 ± 6	31 ± 2	34 ± 1	22.3 ± 1.7	7.8 ± 0.6	2.89 ± 0.07
+ genistein 0.1	7.6 ± 0.8	78 ± 5[a]	30 ± 3	34 ± 2	22.2 ± 1.7	7.8 ± 0.6	2.88 ± 0.08
G10 + K 30 + diazoxide 0.25					20.0 ± 1.5	8.6 ± 0.7	2.37 ± 0.05
+ genistein 0.1					18.2 ± 1.4	9.1 ± 0.6	1.98 ± 0.05[b]

The islets were incubated for 1 h in a medium containing 15 mM glucose or 10 mM glucose (G), 30 mM K and 0.25 mM diazoxide in the absence or presence of 100 μM genistein. At the end of the incubation, cyclic AMP, inositol phosphates and adenine nucleotides were measured as described in the Methods section. Values are means ± s.e.mean for 21–22 batches of islets from 4–5 separate experiments.
[a]$P < 0.05$, [b]$P < 0.01$ versus controls without genistein, by unpaired t test.

cytotoxic effects of genistein have sometimes been reported (Linassier *et al.*, 1990), we can exclude the possibility that the increase in insulin release is due to damage of β-cells for the following reasons. The effect of genistein showed saturation at high concentration, was reversible upon removal of the drug, was dependent on the presence of a metabolizable substrate, and was inhibited by adrenaline or omission of extracellular Ca^{2+}.

Genistein was practically without effect in the presence of a non-stimulatory concentration of glucose. This may mean that the effect of genistein on insulin release can manifest itself only if the concentration of glucose is high enough, or if insulin release is already stimulated. The latter condition is not sufficient because genistein was ineffective when insulin release was stimulated by barium or high K in the absence of glucose, and became effective when glucose was present in the high K medium. On the other hand, glucose is not a prerequisite, since it could be replaced by a mixture of leucine and glutamine. The requirements for the acute effects of genistein are thus an already stimulated release and the availability of a fuel for β-cells.

Blockade of K^+_{ATP} channels in the plasma membrane of β-cells is a key step in the stimulation of insulin release by glucose and by many pharmacological agents, in particular the hypoglycemic sulphonylureas (Ashford, 1990; Henquin, 1990). This blockade causes depolarization of the membrane, with subsequent opening of voltage-dependent Ca^{2+} channels, acceleration of Ca^{2+} influx and rise of the cytosolic Ca^{2+}. Genistein inhibited K^+_{ATP} channels in β-cells, as indicated by the decrease in ^{86}Rb efflux that the drug produced in the presence of a low concentration of glucose, and directly demonstrated by the patch-clamp experiments. This blockade may explain the small depolarization of β-cells produced by genistein in the presence of 3 mM glucose. On the other hand, it cannot explain the potentiation of insulin release for several reasons. First, the effects of genistein and tolbutamide on insulin release were additive, even when tolbutamide was used at a maximally effective concentration that blocks all K^+_{ATP} channels. Second, at a concentration of 100 μM, genistein did not antagonize the inhibition of insulin release brought about by the opening of K^+_{ATP} channels with diazoxide. However, genistein increased insulin release induced by high K in the presence of diazoxide (provided a fuel was present). Third, in contrast to all agents that inhibit K^+_{ATP} channels, genistein did not increase cytoplasmic Ca^{2+} in β cells.

On the contrary, genistein decreased the concentration of cytoplasmic Ca^{2+} in islet cells, both in the presence of 15 mM glucose or when the membrane was depolarized by high K in the presence of diazoxide. This decrease can be ascribed to an inhibition of voltage-dependent Ca^{2+} channels, as shown by the inhibition of the Ca^{2+}-dependent electrical activity induced by glucose, and by the block of voltage-activated Ca^{2+} currents in β-cells.

The potentiation of insulin release by genistein is thus very unusual, in that it occurs in spite of a decrease in cytoplasmic Ca^{2+}. This means that the drug somehow sensitizes the releasing machinery to the action of $[Ca^{2+}]_i$. Three known mechanisms are possible.

First, activation of the cyclic AMP-dependent protein kinase A increases insulin release partly by increasing the efficacy of Ca^{2+} on the exocytotic process (Tamagawa *et al.*, 1985; Jones *et al.*, 1988). In the MIN 6 cell line, genistein concomitantly increased insulin release and cyclic AMP levels (Ohno *et al.*, 1993). This was not the case in normal mouse islets, in which genistein did not affect cyclic AMP levels in the presence or absence of isobutyl-methylxanthine. It is also unlikely that genistein activates the kinase directly, since the drug (in contrast to cyclic AMP) was without effect on insulin release by permeabilized rat islets (S.J. Persaud and P.M. Jones, personal communication).

Second, activation of protein kinase C by phorbol esters increases insulin release while lowering cytoplasmic Ca^{2+} (Gao *et al.*, 1994). However, high concentrations of genistein do not stimulate but weakly inhibit the kinase in cell-free systems (Akiyama & Ogawara, 1991). Moreover, in contrast to phorbol esters (Tamagawa *et al.*, 1985; Jones *et al.*, 1988) genistein does not increase insulin release from permeabilized islets (S.J. Persaud and P.M. Jones, personal communication). This argues against a direct stimulation of PKC by the drug. It is also clear that phorbol esters do not have the effects of genistein on K^+ or Ca^{2+} channels and on the membrane potential of mouse β cells (Bozem *et al.*, 1987; Henquin *et al.*, 1989). The lack of effect of genistein on inositol phosphate production also indicates that genistein does not increase diacylglycerol production, at least from phosphoinositides.

Third, we and others have recently reported that, besides its action on K^+_{ATP} channels and the β-cell membrane potential, glucose can increase insulin release by augmenting the effectiveness of Ca^{2+} on its intracellular targets (Gembal *et al.*, 1992; Sato *et al.*, 1992). The biochemical nature of this novel effect has yet to be established, but several lines of evidence indicate that changes in the ATP/ADP ratio are involved (Gembal *et al.*, 1993). This is not the case for the potentiating action of genistein because the drug was either without effect or slightly decreased the ATP/ADP ratio in islet cells.

Genistein and several other tyrosine kinase inhibitors also inhibit lactate transport (Young *et al.*, 1993). Inhibition of lactate efflux from islet cells has sometimes been reported to increase insulin release (Best *et al.*, 1991), but this observation has not been explained. We do not believe that such an effect mediates the action of genistein because the drug increased insulin release not only in the presence of glucose, but also in the presence of leucine and glutamine which do not increase lactate production in islet cells (Malaisse *et al.*, 1982).

Several recent studies have reported that genistein affects the concentration of cytoplasmic $[Ca^{2+}]_i$ in other cell types. There is general agreement that genistein inhibits the rise in $[Ca^{2+}]_i$ that thrombin produces in platelets, but the underlying mechanisms are still disputed. In one study, the inhibitory effect of genistein was ascribed to an interference with inositol phosphate metabolism (Ozaki *et al.*, 1993). In two other studies, genistein did not affect the initial mobilization of $[Ca^{2+}]_i$ (suggesting that inositol phosphate formation was unaltered) but inhibited the delayed and more sustained rise in Ca^{2+} requiring Ca^{2+} influx (Asahi *et al.*, 1992; Sargeant *et al.*, 1993). A similar observation was made in fibroblasts stimulated with bradykinin (Lee *et al.*, 1993). These studies, therefore, suggest that genistein inhibits the voltage-independent Ca^{2+} influx pathway that is activated upon emptying of intracellular Ca^{2+} stores by agonists acting on membrane receptors. In the present study, voltage-dependent Ca^{2+} channels were inhibited by genistein. A similar effect has previously been observed only in vascular smooth muscle cells (Wijetunge *et al.*, 1992). In pancreatic β-cells, the inhibitory effects of genistein were not restricted to Ca^{2+} channels. The drug also closed K^+channels. Such an effect has not been reported for other cell types. It is thus becoming progressively evident that genistein exerts a number of effects that are not always clearly secondary to an inhibition of tyrosine kinases.

Daidzein and genistin are often used as analogues of genistein that are devoid of activity on tyrosine kinases (Akiyama & Ogawara, 1991). Daidzein has been reported not to mimic the effects of genistein in several systems, e.g. on Ca^{2+} channels or Ca^{2+} fluxes (Wijetunge *et al.*, 1992; Sargeant *et al.*, 1993; Lee *et al.*, 1993), and this lack of effect of daidzein was interpreted as evidence in support of a role of tyrosine kinases in the process affected by genistein. On the other hand, daidzein reproduced the inhibitory effect of genistein on the responses of platelets to thromboxane A_2 (Nakashima *et al.*, 1991). In β-cells, daidzein was slightly less potent than genistein on $K^+$and Ca^{2+} channels, but increased

insulin release in a similar way. Thus, although genistin was without effect, we cannot ascribe the potentiation of insulin release by genistein to an inhibition of tyrosine kinases. The use of other tyrosine kinase inhibitors did not clarify the issue since tyrphostin A47 inhibited insulin secretion, herbimycin A increased it and an erbstatin analogue was without effect.

In conclusion, genistein, which is currently widely used as a selective inhibitor of tyrosine kinases, exerts a number of heretofore unrecognized effects. The present study has several important implications. First, great caution should be exercised before concluding that a biological process inhibited by genistein is under direct control of tyrosine kinases. Second, the mode of action of genistein in pancreatic β-cells is rather unusual. The mechanisms by which the drug increases insulin release in spite of a decrease in $[Ca^{2+}]_i$ and without activating the known potentiating pathways certainly calls for further exploration. Third, whether tyrosine kinases are involved in stimulus-secretion coupling in normal β-cells remains to be established.

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References

ABLER, A., SMITH, J.A., RANDAZZO, P.A., ROTHENBERG, P.L. & JARETT, L. (1992). Genistein differentially inhibits postreceptor effects of insulin in rat adipocytes without inhibiting the insulin receptor kinase. *J. Biol. Chem.*, **267**, 3946–3951.

AKIYAMA, T. & OGAWARA, H. (1991). Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol.*, **201**, 362–370.

ASAHI, M., YANAGI, S., OHTA, S., INAZU, T., SAKAI, K., TAKEUCHI, F., TANIGUCHI, T. & YAMAMURA, H. (1992). Thrombin-induced human platelet aggregation is inhibited by protein-tyrosine kinase inhibitors, ST638 and genistein. *FEBS Lett.*, **309**, 10–14.

ASHFORD, M.L.J. (1990). Potassium channels and modulation of insulin secretion. In *Potassium Channels. Structure, Classification, Function and Therapeutic Potential.* ed Cook, N.S. pp. 300–325. Chichester: Ellis Horwood.

BEST, L., TREBILCOCK, R. & TOMLINSON, S. (1991). Acute stimulation of pancreatic islets by inhibitors of lactic acid transport. *Biochem. Pharmacol.*, **41**, 405–409.

BOZEM, M., NENQUIN, M. & HENQUIN, J.C. (1987). The ionic, electrical, and secretory effects of protein kinase C activation in mouse pancreatic B-cells: studies with a phorbol ester. *Endocrinology*, **121**, 1025–1033.

CLARK, S.A. & CHICK, W.L. (1990). Islet cell culture in defined serum-free medium. *Endocrinology*, **126**, 1895–1903.

GAO, Z.Y., GILON, P.G. & HENQUIN, J.C. (1994). The role of protein kinase C in signal transduction through vasopressin and acetylcholine receptors in pancreatic B-cells from normal mouse. *Endocrinology*, **135**, 191–199.

GARRINO, M.G. & HENQUIN, J.C. (1988). Highly potent and stereoselective effects of the benzoic acid derivative AZ-DF 265 on pancreatic β cells. *Br. J. Pharmacol.*, **93**, 61–68.

GEMBAL, M., DETIMARY, P., GILON, P., GAO, Z.Y. & HENQUIN, J.C. (1993). Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse B cells. *J. Clin. Invest.*, **91**, 871–880.

GEMBAL, M., GILON, P. & HENQUIN, J.C. (1992). Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+-channels in mouse B cells. *J. Clin. Invest.*, **89**, 1288–1295.

GILON, P. & HENQUIN, J.C. (1992). Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell. *J. Biol. Chem.*, **267**, 20713–20720.

HENQUIN, J.C. (1990). Established, unsuspected and novel pharmacological insulin secretagogues. In *New Antidiabetic Drugs* ed. Bailey, C.J. & Flatt, P.R. pp. 93–106. London: Smith-Gordon.

HENQUIN, J.C., DEBUYSER, A., DREWS, G. & PLANT, T.D. (1992). Regulation of K^+ permeability and membrane potential in insulin-secreting cells. In *Nutrient Regulation of Insulin Secretion* ed. Flatt, P.R. pp. 173–191. London: Portland Press.

HENQUIN, J.C., SCHMEER, W., NENQUIN, M. & PLANT, T.D. (1989). Does protein kinase C link glucose metabolism to B-cell membrane depolarization? *Diabetologia*, **32**, 496.

HUGHES, S.J. & ASHCROFT, S.J.H. (1992). Cyclic AMP, protein phosphorylation, and insulin secretion. In *Nutrient Regulation of Insulin Secretion* ed. Flatt, P.R. pp. 271–288. London: Portland Press.

JONES, P.M., PERSAUD, S.J. & HOWELL, S.L. (1991). Protein kinase C and the regulation of insulin secretion from pancreatic B cells. *J. Mol. Endocrinol.*, **6**, 121–127.

JONES, P.M., SALMON, D.M.W. & HOWELL, S.L. (1988). Protein phosphorylation in electrically permeabilised islets of Langerhans. Effects of Ca^{2+}, cyclic AMP, a phorbol ester and noradrenaline. *Biochem. J.*, **254**, 397–403.

LEAHY, J.L. & VANDEKERKHOVE, K.M. (1990). Insulin-like growth factor-I at physiological concentrations is a potent inhibitor of insulin secretion. *Endocrinology*, **126**, 1593–1598.

LEE, K.M., TOSCAS, K. & VILLEREAL, M.L. (1993). Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *J. Biol. Chem.*, **268**, 9945–9948.

LEVITZKI, A., GAZIT, A., OSHEROV, N., POSNER, I. & GILON, C. (1991). Inhibition of protein-tyrosine kinases by tyrphostins. *Methods Enzymol.*, **201**, 347–361.

LINASSIER, C., PIERRE, M., LE PECQ, J.B. & PIERRE, J. (1990). Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. *Biochem. Pharmacol.*, **39**, 187–193.

MALAISSE, W.J., SENER, A., MALAISSE-LAGAE, F., WELSH, M., MATTHEWS, D.E., BIER, D.M. & HELLERSTRÖM, C. (1982). The stimulus-secretion coupling of amino acid-induced insulin release. Metabolic response of pancreatic islets to L-glutamine and L-leucine. *J. Biol. Chem.*, **257**, 8731–8737.

MEISSNER, H.P. & SCHMELZ, H. (1974). Membrane potential of beta cells in pancreatic islets. *Pflügers Arch.*, **351**, 195–206.

NAKASHIMA, S., KOIKE, T. & NOZAWA, Y. (1991). Genistein, a protein tyrosine kinase inhibitor, inhibits thromboxane A_2-mediated human platelet responses. *Mol. Pharmacol.*, **39**, 475–480.

OHNO, T., KATO, N., ISHII, C., SHIMIZU, M., ITO, Y., TOMONO, S. & KAWAZU, S. (1993). Genistein augments cyclic adenosine 3′5′-monophosphate (cAMP) accumulation and insulin release in MIN6 cells. *Endocrine Res.*, **19**, 273–285.

OZAKI, Y., YATOMI, Y., JINNAI, Y. & KUME, S. (1993). Effects of genistein, a tyrosine kinase inhibitor, on platelet functions. Genistein attenuates thrombin-induced Ca^{2+} mobilization in human platelets by affecting polyphosphoinositide turnover. *Biochem. Pharmacol.*, **46**, 395–403.

PLANT, T.D., JONAS, J.C. & HENQUIN, J.C. (1991). Clonidine inhibits ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **104**, 385–390.

PRENTKI, M. & MATSCHINSKY, F.M. (1987). Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol. Rev.*, **67**, 1185–1248.

SANTOS, R.M., ROSARIO, L.M., NADAL, A., GARCIA-SANCHO, J., SORIA, B. & VALDEOLMILLOS, M. (1991). Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflügers Arch.*, **418**, 417–422.

SARGEANT, P., FARNDALE, R.W. & SAGE, S.O. (1993). The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets. *FEBS Lett.*, **315**, 242–246.

SATO, Y., AIZAWA, T., KOMATSU, M., OKADA, N. & YAMADA, T. (1992). Dual functional role of membrane depolarization -Ca^{2+} influx in rat pancreatic B-cell. *Diabetes*, **41**, 438–443.

SORENSON, R.L., BRELJE, T.C. & ROTH, C. (1994). Effect of tyrosine kinase inhibitors on islets of Langerhans: evidence for tyrosine kinases in the regulation of insulin secretion. *Endocrinology*, **134**, 1975–1978.

SWARUP, G., COHEN, S. & GARBERS, D.L. (1982). Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem. Biophys. Res. Commun.*, **107**, 1104–1109.

TAMAGAWA, T., NIKI, H. & NIKI, A. (1985). Insulin release independent of a rise in cytosolic free Ca^{2+} by forskolin and phorbol ester. *FEBS Lett.*, **183**, 430–432.

UEHARA, Y. & FUKAZAWA, H. (1991). Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinases. *Methods Enzymol.*, **201**, 370–379.

UMEZAWA, K. & IMOTO, M. (1991). Use of erbstatin as protein-tyrosine kinase inhibitor. *Methods Enzymol.*, **201**, 379–385.

VAN SCHRAVENDIJK, C.F.H., FORIERS, A., VAN DEN BRANDE, J.L. & PIPELEERS, D.G. (1987). Evidence for the presence of type I insulin-like growth factor receptors on rat pancreatic A and B cells. *Endocrinology*, **121**, 1784–1788.

VAN SCHRAVENDIJK, C.F.H., HEYLEN, L., VAN DEN BRANDE, J.L. & PIPELEERS, D.G. (1990). Direct effects of insulin and insulin-like growth factor-I on the secretory activity of rat pancreatic beta cells. *Diabetologia*, **33**, 649–653.

WENHAM, R.M., LANDT, M. & EASOM, R.A. (1994). Glucose activates the multifunctional Ca^{2+}/Calmodulin-dependent protein kinase II in isolated rat pancreatic islets. *J. Biol. Chem.*, **269**, 4947–4952.

WIJETUNGE, S., AALKJAER, C., SCHACHTER, M. & HUGHES, A.D. (1992). Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **189**, 1620–1623.

YOUNG, S.W., POOLE, R.C., HUDSON, A.T., HALESTRAP, A.P., DENTON, R.M. & TAVARÉ, J.M. (1993). Effects of tyrosine kinase inhibitors on protein kinase-independent systems. *FEBS Lett.*, **316**, 278–282.

ZHANG, A., GAO, Z.Y., GILON, P., NENQUIN, M., DREWS, G. & HENQUIN, J.C. (1991). Vanadate stimulation of insulin release in normal mouse islets. *J. Biol. Chem.*, **266**, 21649–21656.

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Dysfunction of muscarinic M_2 receptors after the early allergic reaction: possible contribution to bronchial hyperresponsiveness in allergic guinea-pigs

[1]Ronald E.J. ten Berge, Ruud E. Santing, Jacob Jan Hamstra, Ad F. Roffel & Johan Zaagsma

Department of Medicinal Chemistry and Molecular Pharmacology, University Centre for Pharmacy, A. Deusinglaan 2, 9713 AW Groningen, The Netherlands

1 Using a guinea-pig model of allergic asthma, in which the animals display early (0-5 h) and late phase (8-23 h after antigen challenge) bronchoconstrictor reactions, the function of prejunctional inhibitory M_2 and postjunctional M_3 receptors in isolated tracheal preparations have been investigated. In addition, cardiac M_2 receptor function *in vitro* and bronchial responsiveness to histamine *in vivo* were evaluated.

2 Sensitivity to inhaled histamine was increased 3.1 fold and 1.6 fold after the early and late allergic reactions (i.e. at 5 h and 23 h after a single ovalbumin challenge), respectively. At 23 h after the last of four allergen challenges, executed on four consecutive days, bronchial hyperresponsiveness to histamine was diminished to 1.3 fold.

3 After the early response, there was no change in cardiac muscarinic M_2 receptor function, since in left atria pD_2 ($-\log EC_{50}$) and E_{max} values of pilocarpine and pK_B values of AQ-RA 741, a selective M_2 receptor antagonist, were not significantly different from controls (unchallenged sensitized animals), and this also applied to methacholine pD_2 values for muscarinic M_3 receptors in tracheal smooth muscle.

4 Prejunctional inhibitory muscarinic M_2 autoreceptors in airway smooth muscle were markedly dysfunctional after the early allergic response, since potentiation of electrically evoked twitch contractions of tracheal preparations by low concentrations of the M_2-selective muscarinic receptor antagonists, gallamine, methoctramine, AQ-RA 741 and AF-DX 116, which is the result of M_2 receptor blockade, was clearly and significantly diminished compared to controls. However, after the late response, both in single and repeatedly challenged animals, twitch potentiation was not significantly different from and similar to controls, indicating restoration of M_2 receptor function during the late allergic reaction.

5 It is concluded that dysfunction of muscarinic M_2 autoreceptors in the airways of sensitized and challenged guinea-pigs is already present after the early allergic reaction, and that it has recovered after the late response. Since histamine-induced bronchoconstriction involves vagal pathways, the present results suggest that bronchial hyperresponsiveness to histamine is partly due to M_2 autoreceptor dysfunction, leading to increased release of acetylcholine.

Keywords: Guinea-pig trachea; prejunctional muscarinic M_2 receptors; receptor dysfunction; electrical field stimulation; muscarinic receptor antagonists; early and late allergic reactions; bronchial hyperresponsiveness

Introduction

Bronchial hyperresponsiveness is one of the most important features of asthma in man (Cockcroft *et al.*, 1977) and in models of asthma in experimental animals (Wanner *et al.*, 1990). However, the mechanisms behind this hyperresponsiveness are barely understood. Whereas in ovalbumin-challenged guinea-pigs sensitivity to histamine, given by aerosol, is significantly increased *in vivo*, the response of the smooth muscle to histamine *in vitro* is unchanged from controls (Stewart & Fennesy, 1983). Alternatively, an altered function of the cholinergic nervous system may play a role, since Hulbert *et al.* (1985) showed that part of the response to histamine in guinea-pigs *in vivo* is mediated via the vagus nerve. This observation was strengthened by Ayala & Ahmed (1989) who demonstrated in man that the muscarinic antagonist, ipratropium bromide, attenuated histamine-induced bronchoconstriction. In addition, antigen provocation results in increased vagal sensitivity, since vagally-induced contraction of airway smooth muscle of challenged animals is potentiated *in vivo* and *in vitro* while responses to exogenous acetylcholine are unchanged (McCaig, 1987; Fryer & Wills-Karp, 1991).

In the airways, the release of acetylcholine from vagus nerve endings is under the inhibitory control of muscarinic autoreceptors (Fryer & Maclagan, 1984) of the M_2 subtype, while postjunctionally muscarinic M_3 receptors are involved in airway smooth muscle contraction (Barnes *et al.*, 1988; Ten Berge *et al.*, 1993). These prejunctional M_2 receptors have been demonstrated in many species including guinea-pig (Fryer & Maclagan, 1984; Faulkner *et al.*, 1986) and man (Minette & Barnes, 1988), and it has been suggested that they become dysfunctional in asthmatics (Ayala & Ahmed, 1989; Minette *et al.*, 1989) and in guinea-pigs after acute viral infection (Fryer & Jacoby, 1991), ozone exposure (Schultheis *et al.*, 1994) and antigen challenge of sensitized animals (Fryer & Wills-Karp, 1991). Such dysfunction may partly explain airway hyperresponsiveness.

Recently, a guinea-pig model of allergic asthma was developed in our laboratory, in which lung function is monitored on-line in unrestrained animals, and in which early (0-5 h) and late phase (8-23 h after antigen challenge) bronchoconstrictor reactions, bronchial hyperresponsiveness and inflammatory cell influxes have been demonstrated both after a single and after repeated antigen challenge (Santing *et al.*, 1992; 1994a). Using this model, in the present study we investigated bronchial reactivity *in vivo* and the function of

[1] Author for correspondence.

prejunctional inhibitory M_2 and postjunctional M_3 receptors *in vitro* using isolated tracheal preparations obtained 6 or 24 h after the single challenge (i.e. after the early or late allergic reaction) or 24 h after completion of four challenges executed on four consecutive days, which latter protocol is similar to that of Fryer & Wills-Karp (1991). Cardiac M_2 receptor function *in vitro* was determined for comparison in isolated atrial preparations.

Methods

Animals

Outbred guinea-pigs of either sex, weighing approximately 300 g, were sensitized to ovalbumin at three weeks of age. A shift to the heat-labile IgE class antibodies (as determined by passive cutaneous anaphylaxis) was obtained by adding aluminium hydroxide powder to a final concentration of 100 mg ml^{-1} to an antigen solution containing 100 μg ovalbumin per ml saline. The antigen solution was injected intraperitoneally (0.5 ml) and intradermally in the proximity of seven lymph nodes in the paws, lumbar regions and neck (70 μl each). This procedure is a modification of the method of Andersson (1980), as described by Van Amsterdam *et al.* (1989). Sensitized animals were operated on in week 3 following sensitization and used experimentally in week 4 to 8, weighing 500–700 g.

All protocols described were approved by the University of Groningen Animal Health Committee, which is responsible for assuring the care and proper use of experimental animals.

Measurement of airway function

Airway function was assessed by pleural pressure (P_{pl}) measurement as described by Santing *et al.* (1992). In brief, a small latex balloon, connected to saline-filled tubing, was implanted surgically inside the thoracic cavity. The cannula was driven subcutaneously and permanently attached to the neck of the animal. After connection via a fluid-filled cannula to a pressure transducer (Gould P23ID, Gould Medical BV, Bilthoven, The Netherlands), P_{pl} (in cmH_2O) was measured by an on-line computer system. Over the time period of experimentation (1 to 5 weeks after surgery) no visual sign of inflammation was observed at the sites of surgery, and baseline P_{pl} values remained stable during repeated measurements; changes in P_{pl} are linearly related to changes in airway resistance and hence can be used as a sensitive index for histamine- and allergen-induced bronchoconstriction (Santing *et al.*, 1992).

Provocation techniques and protocol

On the first day of the experimental protocol, the animals were placed unrestrained inside a specially designed animal provocation cage (Santing *et al.*, 1992), without connection to the pressure transducer. The animals were exposed to three saline aerosols, lasting 3 min each, and separated by 10 min intervals. On day 2 this protocol was repeated, with the animals connected to the measuring system. From day 3 onward, animals were challenged with histamine or ovalbumin aerosols, each time preceded by two saline challenges as described above.

Histamine provocations started with a 25 μg ml^{-1} solution of histamine in saline, followed by increasing dosage steps of 25 μg ml^{-1}. Challenges lasted 3 min each and were separated by 10 min intervals. Animals were challenged until the provocative dose was reached, which was defined as the histamine concentration in the nebulizer that caused a more than two fold increase in the pleural pressure for three or more consecutive minutes. The concentration of histamine giving a two fold increase in pleural pressure (PC_{100}) was calculated by interpolation.

Single allergen provocations were performed at a concentration of 0.1% ovalbumin in saline. The aerosol challenge was continued until an acute 2 fold increase in pleural pressure was measured (generally 2 to 3 min but occasionally longer), or to a maximum of 15 min. After ovalbumin provocation, airway function was continuously measured up to 23 h, in order to assess early and late phase obstructive reactions. One group of animals received ovalbumin challenges on four consecutive days; pleural pressure was monitored during all experiments.

To study bronchial responsiveness to histamine after allergen provocation, histamine inhalations were started at 5 h or at 23 h after ovalbumin challenge, i.e. after completion of the early and late phase, respectively.

In vitro *tracheal responses*

The guinea-pigs were killed 6 or 24 h after a single ovalbumin challenge (i.e. after the early or late allergic reaction), or 24 h after the last of four challenges. Non-challenged IgE-sensitized animals were controls. The animals were killed by a sharp blow on the head and exsanguinated. The tracheae were rapidly removed and placed in Krebs-Henseleit (KH) buffer solution (37°C) of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and D-glucose 5.55, gassed with 5% CO_2 and 95% O_2; pH 7.4. The tracheae were prepared free of serosal connective tissue and single open ring preparations were mounted under isotonic recording in 20.0 ml water-jacketed organ baths (37°C) in KH-solution using a 300 mg load. After equilibration for 45 min, the tracheal smooth muscle was relaxed with 0.1 μM isoprenaline to establish basal tone. The muscle was washed twice rapidly and subsequently contracted by cumulative administration of methacholine (1, 10 and 100 μM). After washing for 60 min a full cumulative concentration-response curve, i.e. until maximum isotonic contraction (100%) was reached, was constructed with methacholine concentrations from 0.01 to 100 μM with 0.5 log unit increments.

Subsequently, after a 60 min washing period, contractions induced by electrical field stimulation (EFS) were elicited via a Grass S 88 stimulator using two platinum electrodes placed on opposite sides of the preparation, 0.7 cm apart. EFS was applied at 1 min intervals for 5 s, at a voltage over the electrodes of 8 V and pulses of 0.5 ms duration at a frequency of 30 Hz. These parameters were chosen based on our previous study (Ten Berge *et al.*, 1993) since they allowed accurate and detailed investigation of prejunctional (M_2) and postjunctional (M_3) receptor function in guinea-pig trachea. Modulation of the twitch contractions was studied with cumulative concentrations of the M_2-selective muscarinic antagonists, gallamine, methoctramine, AQ-RA 741 and AF-DX 116 (Ten Berge *et al.*, 1993).

In vitro *cardiac responses*

The heart was removed and transferred to a preparation dish filled with continuously gassed KH buffer solution at room temperature. The atria were prepared free and threads were tied to the tip of the left atrial appendage and the opposite part of this atrium. After separation of the left and right atrium by cutting the interatrial septum carefully, left atrial preparations were mounted in water-jacketed organ baths, containing 20.0 ml gassed KH solution (32°C), and were adjusted by stretching to produce a resting tension of 500 mg.

Left atria were stimulated by electrical field via platinum electrodes at a frequency of 3 Hz, a pulse duration of 3 ms and a current density of 1.5 times the threshold value (usually 7.5 mA), using a home-made constant current biphasic square wave stimulator. The atria were allowed to equilibrate for at least 30 min. Concentration-response curves (CRCs) were obtained by cumulative addition of the full

muscarinic agonist, methacholine (3 nM–10 μM), or the partial and putatively M_2-selective agonist, pilocarpine (0.01–100 μM). A methacholine CRC preceded the construction of the pilocarpine CRC. In another set of experiments, a control CRC with methacholine was obtained and, subsequently, three CRCs were constructed in the presence of the antagonist AQ-RA 741 (0.01, 0.1, 1.0 μM; 15 min preincubation).

Data analysis and statistics

Bronchial hyperresponsiveness (BHR) to histamine was estimated as the ratio of PC_{100} values pre/post ovalbumin provocation. The magnitude of the early allergic reaction after allergen challenge was expressed as the area under the P_{pl} versus time curve (AUC) between 0 and 5 h after provocation, calculated by trapezoid integration over 5 min periods, thus including both the maximal increase in pleural pressure and the time path of this enhancement (Santing *et al.*, 1994a).

pD_2 values ($-\log EC_{50}$) were estimated for the concentration-response curves of methacholine performed on all tracheal preparations. The maximal potentiation (%) of the twitch contractions as a result of M_2 autoreceptor blockade (twitch height in the absence of muscarinic antagonists = 100%) was determined for the four M_2-selective antagonists. The degree of M_2 receptor dysfunction after the early allergic reaction was defined as (1 – mean potentiation 6 h after ovalbumin/mean control potentiation) × 100%.

Cardiac responses were measured as change in the isometric tension of the electrically paced left atria. pD_2 values were estimated for the concentration-response curves of methacholine and pilocarpine. Competitive antagonism was assessed according to Arunlakshana & Schild (1959). When the slope of the Schild plot did not differ significantly from unity, pK_B values were calculated for each concentration of antagonist according to: $pK_B = -\log([\text{antagonist}]/(DR-1))$.

The results are expressed as means ± s.e.mean. Statistical significance was assessed by paired (*in vivo* data) or unpaired (*in vitro* data) Student's *t* test and *P* values less than 0.05 were considered to be statistically significant.

Table 1 Methacholine pD_2-values on the postjunctional muscarinic M_3 receptor in guinea-pig tracheal smooth muscle preparations from IgE-sensitized animals that were not challenged (controls, 0 × OA) or that were killed for the *in vitro* experiment 6 or 24 h after a single challenge (1 × OA) or 24 h after four ovalbumin challenges on consecutive days (4 × OA)

Treatment	*pD_2 values*
IgE 0 × OA	6.31 ± 0.07 (11)
IgE 1 × OA 6 h	6.22 ± 0.08 (9)
IgE 1 × OA 24 h	6.33 ± 0.06 (9)
IgE 4 × OA 24 h	6.23 ± 0.03 (6)

Results are expressed as means ± s.e.mean of (*n*) determinations, each performed at least in triplicate.

Drugs

The following substances were used: atropine sulphate monohydrate and methacholine chloride (Aldrich, Milwaukee, WI, U.S.A.); (−)-isoprenaline hydrochloride, gallamine triethiodide, pilocarpine hydrochloride, histamine dihydrochloride, ovalbumin (grade III), aluminium hydroxide and tetrodotoxin (Sigma Chemical Co., St. Louis, MO, U.S.A.); AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one) and AQ-RA 741 (11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one) (gifts from Thomae, Biberach an der Riss, Germany); methoctramine tetrahydrochloride (gift from Dr C. Melchiorre, University of Bologna, Italy).

Results

Airway responses in vivo

Guinea-pigs that received ovalbumin between 4 and 8 weeks after sensitization generally developed an early allergic reaction that started with a sharp increase in baseline pleural pressure (5.41 ± 0.22 cmH_2O, mean ± s.e.mean of 24 animals) which was maximal within 20 min and lasted for 2–5 h. This applied to seven out of nine animals that were killed 6 h after antigen challenge and to eight out of nine animals that were killed 24 h after challenge. The late response started at about 8 h after allergen provocation and ended within 24 h after the challenge (Santing *et al.*, 1994a). All animals that received four challenges, executed daily, in an attempt to induce chronic changes in airway function, showed an early and a late phase response, the magnitude of the four allergic reactions being largely identical within one animal.

Six hours after a single allergen challenge, bronchial responsiveness as measured by histamine inhalation (PC_{100} = 104.3 ± 9.5 μg ml^{-1} prior to antigen challenge) was increased 3.1 ± 0.9 fold ($n = 7$; $P < 0.05$). After the late reaction the hyperresponsiveness was 1.6 ± 0.2 fold ($n = 8$; $P < 0.001$; single challenge) or 1.3 ± 0.1 fold ($n = 6$; $P < 0.05$; four challenges).

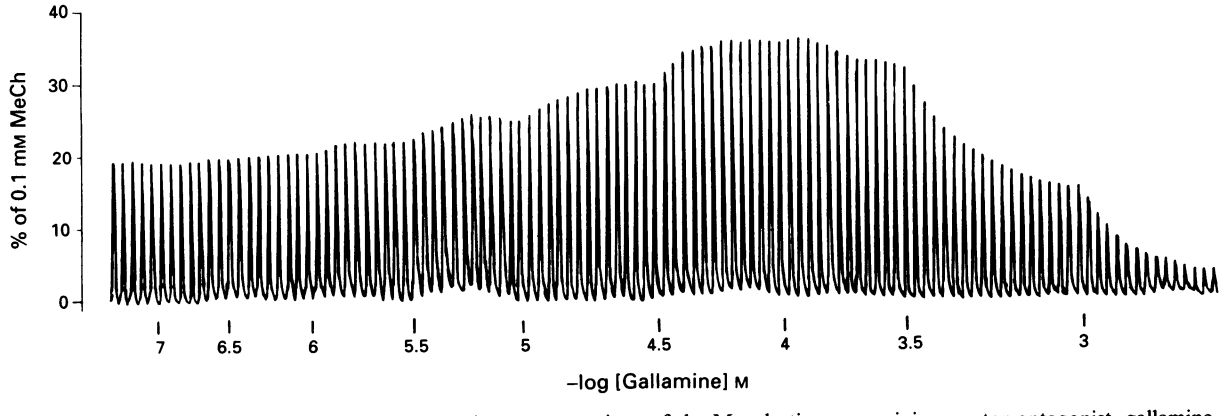
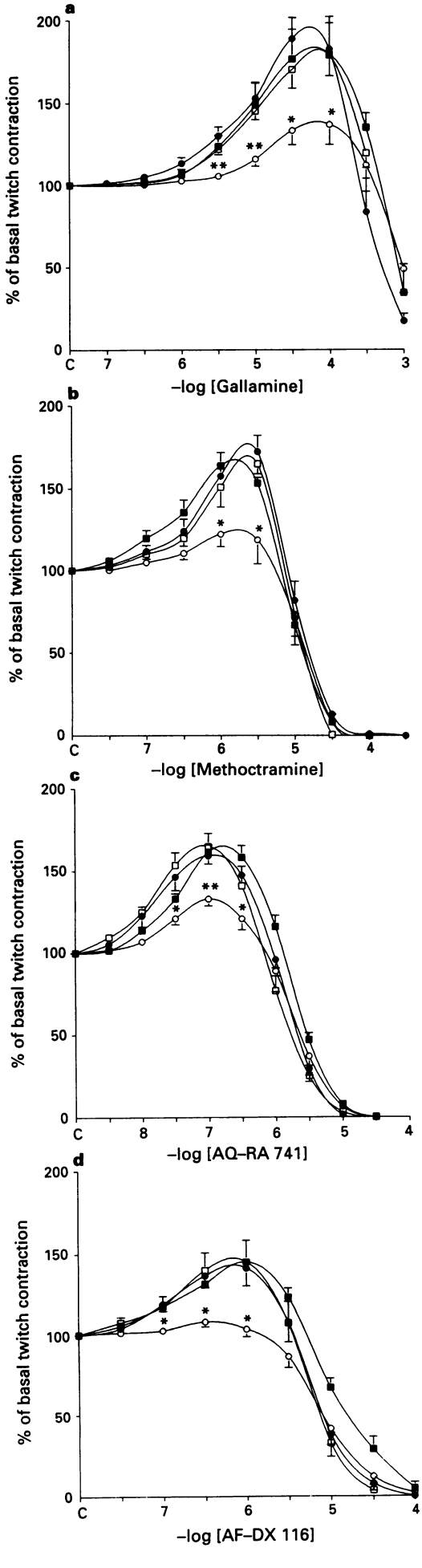


Figure 1 Typical example of the effect of increasing concentrations of the M_2-selective muscarinic receptor antagonist, gallamine, on twitch contractions elicited by electrical field stimulation of a tracheal single ring preparation from an unchallenged IgE-sensitized guinea-pig. Contraction expressed as percentage of maximal methacholine (MeCh)-induced contraction in the preceding concentration-response curve.



Tracheal responses in vitro

Methacholine pD_2 values for contraction of guinea-pig tracheal smooth muscle preparations from the different groups of animals are given in Table 1. No change in muscarinic M_3 receptor responsiveness after the early or late allergic reactions was observed.

Electrical field stimulation of guinea-pig tracheal single ring preparations induced rapid monophasic twitch contractions that could be abolished by atropine (0.1 μM) and tetrodotoxin (0.3 μM), confirming their cholinergic and neurogenic nature, respectively. Furthermore, the ganglion blocker hexamethonium (10 μM) had no effect on the twitch response (data not shown). Low concentrations of the M_2-selective muscarinic receptor antagonists potentiated the response, followed gradually by complete blockade at higher concentrations (see Figure 1 for example).

Figure 2 shows the effects of the M_2-selective muscarinic antagonists on electrical field stimulation-induced twitch contractions in guinea-pig tracheal preparations from IgE-sensitized animals that served as controls (unchallenged) and from those terminated 6 h after a single or 24 h after a single or four consecutive ovalbumin challenges. The maximal potentiation of the twitch contractions in control animals yielded a potency order: gallamine>methoctramine>AQ-RA 741>AF-DX 116. For all antagonists a significant decrease in maximal potentiation was observed after the early allergic reaction but not after the late reaction (Figure 2, Table 2). A significant correlation ($r = 0.8506$; $P = 0.032$) was obtained between the magnitude of the early allergic reaction and the degree of M_2 receptor dysfunction as determined with gallamine for animals killed 6 h after allergen provocation (Figure 3). There was no significant difference in the maximal potentiation of the twitch responses by any antagonist 24 h after one or four allergic bronchoconstrictor episodes (Table 2).

Cardiac responses in vitro

Both methacholine and pilocarpine produced a concentration-dependent negative inotropic response in electrically paced left atrial preparations of unchallenged controls and of guinea-pigs that were killed 6 h after ovalbumin challenge (Table 3). Pilocarpine was a partial agonist with an intrinsic activity of 0.93 in atria from unchallenged and 0.86 in atria from antigen-challenged animals (not significantly different). Methacholine dose-response curves were shifted to the right in a parallel and concentration-dependent fashion by the M_2-selective antagonist AQ-RA 741 (Figure 4). For AQ-RA 741, the slopes of the Schild plots did not significantly differ from unity in both groups of animals, nor were they significantly different between these groups; the pK_B values obtained were not significantly different for cardiac preparations from unchallenged and ovalbumin challenged guinea-pigs (Table 3).

Figure 2 Modulation of twitch contractions (expressed as percentage of the response in the absence of muscarinic antagonists) of electrical field-stimulated guinea-pig tracheal smooth muscle by the M_2-selective muscarinic antagonists, gallamine (a), methoctramine (b), AQ-RA 741 (c) and AF-DX 116 (d). Experiments were performed on single ring preparations from IgE-sensitized animals that were either unchallenged (●) or ovalbumin-challenged, and killed for the *in vitro* experiment 6 h (○) or 24 h after a single (□) or four challenges (■). Results are expressed as means ± s.e.mean of 3–7 determinations, each performed in duplicate or in triplicate. Results from antigen challenged guinea-pigs were compared to those from controls (unchallenged); $*P<0.05$, $**P<0.01$ (Student's unpaired t test).

Table 2 Maximal potentiation of twitch contractions (expressed as percentage of the response in the absence of muscarinic antagonists) of electrical field-stimulated guinea-pig tracheal smooth muscle preparations by M_2-selective muscarinic receptor antagonists (see Table 1 for groups of animals)

	0 × OA	*1 × OA 6 h*	*1 × OA 24 h*	*4 × OA daily 24 h*	*1 × OA 6 h vs 0 × OA*
Gallamine	189.6 ± 16.1 (6)	137.5 ± 12.2 (6)	179.7 ± 12.4 (7)	180.2 ± 19.0 (3)	$P = 0.025$
Methoctramine	173.0 ± 9.8 (4)	122.3 ± 7.5 (5)	165.1 ± 6.1 (5)	164.2 ± 8.2 (4)	$P = 0.018$
AQ-RA 741	159.3 ± 4.8 (5)	132.9 ± 4.2 (6)	165.0 ± 8.0 (6)	161.5 ± 4.3 (5)	$P = 0.007$
AF-DX 116	141.0 ± 10.9 (5)	107.9 ± 3.0 (5)	144.9 ± 6.0 (6)	144.2 ± 3.4 (4)	$P = 0.021$

Results are expressed as means ± s.e.mean of (n) determinations, performed in duplicate or in triplicate. P values indicate significance between maxima from unchallenged IgE-sensitized guinea-pigs and animals that were killed 6 h after antigen challenge (Student's unpaired t test).

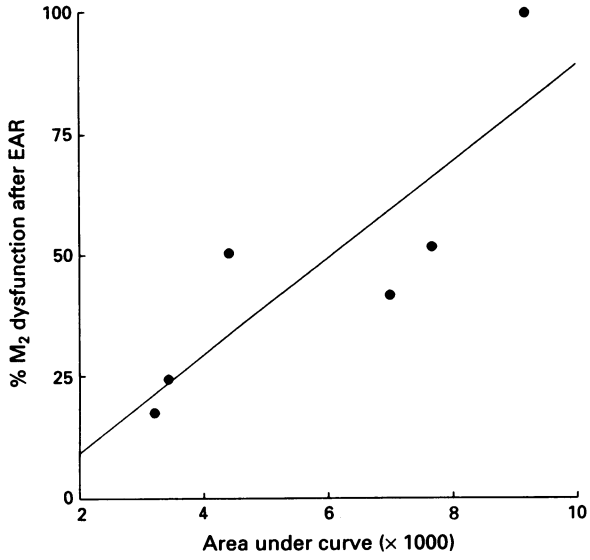


Figure 3 Relationship between the degree of M_2 receptor dysfunction determined *in vitro* with gallamine as the M_2-selective muscarinic antagonist (as described in Methods) and the magnitude of the early allergic reaction (EAR), expressed as area under curve (see Methods). Solid line represents linear regression ($r = 0.8506$; $P = 0.0318$, $n = 6$).

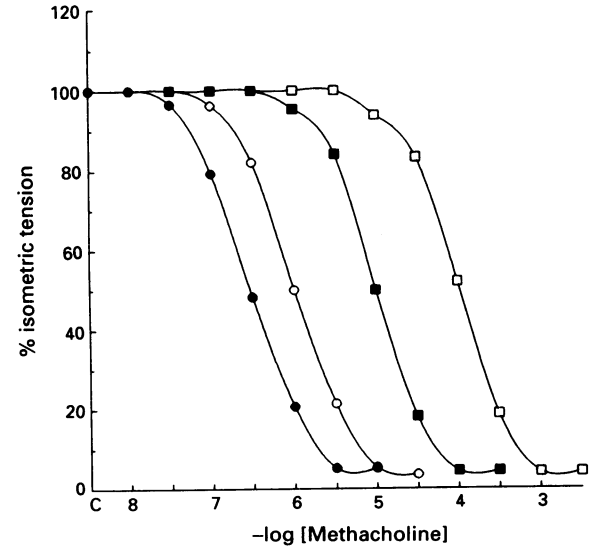


Figure 4 Typical example of antagonism of the methacholine-induced negative inotropic responses of atria from unchallenged IgE-sensitized guinea-pigs by the M_2-selective antagonist, AQ-RA 741 (control (●); 10 nM (○); 0.1 μM (■); 1.0 μM (□)). Contraction expressed as percentage of the response in the absence of muscarinic agonist.

Table 3 Functional parameters for the muscarinic receptor agonists, methacholine and pilocarpine, and the M_2-selective antagonist, AQ-RA 741, on left atrial preparations from unchallenged, IgE-sensitized guinea-pigs and from sensitized animals killed 6 h after allergen (OA) challenge

	Unchallenged	*OA-challenged*
Methacholine pD_2	6.50 ± 0.08 (14)	6.57 ± 0.08 (8)
Pilocarpine pD_2	5.87 ± 0.09 (11)	5.72 ± 0.13 (4)
i.a.[a]	0.93 ± 0.03 (11)	0.86 ± 0.04 (4)
AQ-RA 741 pK_B	8.59 ± 0.05 (9)	8.40 ± 0.07 (12)
slope[b]	1.06 ± 0.15 (3)	1.13 ± 0.06 (4)

Data represent mean values ± s.e.means of (n) determinations.
[a]Maximum response relative to the methacholine maximum (1.00).
[b]Mean slope determined from (n) independent Schild plots.

Discussion

The present study was designed to establish the function of muscarinic prejunctional inhibitory M_2 receptors in guinea-pig isolated airways after an early allergic bronchoconstrictor response (i.e. 6 h after antigen challenge *in vivo*), because it was recently found that these receptors had become dysfunctional *in vivo* 24 h after four consecutive antigen challenges (i.e. after the putative late response) (Fryer & Wills-Karp, 1991). In contrast to that study, M_2 receptor function *in vitro* was found to be normal 24 h after the last of four challenges executed on four consecutive days, and also 24 h after a single allergen challenge, but it was markedly decreased already 6 h after a single challenge. Moreover, bronchial responsiveness to histamine *in vivo* was evaluated and found to be enhanced to a higher extent after the early (3.1 fold) than after the late allergic response (1.6 to 1.3 fold). Since it has been reported that bronchoconstrictor responses to histamine are partly mediated via cholinergic activation of the vagus nerve (Hulbert *et al.*, 1985), the present results suggest that M_2 receptor dysfunction, leading to enhanced acetylcholine release through loss of inhibitory control, contributes to histamine hyperresponsiveness after the early but not after the late response.

Muscarinic receptor function in vitro

The function of muscarinic autoreceptors of the M_2 subtype, which provide a negative feedback control on neurotransmitter release from vagus nerve endings in the airways (Barnes *et al.*, 1988), was investigated by the use of M_2-selective antagonists as described previously (Ten Berge *et al.*, 1993). Since autoreceptors function best, and hence effects of antagonists are most apparent, at higher frequencies of stimulation (McCaig, 1987; Fryer & Wills-Karp, 1991), 30 Hz was chosen to ensure stable twitch contractions that were sensitive to modulation. In IgE-sensitized control animals, the maximal potentiation of the twitch contractions cor-

related with the degree of selectivity for M_2 compared to M_3 muscarinic receptors and was identical to non-sensitized guinea-pigs (Ten Berge *et al.*, 1993). This potentiation, due to selective M_2 receptor blockade, was markedly decreased in tracheal preparations from IgE-sensitized guinea-pigs used 6 h after a single provocation with the antigen ovalbumin, i.e. after the early allergic reaction, indicating for the first time that M_2 receptors are already dysfunctional after the early allergic reaction. However, no differences in M_2 receptor function were observed between guinea-pigs that had been killed 24 h after one or four antigen challenges, i.e. after the late allergic reaction, and controls, suggesting complete restoration of M_2 receptor function during the late response. The decrease in potentiation of the EFS-induced twitch contractions by M_2-selective muscarinic antagonists in sensitized and challenged animals was not due to changes in sensitivity of postjunctional muscarinic M_3 receptors, since methacholine pD_2 values were not different between any two groups of animals. This agrees with previous studies on muscarinic receptor function in allergen-challenged animals, which unanimously indicated unaltered postjunctional sensitivity either with (Fryer & Wills-Karp, 1991; Larsen *et al.*, 1994) or without (Watson *et al.*, 1991; Yang & Biggs, 1991) prejunctional dysfunction measured either *in vivo* or *in vitro*. Furthermore, dysfunction of M_2 receptors on vagus nerve endings in the airways after the early allergic response appears to be a local phenomenon, probably as a consequence of antigen challenge by inhalation, since M_2 receptor function in the heart *in vitro* was completely unchanged 6 h after antigen provocation *in vivo*. This was true for the affinity and intrinsic activity of the partial and *in vivo* M_2-selective (cf. Fryer & Wills-Karp, 1991) muscarinic agonist, pilocarpine, which was used to perceive changes in the agonist binding site of the receptor and its activation, and for the M_2-selective antagonist, AQ-RA 741, which might reveal changes in the antagonist binding site. These results confirm the previous observation of unchanged cardiac muscarinic receptor function *in vivo* under conditions of M_2 receptor dysfunction in the airways (Fryer & Wills-Karp, 1991).

Muscarinic receptor dysfunction and in vivo *airway responses*

In the present study a recently developed guinea-pig model that has been shown to comprise all major features of human allergic asthma was used (Santing *et al.*, 1992; 1994a). This model includes as its key features the single challenge of unanaesthetized unrestrained animals with a low dose of antigen (0.1% ovalbumin) in the absence of a histamine H_1 receptor antagonist (cf. Santing *et al.*, 1994c), the continuous measurement of lung function, and the repeated estimation of bronchial responsiveness under the same conditions (Santing *et al.*, 1992; 1994a). As a result, guinea-pigs showed early (0–5 h) and late phase (8–23 h after challenge) bronchoconstrictor responses, and bronchial hyperresponsiveness to histamine that was already present and more marked after the early than after the late response. These observations are similar to those in asthmatic subjects (Cockcroft & Murdock, 1987; Durham *et al.*, 1988; Bernstein *et al.*, 1992) where bronchial hyperreactivity was increased 3.1 to 3.5 fold before and 1.8 to 2.3 fold after the late response. In addition, this model incorporates an influx of inflammatory cells into the lungs (Santing *et al.*, 1994a). Interestingly, the more marked bronchial hyperreactivity to histamine after the early reaction was associated with a substantial dysfunction of prejunctional inhibitory M_2 autoreceptors in the airways, whereas the lower histamine bronchial hyperreactivity after the late response was accompanied by normal autoreceptor function. This was also the case with animals that were challenged four times on four consecutive days. It is therefore tempting to speculate that part of the histamine bronchial hyperreactivity after the early response is due to the fact that the dysfunctional M_2 receptors no longer efficiently inhibit acetylcholine release upon nerve activation, since histamine-induced bronchoconstriction *in vivo* has been attributed, in part, to vagal cholinergic pathways (Vidruk *et al.*, 1977; Holtzman *et al.*, 1980). In support of that, we have recently shown that ipratropium bromide, given by inhalation, partially inhibits the histamine-induced bronchoconstriction of non-challenged guinea-pigs whereas at 6 h after antigen challenge, i.e. after the early allergic reaction, *hyper*responsiveness to histamine was already inhibited by a ten fold lower dose of ipratropium. These findings clearly suggested that an exaggerated vagal reflex contributes to the allergen-induced bronchial hyperreactivity to histamine (Santing *et al.*, 1994b). Moreover, in the present study we found a significant correlation between the magnitude of the early allergic response and the extent of M_2 receptor dysfunction *in vitro*. These data would suggest that M_2 receptor dysfunction developing during the early allergic response contributes *to* this response. Alternatively, both phenomena may result from a common origin, such as the influx and activation of inflammatory cells after allergen provocation. These cells are known to release constrictor mediators that contribute to the early response (Daffonchio *et al.*, 1987), and other cell products that interfere with M_2 receptor function (see Fryer & Jacoby, 1993 and references cited therein). These include macrophage and lymphocyte neuraminidase (which may cleave sialic acid residues necessary for agonist binding to muscarinic M_2 but not M_3 receptors), eosinophil poly-cationic proteins (like major basic protein and eosinophil peroxidase, which may act as allosteric antagonists of M_2 but not of M_3 receptors) and oxygen free radicals (which may damage receptor structure, Barnes, 1990); notably, among these cell types only eosinophils were increased in number after the early reaction in our guinea-pig model (Santing *et al.*, 1994a). Regardless of what mechanism is responsible for M_2 receptor dysfunction in the present study, it is clear that it is readily reversible between 6 and 24 h after antigen challenge. This contrasts with the study of Fryer & Wills-Karp (1991), who only investigated and found M_2 receptor dysfunction 24 h after provocation. This difference may be due to the higher number and percentage of resident eosinophils and/or the more severe sensitization and provocation protocols possibly leading to a higher extent of airway inflammation in the earlier study, which may have been IgG- rather than IgE-mediated in contrast to our model (Andersson, 1980; Van Amsterdam *et al.*, 1989) and human asthma (Cockcroft *et al.*, 1979).

In summary, the present study has shown that prejunctional inhibitory muscarinic M_2 autoreceptors are already dysfunctional 6 h after antigen challenge of sensitized guinea-pigs, and that at the same time bronchial responsiveness to histamine is also markedly enhanced. When this M_2 receptor defect is restored 24 h after challenge, histamine bronchial hyperreactivity is also clearly diminished. These results suggest that bronchial hyperreactivity to histamine is partly due to M_2 receptor dysfunction, resulting in increased acetylcholine release from vagal nerve endings. This may equally apply to man, since dysfunction of these receptors has been observed in stable mild human asthmatics (Ayala & Ahmed, 1989; Minette *et al.*, 1989). It remains to be established, however, whether or not M_2 receptor dysfunction in such patients is reversible with time, since a recent study suggests that these receptors are at least in part functioning in stable asthmatic airways (Okayama *et al.*, 1994).

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References

ANDERSSON, P. (1980). Antigen-induced bronchial anaphylaxis in actively sensitized guinea-pigs. *Allergy*, **35**, 65–71.

ARUNLAKSHANA, A. & SCHILD, H.O. (1959). Some quantitive uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

AYALA, L.E. & AHMED, T. (1989). Is there loss of a protective muscarinic receptor mechanism in asthma? *Chest*, **96**, 1285–1291.

BARNES, P.J. (1990). Reactive oxygen species and airway inflammation. *Free Radical Biol. Med.*, **9**, 235–243.

BARNES, P.J., MINETTE, P. & MACLAGAN, J. (1988). Muscarinic receptor subtypes in airways. *Trends Pharmacol. Sci.*, **9**, 412–416.

BERNSTEIN, D.I., PLOYSONGSANG, Y., MITTMAN, R.J., PIYAMAHUNT, A. & BERNSTEIN, I.L. (1992). The relationship between airway responsiveness measured before and after the allergen-induced late asthmatic response. *Chest*, **101**, 437–441.

COCKCROFT, D.W. & MURDOCK, K.Y. (1987). Changes in bronchial responsiveness to histamine at intervals after allergen challenge. *Thorax*, **42**, 302–308.

COCKCROFT, D.W., RUFFIN, R.E., DOLOVICH, J. & HARGREAVE, F.E. (1977). Allergen-induced increase in non-allergic bronchial reactivity. *Clin. Allergy*, **7**, 503–513.

COCKCROFT, D.W., RUFFIN, R.E., FRITH, P.A., CARTIER, A., JUNIPER, E.F., DOLOVICH, J. & HARGREAVE, F.E. (1979). Determinants of allergen-induced asthma: dose of allergen, circulating IgE antibody concentration, and bronchial responsiveness to inhaled histamine. *Am. Rev. Respir. Dis.*, **120**, 1053–1058.

DAFFONCHIO, L., LEES, I.W., PAYNE, A.N. & WHITTLE, B.J.R. (1987). Pharmacological modulation of bronchial anaphylaxis induced by aerosol challenge in anaesthetized guinea-pigs. *Br. J. Pharmacol.*, **91**, 701–708.

DURHAM, S.R., CRADDOCK, C.F., COOKSON, W.O. & BENSON, M.K. (1988). Increases in airway responsiveness to histamine precede allergen-induced late asthmatic responses. *J. Allergy Clin. Immunol.*, **82**, 764–770.

FAULKNER, D., FRYER, A.D. & MACLAGAN, J. (1986). Postganglionic muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.*, **88**, 181–187.

FRYER, A.D. & JACOBY, D.B. (1991). Parainfluenza virus infection damages inhibitory M_2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.*, **102**, 267–271.

FRYER, A.D. & JACOBY, D.B. (1993). Effect of inflammatory cell mediators on M_2 muscarinic receptors in the lungs. *Life Sci.*, **52**, 529–536.

FRYER, A.D. & MACLAGAN, J. (1984). Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.*, **83**, 973–978.

FRYER, A.D. & WILLS-KARP, M. (1991). Dysfunction of M_2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J. Appl. Physiol.*, **71**, 2255–2261.

HOLTZMAN, M.J., SHELLER, J.R., DIMEO, M., NADEL, J.A. & BOUSHEY, H.A. (1980). Effect of ganglionic blockade on bronchial reactivity in atopic subjects. *Am. Rev. Respir. Dis.*, **122**, 17–25.

HULBERT, W.C., MCLEAN, T., WIGGS, B., PARÉ, P.D. & HOGG, J.C. (1985). Histamine dose-response curves in guinea pigs. *J. Appl. Physiol.*, **58**, 625–634.

LARSEN, G.L., FAME, T.M., RENZ, H., LOADER, J.E., GRAVES, J., HILL, M. & GELFAND, E.W. (1994). Increased acetylcholine release in tracheas from allergen-exposed IgE-immune mice. *Am. J. Physiol.*, **266**, L263–L270.

MCCAIG, D.J. (1987). Comparison of autonomic responses in the trachea isolated from normal and albumin-sensitive guinea-pigs. *Br. J. Pharmacol.*, **92**, 809–816.

MINETTE, P.A. & BARNES, P.J. (1988). Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea-pig airways. *J. Appl. Physiol.*, **64**, 2532–2537.

MINETTE, P.A.H., LAMMERS, J.-W.J., DIXON, C.M.S., MCCUSKER, M.T. & BARNES, P.J. (1989). A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J. Appl. Physiol.*, **67**, 2461–2465.

OKAYAMA, M., SHEN, T., MIDORIKAWA, J., LIN, J.-T., INOUE, H., TAKISHIMA, T. & SHIRATO, K. (1994). Effect of pilocarpine on propranolol-induced bronchoconstriction in asthma. *Am. J. Respir. Crit. Care Med.*, **149**, 76–80.

SANTING, R.E., MEURS, H., OLYMULDER, C.G., OOSTEROM, W.C., BROUWER, F. & ZAAGSMA, J. (1994a). Relationships between allergen-induced early and late phase airway obstructions, bronchial hyperreactivity and inflammation in conscious, unrestrained guinea pigs. *J. Allergy Clin. Immunol.*, **93**, 1021–1030.

SANTING, R.E., MEURS, H., VAN DER MARK, TH.W., REMIE, R., OOSTEROM, W.C., BROUWER, F. & ZAAGSMA, J. (1992). A novel method to assess airway function parameters in chronically instrumented, unrestrained guinea-pigs. *Pulmon. Pharmacol.*, **5**, 265–272.

SANTING, R.E., PASMAN, Y., OLYMULDER, C.G., ROFFEL, A.F., MEURS, H. & ZAAGSMA, J. (1994b). A cholinergic reflex mechanism contributes to allergen-induced bronchial hyperreactivity in permanently instrumented, unrestrained guinea-pigs. *Br. J. Pharmacol.* (in press).

SANTING, R.E., SCHRAA, E.O., WACHTERS, A., OLYMULDER, C.G., ZAAGSMA, J. & MEURS, H. (1994c). Role of histamine in allergen-induced asthmatic reactions, bronchial hyperreactivity and inflammation in unrestrained guinea pigs. *Eur. J. Pharmacol.*, **254**, 49–57.

SCHULTHEIS, A.H., BASSETT, D.J.P. & FRYER, A.D. (1994). Ozone-induced airway hyperresponsiveness and loss of neuronal M_2 muscarinic receptor function. *J. Appl. Physiol.*, **76**, 1088–1097.

STEWART, A.G. & FENNESSY, M.R. (1983). A model of bronchial hyperreactivity using ovalbumin-sensitized guinea-pigs. *Clin. Exp. Pharmacol. Physiol.*, **10**, 205.

TEN BERGE, R.E.J., ROFFEL, A.F. & ZAAGSMA, J. (1993). The interaction of selective and non-selective antagonists with pre- and postjunctional muscarinic receptor subtypes in the guinea pig trachea. *Eur. J. Pharmacol.*, **233**, 279–284.

VAN AMSTERDAM, R.G.M., BROUWER, F. & ZAAGSMA, J. (1989). Analysis of the β-adrenoceptor mediated inhibition of IgG_1 and IgE dependent guinea-pig anaphylactic tracheal smooth muscle contraction. *Agents Actions*, **26**, 48–51.

VIDRUK, E.H., HAHN, H.L., NADEL, J.A. & SAMPSON, S.R. (1977). Mechanisms by which histamine stimulates rapidly adapting receptors in dog lungs. *J. Appl. Physiol.*, **43**, 397–402.

WANNER, A., ABRAHAM, W.M., DOUGLAS, J.S., DRAZEN, J.M., RICHERSON, H.B. & SRI RAM, J. (1990). Models of airway hyperresponsiveness. NHLBI Workshop Summary. *Am. Rev. Respir. Dis.*, **141**, 253–257.

WATSON, N., OWEN, R., BARNES, P.J. & MACLAGAN, J. (1991). Pulmonary muscarinic receptors are functionally unaffected by ovalbumin sensitisation. *Am. Rev. Respir. Dis.*, **143**, A751.

YANG, Z.-J. & BIGGS, D.F. (1991). Muscarinic-receptor functioning in tracheas from normal and ovalbumin-sensitive guinea pigs. *Can. J. Physiol. Pharmacol.*, **69**, 871–876.

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Acute effects of the β_3-adrenoceptor agonist, BRL 35135, on tissue glucose utilisation

Yong-Ling Liu & [1]Michael J. Stock

Department of Physiology, St George's Hospital Medical School, Tooting, London, SW17 0RE

1 The acute effects of BRL 35135 (BRL) on tissue glucose utilisation index (GUI) *in vivo* were investigated in anaesthetized rats by use of 2-deoxy-[^{3}H]-glucose.

2 Intravenous injection of BRL caused a dose-dependent increase in GUI in skeletal muscle, and white and brown adipose tissue; plasma insulin and fatty acid concentrations were also increased. Chronic treatment with BRL added to the diet caused a 34 fold increase in basal GUI of brown adipose tissue (BAT), but had no effect on GUI in other tissues. After chronic treatment, the acute tissue response to an intravenous maximal dose of BRL had disappeared completely in all tissues apart from the soleus muscle.

3 A high dose (20 mg kg^{-1}) of the non-selective β-antagonist, propranolol, inhibited the acute effect of BRL on GUI in BAT, but failed to affect GUI in muscle. A lower dose (1 mg kg^{-1}) of the antagonist also inhibited the BAT response, but had little or no effect on the response in Type I (working) muscles such as soleus and adductor longus (ADL), and potentiated the response in Type II (non-working) muscles such as tibialis and extensor digitorium longus (EDL).

4 A low dose (1 mg kg^{-1}) of the selective β_1-antagonist, atenolol, had no effect on the BRL response but the same dose of the selective β_2-antagonist, ICI 118551, potentiated significantly the effect of BRL on GUI in most muscles without altering plasma insulin levels.

5 It is concluded that: (i) the heterogeneous tissue responses of different muscle fibre types in the presence of β-antagonists indicates that BRL affects muscle GUI directly, in addition to effects mediated by increases in plasma insulin concentration; (ii) the resistance of the BRL response to conventional β-adrenoceptor antagonists implicates an atypical adrenoceptor mediating the GUI response in skeletal muscle, but this may not be identical to the adipose tissue β_3-adrenoceptor; (iii) the potentiation of BRL responses by ICI 118551 indicates an inhibitory β_2-adrenoceptor-mediated component in the muscle GUI response to BRL.

Keywords: 2-Deoxy-glucose; muscle; brown adipose tissue; β-adrenoceptors; BRL 35135; atenolol; ICI 118551; glucose; insulin; fatty acids; body temperature; diabetes

Introduction

Most functional responses to β-adrenoceptor agonists can be reconciled with the involvement of β_1 or β_2-adrenoceptors (βAR) or a mixture of the two, but there is now considerable evidence for the existence of a third, or atypical βAR-subtype (Zaagsma & Nahorski, 1990; Zaagsma & Hollenga, 1991). Some of the strongest evidence for an atypical βAR came from studies with the first of a series of novel thermogenic anti-obesity drugs (e.g. BRL 26830, BRL 35135). The active metabolites of these compounds were found to be 20 to 400 times more potent in activating brown adipose tissue (BAT) thermogenesis than as agonists of atrial rate (β_1) and tracheal relaxation (β_2; Arch *et al.*, 1984). These potency ratios, and the low pA_2 values obtained for inhibition of BAT thermogenesis by conventional βAR antagonists (Arch *et al.*, 1984; Stock & Sudera, 1989) suggested that BAT possessed an atypical βAR. Subsequently, a human gene was identified that encodes for a third (i.e. β_3) βAR (Emorine *et al.*, 1989), and cells transfected with this receptor are 10 times more sensitive to noradrenaline than adrenaline, resistant to blockade by conventioanl βAR antagonists and highly sensitive to the novel, thermogenic βAR agonists. A very similar gene has also been identified in the rat (Granneman *et al.*, 1991) and mouse (Nahmias *et al.*, 1991), and the atypical βAR in BAT is now thought to be the β_3-subtype. As a result, the thermogenic BAT-selective agonists (such as BRL 35135) are commonly referred to as β_3AR agonists.

As might be expected of potent thermogenic agonists, the novel β_3AR agonists are highly effective in reducing body weight and fat in genetically obese rats and mice (Smith *et al.*, 1990). However, these animals (e.g. ob/ob and db/db mice and fa/fa rats) are also used as models of insulin resistance and diabetes (i.e. NIDDM), and chronic administration of BRL 35135 results in improvements in glucose tolerance and glycaemic control (Smith *et al.*, 1990). These chronic changes suggest the β_3-adrenoceptor agonists normalize glucose tolerance by partially reversing insulin resistance in peripheral tissues. The mechanisms whereby these thermogenic agonists increase insulin sensitivity, and the contribution of skeletal muscle to this have yet to be elucidated. In genetically lean, non-diabetic animals a single dose of BRL 35135 produces a significant increase in glucose tolerance, and this has been ascribed to the rapid rise in plasma insulin seen within 30 min of administration (Smith *et al.*, 1990). However, an earlier study (Challis *et al.*, 1988) on the effects of BRL 26830 on lactate and glycogen metabolism indicates a possible direct effect on muscle via an atypical βAR. More recently, the identification of atypical ligand binding sites (Roberts *et al.*, 1993; Sillence *et al.*, 1993) suggests that any direct effects of these β_3-agonists on muscle glucose metabolism are likely to involve this atypical βAR. Whether this atypical binding site corresponds to the β_3AR is debatable, since molecular studies have failed to detect the β_3AR in skeletal muscle (Granneman *et al.*, 1991; Krief *et al.*, 1993).

In order to investigate further the β-adrenoceptor control of muscle glucose metabolism, it was decided to determine the acute *in vivo* effect of BRL 35135 on glucose utilisation by skeletal muscle, BAT and other tissues in rats by use of a

[1] Author for correspondence.

radioactive tracer, 2-deoxy-[^{3}H]-glucose. Having established a dose-dependent effect on muscle glucose utilisation, maximal (ED_{100}) doses were tested in rats after chronic treatment with BRL 35135. Subsequent experiments involved the use of non-selective and selective βAR antagonists in an attempt to dissect pharmacologically the βAR subtype(s) involved in mediating the acute increases in glucose utilization produced by BRL 35135. The results indicate that BRL 35135 stimulates glucose utilisation in muscle via increases in plasma insulin, plus a direct activation of an atypical βAR. However, the latter effect is attenuated by a concomitant weak inhibitory action that appears to involve β_2AR activation.

Methods

Animals

For all acute studies of glucose utilisation index (GUI), male Wistar rats (220–250g) were obtained from the Biological Research Facility (St George's Hospital Medical School) and used without further treatment. For the chronic experiment, younger rats (100 g) from the same colony were housed in pairs in a room maintained at a temperature of 22°C with a controlled light cycle (07 h 00 min–19 h 00 min) and free access to water. The control animals were fed a powdered stock diet (PRD; Christopher Hill, Dorset, U.K.), and the experimental groups fed the same diet containing BRL 35135 for two weeks before carrying out acute measurements of GUI. The concentration in the diet (5 mg kg^{-1} diet), was designed to provide an approximate dose of 0.5 mg day^{-1} kg^{-1} body weight.

Measurement of tissue GUI

The rats were anaesthetized with urethane (1.2 g kg^{-1}, i.p.) and the right jugular vein cannulated. Rats were kept warm with overhead bench lamps positioned to maintain a constant colonic temperature of 37°C during surgical preparation and until the start of the experiment. Colonic temperature was recorded every 10 min in control and experimental animals and experiments were started approximately 45 min after induction of anaesthesia. In all experiments, BRL 35135 (BRL) or vehicle (saline) was given intravenously 20 min before the intravenous injection of 2-deoxy-[^{3}H]-glucose (2DG). All βAR-antagonists were given subcutaneously 20 min before BRL. 2DG (30 μCi; 20 Ci mmol^{-1}) was injected in 200 μl of saline. Serial blood samples (50 μl) were taken through the same cannula at 1, 3, 5, 10, 20, 40 and 60 min after the 2DG injection. Samples were immediately deproteinized in $Ba(OH)_2/ZnSO_4$ as described by Ferré *et al.* (1985) and centrifuged. The supernatant was used for the determination of blood glucose and plasma radioactivity. A final, heparinized blood sample (1 ml) was taken at 60 min and the plasma used for the determination of NEFA and insulin.

Rats were killed 1 h after the administration of the 2DG by injecting 0.5 ml of 30% KCl and the following tissues were dissected, freeze-clamped and stored in liquid N_2 prior to extraction: gastrocnemius, soleus, tibialis anterior (TA), extensor digitorum longus (EDL), adductor longus (ADL), diaphragm, heart, cerebral cortex, epididymal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT). Tissues were digested in 0.5 ml 1M NaOH for 45 min at 60°C and neutralized with 0.5 ml 1M HCl. One aliquot (200 μl) of the digest was added to 1 ml of $Ba(OH)_2/ZnSO_4$ and another (200 μl) was added to 1 ml of 6% $HClO_4$. After centrifugation, 800 μl of each supernatant was added to 10 ml scintillation cocktail (Beckman Ready Value) and the radioactivity determined (Beckman LS6000). Tissue glucose utilization index (GUI) was calculated as described by Issad *et al.* (1987) by dividing the radioactivity (d.p.m.) of 2DG-6-phosphate in the tissues by the calculated 60 min integral of the ratio of blood 2DG/blood glucose (d.p.m. ng^{-1}). As in Issad *et al.* (1987), the value obtained was not corrected by the discrimination factor for 2-deoxy-glucose in metabolic pathways (see Ferré *et al.*, 1985). The results were expressed as ng glucose min^{-1} mg^{-1} wet weight of tissue.

Blood metabolite and hormone measurements

Glucose was measured in the supernatant of the deproteinized blood samples with a glucose oxidase assay kit (Boehringer, Germany). Plasma non-esterified fatty acids (NEFA) were determined by an enzymatic colorimetric method (NEFAC test kit; Wako, Germany), and plasma insulin concentration was measured with a radioimmunoassay kit (Novo, Denmark) with rat insulin standards.

Materials

Radiolabelled 2-deoxy-[^{3}H]-glucose (2DG) was purchased from Amersham International (Amersham, U.K.), and atenolol and propranolol were purchased from Sigma (Poole, U.K.). BRL 35135 (BRL) (**R*,R***-(±)-methyl-4-[2-[2-hydroxy-2-(3-chlorophenyl) ethyl-amino] -propyl] -phenoxyacetate hydrobromide) and ICI 118551 (erythro-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol) were supplied by SmithKline Beecham (Epsom, U.K.) and ICI Pharmaceuticals (Macclesfield, U.K), respectively. The drugs were racemic mixtures of enantiomers and were prepared for injection by dissolving in saline.

Statistics

Results have been presented as mean values ± s.e.mean. The statistical significance of differences between control and experimental groups was assessed by Student's unpaired *t* test; all probabilities quoted are two-tailed.

Results

In order to determine the dose-response characteristics of BRL on muscle glucose utilisation (GUI), the effect of the maximal thermogenic dose (10 μg kg^{-1}; Carlisle & Stock, 1993a) was compared with the effects of submaximal (2, 4 and 8 μg kg^{-1}) and supramaximal (100 μg kg^{-1}) thermogenic doses. In this first experiment (Expt. 1), the measurement of GUI in the 30 rats receiving different doses of BRL took over a month, and so a large number (26) of saline-treated animals were tested simultaneously in order to control for day-to-day variations in basal GUI. The results for the six muscles studied (Table 1) show that maximal stimulation of GUI occurred at doses of 8 or 10 μg kg^{-1}, with no further stimulation at the higher dose of 100 μg kg^{-1}. The degree of stimulation at the maximum dose varied between the different muscles from 70–160%; on average, GUI doubled. Since 10 μg kg^{-1} BRL appeared to be the ED_{100} dose for both muscle GUI and thermogenesis, this dose was used for all subsequent experiments.

A more detailed experiment (Expt. 2) using the 10 μg kg^{-1} dose of BRL was carried out, and this showed (Figure 1) that GUI was increased by the drug in all tissues studied, apart from brain (no effect) and heart (significant 44% decrease). The increases (% saline values) in 'working' muscles (e.g. soleus = 134%, ADL = 127%, diaphragm = 240%) tended to be greater than those seen in 'non-working' muscles (e.g. gastrocnemius = 50%, EDL = 77%, TA = 90%). GUI in epididymal WAT was increased by 65%, but the largest increase in any tissue was seen in interscapular BAT where GUI was over 12 times greater than in saline-treated rats. The activation of BAT by BRL was also evident from the recordings of colonic temperature (Figure 2), which showed a progressive rise throughout the experiment to pro-

Table 1 Effect of different doses (0–100 μg kg^{-1}, i.v.) of BRL 35135 on muscle GUI (Expt. 1)

	Saline	*BRL2*	*BRL4*	*BRL8*	*BRL10*	*BRL100*
Gas	2.2 ± 0.2	3.2 ± 0.3	3.3 ± 0.5	3.4 ± 0.4	3.8 ± 0.3	3.9 ± 0.4
Sol	4.4 ± 0.2	4.6 ± 0.4	7.1 ± 0.5	7.5 ± 1.1	9.0 ± 1.0	8.9 ± 1.5
TA	2.4 ± 0.2	3.2 ± 0.4	3.8 ± 0.5	5.9 ± 0.5	4.8 ± 0.7	4.4 ± 0.3
EDL	2.7 ± 0.2	3.6 ± 0.4	4.1 ± 0.3	5.9 ± 0.7	4.7 ± 0.3	5.00 ± 0.8
ADL	5.9 ± 0.4	5.7 ± 0.5	8.7 ± 0.5	8.9 ± 1.4	10.1 ± 0.8	9.8 ± 1.4
DIA	9.3 ± 0.7	13.5 ± 1.9	16.9 ± 2.6	22.7 ± 3.1	24.1 ± 2.0	19.8 ± 2.9

GUI expressed as ng glucose min^{-1} mg^{-1} wet weight. Mean values ± s.e.mean ($n = 26$ for saline; $n = 6$ for BRL doses). Gas = gastrocnemius; Sol = soleus; TA = tibialis anterior; EDL = extensor digitorium longus; ADL = adductor longus; Dia = diaphragm.

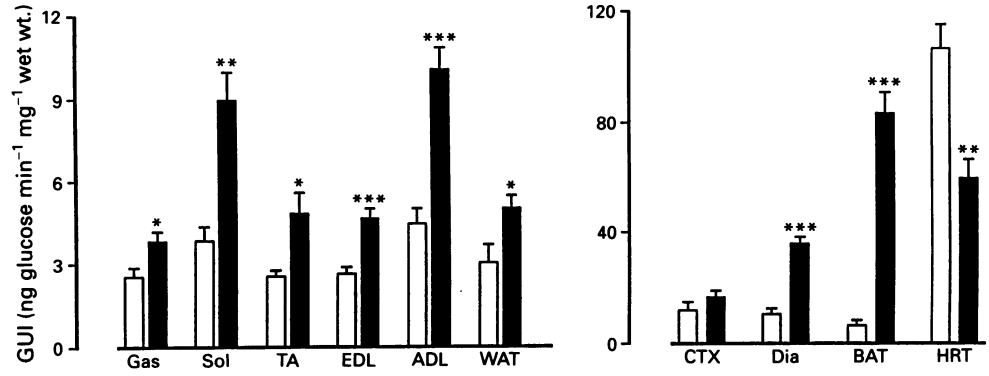


Figure 1 Expt. 2: Tissue glucose utilization index (GUI) in rats receiving vehicle (open columns) and a single dose (10 μg kg^{-1}) of BRL 35135 (solid columns). Mean values with s.e.mean ($n = 6$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle (unpaired t test). Gas = gastrocnemius; Sol = soleus; TA = tibialis anterior; EDL = extensor digitorium longus; ADL = adductor longus; WAT = epididymal white adipose tissue; CTX = cerebral cortex; Dia = diaphragm; BAT = interscapular brown adipose tissue; HRT = heart.

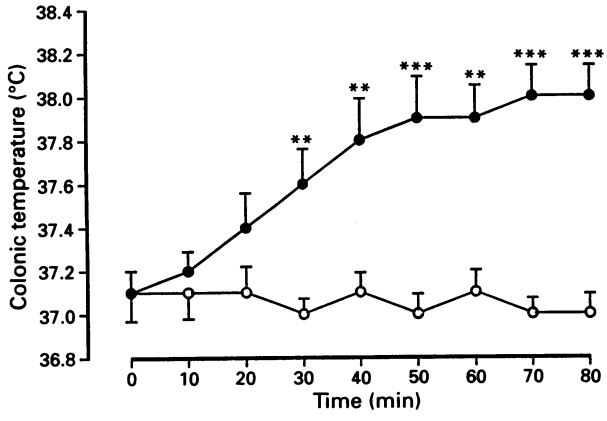


Figure 2 Expt. 2: Colonic temperature in rats following injection (10 μg kg^{-1}, i.v.) of BRL 35135 (●) or vehicle (○) at 0 min; 2DG was injected at 20 min. Mean values ± s.e.mean ($n = 6$). **$P<0.01$, ***$P<0,001$ compared to vehicle (unpaired t test).

duce eventually a 1°C difference between treated rats and those receiving saline.

Chronic treatment with BRL as a dietary admixture (5mg kg^{-1} diet) for 2 weeks (Expt. 3) had no apparent effect on basal GUI in any tissue apart from a 34 fold increase in BAT (Table 2). The acute response to BRL in the rats fed the control diet was very similar to that seen in the previous experiment, although the increase in WAT was not significant on this occasion. Blood glucose (average of the 7 samples taken over 60 min) and plasma insulin (final 60 min sample) was not significantly affected by the injection of BRL, but plasma NEFA at the end of the experiment were increased by 104% and the rise in colonic temperature was 0.5°C greater than in saline-treated rats. Contrasting with these results, the acute response to the agonist in the rats chronically treated with BRL had completely disappeared, apart from the 50% increase seen in soleus, which was approximately half that seen in control rats (106% increase). The only other significant response was the rise in colonic temperature.

The large, but non-significant increase in plasma insulin seen in the terminal (60 min) sample taken from control rats after injection of BRL prompted a more detailed investigation into the time course of the insulin response. The experiment (Expt. 4) followed a similar time-schedule to the other acute experiments except that 2DG was not injected at time zero, and blood samples for insulin analysis were taken at -30, -20, 0, 30 and 60 min. The changes in plasma insulin are shown in Figure 3, and include results from rats pretreated with a high dose of propranolol (20 mg kg^{-1}, s.c.) 10 min before BRL (10 μg kg^{-1}, i.v.). Plasma insulin rose by more than 2.5 fold within 20 min following BRL, and remained elevated for the next 60 min; pre-treatment with propranolol had no effect on the insulin response.

The effect of high (20 mg kg^{-1}) and low (1 mg kg^{-1}) pretreatment doses of propranolol (using the same timing and sequence of injections as for Expt. 4) on tissue GUI responses to BRL (Expt. 5) is shown in Table 3. The high dose of propranolol had no significant effect on GUI in rats injected with BRL, apart from interscapular BAT and brain where GUI was depressed by 56 and 22% respectively. Blood glucose, plasma insulin and NEFA (60 min samples), and the rise in colonic temperature were also unaffected by propranolol. In addition, it should be noted that treatment with propranolol alone had no effect on basal GUI in any tissue or on any of the other parameters studied apart from a small, non-significant decrease in brain ($n = 3$; results not shown). A significant inhibition of the GUI response in BAT was also seen with the low dose (1 mg kg^{-1}) of propranolol, but more surprising was the observation that this dose of the antagonist potentiated the effects of BRL in several muscles.

Table 2 Effect of chronic dietary treatment with BRL 35135 on tissue GUI and other parameters in response to an acute dose of BRL 35135 (Expt. 3)

	Control Diet		*BRL diet*	
	Saline	*BRL*	*Saline*	*BRL*
GUI (ng min^{-1} mg^{-1})				
Gas	1.7 ± 0.2	2.9 ± 0.5*	2.1 ± 0.4	2.5 ± 0.3
Sol	4.8 ± 0.4	9.9 ± 1.8*	5.0 ± 0.4	7.5 ± 0.9*
TA	1.9 ± 0.1	3.3 ± 0.5*	2.1 ± 0.1	2.8 ± 0.4
EDL	2.0 ± 0.2	3.9 ± 0.6*	2.6 ± 0.2	2.8 ± 0.4
ADL	7.1 ± 0.9	12.9 ± 1.5**	9.2 ± 1.1	8.3 ± 1.2
Dia	8.2 ± 0.4	28.7 ± 4.8**	12.4 ± 1.7	19.1 ± 3.0
HRT	83.6 ± 9.4	40.8 ± 8.4**	82.3 ± 7.0	80.1 ± 11.2
WAT	6.7 ± 1.2	9.4 ± 1.7	9.3 ± 2.1	8.6 ± 1.7
BAT	5.7 ± 1.2	72.5 ± 5.3***	193.8 ± 15.7	192.9 ± 12.4
CTX	17.1 ± 2.9	16.9 ± 2.2	16.6 ± 2.2	16.2 ± 1.2
Other parameters				
Glucose (mM)	7.97 ± 0.45	7.05 ± 0.28	6.47 ± 0.1	7.31 ± 0.38
Insulin (ng^{-1} ml^{-1})	4.20 ± 0.30	8.50 ± 2.60	3.60 ± 0.30	4.20 ± 0.80
NEFA (mEq l^{-1}	0.45 ± 0.04	0.92 ± 0.06***	0.51 ± 0.06	0.50 ± 0.07
dTc (°C)	0.13 ± 0.07	0.70 ± 0.17*	0.15 ± 0.06	0.50 ± 0.08**

Mean values ± s.e.mean ($n = 6$ per group) after injection of either saline or BRL 35135 (10 μg kg^{-1}). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to respective saline group (unpaired t test). Gas = gastrocnemius; Sol = soleus; TA = tibialis anterior; EDL = extensor digitorium longus; ADL = adductor longus; Dia = diaphragm; HRT = heart; WAT = epididymal white adipose tissue; BAT = interscapular brown adipose tissue; CTX = cerebral cortex; NEFA = non-esterified fatty acids; dTc = average increase in colonic temperature over pre-injection temperature.

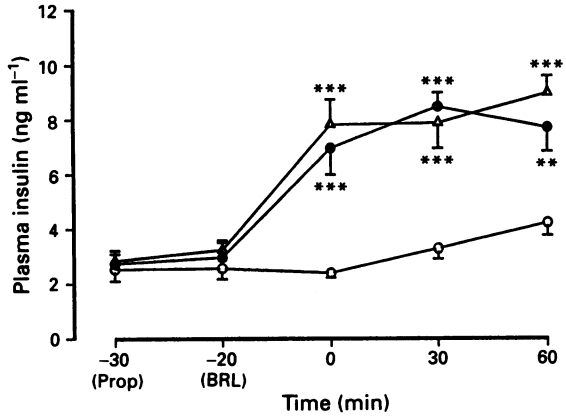


Figure 3 Expt. 4: Plasma insulin responses following injection of vehicle (○) or BRL 35135 (10 μg kg^{-1}, i.v. at −20 min) with (△) or without (●) pretreatment with propranolol (prop, 20 mg kg^{-1}, s.c. at −30 min); 0 min = normal time-point when 2DG injected in other experiments. Mean values ±s.e.mean (n = 7). **$P<0.01$, ***$P<0.001$ compared to vehicle (unpaired t test).

Table 3 Effect of high (20 mg kg^{-1}) and low (1 mg kg^{-1}) dose propranolol on tissue GUI and other responses to BRL 35135 (Expt. 5)

	BRL	*BRL/Prop* 20 mg	*BRL/Prop* 1 mg
GUI (ng min^{-1} mg^{-1})			
Gas	3.0 ± 0.2	2.6 ± 0.3	3.4 ± 0.2
Sol	7.6 ± 0.6	6.4 ± 0.6	6.2 ± 0.5
TA	4.4 ± 0.5	3.8 ± 0.5	6.7 ± 0.8*
EDL	4.3 ± 0.4	3.7 ± 0.3	6.5 ± 0.6*
ADL	10.1 ± 0.7	7.8 ± 0.8	8.80 ± 1.0
Dia	20.4 ± 1.9	19.1 ± 1.9	39.0 ± 4.6**
HRT	33.3 ± 3.6	23.4 ± 2.6	42.6 ± 9.6
WAT	5.9 ± 0.7	4.0 ± 0.7	6.1 ± 0.5
BAT	77.0 ± 4.7	33.9 ± 5.8***	41.7 ± 7.2**
CTX	15.6 ± 1.3	12.1 ± 0.5*	17.1 ± 0.8
Other parameters			
Glucose (mM)	7.78 ± 0.37	7.11 ± 0.29	7.57 ± 0.52
Insulin (ng ml^{-1})	7.88 ± 1.9	8.97 ± 0.63	8.60 ± 2.2
NEFA (mEq l^{-1})	0.74 ± 0.06	0.65 ± 0.10	0.77 ± 0.10
dTc (°C)	0.71 ± 0.12	0.54 ± 0.08	0.62 ± 0.09

Mean values ± s.e.mean ($n = 6$ per group)
*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to BRL (unpaired t test).
Abbreviations as for Table 2.

Compared to BRL alone, GUI was increased significantly by 52, 51 and 91% in tibialis, EDL and diaphragm, respectively. There were small, non-significant changes in gastrocnemius (13% stimulation) and in soleus and ADL (18 and 12% inhibition, respectively). As with the high dose, the low dose of propranolol had no effect on blood glucose, plasma insulin, plasma NEFA or colonic temperature.

Given the unexpected effects of low dose propranolol on the effects of BRL on GUI in certain muscles, it was decided to attempt to determine the βAR-subtypes responsible by use of similar low doses of the selective antagonists atenolol (β_1AR) and ICI 118551 (β_2AR) on tissue glucose utilization responses to BRL 35135. For this experiment (Expt. 6), the same protocol and injection sequence for the antagonists as used for Expt. 5 was followed. Atenolol had no effect on the GUI response to BRL in any tissue studied (Table 4). However, the selective β_2AR antagonist, ICI 118551, significantly potentiated the effect of BRL on GUI in soleus (127% greater than BRL alone), tibialis (88%), EDL (79%), ADL (157%) and diaphragm (149%). ICI 118551 had no effect on GUI in gastrocnemius, heart, WAT, BAT or brain, and neither atenolol nor ICI 118551 altered blood glucose, plasma insulin, plasma NEFA or colonic temperature.

Discussion

Skeletal muscle is considered to be the principal site of insulin-stimulated glucose disposal (DeFronzo *et al.*, 1983), but the tissue displays significant heterogeneity due to the presence of at least three muscle fibre types that have distinct biochemical and contractile properties. There are substantial differences in rates of glucose uptake in muscles composed predominantly of the different fibre types (Bonen *et al.*, 1981, Richter *et al.*, 1984; Ploug *et al.*, 1987), with glucose uptake being highest in 'working' muscles composed primarily of slow-twitch oxidative (Type I) fibres, followed by 'non-

Table 4 Effect of atenolol and ICI 118551 on tissue GUI and other responses to BRL 35135 (Expt. 6)

	BRL	*Aten/BRL*	*ICI/BRL*
GUI (ng min^{-1} mg^{-1})			
Gas	3.1 ± 0.3	2.9 ± 0.2	3.4 ± 0.4
Sol	7.1 ± 1.0	5.2 ± 0.7	16.1 ± 2.1**
TA	4.2 ± 0.5	3.7 ± 0.4	7.9 ± 1.1**
EDL	4.2 ± 0.5	3.5 ± 0.3	7.5 ± 0.9**
ADL	7.9 ± 0.9	6.9 ± 0.9	20.3 ± 3.0***
Dia	18.5 ± 2.5	19.6 ± 1.0	46.1 ± 4.1***
HRT	41.0 ± 5.6	45.6 ± 7.4	43.8 ± 8.5
WAT	8.1 ± 1.6	7.7 ± 0.8	6.5 ± 1.0
BAT	77.5 ± 9.8	56.8 ± 12.0	82.7 ± 12.9
CTX	19.0 ± 1.8	14.8 ± 0.9	18.8 ± 2.3
Other parameters			
Glucose (mM)	9.40 ± 0.61	7.42 ± 0.50	7.87 ± 0.60
Insulin (ng ml^{-1})	9.00 ± 1.80	6.75 ± 2.00	8.25 ± 2.80
NEFA (mEq l^{-1})	0.52 ± 0.05	0.75 ± 0.06	0.66 ± 0.05
dTc (°C)	0.67 ± 0.10	0.68 ± 0.08	0.73 ± 0.09

Atenolol (Aten, 1 mg kg^{-1}) and ICI 118551 (1 mg kg^{-1}) given 20 min before BRL 35135 (10 μg kg^{-1}). Mean values ± s.e.mean ($n = 8$ per group, except $n = 6$ for insulin data).
**$P < 0.01$, ***$P < 0.001$ compared to BRL (unpaired t test).
Abbreviations as for Table 2.

working' muscles composed of fast-twitch oxidative-glycolytic and fast-twitch glycolytic (Type II) fibres. The results from the present study are consistent with previous findings, with the higher rates of glucose uptake being seen in oxidative muscles (e.g. soleus, ADL and diaphragm), and it should be emphasised that these differences between 'working' and 'non-working' muscles were still obvious, even though the animals were anaesthetized. This helps justify the decision to use anaesthetized, rather than conscious rats for these studies, and allows future experiments to be undertaken with more intensive measurements and/or invasive procedures. In addition, it should be noted that blood glucose remained unchanged throughout all experiments, and was not in itself responsible for influencing the GUI responses to drug treatment.

Although the 'working' muscles showed the largest increases in GUI response to BRL, all the other muscles exhibited significant increases. However, the largest tissue response was seen in BAT, where there was over a 12 fold increase in glucose utilisation. Given the well-known selectivity of BRL for BAT and the β_3AR, the magnitude of this response and the simultaneous rise in body temperature (i.e. thermogenesis) was perhaps not surprising. Furthermore, Marette *et al.* (1989) have shown βAR stimulation by noradrenaline of glucose transport of brown adipocytes *in vitro* in the absence of insulin, as well as potentiation of the response to insulin, and independent effects of insulin and noradrenaline on glucose transporters in BAT have also been reported (Omatsu-Kanbe & Kitasato, 1992). However, BAT thermogenesis relies mainly on lipolysis and oxidation of fatty acids, rather than increased glucose oxidation (Isler *et al.*, 1987), and it is assumed that the increased glucose utilisation is due to increased lipogenesis. BAT has a high lipogenic capacity and, paradoxically, both lipolysis and *de novo* triglyceride synthesis are markedly increased when thermogenesis is activated (see Bukowiecki, 1986).

Lipogenesis may also account for the increased GUI of white adipose tissue, although βAR activation of lipolysis is likely to predominate and account for the large rise in plasma NEFA. The increase in plasma NEFA levels after BRL would be expected to suppress tissue glucose utilization via the glucose/fatty acid cycle, but this effect of raised NEFA was not seen. The exception was heart in which, unlike all other tissues studied, GUI was decreased by BRL. This is probably explained by the observation that heart glucose utilisation is more sensitive, or responds more rapidly to elevated plasma NEFA levels than skeletal muscles (Holness *et al.*, 1990; Wisneski *et al.*, 1985).

Although skeletal muscle is recognised as a major site for glucose disposal under normal free-living conditions, it is much less important in the resting, or anaesthetized state and raises questions about the metabolic mechanisms producing the increased GUI following BRL treatment. The role of skeletal muscle in sympathetically-mediated thermogenesis is debatable, and the so-called 'hot-pipe', or vasomotor theory of muscle thermogenesis (Colquhoun & Clarke, 1991) depends on the αAR-mediated vasoconstrictor effects of noradrenaline rather than any βAR-mediated effects, which inhibit vascular thermogenesis. However, some studies have indicated that βAR-agonists may produce a thermogenic response in skeletal muscle. Thurlby & Ellis (1986) determined the effects of the β_3AR-agonist, BRL 26830, on oxygen uptake of the hindlimb *in situ* and found a 33% increase in oxygen extraction and implied that the βAR responsible for thermogenesis in skeletal muscle might be similar to the atypical βAR (since classified as the β_3AR) that mediates the lipolytic responses to BRL 26830. The ligand binding studies of Roberts *et al.* (1993) and Sillence *et al.* (1993) have confirmed the presence of an atypical βAR in skeletal muscle, and this could help explain the large increases in muscle GUI following treatment with BRL, although it is not known if the greater GUI results from increased glucose storage (as glycogen) or increased glucose oxidation (i.e. thermogenesis).

It has been shown that chronic treatment with BRL to insulin-resistant rodents results in a dose-related improvement in glucose tolerance, normalization of blood glucose and improved insulin sensitivity (Smith *et al.*, 1990). The precise mechanisms whereby BRL improves insulin sensitivity in insulin-resistant rodents have not been identified, although studies with the analogue, BRL 26830, indicate that changes in both insulin receptor binding the post-receptor actions are involved (Rochet *et al.*, 1988). In contrast to these previous investigations using insulin-resistant animal models, chronic treatment of the normal Wistar rats used here failed to affect basal blood glucose and insulin levels. Moreover, the GUI response to acute administration of BRL after two weeks chronic treatment had disappeared completely. This might have been due to the failure of BRL to increase plasma insulin, although this would not explain why acute injection of BRL was still capable of producing a 50% increase in soleus muscle GUI. This provided the first indication that, apart from stimulating pancreatic insulin release, BRL might also influence muscle GUI by an insulin-independent mechanism. Failure to produce an acute response in BAT was almost certainly due to the fact that chronic treament

has already induced very large, probably maximal increases in basal GUI, equivalent to 34 times basal GUI in the control group. The marked suppression of the acute effects of BRL on skeletal muscle glucose uptake after chronic treatment implicates βAR desensitization or down-regulation, but does not explain why this happened in muscle and not in BAT. A possible explanation relates to the fact that chronic treatment with βAR-agonists causes BAT hyperplasia (see Himms-Hagen, 1990) and the greater number of maximally-active brown adipocytes in a given tissue mass accounts for the large increase in basal GUI.

It has been claimed (Smith *et al.*, 1990) that the ability of a single dose of BRL to improve glucose tolerance in lean rats and mice is dependent upon its potent insulin secretagogue activity, and this is supported by the observation that BRL 35135 and BRL 26830 are inactive in streptozotocin-diabetic rats (Smith *et al.*, 1990; Yoshida, 1992). The present results and those of Abe *et al.* (1993) confirm this effect on pancreatic insulin release, with elevated insulin concentrations appearing within 20 min of administration. However, it can be argued that this may not be the only mechanism by which BRL affects muscle glucose utilisation. Firstly, Abe *et al.*(1993) used 2DG to measure the rate constant of glucose uptake in muscle while infusing BRL, and found that this could be increased by doses of BRL that failed to produce detectable changes in plasma insulin. Similarly, in Expt. 3 a single injection of BRL caused a marked 50% increase in soleus GUI without any change in plasma insulin levels following chronic treatment with the agonist. Finally, there is the observation that low-dose propranolol and ICI 118551 produced variable, and qualitatively different GUI responses to BRL in different tissues without altering the plasma insulin response. If βAR stimulation of pancreatic insulin release was the sole mechanism by which BRL affected GUI, the βAR-antagonists would be expected to produce both quantitatively and qualitatively similar responses to the humoral stimulus in all tissues. Thus, these observations suggest that BRL 35135 can stimulate glucose uptake by direct activation of muscle βAR in addition to the stimulation resulting from βAR stimulation of pancreatic insulin release. The *in vitro* soleus muscle experiments of Abe *et al.* (1993) have confirmed that BRL 37344 (the active metabolite of BRL 35135) will stimulate glucose uptake in the absence of insulin, and preliminary results from this laboratory (Y.-L. Liu & M.J. Stock, unpublished data) have confirmed this for soleus and EDL in the presence of a fixed, low concentration of insulin.

Although these experiments show that BRL might exert direct βAR-mediated effects on muscle GUI, they raise certain questions about the nature of the receptor subtype involved. The failure of high doses (20 mg kg^{-1}) of propranolol to affect the muscle GUI response to BRL strongly suggests the involvement of an atypical βAR. One of the main features of the β_3AR is its weak affinity for conventional βAR-antagonists (i.e. the antagonists have a low pA_2 for the adrenoceptor; Arch & Kaumann, 1993), and it is possible to use low *in vivo* doses of these antagonists that are sufficient to block β_1AR and/or β_2AR responses to non-selective agonists without affecting β_3AR responses, such as thermogenesis. For example, Carlisle & Stock (1992, 1993b) used the same low doses and routes of administration of propranolol, atenolol and ICI 118551 as used here to 'dissect' out the βAR subtypes responsible for the conflicting thermoregulatory responses to non-selective βAR-agonists. The doses used had no effect alone, but were sufficient to inhibit β_1AR and β_2AR responses to isoprenaline. Conversely, a high (20 mg kg^{-1}) dose of ICI 118551 was found to have no effect on *in vivo* skeletal muscle rubidium uptake (Cartaña & Stock, 1994), thus indicating that the antagonist is unlikely to have any partial agonist activity on muscle. Adopting the same approach here, and using a 1 mg kg^{-1} dose of propranolol resulted in some paradoxical tissue responses, with GUI responses to BRL in gastrocnemius, soleus and ADL showing complete resistance to the antagonist, whereas responses in tibialis, EDL and diaphragm were markedly potentiated by the antagonist.

The resistance of the muscle GUI response to the high dose of propranolol supports the notion of an atypical receptor involvement, but the results with low-dose propranolol suggested that the normal response to BRL includes an inhibitory component, i.e. the potentiation of GUI by low-dose propranolol was due to disinhibition of atypical βAR activation. Since propranolol is a non-selective antagonist, it was not possible to ascribe this to the β_1AR or the β_2AR, and so in the next experiment the selective antagonists, atenolol and ICI 118551, were used. Atenolol had no effect on the GUI response to BRL, whereas pretreatment with ICI 118551 significantly potentiated GUI in all muscle fibre types. This indicates that BRL 35135 has β_2AR-agonist activity and that in the absence of propranolol and ICI 118551 the β_2AR activity exerts an inhibitory effect on muscle glucose utilization which offsets the stimulation due to activation via the atypical βAR. It seems unlikely that the β_2AR-inhibitory effect on GUI could be due to increases in muscle blood flow since: (a) ICI 118551 would be expected to reduce rather than increase blood flow and, (b) if anything, vasodilatation would stimulate rather than inhibit glucose utilisation (James *et al.*, 1985; Baron *et al.*, 1994). Thus, these are probably direct effects of BRL on muscle, and loss of selectivity and increased β_2AR inhibition would also explain why the *in vitro* stimulation of muscle 2DG uptake described by Abe *et al.* (1993) was attenuated at high concentrations of BRL 37344. The simultaneous stimulation and inhibition of GUI via two different βAR-subtypes in muscle is also difficult to reconcile with both having common post-receptor mechanisms involving G protein and adenylyl cyclase activation. However, multifunctional signalling by G protein-linked receptors is possible (for review, see Milligan, 1993) and simultaneous activation of glucose transport via the atypical βAR and glycogenolysis via the β_2AR could explain the effects of BRL observed here.

It is tempting to assume that the atypical βAR responsible for mediating the increase in muscle GUI is the β_3AR found in adipose tissue. However, there are some notable differences between the effects of the βAR antagonists on the BRL response in adipose tissue compared to muscle. For example, there was significant inhibition of the BRL response in BAT by the high and low use of propranolol, but no inhibition by the high and low doses in any muscle. The different sensitivity to βAR antagonists of the GUI responses in BAT by the high and low dose of propranolol, but no inhibition by the high and low doses in any muscle. The different sensitivity to βAR antagonists of the GUI responses in BAT and muscle suggest that the atypical receptor in muscle may not be a β_3AR. The same argument could be used for the pancreatic release of insulin, since plasma insulin levels were also more resistant to propranolol than BAT though there is an atypical ligand-binding site (Robers *et al.*, 1993; Sillence *et al.*, 1993). However, the *in vivo* GUI responses described here could result from a complex interplay between a variety of factors, and it would be dangerous to conclude that another βAR-subtype is involved until more detailed *in vitro* and molecular studies have been carried out.

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References

ABE, H., MINOKOSHI, Y. & SHIMAZU, T. (1993). Effect of a β_3-adrenergic agonist, BRL 35135A, on glucose uptake in rat skeletal muscle *in vivo* and *in vitro*. *J. Endocrinol.*, **139**, 479–486.

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNIT, M.V., THODY, V., WILSON, C. & WILSON, S. (1984). Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature.*, **309**, 163–165.

ARCH, J.R.S. & KAUMANN, A.J. (1993). β_3 and atypical β-adrenoceptors. *Med. Res. Ev.*, **13**, 663–729.

BARON, A.D., STEINBERG, H., BRECHTEL, G. & JOHNSON, A. (1994). Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. *Am. J. Physiol.*, **266**, E248–E253.

BONEN, A., TAR, M.H. & WATSON-WRIGHT, W.M. (1981). Insulin binding and glucose uptake differences in rodent muscles. *Diabetes*, **30**, 702–704.

BUKOWIECKI, L.J. (1986). Lipid metabolism in brown adipose tissue. In *Brown Adipose Tissue*. ed Trayhurn, P. & Nicholls, D.G. pp. 105–121. London: Edward Arnold.

CARLISLE, H.J. & STOCK, M.J. (1992). Potentiation of thermoregulatory responses to isoproterenol by β-adrenergic antagonists. *Am. J. Physiol.*, **263**, R915–923.

CARLISLE, H.J. & STOCK, M.J. (1993a). Thermoregulatory responses to β-adrenergic agonists at low ambient temperatures in the rat. *Exp. Physiol.*, **78** 775–786.

CARLISLE, H.J. & STOCK, M.J. (1993b). Thermoregulatory effects of beta-adrenoceptors: effects of selective agonists and the interaction of antagonists with isoproterenol and BRL 35135 in the cold. *J. Pharmacol. Exp. Ther.*, **266**, 1446–1453.

CARTAÑA, J. & STOCK, M.J. (1994). Effects of clenbuterol and salbutamol on tissue rubidium uptake in vivo. *Metabolism*, (in press).

CHALLIS, R., LEIGHTON, B., WILSON, S., THURLBY, P. & ARCH, J. (1988). An investigation of the β-adrenoceptor that mediates metabolic response to the novel agonist BRL 28410 in rat soleus muscle. *Biochem. Pharmacol.*, **37**, 947–950.

COLQUHOUN, E.Q. & CLARKE, M.G. (1991). Open question: has thermogenesis in muscle been overlooked and misinterpreted? *News Physiol. Sci.*, **6**, 256–259.

DEFRONZO, R.A., FERRANNINI, E., HENDLER, R., FELIG, P. & WAHREN, J. (1983). Regulation of splanchnic and peripheral glucose uptake by insulin and hyperglycemia in man. *Diabetes*, **32**, 35–45.

EMORINE, L.J., MARULLO, S., BRIEND-SUTREN, M.M., PATEY, G., TATE, K., DELARIER-KLUTCHKO, C. & STROSBERG, A.D. (1989). Molecular characterisation of the human β_3-adrenergic receptor. *Science*, **245**, 1118–1121.

FERRÉ, P., LETURQUE, A., BURNOL, A.F., PÉNICAUD, L. & GIRARD, J. (1985). A method to quantify glucose utilization in vivo in skeletal muscle and white adipose tissue of the anaesthetized rat. *Biochem. J.*, **228**, 103–110.

GRANNEMAN, J.G., LAHNERS, K.N. & CHAUDHRY, A. (1991). Molecular cloning and expression of the rat beta 3-adrenergic receptor. *Mol. Pharmacol.*, **40**, 895–899.

HIMMS-HAGGEN, J. (1990). Brown adipose tissue thermogenesis: role in thermoregulation, energy regulation and obesity. In *Thermoregulation – Physiology & Biochemistry*. ed Schonbaum, E. & Lomax, P. pp 327–414. New York: Pergamon.

HOLNESS, M.J. & SUGDEN, M.C. (1990). Glucose utilization in heart, diaphragm and skeletal muscle during the fed-to-starved transition. *Biochem. J.*, **270**, 245–249.

ISLER, D., HILL, H.P. & MEIER, M.K. (1987). Glucose metabolism in isolated brown adipocytes under β-adrenergic stimulation, *Biochem. J.*, **245**, 789–793.

ISSAD, T., PÉNICAUD, L., FERRÉ, P., KANDÉ, J., BAUDON, M.A. & GIRARD, J. (1987). Effects of fasting on tissue glucose utilization in conscious resting rats. *Biochem. J.*, **246**, 241–244.

JAMES, D.E., JENKINS, A.B. & KRAEGEN, E.W. (1985). Heterogenity of insulin action in individual muscles *in vivo*: euglycaemic clamp studies in rats. *Am. J. Physiol.*, **248**, E567–574.

KRIEF, S., LONNQVIST, F., RAIMBAULT, S., BAUDE, B., VAN SPRONSEN, A., ARNER, P., STROSBERG, A.D., RICQUIER, D. & EMORINE, L.J. (1993). Tissue distribution of beta 3-adrenergic receptor mRNA in man. *J. Clin. Invest.*, **91**, 344–449.

MARETTE, A. & BUKOWIECKI, L.J. (1989). Stimulation of glucose transport by insulin and norepinephrine in isolated rat brown adipocytes. *Am. J. Physiol.*, **257**, C714–721.

MILLIGAN, G. (1993). Mechanisms of multifunctional signalling by G protein-linked receptors. *Trends. Pharmacol. Sci.*, **14**, 239–244.

NAHMIAS, C., BLIN, N., ELALOUF, J.M., MATTEI, M.G., STROSBERG, A.D. & EMORINE, L.J. (1991). Molecular characterization of the mouse β_3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO*, **10**, 3721–3727.

OMATSU-KANBE, M. & KITASATO, H. (1992). Insulin and noradrenaline independently stimulate the translocation of glucose transporters from intracellular stores to the plasma membrane in mouse brown adipocytes. *FEBS Lett.*, **314**, 246–250.

PLOUG, T., GALBO, H., VINTEN, J., JORGENSEN, M. & RICHTER, E.A. (1987). Kinetics of glucose transport in rat skeletal muscle: effects of insulin and contractions. *Am. J. Physiol.*, **253**, E12–20.

RICHTER, E.A., GARRETO, L.P., GOODMAN, M.N. & RUDERMAN, N.B. (1984). Enhanced muscle glucose metabolism after exercise: modulation by local factors. *Am. J. Physiol.*, **246**, E476–482.

ROBERTS, S.J., MOLENAAR, P. & SUMMERS, R.J. (1993). Characterization of propranolol-resistent (−)-[^{125}]-cyanopindolol binding sites in rat soleus muscle. *Br. J. Pharmacol.*, **109**, 344–352.

ROCHET, N., TANTI, J.F., GREMEAUX, T., VAN OBBERGHEN, E. & MARCHAND-BRUSTEL, Y. (1988). Effect of the thermogenic agent BRL 26830A on insulin receptors in obese mice. *Am. J. Physiol.*, **255**, E101–109.

SILLENCE, M.N., MOORE, N.G., PEGG, G.G. & LINDSAY, D.B. (1993). Ligand binding properties of putative β_3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes. *Br. J.Pharmacol.*, **109**, 1157–1163.

SMITH, S.A., SENNITT, M.V. & CAWTHORNE, M.A. (1990). BRL 35135: an orally active antihyperglycaemic agent with weight reducing effects. In *New Anti-Diabetic Drugs*. ed Bailey, C.J. & Flatt, P.R. pp.177–189. London: Smith–Gordon.

STOCK, M.J. & SUDERA, D.K. (1989). Schild analysis of β-adrenoceptor antagonist inhibition of brown adipocyte respiratory response to novel β-adrenoceptor agonists. *Br. J. Pharmacol.*, **96**, 741P.

THURLBY, P.L. & ELLIS, R.D.M. (1986). Differences between the effects of noradrenaline and the β-adrenoceptor agonist BRL 28410 in brown adipose tissue and hind limb of the anaesthetized rat. *Can. J. Physiol. Pharmacol.*, **64**, 1111–1114.

WISNESKI, J.A., GERTZ, E.W., NEESE, R.A., GRUENKE, L.D., MORRIS, D.L. & CRAIS, J.C. (1985). Metabolic fate of extracted glucose in normal human myocardium. *J. Clin. Invest.*, **76**, 1819–1827.

YOSHIDA, T. (1992). The antidiabetic β_3-adrenoceptor agonist BRL 26830A works by release of endogenous insulin. *Am. J. Clin. Nutr.*, **55**, 237–413.

ZAAGSMA, J. & HOLLENGA, C.H. (1991). Distribution and function of atypical β_3-adrenoceptors. In *Adrenoceptors: Structure, Mechanism, Function*. ed Szabadi, E. & Bradshaw, C.M. pp. 47–58. Basle: Birkhausverlag.

ZAAGSMA, J. & NAHORSKI, S. (1990). Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical β_3-adrenoceptor? *Trends Pharmacol. Sci.*, **11**, 3–7.

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Platelet-activating factor: the effector of protein-rich plasma extravasation and nitric oxide synthase induction in rat immune complex peritonitis

Ana Angelica Steil, Maria del Carmen Garcia Rodriguez, Andrés Alonso, [1]Mariano Sanchez Crespo & *Lisardo Bosca

Departamento de Bioquimica y Fisiologia-CSIC, Facultad de Medicina, 47005-Valladolid, Spain and *Instituto de Bioquimica-CSIC, Facultad de Farmacia, Universidad Complutense, 28040-Madrid, Spain

1 The involvement of platelet-activating factor (PAF) in immune complex-induced/polymorphonuclear-mediated tissue injury was studied by use of a reverse passive Arthus (RPA) model in the peritoneal cavity of rats.

2 Extravasation of protein-rich plasma, accumulation of polymorphonuclear leukocytes (PMN), and the production of nitric oxide (NO) by resident peritoneal mononuclear phagocytes were assayed.

3 Treatment of rats with either UR-12460 or BB-823, two compounds which possess different chemical structures, but elicit the same antagonistic effect on the PAF receptor, abrogated protein-rich plasma extravasation. In contrast, they did not show any effect on the accumulation of PMN.

4 Inhibition of NO production with both N^G-mono methyl-L-arginine and N^G-nitro-L-arginine failed to prevent protein-rich plasma extravasation.

5 The production of NO by peritoneal adherent cells following RPA was measured in cells maintained for 2 to 28 h in culture, and it was significantly increased in cells removed as early as 15 min after RPA induction, as compared to controls.

6 Addition of 10 nM PAF to the culture medium reduced the generation of NO by peritoneal cells from RPA rats, whereas this mediator enhanced NO production in cells from naive control animals.

7 Treatment with either UR-12460 or BB-823 prior to the induction of RPA produced an almost complete inhibition of NO production.

8 Assay of nitric oxide synthase activity in cell homogenates from peritoneal cells showed that the activity was due to the inducible form of the enzyme.

9 Study by Northen blotting of mRNA coding for the inducible NO synthase (iNOS) showed transcription at 6 and 18 h after the induction of RPA, which was inhibited in UR-12460-treated rats.

10 These data indicate that PAF is the main mediator of the early plasma leakage observed in RPA, and also that PAF is implicated in the triggering of long-term changes via induction of specific genes, as judged from its ability to promote the expression of iNOS.

Keywords: Arthus reaction; complement; cytokines; endotoxin; immune complexes; inflammatory oedema; nitric oxide; PAF; polymorphonuclear leukocytes; shock; tumour necrosis factor; vascular endothelial-cells

Introduction

The Arthus reaction was first described at the turn of the century as an acute inflammatory and haemorrhagic reaction produced in the skin of rabbits when a local injection of horse serum was administered to previously sensitized animals (Arthus, 1903). Experimentally it is convenient to investigate the reversed passive Arthus reaction (RPA), which is elicited by a local injection of antibody and an intravenous injection of its cognate antigen. This model is a good counterpart of immune complex peritonitis, when developed in the peritoneal cavity. The sequence of events that occur in the RPA reaction includes formation of immune complexes in the microvessel wall, activation of the complement cascade, migration and adherence of polymorphonuclear leukocytes (PMN) to the endothelial cells, which results in an increase of microvascular permeability (Wedmore & Williams, 1981), and release of PAF from neutrophils which acts on endothelial cells to cause further leakage (Hellewell & Williams, 1986; Warren *et al.*, 1989a; Rossi *et al.*, 1992; Tavares de Lima *et al.*, 1992). Since tissue injury in RPA is complement-dependent and largely neutrophil-mediated, detailed attention has been dedicated to ascertain the mechanisms which in addition to generation of C5a might lead to recruitment of polymorphonuclear leukocytes. Thus, it has been shown that tumour necrosis factor-α (Warren *et al.*, 1989b), interleukin (IL)-1β (Warren, 1992), cytokines related to IL-8/neutrophil activating protein 1 (Collins *et al.*, 1991), and products of oxidative metabolism of arachidonate via 5-lipoxygenase (Zhang *et al.*, 1992) are involved.

Recent therapeutic approaches to the Arthus reaction focus on the use of recombinant soluble human complement receptor type I (Rossi *et al.*, 1992) to control the activation of the complement cascade, the utilization of a recombinant soluble form of human FcγRII (FcγR, receptor for the Fc portion of IgG) genetically engineered to interfere with neutrophil activation via FcγRII, and the ensuing release of inflammatory mediators (Ierino *et al.*, 1993); and the utilization of recombinant selectin chimeric molecules to block PMN recruitment (Mulligan *et al.*, 1993). Another step for pharmacological modulation might be the antagonism of mediators released from PMN that could be particularly active either on their own or because of their central position

[1] Author for correspondence.

in the triggering of other mediators. Recent studies have emphasized the involement of nitric oxide in the production of tissue injury in a model of alveolitis and dermal vasculitis analogous to RPA (Mulligan *et al.*, 1991; 1992), whereas there has been some controversy regarding the possible involvement of eicosanoids in the mediation of plasma leakage in RPA (Boughton-Smith *et al.*, 1993). The purpose of this study was to assess the effect of blocking receptors for the lipid mediator platelet-activating factor on events of the RPA that occur at different times after antigen challenge.

Methods

Materials and animals

Male pathogen-free Wistar rats (200–300 g) were obtained from Valladolid Medical School Animal Facility. IgG antibodies were raised in rabbits by subcutaneous injections of ovalbumin in complete Freund's adjuvant followed by booster intramuscular injections until a positive reaction in radial immunodiffusion could be observed at a dilution of antiserum 1/16. IgG2 antibodies were purified by sequential precipitation with octanoic acid and ammonium sulphate. The PAF receptor antagonist UR-12460 (1-[(N-diphenylmethyl) aminoacetyl]-4-[(2-methyl-3-pyridyl)cyanomethyl] piperazine) was obtained from URIACH, S.A., Barcelona, Spain, and solubilized in saline solution (Carceller *et al.*, 1993). The PAF receptor antagonist BB-823 (N-4-(1-H-2-methylimidazo[4,5-c]pyridylmethyl)phenylsulphonyl-L-leucinyl ethyl ether) was a gift from British Bio-technology Ltd., Oxford, U.K., and was solubilized by stoichiometric mixture with 1N HCl to form the hydrochloride salt, and dilution with saline solution (Whittaker *et. al.*, 1992). 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), N^G-nitro-L-arginine methyl ester (L-NAME), L-NOARG (N^G-nitro-L-arginine), L-NMA (N^G-methyl-L-arginine), molsidomine and isosorbide dinitrate were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Induction of the reverse passive Arthus reaction

Rats were injected i.p. with 1 mg of specific antibody and i.v. with 10 mg ovalbumin and 20 mg kg^{-1} of Evans blue dye to study extravasation of protein-rich plasma. The doses of antibody and antigen used produced an extravasation response near the maximum, which is comparable to that observed in a passive anaphylaxis model with IgE sensitizing antibody (Pellón *et al.*, 1994). The concentration of Evans blue in the peritoneal cavity was determined spectrophotometrically at a wavelength of 620 nm in the fluid obtained by washing the peritoneal cavity with 10 ml of phosphate-buffered saline solution. The results were compared with a standard curve prepared with known amounts of Evans blue dye, and the content of each sample was expressed as μg Evans blue per ml. The experimental protocol was approved by our Institutional Review Board.

Assay of polymorphonuclear accumulation into the peritoneal cavity

Neutrophil influx was evaluated by measurement of myeloperoxidase (MPO) activity colorimetrically in the lysate of cells obtained from the peritoneal cavity. MPO activity in the cell lysate was measured by the change in optical absorbance at 460 nm resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine (Henson *et al.*, 1978). The results were expressed as number of PMN obtained by comparison with a standard curve of the MPO activity of known numbers of PMN from peritoneal exudate after 6 h of RPA (Zhang *et al.*, 1992).

Assay of NO production by peritoneal macrophages ex vivo

Peritoneal cells obtained 15 min after induction of the Arthus reaction in rats were resuspended in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin, 0.5 mM L-arginine, 2mM glutamine and 10% heat-inactivated foetal calf serum. Non-adherent cells were removed two hours after challenge, and the production of NO was assessed after different times of incubation at 37°C in an atmosphere containing 5% CO_2.

Determination of NO and nitrite

NO released from macrophage cultures was determined by the accumulation of nitrite (Green *et al.*, 1982). The cell cultures (5×10^5 cells in 1 ml of phenol red-free medium) were filled with 100 μl of a solution of 1 mM sulphanilic acid and 100 mM HCl (final concentration). After incubation for 5 min the medium was aspirated and centrifuged in an Eppendorf microcentrifuge; 50 μl of naphthylenediamine (1 mM in the assay) was added to the samples, and the reaction was completed after 15 min of incubation. The absorbance at 548 nm was compared with a standard of $NaNO_2$, and the production of NO was expressed as nmol of NO_2 mg^{-1} protein.

Assay of NOS activity

NOS activity was measured in 105,000 *g* supernatants from homogenates of 10^7 cells in a medium containing 20 nM Tris, 20 mM KCl, 100 μM EGTA, 10 μM (6R)-5,6,7,8-tetrahydrobiopterin, 0.5 mM dithioerythritol (DTT), 10 μg ml^{-1} of leupeptin and 0.5 mM phenylmethylsulphonyl fluoride, pH 7.5. After partial purification by 2',5'-ADP-sepharose chromatography (0.5 ml of gel), the activity was determined by the production of [^{14}C]-citrulline from [U-^{14}C]-arginine (Schmidt *et al.*, 1992). The reaction was started by the addition of 50 μM [U-^{14}C]-arginine (0.3 μCi) to a mixture that contained 20 mM Tris, 0.5 mM NADPH, 0.1 mM DTT, and 10 μM (6R)-5,6,7,8-tetrahydrobiopterin (200 μl of incubation volume). After 10 min of incubation at 30°C, the reaction was stopped by addition of 1 ml ice-cold 10 mM EGTA, 0.1 mM citrulline, 10 mM PIPES, pH 5.5. One ml of this mixture was applied to a 1 ml Dowex AG50W-X8 column (Na^+-form), and the [U-^{14}C]-citrulline was eluted in 2 ml of water. After counting the emerging radioactivity, the enzyme activities were calculated as the differences of product formation in the absence or presence of 0.25 mM L-NMA in the reaction mixture. The Ca^{2+}-dependence of NOS was assayed in the presence of 200 μM $CaCl_2$ and 10 nM calmodulin in the reaction mixture. One unit of NOS activity was defined as the amount of protein that released 1 nmol of [U-^{14}C]-citrulline per min.

Preparation of total RNA and Northern blot analysis of NOS

Total cellular RNA was prepared from cell cultures (10^7 cells) following the guanidium isothiocynate method (Chomczynski & Sacchi, 1987). Aliquots of total RNA (5 μg) were denatured at 65°C for 15 min in 5% formaldehyde, 50% formamide and 8% glycerol and then were size-separated by electrophoresis in a 0.9% agarose gel containing 2% formaldehyde and 3-(N-morpholine) propane sulphonic acid (MOPS) buffering system (Maniatis *et al.*, 1989). After transference of the RNA to Nytran membranes (NY 13-N; Schleicher & Schuell) with 10 × SSC (10 × SSC is 1.5 M NaCl, 0.3 M sodium citrate, pH 7.4), the membranes were prehybridized for 6 h at 42°C in 50% formamide, 0.25 M NaCl, 0.1 M sodium phosphate, 7% SDS and 0.01% of salmon sperm. An 817 bp fragment (nucleotides 1 to 817)

from the complementary DNA of macrophage NOS was random primed labelled (45% efficiency) and was used to detect the level of transcription (Xie *et al.*, 1992). The membranes were washed once with 0.1 × times standard saline citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 10 min, and twice at 50°C for 30 min, followed by exposure to an X-ray film (Kodak, X-OMAT). Quantitation of the film was performed by laser densitometry (Molecular Dynamics, Kemsing, U.K.) using the hybridization with a β-actin probe (0.6 kb EcoRI/HindIII fragment isolated from VC18 vector) as internal control to demonstrate the integrity of the RNA, the equivalence of loading, and the specificity of mRNA induction.

Statistical analyses

Data are expressed as the mean ± s.e.mean. For comparison of two groups of samples normally distributed, Student's two-tailed *t* test was used to analyze differences for significance. For comparison of two groups of samples not normally distributed, the Mann-Whitney U test was used. Changes of a variable measured during a period of time were analyzed by one way analysis of variance (ANOVA). Statistical procedures were performed using a data base and statistical package (Instat, GraphPAD Software Inc., San Diego, CA, U.S.A.), $P<0.05$ was considered significant.

Results

Protein-rich plasma extravasation in RPA

Induction of RPA produced a marked increase of the accumulation of Evans blue dye in the peritoneal cavity of rats. This was already apparent at 15 min, but maximal accumulation occurred at 30-60 min (solid columns in Figure 1). Attempts to follow extravasation at later times showed a decay of the Evans blue dye (EB) in the peritoneal lavage fluid, even when the experiment was modified to perform the injection of EB at 3 h after challenge and the collection of the peritoneal exudate carried out 1 h thereafter (4.92 ± 0.24 μg ml^{-1} in a group of 3 rats). These findings were interpreted as evidence that extravasation is an early event in RPA, that subsides within 1-2 h after RPA challenge. When the rats were pretreated with an i.v. dose of 5 mg kg^{-1} of UR-12460 10 min prior to the induction of RPA, a reduction of extravasation to levels similar to those detected in vehicle-treated animals was observed at 30 min; however, the inhibition was less marked when rats were killed 60 min after challenge. This could indicate either recruitment of other extravasation-inducing mediators or metabolism of the drug. To ascertain whether the effect of UR-12460 was a consequence of its purported PAF receptor antagonist effect, experiments were also performed with BB-823, a PAF receptor antagonist with a chemical structure unrelated to UR-12460. As indicated by the cross-hatched column in Figure 1, BB-823 showed a similar protective effect on extravasation. Treatment with the L-arginine analogues L-NOARG and L-NMA alone or in combination at doses as high as 100 mg kg^{-1}, i.p., prior to challenge with antigen and antibody gave no protection (Table 1). Moreover, to substantiate that these drugs were acting on the NO system, mean arterial pressure was measured under the conditions described by Pellón *et al.* (1994) in three rats after L-NMA treatment and found to be significantly higher than in vehicle-treated rats. Attempts to enhance extravasation by the NO-generating compounds molsidomine and isosorbide dinitrate at doses of 15 mg kg^{-1} in combination with 200 mg kg^{-1} L-arginine did not influence extravasation.

Table 1 Effect of NO synthase inhibitors on Evans blue dye extravasation

Treatment	*Evans blue dye* (μg ml^{-1} peritoneal fluid)	n
Anti-ovalbumin antibody	4.1 ± 0.6	9
RPA	14.8 ± 2.4	8
RPA + 100 mg kg^{-1} L-NOARG	15.0 ± 3.3*	4
RPA + 100 mg kg^{-1} L-NMA	13.2 ± 2.4*	4
RPA + 100 mg kg^{-1} L-NOARG + L-NMA	14.5 ± 3.0*	4

NO synthase (L-NOARG = N^G-nitro-L-arginine methylester; L-NMA = N^G-methyl-L-arginine) inhibitors were i.p. injected 10 min before the induction of RPA. The peritoneal exudate was obtained 30 min after RPA or antibody administration. Data represent mean ± s.e.mean. *Not significant as compared to RPA animals.

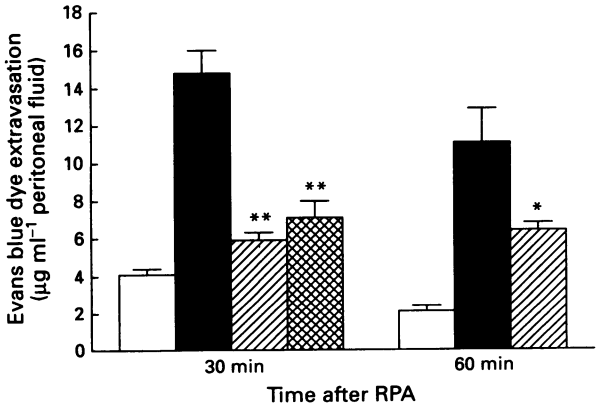


Figure 1 Extravasation of Evans blue dye in the peritoneal cavity during RPA and effect of PAF-receptor antagonists. The peritoneal exudate from rats was obtained at the times indicated after local injection of anti-ovalbumin antibody (negative control, open columns), local antibody and intravenous antigen (RPA animals, solid columns), or local antibody and intravenous antigen preceded by i.p. injection of 5 mg kg^{-1} UR-12460 10 min prior to challenge (hatched columns), or 5 kg^{-1} BB-823 (cross-hatched column). Results represent mean ± s.e.mean of 7 to 9 animals in each group. $^{**}P<0.01$, $^{*}P<0.05$.

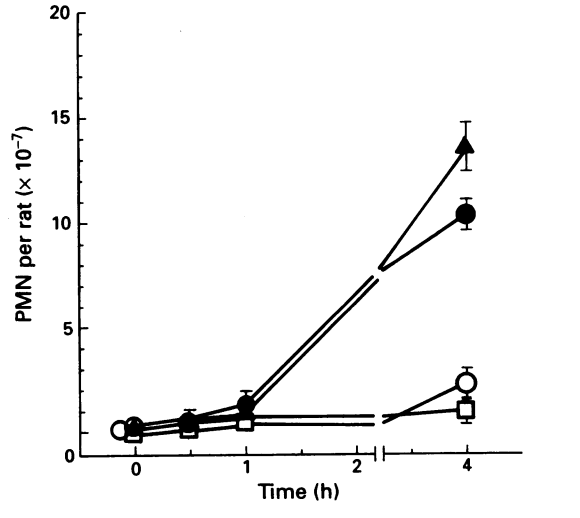


Figure 2 Effect of UR-12640 on the infiltration of PMN in the peritoneal cavity. Saline-treated rats (□), antibody-treated rats (○), RPA rats (●) and RPA rats pretreated with 5 mg kg^{-1} UR-12460 (▲) were killed at the times indicated for collection of peritoneal cells. The number of infiltrating PMN was assessed from the assay of myeloperoxidase activity.

Accumulation of PMN in the peritoneal cavity

Assay of myeloperoxidase (MPO) activity as an index of PMN accumulation in the peritoneal cavity showed that infiltration by PMN was a delayed event as compared to plasma leakage, with little infiltration of PMN up to 1 h and a significant increase at 4 h (Figure 2). Treatment with UR-12460 did not influence the number of infiltrating PMN. When PAF was injected at doses up to 10 $\mu g\,kg^{-1}$ i.p., it did not induce PMN recruitment in the peritoneal cavity, and higher doses induced haemorrhagic necrosis of the small intestine, which is most probably due to its ability to induce widespread endothelial damage (Pellón *et al.*, 1994).

Nitrite production and iNOS expression in RPA

Adherent peritoneal cells from RPA rats showed an enhanced *ex vivo* production of NO as compared to cells obtained from control animals receiving either antigen or antibody alone (Figure 3). This enhanced production occurred after 8 h in culture, with a linear increase up to 18 h and a less prominent increase up to 28 h. Both this pattern of NO production and its magnitude suggested the involvement of the inducible form of NO synthase, and would be in keeping with the above mentioned lack of effect of the modulation of NO production on the early phase of RPA. The involvement of the inducible form of NOS was confirmed by measuring the activity in the presence and absence of calcium/calmodulin. As shown in Table 2, the result was almost the same under either condition, which points to the singular involvement of the inducible form of the enzyme. To determine the optimal period of time after induction of RPA required to show an enhanced *in vitro* nitrite production, peritoneal cells were collected either 15 or 60 min after challenge. As shown in Figure 4, no significant difference was observed at either time, which indicates the dependency of enhanced NO production on events occurring within 15 min after induction of RPA. Treatment of rats prior to the induction of RPA with either UR-12460 or BB-823 showed no enhancement of nitrite production as compared to controls (Figure 3). Since these compounds possess different chemical structures, but elicit the same pharmacological effect on the PAF receptor, the results strongly suggest a role for PAF on the induction of NO synthase in RPA. Moreover, adherent peritoneal cells collected from rats that had been injected i.p. with PAF at the dose of 5 $\mu g\,kg^{-1}$ showed a production of NO of 18 ± 2 nmol mg^{-1} protein after 24 h in culture. In agreement with the production of NO by adherent cells after RPA, the levels of iNOS mRNA increased 14 fold after 18 h of culture in animals challenged with antibody plus antigen. This increase could be observed at 6 h after the initiation of the culture, but was most prominent at 18 h. The appearance of iNOS mRNA was inhibited more than 90% by pretreatment of the animals with UR-12460 (Figure 5).

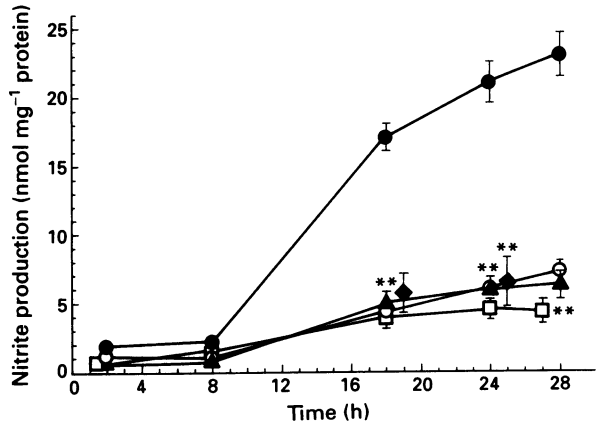


Figure 3 Production of NO by adherent peritoneal cells in culture after induction of RPA. Peritoneal cells were collected by washing the peritoneum with 40 ml of DMEM, followed by centrifugation and resuspension in 10 ml of the same medium supplemented with antibiotics. At that time cells from each rat were distributed in aliquots and maintained at 37°C to allow the collection of adherent cells. NO_2-production was assayed in duplicate samples at the times indicated. Control animals (□); rats treated with i.p. antibody alone (○); animals treated with i.p. antibody and i.v. antigen (●); RPA animals pretreated with 5 mg kg^{-1} UR-12460, i.p., 10 min before elicitation of RPA (▲); at 18 and 24 h (◆) indicate rats receiving 5 mg kg^{-1} BB-823, under similar conditions to UR-12460-treated rats. Data represent mean ± s.e.mean of 9 to 12 animals in each group. $^{**}P > 0.001$.

PAF enhances nitrite production by control peritoneal cells and abrogates the response after RPA

Experiments carried out with adherent cells from control rats showed that the production of nitrite can be elicited by PAF with a bell-shaped pattern of dose-dependency (Figure 6). This was abrogated by the inclusion of UR-12460 in the medium (Figure 6), suggesting again that PAF might induce the production of NO via the inducible form of NOS by signalling through its receptor. When adherent peritoneal cells obtained 1 h after RPA were incubated with different concentrations of PAF, (Figure 6) , a dose-dependent inhibition of nitrite production was observed. The time-dependency of this effect was studied with PAF at the dose of 10 nM.

PAF induced a diminution of nitrite formation by adherent peritoneal cells from RPA rats at all of the times

Table 2 iNOS activity in *ex vivo* cultured adherent peritoneal cells

Treatment	*NOS activity* (%) + *0.5 mM EGTA*	+ Ca^{2+}/*Calmodulin*
None	3	4
RPA	100	96
Antibody	5	5

Animals were treated as indicated and peritoneal cells were cultured for 8 h. iNOS activity was measured by the production of [^{14}C]-citrulline from [^{14}C]-arginine. Results show the means of two cultures assayed in duplicate and expressed as percentage of the maximal activity (12.1 nmol min^{-1} mg^{-1} protein).

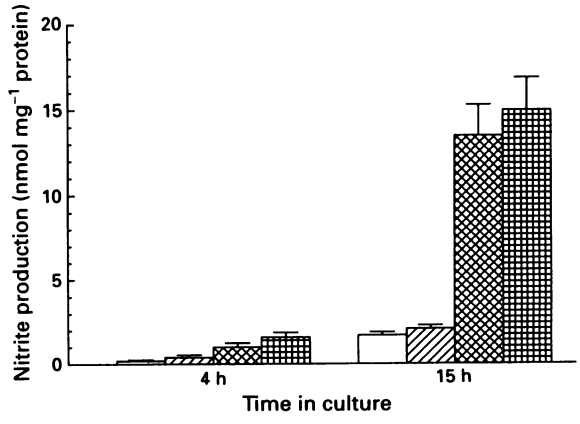


Figure 4 Influence of the time elapsed after RPA on nitrite production by rat adherent peritoneal cells. Rats were challenged with saline solution (open column), antibody (hatched columns), or antigen and antibody, and removed from the peritoneal cavity 15 (cross-hatched columns) or 60 min (checkered columns) thereafter. Nitrite production was assessed 4 and 15 h after removal of non-adherent cells.

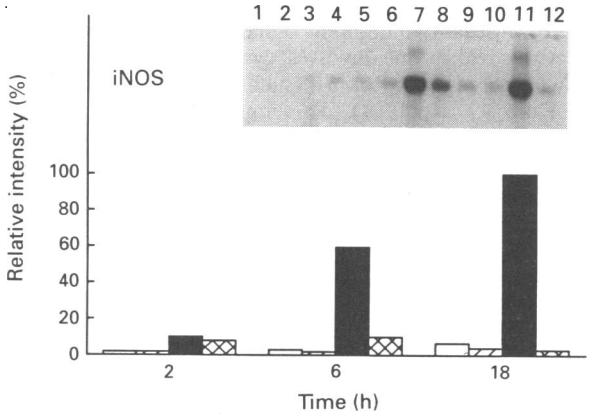


Figure 5 iNOS mRNA levels in RPA. Rats were injected with saline (lanes 1, 5 and 9), antibody (lanes 2, 6 and 10), or antibody and antigen in the absence (lanes 3, 7 and 11) or in the presence of 5 mg kg^{-1} of UR-12460 (lanes 4, 8 and 12). Adherent peritoneal cells were cultured *ex vivo* for 2 (lanes 1-4), 6 (lanes 5-8), and 18 h (lane 9-12), and used for the analysis of iNOS and mRNA using a specific probe. Quantitative analyses of mRNA levels were obtained by densitometry of the bands after normalization for the β-actin content, and are shown in the histogram.

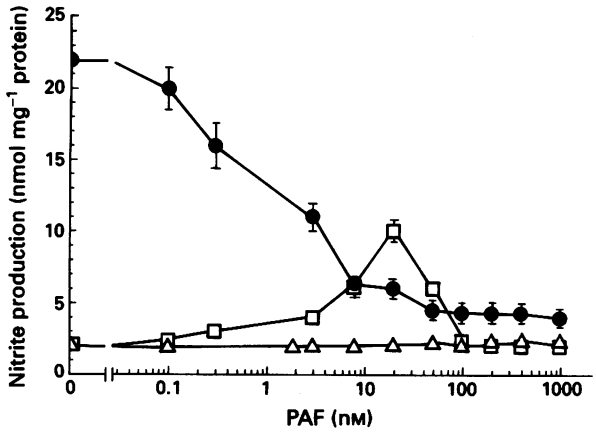


Figure 6 Effect of PAF on the production of nitrite by adherent peritoneal cells after RPA: dose-response. Rats treated to induce RPA (●); rats receiving saline solution alone (□); peritoneal cells from rats treated with saline solution incubated *in vitro* with different concentrations of PAF and 1 μM UR-12460 (△). The measurement of nitrite was carried out after 15 h in culture.

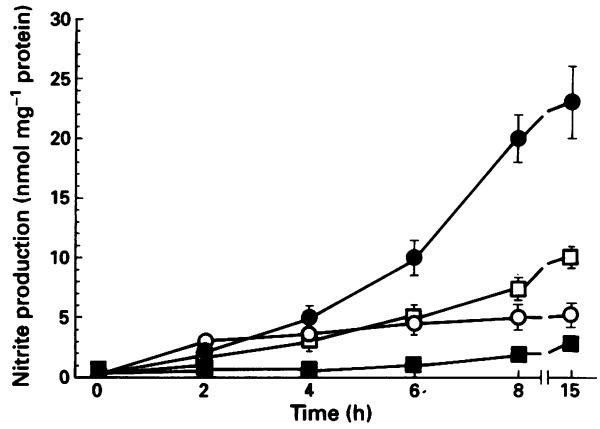


Figure 7 Effect of PAF on the production of nitrite by adherent peritoneal cells after RPA: Time course. Peritoneal cells were collected 15 min after induction of RPA (○, ●) or after saline injection (□, ■). The production of nitrite was assessed in adherent cells after culture for the times indicated in the presence of 10 nM PAF (open symbols) or vehicle (closed symbols).

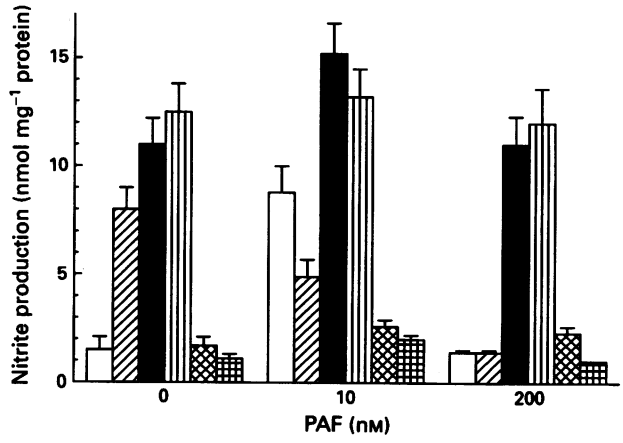


Figure 8 Effect of different agonists on the production of nitrite induced by PAF. Adherent peritoneal cells were incubated in the presence of different concentrations of PAF and the additions indicated. The production of nitrite was assessed 15 h after separation of adherent cells. No addition (open columns), 200 ng ml^{-1} phorbol dibutyrate (hatched columns), 20 μg ml^{-1} *E. coli* lipopolysaccharide (solid column), 1 nM epidermal growth factor (vertically lined columns), 1 μM UR-12460 (cross-hatched columns), 1 mM N^Gnitro-L-arginine methylester/N^G-nitro-L-arginine (checkered columns).

studied, whereas this concentration of PAF enhanced nitrite production in control saline-treated rats (Figure 7).

Some characteristics of the transduction pathway used for PAF to elicit NO production were studied with the use of other agonists, and are shown in Figure 8. Whereas phorbol dibutyrate showed a slight inhibition of the production of nitrites elicited by PAF, lipopolysaccharide (LPS) and epidermal growth factor (EGF) showed either additive effect or its own effect when PAF was used at doses that do not induce NO production.

Discussion

The data presented herein indicate a prominent role for PAF in the RPA model of rat immune complex peritonitis, which confirms earlier findings in the rabbit skin model (Hellewell & Williams, 1986; Rossi *et al.*, 1992), and in rat alveolitis (Warren *et al.*, 1989a; Tavares de Lima *et al.*, 1992). The release of PAF is an early event, and can probably be explained by the stimulation of PMN by immune complexes and/or complement-derived components in the microvasculature (Sánchez Crespo *et al.*, 1980). Endothelial cells are one of the targets for PAF, as judged from the early appearance of protein-rich plasma leakage. In fact, it has recently been reported that exposure of confluent endothelial cells to PAF results in a rapid and concentration-dependent redistribution of protein kinase C, rearrangement of cytoskeleton, and increase in the transendothelial flux of [^{125}I]-albumin (Bussolino *et al.*, 1994). On this basis, PAF-induced extravasation seems to be a clearcut component of the inflammatory response, and not a mere consequence of physical interactions between neutrophils and endothelial cells in which selectin-dependent adherent interactions seem to be involved (Lorant *et al.*, 1993). In contrast, our data are not consistent with a role for PAF on the cascade of events leading to the migration of PMN into the peritoneal cavity, and they would indirectly support current views on the

mechanisms of recruitment of PMN in this model, which involves C5a, leukotrienes and tumour necrosis factor-α (TNF-α) (Warren *et al.*, 1989b; Zhang *et al.*, 1992).

Peritoneal macrophages are another target for PAF in RPA as judged from the effect of both PAF and PAF-receptor antagonists on nitrite production under a wide array of experimental conditions including *ex vivo* experiments with adherent peritoneal cells from RPA rats and *in vitro* studies with PAF and other agonists. PAF showed a great ability to down regulate the production of NO in adherent peritoneal cells from both RPA and PAF-treated rats, whereas by itself it elicited NO production. This may be the consequence of antagonistic signalling triggered by previous exposure to an agonist, as observed in hepatocytes and peritoneal macrophages treated with LPS and phorbol ester (Hortelano *et al.*, 1992), which is explained by an effect on protein kinase C. In support of this view there may be mentioned the ability of PAF to stimulate calcium influx and phosphoinositide hydrolysis in mononuclear phagocytes at very low concentrations (Randriamampita & Tsien, 1993), the inhibitory effect observed when PAF was used in combination with phorbol ester, an agonist acting via protein kinase C; and the additive effect obtained when agonists acting on other signalling pathways were used, i.e. LPS and EGF.

The contribution of infiltrating PMN to the increased production of NO by peritoneal cells in *ex vivo* experiments seems unlikely, since the cell population was collected before infiltration of PMN had occurred, and also because the assay of nitrite production was carried out with adherent cells. In addition, the production of NO by PMN has been a matter of debate (Stuehr & Nathan, 1989; Schattner *et al.*, 1990; Stewart *et al.*, 1993), and it has been suggested that the NOS in PMN may represent a third type of enzyme intermediate between that of the inducible form and the calcium/calmodulin dependent constitutive form (Yui at al., 1990; Hiki *et al.*, 1991). Interestingly, a recent study has shown that the production of reactive nitrogen intermediates by human PMN in response to phorbol esters is greatly enhanced by azide and catalase, which strongly suggests that the alleged nitric oxide synthase activity detected under these conditions rather represents the catalase-catalyzed conversion of azide to nitrite (Klebanoff & Nathan, 1993).

Since iNOS induction is a delayed event involving nuclear signalling, this should be analyzed in the light of recent reports linking PAF receptor signalling and gene expression. Thus, PAF stimulates transcription of genes possessing the Fos-Jun/AP-1 responsive sequence (Squinto *et al.*, 1989; Mazer *et al.*, 1991), and the accumulation of collagenase type I mRNA in corneal epithelial cells (Bazan *et al.*, 1993). It has recently been shown that the level of iNOS mRNA is controlled by at least two different signalling cascades which may act synergistically, one involves cyclic AMP and the other one is triggered by cytokines such as IL-1β (Mühl *et al.*, 1994; Kunz *et al.*, 1994). This raises the question of the possible relationship of PAF-triggered iNOS induction to any of these pathways.

The biological role for NO synthase induction in RPA might be linked to either the induction of tissue damage or to the reparative phase of inflammation. Initial studies in rat immune complex alveolitis linked NO production to generation of peroxynitrite and to extravasation of protein-rich plasma, even though a diminished recruitment of PMN was not observed (Mulligan *et al.*, 1991; 1992). Our data differ from these results since we have not observed any effect on the extent of plasma extravasation by either inhibition of NO production with pharmacological tools or enhancement of NO production by NO-inducers. These discrepancies could be due to differences in either experimental conditions or properties of the tissues studied, which differ in both cellular components and vascularity. In keeping with the difficulty of assigning a damaging role to NO production in tissue injury there are a number of reports emphasizing its role as an oxygen radical scavenger (Wink *et al.*, 1993; Choi, 1993), and its protective role in hypoxia-induced intestinal injury in rats (Caplan *et al.*, 1994). Alternative roles for NO in immune-mediated inflammation could be regulation of blood flow in the reparative phase of inflammation (Moncada *et al.*, 1991; Pons *et al.*, 1993), or reduction of leukocyte adherence and emigration after the initial injury (Arndt *et al.*, 1993). Another function for NO not apparently related to inflammatory damage could be the recently reported synergistic effect with calcium in gene transcription linked to an increase in binding activity of the AP-1 transcription factor comprising the Fos and Jun family proteins (Peunova & Enikolov, 1993).

Finally, the possibility that PAF could also play a significant role in the production of NO in other models of tissue injury is strongly suggested by the finding of significant attenuation of the induction of NO synthase produced by bacterial LPS in anaesthetized rats treated with the PAF receptor antagonist WEB 2086 (Szabo *et al.*, 1993).

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References

ARNDT, H., RUSSEL, J.B., KUROSE, I., KUBES, P. & GRANGER, D.M. (1993). Mediators of leukocyte adhesion in rat mesenteric venules elicited by inhibition of nitric oxide synthesis. *Gastroenterology*, **105**, 675–680.

ARTHUS, M. (1903). Injections repetées de serum de cheval chez le lapin. *C.R. Soc. Biol. (Paris)*, **55**, 817–820.

BAZAN, H.E.P, TAO, Y. & BAZAN, N.G. (1993). Platelet-activating factor induces collagenase expression in corneal epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8678–8682.

BOUGHTON-SMITH, N.K., DEAKIN, A.M., FOLLEFANT, R.L., WHITTLE, B.J.R. & GARLAND, L.G. (1993). Role of oxygen radicals and arachidonic acid metabolites in the reverse passive Arthus reaction and carrageenin paw oedema in the rat. *Br. J. Pharmacol.*, **110**, 896–902.

BUSSOLINO, F., SILVAGNO, F., GARBARINO, G., COSTAMAGNA, C., SANAVIO, F., ARESE, M., SOLDI, R., AGLIETTA, M., PESCARMONA, G., CAMUSSI, G., & BOSIA, A. (1994). Human endothelial cells are targets for platelet-activating factor (PAF). Activation of α and β protein kinase C isozymes in endothelial cells stimulated by PAF. *J. Biol. Chem.*, **269**, 2877–2886.

CAPLAN, M.S., HEDLUND, E., HILL, N., & MACENDRICK. (1994). The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology*, **106**, 346–352.

CARCELLER, E., MERLOS, M. GIRAL, M., ALMANSA, C., BARTROLI, J., GARCIA-RAFANELL, J. & FORN, J. (1993). Synthesis and structure-activity relationships of 1-acyl-4-[(methyl-3-pyridyl) cyanomethyl]piperazines as PAF antagonists. *J. Med. Chem.*, **36**, 2984–2997.

CHOI, D.W. (1993). Nitric oxide: foe or friend to the injured brain. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9741–9743.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem.*, **162**, 156–159.

COLLINS, P.D., JOSE, P.J. & WILLIAMS, T.J. (1991). The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationships between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein. *J. Immunol.*, **146**, 677–684.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNEBAUM, S.R. (1982). Analysis of nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

HELLEWELL, P.G. & WILLIAMS, T.J. (1986). A specific antagonist of platelet-activating factor suppresses oedema formation in an Arthus reaction but not oedema induced by leukocyte chemoattractants in rabbit skin. *J. Immunol.*, **137**, 302–307.

HENSON, P.M., ZANOLARI, B., SCHWARTZMAN, N.A. & HONG, S.R. (1978). Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J. Immunol.*, **121**, 851–855.

HIKI, K., YUI, Y., HATTORI, R., EIZAWA, H., KOSUGA, K. & KAWAI, C. (1991). Three regulation mechanisms of nitric oxide synthase. *Eur. J. Pharmacol.*, **206**, 163–164.

HORTELANO, S., GENARO, A.M. & BOSCA, L. (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. Antagonism with the induction elicited by lipopolysaccharide. *J. Biol. Chem.*, **267**, 24937–24940.

IERINO, F.L., POWELL, M.S., McKENZIE, I.F.G. & HOGARTH, P.M. (1993). Recombinant soluble human Fc gammaRII. Production, characterization and inhibition of the Arthus reaction. *J. Exp. Med.*, **178**, 1617–1628.

KLEBANOFF, S.J. & NATHAN, C.F. (1993). Nitrite production by stimulated human polymorphonuclear leukocytes supplemented with azide and catalase. *Biochem. Biophys. Res. Commun.*, **197**, 192–196.

KUNZ, D., MÜHL, H., WALKER, G., & PFEILSCHIFTER, J. (1994). Two distinct signalling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 5387–5391.

LORANT, D.E., TOPHAM, M.K., WHATLEY, R.E., McEVER, R.P., McINTYRE, T.M., PRESCOTT, S.M. & ZIMMERMAN, G.A. (1993). Inflammatory roles of P-selectin. *J. Clin. Invest.*, **92**, 559–570.

MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. (1989). *Molecular Cloning: A Laboratory Manual.* pp 7.1–7.79. New York: Cold Spring Harbor Laboratory Press.

MAZER, B., DOMENICO, J., SAWAMI, H. & GELFAND, E.W. (1991). Platelet-activating factor induces an increase in intracellular calcium and expression of regulatory genes in human lymphoblastoid cells. *J. Immunol.*, **146**, 1914–1920.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–141.

MÜHL, H., KUNZ, D., & PFEILSCHIFTER, J. (1994). Expression of nitric oxide synthase in rat glomerular mesangial cells mediated by cyclic AMP. *Br. J. Pharmacol.*, **112**, 1–8.

MULLIGAN, M.S., HEVEL, J.M. MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6338–6342.

MULLIGAN, M.S., MONCADA, S. & WARD, P.A. (1992). Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis. *Br. J. Pharmacol.*, **197**, 1159–1162.

MULLIGAN, M.S., WATSON, S.R., FENNIE, C. & WARD, P.A. (1993). Protective effects of selectin chimeras in neutrophil-mediated lung injury. *J. Immunol.*, **151**, 6410–6417.

PELLON, M.I., STEIL, A.A., FURIO, V. & SANCHEZ CRESPO, M. (1994). Study of the effector mechanism involved in the production of haemorrhagic necrosis of the small intestine in rat passive anaphylaxis. *Br. J. Pharmacol.*, **112**, 1101–1108.

PEUNOVA, N. & ENIKOLOV, G. (1993). Amplification of calcium-induced gene transciption by nitric oxide in neuronal cells. *Nature*, **364**, 450–453.

PONS, F., WILLIAMS, T.J. & WARREN, J.B. (1993). Nitric oxide, but not interleukin-1, mediates the local blood flow response to lipopolysaccharide in rabbit skin. *Eur. J. Pharmacol.*, **239**, 23–30.

RANDRIAMAMPITA, C. & TSIEN, R.Y. (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature.*, **364**, 809–813.

ROSSI, A.G., NORMAN, K.E., DONIGI-GALE, D., SHOUPE, T.S., EDWARDS, R., & WILLIAMS, T.J. (1992). The role of complement, platelet-activating factor and leukotriene B_4 in the reversed passive Arthus reaction. *Br. J. Pharmacol.*, **107**, 44–49.

SÁNCHEZ-CRESPO, M., ALONSO, F, & EGIDO, J. (1980). Platelet-activating factor in anaphylaxis and phagocytosis. Release from polymorphonuclear leukocytes and monocytes during phagocytosis and by ionophore A23187. *Immunology.*, **40**, 645–655.

SCHATTNER, M.A., GEFFNER, J.R., ISTURIZ, M.A. & LAZZARI, M.A. (1990). Inhibition of human platelet activation by polymorphonuclear leukocytes. *Br. J. Pharmacol.*, **101**, 253–256.

SCHMIDT, H.H., WARNER, T.D., NAKANE, M., FORSTERMAN, U. & MURAD, F. (1992). Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Mol. Phamacol.*, **41**, 615–624.

SQUINTO, S.P., BLOCK, A.L., BRAQUET, P. & BAZAN, N.G. (1989). Platelet-activating factor stimulates a Fos/Jun/AP-1 transcriptional signalling system in human neuroblastoma cells. *J. Neurosci. Res.*, **24**, 558–566.

STEWART, A.G., DUSTING, G.J., GIARRACCA, R.G., HARRIS, T., LIM, Y.M. & SOBEY, C.G. (1993). Nitrite is produced by elicited but not by circulating neutrophils. *Med. Inflam.*, **2**, 349–356.

STUEHR, D.J. & NATHAN, C.F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumour target cells. *J. Exp. Med.*, **169**, 1543–1555.

SZABO, C., WU, C.C., MITCHELL, J.A., GROSS, S.S., THIEMERMANN, C. & VANE, J.R. (1993). Platelet-activating factor contributes to the induction of nitric oxide synthase by bacterial lipopolysaccharide. *Circ. Res.*, **73**, 991–999.

TAVARES DE LIMA, W., SIROIS, P. & JANCAR, S. (1992). Immune-complex alveolitis in the rat: evidence for platelet-activating factor and leukotrienes as mediators of the vascular lesions. *Eur. J. Pharmacol.*, **213**, 63–70.

WARREN, J.S. (1992). Relationship between interleukin-1β and platelet-activating factor in the pathogenesis of acute immune complex alveolitis in the rat. *Am. J. Pathol.*, **141**, 551–560.

WARREN, J.S., MANDEL, D.M., JOHNSON, K.J. & WARD, P.A. (1989a). Evidence for the role of platelet-activating factor in immune complex vasculitis. *J. Clin. Invest.*, **83**, 669–679.

WARREN, J.S., YABROFF, K.R., REMICK, D.G., KUNKEL, S.L., CHENSUE, S.W., KUNKEL, R.G., JOHNSON, K.L. & WARD, P.A. (1989b). Tumour necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. *J. Clin. Invest.*, **84**, 1873–1882.

WEDMORE, C.V. & WILLIAMS, T.J. 1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289**, 646–650.

WHITTAKER, M., BEAUCHAMP, C.L., BOWLES, S.A., CACKETT, K.S., CHRISTODOULOU, M.S., GALLOWAY, W.A., LONGSTAFF, D.S., McGUINNESS, G.P., MILLER, A., TIMMIS, D.J. & WOOD, L.M. (1992). BB–823, A PAF receptor antagonist with picomolar activity. *Pharmacol. Commun.*, **1**, 251–257.

WINK, D.A., HANBAUER, I., KRISHNA, M.C., DEGRAFF, W., GAMSON, J. & MOTCHELL, J.B. (1993). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9813–9817.

XIE, Q-W., CHO, H.J., CALAYCAY, R.A., MUMFORD, R.A., SWIDEREK, K.M., DEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science (Wash. D.C.)*, **256**, 225–228.

YUI, Y., HATTARI, R., KOSUGA, K., EIZAWA, H., HIKI, K., OHKAWA, S. & OHNISHI, K. (1990). Calmodulin independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J. Biol. Chem.*, **266**, 3369–3371.

ZHANG, Y., RAMOS, B.J. & JAKSCHIK, B.A. (1992). Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science, (Wash. D.C.)*, **258**, 1957–1959.

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Role of endothelium in the human uterine arteries during normal menstrual cycle

[1]Hiroshi Azuma, *Satoshi Obayashi, Hidehisa Hamasaki, *Takao Koyama & *Takeshi Aso

Department of Medicinal Chemistry, Institute for Medical and Dental Engineering and *Department of Gynecology and Obstetrics, Tokyo Medical and Dental University, 2-3-10 Surugadai, Kanda, Chiyoda-ku, Tokyo 101, Japan

1 The present experiments were designed to investigate the role of endothelium in the human uterine arteries during the normal menstrual cycle.

2 Acetylcholine (ACh) produced a concentration-dependent relaxation response during the higher level of plasma 17β-oestradiol (E_2) (follicular and luteal phases, $E_2 = 131.9 \pm 15.9$ pg ml^{-1}, $n = 13$; group I). However, the agent did not produce a definite relaxation, but produced a slight contraction during the ovulatory and menstruation phases ($E_2 = 19.8 \pm 2.9$ pg mg^{-1}, $n = 5$; group II). During the follicular and luteal phases ($E_2 = 181.1 \pm 9.0$ pg ml^{-1}, $n = 6$), ACh produced a slight contraction, but not relaxation in 6 cases (group III). Relaxation in response to A23187 in group II was not different from that in group I, while it was significantly ($P<0.05$ and $P<0.005$) reduced in group III. Sodium nitroprusside (SNP)-induced relaxation was similar in the three groups.

3 Correlation between the maximum response to ACh and the plasma E_2 was highly significant ($\gamma = 0.8142$, $P<0.001$) in 18 cases of groups I and II, but not in all 24 cases including group III ($\gamma = 0.1183$, NS).

4 Relaxations in response to ACh in group I or A23187 in all groups were abolished after removal of the endothelium. In group I, ACh- and A23187-induced relaxations were greatly inhibited by methylene blue or N^G-nitro-L-arginine (L-NOARG) and partially inhibited by indomethacin. None of these treatments except for methylene blue modified the SNP-induced relaxation, which was significantly inhibited by methylene blue.

5 The A23187-induced relaxation was hardly affected by methylene blue or L-NOARG in group III, but was partially inhibited by these agents in group II. The effect of indomethacin in inhibiting the A23187 induced-relaxation was most potent (58.9%) in group III and least (16.9%) in group I.

6 There were no histological changes in 14 cases out of 18 (groups I and II), but very slight intimal thickening was observed in 4 cases in group I. On the other hand, severe intimal thickening was observed in all 6 cases in group III.

7 These results indicate that, in human uterine artery strips, ACh and A23187 cause endothelium-dependent relaxations, which are mediated mainly through EDRF/NO in group I, mainly prostacyclin (PGI_2) in group III, or both in group II. It is suggested that lack of the production/release of EDRF/NO and/or of interaction between EDRF/NO and PGI_2 might play a role in the formation of intimal thickening in human uterine arteries.

Keywords: Human uterine artery; menstrual cycle; endothelium-derived relaxing factor (EDRF/NO); prostaglandin I_2; plasma 17β-oestradiol; intimal thickening

Introduction

Vascular tone is regulated by a variety of neural and humoral factors. Furchgott & Zawadzki (1980) showed that acetylcholine (ACh) produced an endothelium-dependent vasodilatation and they called the relaxation producing substance endothelium-derived relaxing factor (EDRF). More recently, it has been proposed that EDRF is nitric oxide (NO) (Palmer *et al.*, 1986; 1987). NO causes vasodilatation by activating the soluble guanylate cyclase and in turn, by elevating guanosine 3':5'-cyclic monophosphate (cyclic GMP). The release of NO from endothelial cells is stimulated by a variety of agents such as ACh and calcium ionophore A23187. In addition to the vasodilator action, EDRF/NO prevents platelet adhesion and aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987) and is thought to be one of the important factors in inhibiting the development of atherosclerosis. There are many reports indicating that endothelium-dependent relaxation is produced in the various kinds of blood vessels but little is known about the role of endothelium in human uterine arteries.

Change in uterine blood supply occurs during menstruation, pregnancy, and the menopause. These changes are thought to be affected by various hormones, especially by ovarian steroids. Weiner *et al.* (1991) reported that the increase in the plasma level of 17β-oestradiol (E_2) during pregnancy decreased the reactivity of blood vessels to noradrenaline through the increased EDRF release. Several reports show that E_2 treatment enhances ACh-stimulated endothelium-dependent relaxation (Bell & Coffey, 1982; Weiner *et al.*, 1989).

The present experiments were designed to investigate the role of endothelium in human uterine arteries during the normal menstrual cycle.

Methods

Human uterine arteries

Human uterine arteries were obtained at operation from 24 patients (range of age; 32 to 52, mean age; 43.5 ± 1.1 years

[1] Author for correspondence.

old) ungergoing surgery on routine benign gynaecological indications with informed consent for this study. All the women had regular menstrual cycles and were otherwise healthy, normotensive and without drug treatment. The abdominal hysterectomies were carried out within 45 min after clamping of the proximal portion of the ascending uterine arteries and during the various phases of the menstrual cycle under thiopentone-isoflurane anaesthesia. The ascending branches (2.5~3.0 mm in outer diameter) of the uterine arteries embedded in the parametrium were dissected free from the surrounding connective tissue in oxygenated and ice-cold modified Krebs solution. Special care was taken to avoid stretching or other type of injury during the operation and dissection. The arteries were cut into transverse strips, 2.5 mm in width and 7 mm in length. In some strips, the endothelium was removed mechanically by gently rubbing the luminal surface with a cotton swab. The complete removal of endothelial cells was confirmed by scanning electron microscopy.

Measurement of mechanical responses

The mechanical responses were measured according to the methods described previously (Azuma *et al.*, 1992; Tsujii *et al.*, 1992). In brief, the transverse strip was mounted vertically in an organ bath containing 5 ml of modified Krebs solution, continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. Special care was taken to avoid unintentional rubbing of the intraluminal surface. One end of each strip was secured to the bottom of the organ bath, and the other was attached to a force-displacement transducer (TB-611T, Nihon Kohden Kogyo Co.). Isometric changes in tension were recorded on a pen-writing oscillograph (R-64, Rikadenki Kogyo Co.). The length of the strips was adjusted several times until a stable tension of 1g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period the bathing solution was replaced every 20 min with fresh solution. The composition (mM) of modified Krebs solution was as follows: NaCl 115.0, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.

After 60 min of equilibration, 10^{-6}M ACh was added to all strips during the contraction caused by 10^{-6}M noradenaline (NA) to check the presence of functional endothelium. After this, in one series of experiments, cumulative concentration-relaxation curves to ACh (10^{-9}–3×10^{-6}M), SNP (10^{-9}–3×10^{-6}M) and A23187 (10^{-9}–3×10^{-6}M) were constructed in that order during the contraction caused by 10^{-6}M NA. In another series of experiments, the relaxations induced by the submaximum concentrations of ACh (10^{-6}M), SNP (10^{-6}M) and A23187 (10^{-6}M) were determined in the presence or absence of 10^{-5}M indomethacin (cyclo-oxygenase inhibitor), 10^{-5}M methylene blue (guanylate cyclase inhibitor) or 10^{-4}M L-NOARG (NO synthase inhibitor), or without endothelium.

A diffusion barrier mechanism of the thickened intima to explain the decreased endothelium-dependent responses in group III was ruled out in a 'sandwich' experiment (Niimi *et al.*, 1994). In brief, a transverse specimen of group III without endothelium was mounted with a longitudinal strip of group I possessing an endothelium so that their luminal surfaces faced each other, for isometric tension recording. Transverse strips of groups I and III without endothelium were each mounted with longitudinal strips of group I with endothelium. With ACh (10^{-6}M) added 20 min after inducing contraction with 10^{-6}M NA, each transverse strip was relaxed in response to the production/release of EDRF/NO and PGI_2 from the endothelium of the longitudinal strip of group I. The degree of relaxation was then compared between the two groups to determine whether the thickened intima functioned as a diffusion barrier to EDRF/NO and PGI_2.

Determination of plasma 17β-oestradiol (E_2), total cholesterol and triglyceride

In order to determine plasma E_2 levels, total cholesterol and triglyceride, blood sampling was performed in the early morning on the day before the operation. The plasma concentration of E_2 was measured by specific radioimmunoassay technique (Lu *et al.*, 1981). The plasma levels of total cholesterol and triglyceride were determined by the cholesterol oxidase (Allain, 1974) and glycerol kinase (Fletcher, 1968) methods, respectively. The normal values in our hospital are 128–249 mg dl^{-1} for total cholesterol and 55–159 mg dl^{-1} for triglyceride.

Light microscopy

All the human uterine arteries isolated were fixed in a 10% neutral solution of formaldehyde. Ring preparations were embedded in paraffin after dehydration with ethanol. Thin sections were stained with haematoxylin-eosin (HE) and the Elastica-Van Gieson method for light microscopic analysis (Azuma *et al.*, 1990; 1992).

In order to confirm the endothelial origin of the lining cells, thin sections were made from formalin-fixed, paraffin-embedded vessels and stained with von Willebrand factor antibody (rabbit antihuman von Willebrand factor, Dako) using the labelled streptavidin-biotin staining technique according to the method described by Niimi *et al.* (1994).

Scanning electron microscopy

Scanning electron microscopic examination was performed according to the method described previously (Azuma *et al.*, 1990). Briefly, uterine arteries were isolated and fixed with phosphate-buffered saline (NaCl 136.8 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM and KH_2PO_4 1.5 mM, pH 7.4) containing 2% glutaraldehyde. After fixation the arteries were placed in phosphate-buffered saline (pH 7.4) containing 1% osmium tetroxide for 2 h at 4°C, specimens were dehydrated in an ascending series of ethanol solution, processed through isoamyl acetate, critical-point-dried with carbon dioxide and coated with gold. The ultrastructure of the intraluminal surface was observed by means of a scanning electron microscope (ESM-3200, Elionix).

Chemicals

The following chemicals were used in the present experiments: acetylcholine chloride (ACh, Daiichi Pharmaceutical Co.), (−)-noradrenaline bitartrate (NA), calcium ionophore A23187, sodium nitroprusside (SNP), indomethacin, methylene blue (all from Sigma), N^G-nitro-L-arginine (L-NOARG, Protein Research Foundation). All chemicals were dissolved in distilled water and kept frozen at −80°C until use (10^{-2} M stock solution) except for A23187, L-NOARG and indomethacin which were dissolved in dimethylsulphoxide (DMSO, Art. 2950, Merck). DMSO was present in a final concentration of 0.1% in the experiments with these agents, and this concentration had no effect on any parameter tested.

Calculation and statistical analysis

Relaxation responses to ACh, A23187 and SNP were given as a percentage of the 10^{-6}M NA-induced contraction. Values are expressed as mean ± s.e.mean. The statistical analyses were carried out by a two-way random block repeated-measures analysis of variance (ANOVA). If the mean values were found to be different at $P<0.05$ by means of ANOVA, either Scheffe or Student's *t*-test was applied. Correlation of two variates was expressed and tested by the Spearman rank correlation coefficient. The difference was regarded as significant at $P<0.05$.

Results

Relaxation responses of uterine arterial strips to ACh, A23187 and sodium nitroprusside (SNP)

ACh was selected as it produces endothelium-dependent relaxation by a receptor-mediated mechanism (Furchgott & Zawadzki, 1980); the calcium ionophore A23187 was used as it is an endothelium-dependent relaxant that is receptor-independent in action (Furchgott, 1981); SNP directly relaxes the vascular smooth muscle by releasing NO as an active substance from its own structure (Kowulak *et al.*, 1992).

ACh did not produce relaxation in all the uterine artery strips. Some of the strips were relaxed in response to ACh as shown in Figure 1. Figure 2 shows the relationships between the maximum responses to ACh and the day of the cycle. In 13 cases out of 24, ACh produced a relaxant response during the follicular and luteal phases (classified as group I and shown by the open circles). However, ACh did not produce a definite relaxation, but produced a slight contraction during the ovulatory and menstruation phases (classified as group II and shown by closed circles, $n = 5$). ACh produced a slight contraction, but not relaxation in 6 cases during the follicular and luteal phases (classified as group III and shown by the crosses). On the contrary, the relaxation response to A23187 in group II was not different from that in group I, while it was significantly ($P < 0.05$ and $P < 0.005$ vs. group I) decreased in group III. The relaxation induced by SNP was similar in the three groups (Figures 3 and 6). As shown in Figure 4, the correlation between the maximum response to ACh and plasma concentration of 17β-oestradiol (E_2) was highly significant ($\gamma = 0.8142$, $P < 0.001$) in 18 cases of groups I and II, but not in all 24 cases including group III ($\gamma = 0.1183$, NS). The mean values of plasma E_2 in groups I, II and III were 131.9 ± 15.9 ($n = 13$), 19.8 ± 2.9 ($n = 5$) and 181.1 ± 9.0 ($n = 6$) pg ml^{-1}, respectively. The plasma E_2 level in groups I and II was significantly ($P < 0.005$) higher than that in group II.

Relaxations induced by ACh (10^{-6}M) and A23187 (10^{-6}M) in group I were abolished after endothelial removal (data not shown) and greatly inhibited but not abolished by 10^{-4}M L-NOARG or 10^{-5}M methylene blue and partially inhibited by 10^{-5}M indomethacin. None of these treatments except for methylene blue modified the SNP (10^{-6}M)-induced relaxation, which was significantly attenuated by 10^{-5}M methylene blue (Figure 5). The relaxation in response to A23187 in groups II and III was again abolished after the endothelial removal. On the other hand, the A23187-induced relaxation

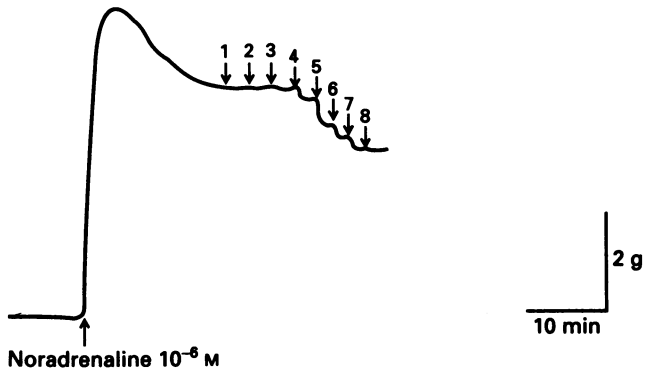


Figure 1 Representative trace of cumulative concentration-relaxation response to acetylcholine in human uterine artery strip precontracted with noradrenaline (10^{-6}M). Numbers (1–8) indicate the concentration of acetylcholine, that is 1,2,3,4,5,6,7 and 8 indicate 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M acetylcholine, respectively.

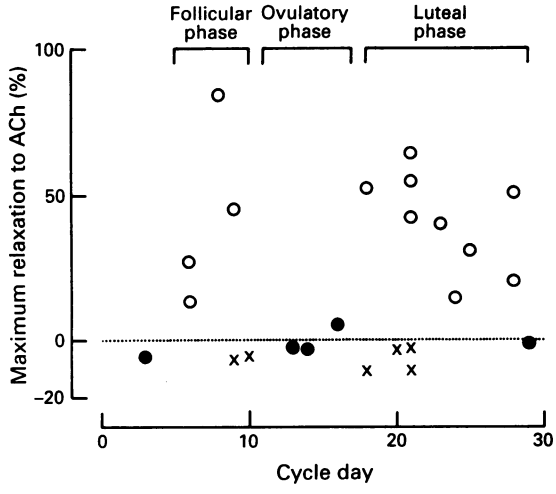


Figure 2 Relationship between the maximum response to acetylcholine (ACh) and the day of the cycle: (○) group I; (●) group II and (×) group III (see text). The days of the menstrual cycle were divided as follows: follicular phase: days 5–10 of cycle; ovulatory phase: days 11–17 of cycle and luteal phase: days of 18–29 of cycle.

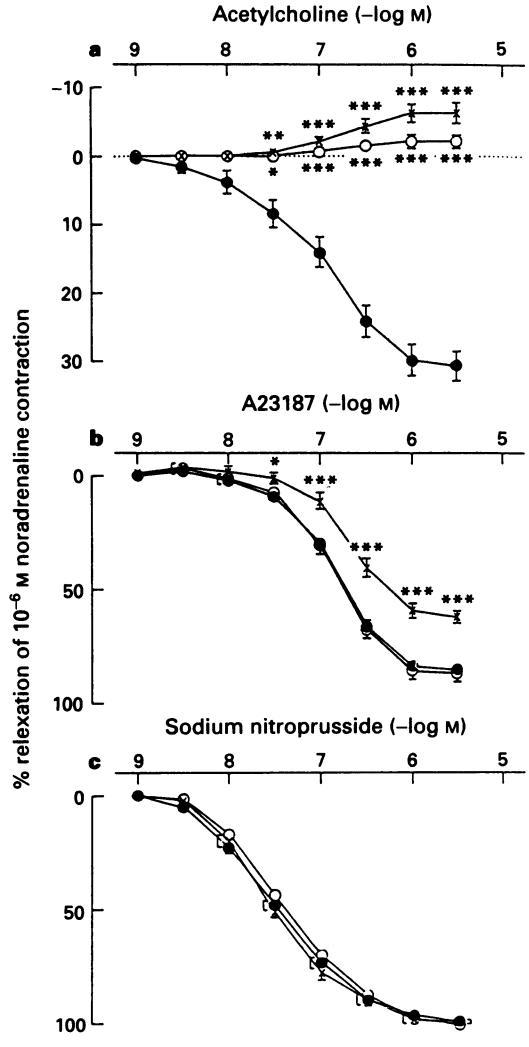


Figure 3 The concentration-response curves for acetylcholine (a), A23187 (b) and sodium nitroprusside (c) in human isolated uterine artery strips: (○) group I ($n = 13$); (●) group II ($n = 5$) and (×) group III ($n = 6$). Values are mean ± s.e.mean. Significant difference vs. corresponding values in group I at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$, respectively. The magnitude of precontraction with NA (10^{-6}M) was determined to be 3.9 ± 0.4 g ($n = 13$) in group I, 4.1 ± 0.3 g ($n = 5$) in group II and 4.8 ± 0.6 g ($n = 6$) in group III. These values were not statistically significantly different from each other.

was hardly affected by 10^{-5}M methylene blue or 10^{-4}M L-NOARG in group III, but was partially inhibited by these agents in group II (data not shown). As shown in Figure 6, A23187-induced relaxations were attenuated to a different degree in different groups, but not abolished by the 20 min-pretreatment with 10^{-5}M indomethacin. The effect of indomethacin in inhibiting the relaxation induced by A23187 was most potent (58.9%) in group III and least (16.9%) in group I. However, indomethacin and the endothelial removal failed to modify the SNP-induced relaxation also in groups II and III.

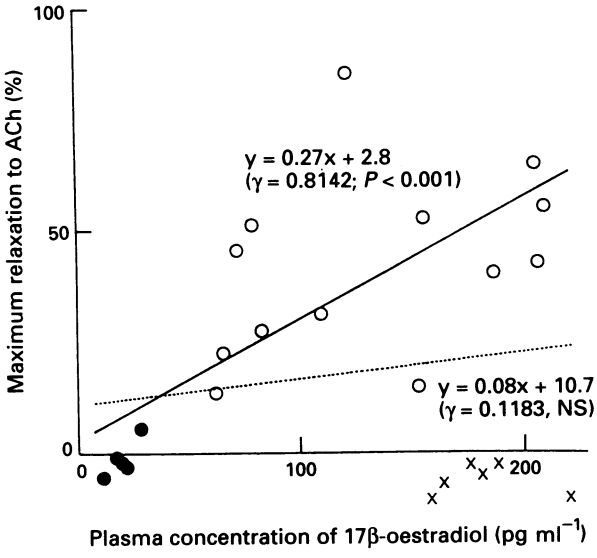


Figure 4 Correlation between the maximum response to acetylcholine (ACh) and the plasma concentration of 17β-oestradiol (E_2). Correlation was examined by Spearman rank correlation coefficient ($\gamma = 0.8142$), which was significant at $P<0.001$ among 18 cases of groups I (○) and II (●). The correlation was not significant ($\gamma = 0.1183$) in all 24 cases including group III (×). Regression equation was obtained by the least-square method. The mean values of plasma E_2 in groups I, II and III were: 131.9 ± 15.9 ($n = 13$), 19.8 ± 2.9 ($n = 5$) and 181.1 ± 9.0 pg ml^{-1} ($n = 6$), respectively.

In the sandwich experiment, both the transverse strips of groups I and III, in which endothelium had been removed, exhibited the same degree of relaxation in response to EDRF/NO and PGI_2 released from the endothelium of the longitudinal strips of group I by ACh (data not shown).

There were no age differences among three groups (44.0 ± 1.5 for group I, 43.8 ± 3.1 for group II and 42.3 ± 1.5 for group III).

Morphological and histological findings

All arterial specimens were examined morphologically and histologically. In 18 cases (group I and II) out of 24, endothelial cells were arranged parallel to the blood stream

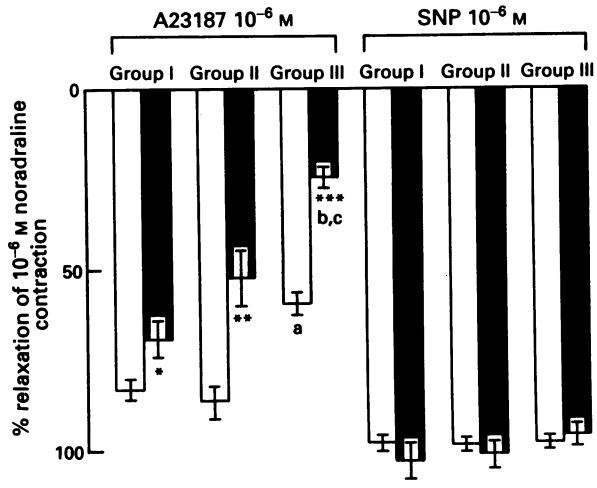


Figure 6 Comparison of the relaxation responses to A23187 and sodium nitroprusside (SNP) in groups I, II and III, and effect of indomethacin on the relaxations. Results are given as means ± s.e.mean of 5 to 13 experiments. $^{a}P<0.05$ vs. corresponding value in groups I and II, $^{b}P<0.005$ vs. corresponding value in group I, $^{c}P<0.01$ vs. corresponding value in group II. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$, respectively vs. each control. Open columns: control; solid columns: in the presence of 10^{-5}M indomethacin.

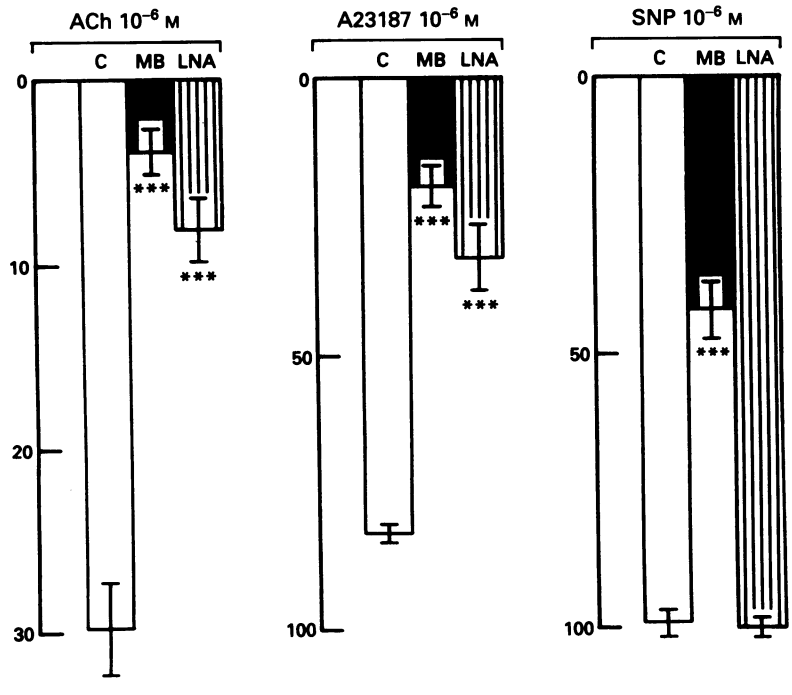


Figure 5 Effects of methylene blue (MB) and N^{G}-nitro-L-arginine (LNA) on the acetylcholine (ACh)-, A23187- and sodium nitroprusside (SNP)-induced relaxations in group I. Results are given as means ± s.e.mean of 5 to 12 experiments. Significant difference at $^{***}P<0.005$ vs. corresponding controls (C).

and their morphology appeared to be normal. There were no histological changes in 14 cases out of 18 (groups I and II), but very slight intimal thickening was observed in 4 cases of group I. On the other hand, severe intimal thickening was observed in all 6 cases of group III. It should be noted that uterine artery strips in these 6 cases were not relaxed by ACh in spite of the higher level of plasma E_2 and there was significantly less response to A23187. The representative histological findings of the cross sections are shown in Figures 7a and b. Endothelial cells in group III were morphologically different from normal ones; they were irregularly arranged in this group, but to different degrees. Cells lining the innermost surface were of endothelial origin, since they were positively stained for von Willebrand factor.

Plasma concentration of total cholesterol and triglyceride

The plasma concentrations of total cholesterol and triglyceride could be determined in 11 cases of group I, 4 of group II and 6 of group III. The plasma total cholesterol levels of groups I, II and III were 162.4 ± 8.3 mg dl^{-1} (143–198 mg dl^{-1}), 159.5 ± 9.2 mg dl^{-1} (136–178 mg dl^{-1}) and 167.5 ± 11.8 mg dl^{-1} (133–203 mg dl^{-1}), respectively. These values were not statistically different from each other and within the normal range. In addition, the triglyceride level was determined to be 94.5 ± 12.5 mg dl^{-1} (64–184 mg dl^{-1}) for group I, 56.0 ± 7.6 mg dl^{-1} (43–77 mg dl^{-1}) for group II and 104.0 ± 29.1 mg dl^{-1} (36–208 mg dl^{-1}) for group III. Again, these values were not different from each other significantly and were within the normal range, although the value in group II appeared to be lower than that in the other 2 groups. The normal values in our hospital are 128–249 mg dl^{-1} for total cholesterol and 55–159 mg dl^{-1} for triglyceride.

Discussion

The present experiments were carried out to investigate the role of endothelium in the human uterine artery during the normal menstrual cycle.

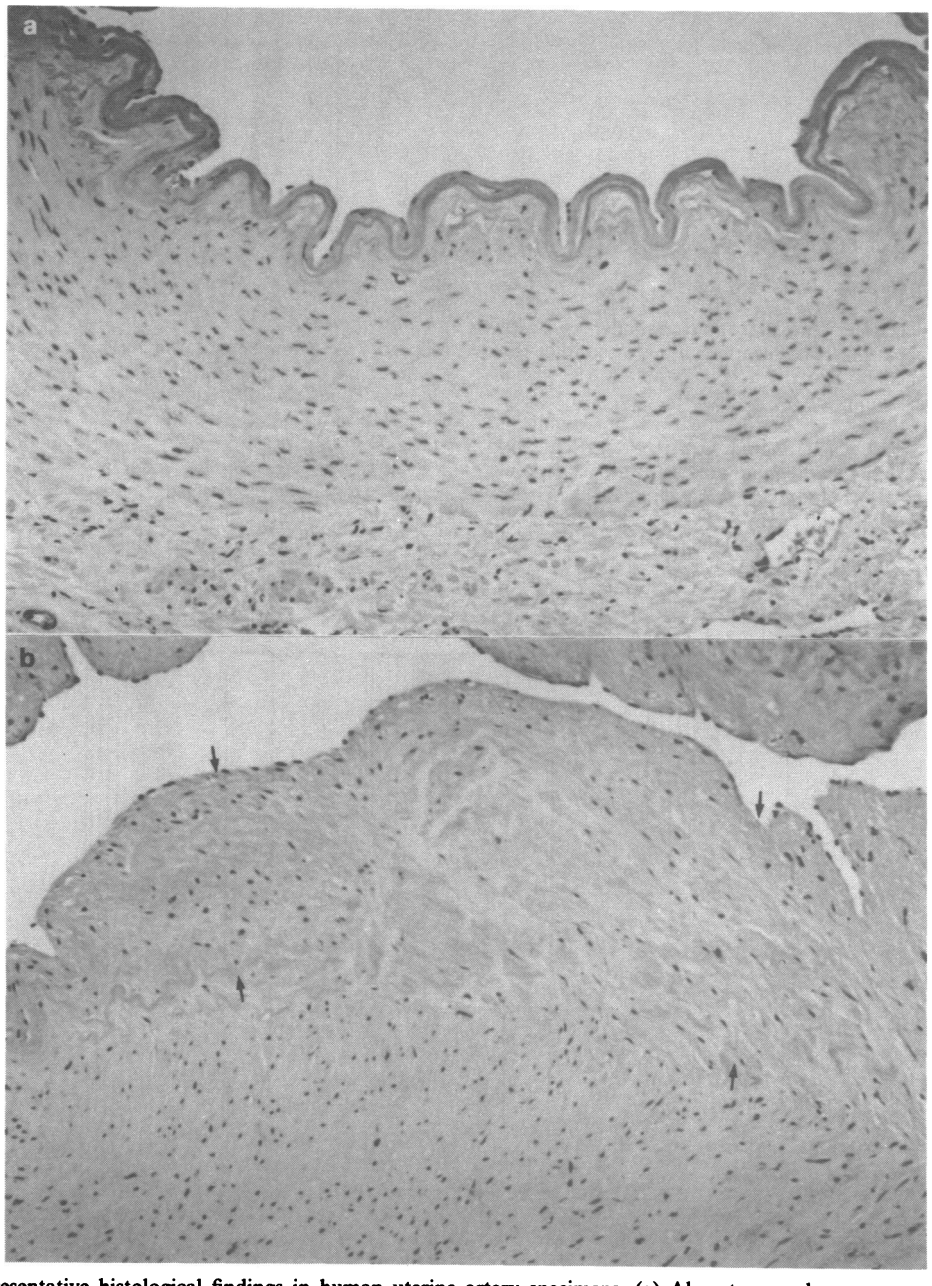


Figure 7 Representative histological findings in human uterine artery specimens. (a) Almost normal appearance (H E staining, original magnification × 100) (O.K., 44 years old in group I). (b) Severe intimal thickening can be observed (H E staining, original magnification × 100) (M.N., 37 years old in group III). Arrows indicate thickened intima.

ACh produced an endothelium-dependent relaxation when plasma E_2 concentration was higher (follicular and luteal phases: plasma $E_2 = 131.9 \pm 15.9$ pg ml^{-1}, $n = 13$: group I). During the lower level of E_2 (ovulatory and menstruation phases: plasma $E_2 = 19.8 \pm 2.9$ pg ml^{-1}, $n = 5$), ACh produced a slight contraction of the uterine artery strips (group II). Correlation between the response to ACh and the plasma concentration of E_2 was highly significant ($r = 0.8142$, $P < 0.001$) in 18 cases on groups I and II. In group II, A23187 and SNP each produced the same magnitude of relaxation as that in group I. Therefore, the lack of response to ACh in group II occurs at a time when the ability of the underlying vascular smooth muscle to relax, the ability of the endothelial cells to release/produce EDRF/NO and the sensitivity of vascular smooth muscle to NO remain unchanged. It is well established that ACh produces endothelium-dependent relaxation by a receptor mediated mechanism (Furchgott & Zawadzki, 1980): A23187 is an endothelium-dependent relaxant that is receptor-independent in action (Furchgott, 1981): SNP directly relaxes the vascular smooth muscle by releasing NO as an active substance from its own structure (Kowulak *et al.*, 1992). On the basis of these findings, it seems likely that the functional muscarinic receptors on the endothelial cells are decreased or disappear during the lower level of plasma E_2 (group II). Rainbow *et al.* (1980) have reported that E_2 increases the number of muscarinic receptors in hypothalamic nuclei of rats. It has also been demonstrated that E_2-treatment enhances the endothelium-dependent relaxation response of vascular preparations to ACh (Bell & Coffey, 1982; Weiner *et al.*, 1989). According to Weiner *et al.* (1991), the increase in the plasma E_2 level during pregnancy leads to the increased release of EDRF/NO induced by ACh. These demonstrations seem to support our speculation described above. However, the precise mechanism changing the density of functional muscarinic receptors by change in the plasma E_2 levels remains to be clarified. We performed an *in vitro* experiment to test whether or not an overnight (for 14 h) incubation of group II strips with 300 pg ml^{-1} of E_2 at 37°C in Eagle's minimum essential medium (MEM) restores the relaxation response to ACh. However, the incubation with E_2 failed to recover the relaxation (Azuma: unpublished observation). This finding may indicate that the incubation time was too short to express functioning muscarinic receptors *in vitro*, E_2 may act as a regulatory factor for the expression of muscarinic receptors in combination with unspecified plasma factor(s), or the expression may be regulated by metabolite(s) of E_2, but not by E_2 itself.

The relaxations in response to ACh and A23187 in group I appeared to be mediated mainly through EDRF/NO and partially PGI_2, since the relaxations were greatly inhibited by methylene blue or L-NOARG and partially by indomethacin. The concentrations of indomethacin (10^{-5}M), methylene blue (10^{-5}M) and L-NOARG (10^{-4}M) were sufficient to inhibit cyclo-oxygenase (Furchgott & Zawadzki, 1980), guanylate cyclase (Ignarro *et al.*, 1986) and NO synthase (Kobayashi & Hattori, 1990), respectively.

In group III, ACh did not produce a relaxation in spite of the higher level of E_2. A23187-induced relaxation was also greatly decreased without reduction in the SNP-induced relaxation. In addition, the effect of indomethacin in inhibiting the A23187-induced relaxation was most potent, while methylene blue and nitroarginine showed no effect on the relaxation in this group. Using the sandwich experiment, we ruled out the possibility of the diffusion barrier mechanism by which thickened intima inhibits diffusion of EDRF/NO and PGI_2 into the medial smooth muscle. These results lead us to speculate that the ability of endothelial cells to produce/release EDRF/NO is greatly impaired and the relaxation induced by A23187 is mediated mainly through PGI_2 in group III.

The mechanism causing atherosclerosis is complex and poorly understood. However, it is well established that injury or dysfunction of endothelial cells, together with resultant platelet products, is one of the important mechanisms (Schwartz *et al.*, 1981; Ross, 1986). We have demonstrated that the myointimal proliferative response after removal of the endothelium is brought about at least partly by the decreased release/production of EDRF/NO by the regenerated endothelial cells (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994). According to Garg & Hassid (1989), the endogenous EDRF/NO may function as an inhibitory modulator of vascular smooth muscle cell proliferation. In addition, we have provided evidence indicating that EDRF/NO is an antiaggregating substance which may also modulate the release of platelet products such as platelet-derived growth factor (PDGF) (Azuma *et al.*, 1986). In the present experiments, severe intimal thickening was observed in all 6 cases in group III. As described above, the ability of endothelial cells to produce/release EDRF/NO was greatly impaired and the relaxation in response to A23187 was mediated mainly through PGI_2 in this group. Thus, it seems likely that the intimal thickening is formed partly due to the dysfunction of endothelial cells to produce/release EDRF/NO through unknown mechanisms, which remain to be investigated. In addition, synergism in inhibiting the platelet aggregation has been reported between EDRF/NO and PGI_2 (Radomski *et al.*, 1988). Therefore, diminution or lack of this interaction between EDRF/NO and PGI_2 in group III may also be important in the formation of the intimal thickening.

High cholesterol and triglyceride are thought to be risk factors of atherosclerosis. However, the plasma levels of these substances were not statistically different among the three groups and were within the normal range.

Therefore, the cause of the intimal thickening in group III could not be accounted for by changes in the plasma cholesterol and triglyceride levels.

Further studies are in progress in our laboratories to clarify the precise mechanism causing the intimal thickening during the normal reproductive age.

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References

ALLAIN, C.C (1974). Enzymatic determination of total serum cholesterol. *Clin. Chem.*, **20**, 470–475.

AZUMA, H., FUNAYAMA, N., KUBOTA, T. & ISHIKAWA, M. (1990). Regeneration of endothelial cells after balloon denudation of the rabbit carotid artery and changes in responsiveness. *Jpn. J. Pharmacol.*, **52**, 541–552.

AZUMA, H., ISHIKAWA, M. & SEKIZAKI, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.*, **88**, 411–415.

AZUMA, H., NIIMI, Y. & HAMASAKI, H. (1992). Prevention of intimal thickening after endothelial removal by a nonpeptide angiotensin II receptor antagonist, losartan. *Br. J. Pharmacol.*, **106**, 665–671.

BELL, C. & COFFEY, C. (1982). Factors influencing oestrogen-induced sensitization to acetylcholine of guinea-pig uterine artery. *J. Reprod. Fertil.*, **66**, 133–137.

FLETCHER, M.J. (1968). Colorimetric method for estimation of serum triglycerides. *Clin. Chem. Acta.*, **22**, 393–397.

FURCHGOTT, R.F. (1981). The requirement for endothelial cells in the relaxation of arteries by acetylcholine and some vasodilators. *Trends Pharmacol. Sci.*, **2**, 173–176.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.

IGNARRO, L.J., HARBINSON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Dissimilarities between methylene blue and cyanide on relaxation and cyclic GMP formation in endothelium-intact intrapulmonary artery caused by nitrogen oxide-containing vasodilators and acetylcholine. *J. Pharmacol. Exp. Ther.*, **236**, 30–36.

KOBAYASHI, Y. & HATTORI, K. (1990). Nitroarginine inhibits endothelium-derived relaxation. *Jpn. J. Pharmacol.*, **52**, 167–169.

KOWULAK, E.A., SETH, P. & FUNG, H. (1992). Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **262**, 916–922.

LU, J.K.H., GILMAN, D.P., MELDRUM, D.R., JUDD, H.L. & SAWYER, C.H. (1981). Relationship between circulating estrogens and the central mechanisms by which ovarian steroids stimulate luteinizing hormone secretion in aged and young female rats. *Endocrinol.*, **108**, 836–841.

NIIMI, Y., AZUMA, H. & HIRAKAWA, K. (1994). Repeated endothelial removal augments intimal thickening and attenuates EDRF release. *Am. J. Physiol.*, **266**, (*Heart Circ. Physiol.*, **35**), H1348–H1356.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1986). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). The antiaggregating properties of vascular endothelium: interaction between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.

RAINBOW, T.C., DERGROFF, V., LUINE, V. N. & MCEWEN, B. S. (1980). Estradiol-17β increases the number of muscarinic receptors in hypothalamic nuclei. *Brain Res.*, **198**, 239–243.

ROSS, R. (1986). The pathogenesis of atherosclerosis-an update. *N. Engl. J. Med.*, **314**, 488–500.

SCHWARTZ, S.M., GAJDUSEK, C.M. & SELDEN, S.C. (1981). Vascular wall growth control: the role of the endothelium. *Arteriosclerosis*, **1**, 106–171.

TSUJII, T., AZUMA, H., YAMAGUCHI, T. & OSHIMA, H. (1992). A possible role of decreased relaxation mediated by β-adrenoreceptors in bladder outlet obstruction by benign prostatic hyperplasia. *Br. J. Pharmacol.*, **107**, 803–807.

WEINER, C.P., KANG, Z.L., THOMPSON, L., HERRIG, J. & CHESTNUT, D. (1991). Effect of pregnancy on endothelium and smooth muscle: their role in reduced adrenergic sensitivity. *Am. J. Physiol.*, **261**, 1275–1283.

WEINER, C., MARTINEZ, E., ZHU, L.K., GHODSI, A. & CHESTNUT, D. (1989). In vitro release of endothelium-derived relaxing factor by acetylcholine is increased during the guinea pig pregnancy. *Am. J. Obstet. Gynecol.*, **161**, 1599–1605.

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Anaesthetic modulation of nicotinic ion channel kinetics in bovine chromaffin cells

[1]P. Charlesworth & C.D. Richards

Department of Physiology, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London NW3 2PF

1 We have investigated the action of the anaesthetics methoxyflurane, methohexitone and etomidate on the nicotinic acetylcholine receptor channel of bovine adrenal chromaffin cells using the whole cell patch clamp technique.

2 Spectral analysis of macroscopic currents evoked by 25 μM carbachol revealed that each of the agents tested reduced the lifetime of the channel open state in a dose-dependent manner. The whole cell current was inhibited in a concentration-dependent fashion by each agent.

3 Channel gating parameters were calculated from single channel studies and the results used to test models explaining the modulation of nicotinic acetylcholine receptor channels by anaesthetics.

4 Each of the agents studied reduced the mean channel open time in a concentration-dependent manner. Anaesthetic concentrations reducing mean open time by 50% were: 370 μM methoxyflurane, 30 μM methohexitone or 23 μM etomidate.

5 Methohexitone and etomidate produced an increase in the number of brief closures within bursts, while no such increase was observed with methoxyflurane. Despite these inter-burst gaps, mean burst length was reduced by each of the agents tested.

6 It is concluded that a simple sequential blocking model fails to account for the action of these anaesthetics. An extended model, in which blocked channels can close, may be applicable.

Keywords: Anaesthetics; nicotinic receptors; patch clamp; bovine chromaffin cells

Introduction

Barbiturates, local anaesthetics and volatile agents have been shown to modify the decay of muscle endplate currents (Kordas, 1970; Adams, 1976; Gage & Hamill, 1976; Torda & Gage, 1977; Sokoll *et al.*, 1989). The suggestion that this effect of anaesthetics is the result of a selective blockade of open channels has been investigated in many subsequent studies (see for example Neher & Steinbach, 1978; McLarnon *et al.*, 1986; Wachtel & Wegrzynowicz, 1992). A simple sequential model, in which only open channels are blocked, and blocked channels can only open, is however inadequate to explain the action of most agents studied (Neher, 1983; Gage & Mackinnon, 1985; Charlesworth *et al.*, 1992).

Recent studies at the single channel level (Dilger & Brett, 1991; Dilger *et al.*, 1992; Murrell *et al.*, 1991) have shown that the actions of primary alcohols and volatile anaesthetics on muscle acetylcholine receptors are reasonably well fitted by an extension of the simple sequential block model, which allows blocked channels to close without first releasing the blocking drug.

In this study we have examined the effect of methoxyflurane, methohexitone and etomidate on the gating kinetics of nicotinic acetylcholine receptor channels in bovine chromaffin cells. These provide a useful model for this study. They express a nicotinic receptor of a neuronal type (Higgins & Berg, 1987; Nooney *et al.*, 1992), whilst most previous patch clamp studies of anaesthetic action have used the muscle receptor. Also, studies in this and other laboratories have shown that catecholamine secretion from chromaffin cells evoked by carbachol is markedly more sensitive to anaesthetics than that evoked by direct depolarization. This clearly indicated a direct effect of anaesthetics on the activation of this nicotinic acetylcholine receptor (Takara *et al.*, 1986; Yashima *et al.*, 1986; Pocock & Richards 1987; 1988). This study examines the mechanism of this effect at the ion channel level.

[1] Author for correspondence.

Methods

Bovine chromaffin cells were isolated by enzymic digestion as described previously (Charlesworth *et al.*, 1992). After isolation and purification (see Waymire *et al.*, 1983), the cells were plated on poly-L-lysine coated glass coverslips at a density of 0.5×10^6 ml^{-1}. Cultures were maintained in a 1:1 mixture of Dulbecco's minimum essential medium and Ham's F12 medium supplemented with 2-10% newborn bovine serum and kept at 37°C in a humidified atmosphere containing 5% CO_2.

Experiments were carried out using the whole cell patch-clamp recording technique of Hamill *et al.* (1981). The external bathing medium for all experiments (Locke's solution) contained (mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 15, glucose 5.5. Tetrodotoxin (TTX, 1 μM) was used to suppress any voltage gated Na channel activity.

Two recording electrode solutions were used. For experiments with methoxyflurane the composition was (mM): KCl 120, NaCl 20, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11; pH 7.2. For experiments with methohexitone and etomidate, in which there was the possibility of a chloride conductance being activated, the following solution was used to fill electrodes: K glutamate 120, Na glutamate 20, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11; pH 7.2. Since E_{Cl} for this solution is similar to the holding potential used for whole cell experiments any chloride conductance activated would have negligible amplitude and so would not interfere with analysis of the nicotinic channel current.

Anaesthetics were directly dissolved in the bathing Locke solution and applied by microperfusion with a U-shaped glass capillary similar to that described by Krishtal & Pidoplichko (1980). The U-tube was connected to solution reservoirs via PTFE tubing and a rheodyne valve. The reservoirs were stoppered, with only a fine bore capillary allowing contact with the air in order to establish the pressure head. This arrangement minimized loss of volatile anaesthetic (methoxyflurane). Methoxyflurane was obtained from Abbott Laboratories, etomidate from Janssen, and methohexitone from Eli Lilly.

Patch pipettes were made from thick-walled filamented borosilicate glass (Clarke Electromedical GC150F) using a List L/M 3PA electrode puller and had tip diameters of 1–2 μM. The electrode tips were fire polished on a microforge before filling with recording solution. Filled electrodes were dipped briefly in Sigmacote before use. Electrode resistances ranged from 3–15 MΩ and the series resistance in whole cell mode was usually between 10 and 20 MΩ. Voltage errors due to series resistance were very small and were not corrected. All experiments were performed at room temperature (18–24°C).

Recording and analysis

Signals were recorded with an Axopatch 1D amplifier and were stored on tape cassettes (recording bandwidth d.c. to 20 kHz, although signals were usually filtered at 2 kHz before storage on tape) using a modified Sony DAT recorder.

Records of nicotinic acetylcholine receptor channel currents activated by carbachol were analysed to determine mean current flow and kinetic parameters of channel gating. Two analytical approaches were used, appropriate to the two different types of experiment performed.

Spectral analysis of whole cell current noise

For analysis of carbachol (25 μM)-induced current fluctuations the signal from the tape recorder was filtered with a 7-pole Cauer elliptical filter (Frequency Devices model 675, bandwidth DC-1 kHz) and digitised at 2.56 kHz. This filter has a very steep attenuation rate above the cutoff frequency (−94 dB at 2 kHz, −31 dB at the Nyqvist frequency (1.28 kHz)) eliminating aliasing effects. Power spectral densities were calculated on 1024 point (0.4s) data sections, and the mean power spectrum was calculated by averaging the spectra from 50–75 consecutive time blocks. The mean background noise spectrum obtained prior to application of agonist was subtracted from this to obtain the spectrum due to agonist alone.

This distribution was then fitted by either a single or double Lorentzian function using a least-squares Levenberg-Marquardt algorithm with proportional weighting of the data points. In some cases, the spectrum was truncated at the high frequency end. This was done when the signal:noise ratio was low (less than 2:1) and the error in the data points of the background subtracted noise spectrum was correspondingly high. The quality of the fitted curve was judged from the value of χ^2 and the random distribution of residuals. The amplitude of single channel currents was estimated from the parameters derived from the fitted power spectra and the mean inward current (see Anderson & Stevens, 1973; Cull-Candy *et al.*, 1988).

Single channel events

For the analysis of single channel, events segments of data (15–300s) were replayed, low-pass filtered at 1 kHz (four-pole Bessel) and digitised at 5 kHz. An all-points histogram of current amplitude was constructed and Gaussian distributions fitted to the peaks. The detection threshold for channel opening was then set at half of the mean single channel amplitude, determined from the Gaussian curve fit. After automatic detection of channel openings was complete, each transition was inspected. Obvious noise artifacts were reclassified as closed states if brief, or rejected if of longer duration. This was done so as not to bias the closed state distribution unduly. Multiple channel openings were infrequent and were rejected from the transition list. The transition list was not corrected for missed events.

After editing of the open/closed state transition list, frequency distribution histograms of open and closed state durations were constructed on logarithmic abscissa and square root ordinate scales using Pstat (Axon instruments). Histograms were then fitted to the sum of two exponential functions using a simplex algorithm and the method of maximum likelihood.

Bursts of openings separated by brief closures were analysed by first calculating a critical closed time duration (t_{crit}) below which a closure is classified as being a closure within a burst (Colquhoun & Sakmann, 1983; Clapham & Neher, 1984). This was calculated from the parameters of the two exponential components of the curve fit to each closed time histogram using an iterative computer routine. This ensured that the proportion of short intervals misclassified as closures between bursts was equal to the proportion of long intervals misclassified as closures within a burst. The mean burst duration and the mean duration of closures within bursts was calculated from such analysis. Throughout, burst length is defined as the total length of openings and closures within a single burst.

The spectral noise analysis (SPAN) and channel transition detection (PAT) programmes were kindly supplied by Dr John Dempster, University of Strathclyde.

Statistical analysis

Combined values are shown as mean ± s.e.mean. Control-anaesthetic differences were assessed using paired *t* tests and were considered statistically significant when $P<0.05$.

Results

Noise analysis

Whole cell currents were evoked by application of carbachol 25 μM. Associated with the inward current, a large increase in noise was observed, due to fluctuations in the number of open channels. Spectra calculated from these currents were well fitted by a single Lorentzian component. In some cases, a marginally better fit could be obtained using the sum of two components, however the second, high frequency, component was rarely more than 1% of the magnitude of the main component. For simplicity, control spectra were routinely fitted to single Lorentzian functions and the mean corner frequency of these was 19.4 ± 1.2Hz ($n = 10$, holding potential −80 mV), corresponding to a time constant for channel closure of 8.2 ms for the simple model of Anderson & Stevens (1973). This assumes that the level of receptor saturation is low, which is valid, since in these cells maximal currents are elicited by carbachol concentrations in excess of 200 μM.

The effect of methoxyflurane (100–500 μM), methohexitone (20–100 μM) and etomidate (10–50 μM) on whole cell current noise evoked by carbachol 25 μM was examined. In the presence of all concentrations of each anaesthetic, spectra were well fitted by a single Lorentzian function. The corner frequency of the fitted curve increased with increasing anaesthetic concentration for each of the agents studied (see Table 1 and Figure 1), revealing a dose-dependent reduction in the time constant for channel closure. The mean inward current generated by application of agonist was inhibited by each agent with approximate IC_{50}s of: 300 μM methoxyflurane, 30 μM methohexitone and 35 μM etomidate. None of the anaesthetics altered the single channel conductance calculated from the parameters of the fitted Lorentzian curves, which was 32.3 ± 0.6 pS ($n = 41$).

Single channel studies

Small chromaffin cells were selected for whole cell patch clamp recording (3–5pF, mean 4.30 pF, $n = 25$. Assuming a specific capacitance of 1 μm cm^{-2} this corresponds to cell diameters in the range 9–13 μm). With the pipette and bath solutions described in the methods, there was no detectable channel activity at potentials more negative than −60 mV

Table 1 The parameters of the Lorentzian curves fitted to power spectra recorded in the presence and absence of anaesthetic (−80 mV holding potential)

	f_c (Hz)	τ (ms)
Control (4)	18.1 ± 1.6	8.8
Methoxyflurane 100 μM (3)	21.2 ± 1.3	7.5
Methoxyflurane 250 μM (3)	30.9 ± 2.7	5.2
Methoxyflurane 500 μM (2)	63.8 ± 0.2	2.5
Control (3)	20.3 ± 2.6	7.8
Methohexitone 20 μM (3)	31.0 ± 3.7	5.1
Methohexitone 50 μM (3)	46.6 ± 4.0	3.4
Methohexitone 100 μM (3)	77.7 ± 6.90	2.0
Control (3)	20.3 ± 1.6	7.8
Etomidate 10 μM (2)	31.4 ± 4.2	5.1
Etomidate 25 μM (3)	40.1 ± 1.6	4.0
Etomidate 50 μM (3)	42.7 ± 3.0	3.7

Values of *n* are in parentheses.

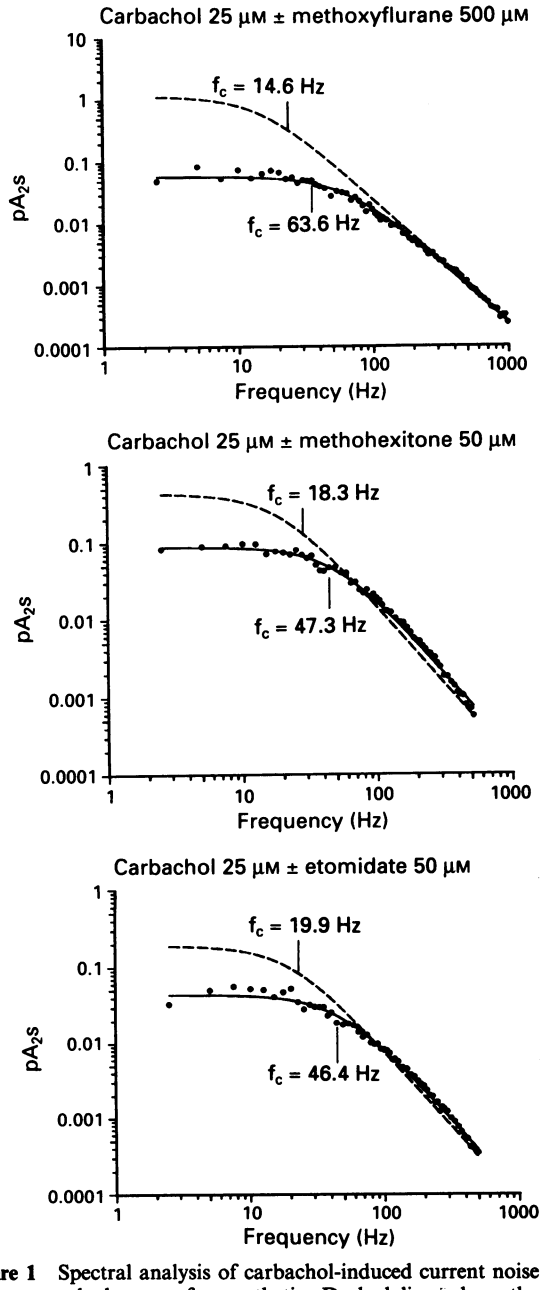


Figure 1 Spectral analysis of carbachol-induced current noise in the presence and absence of anaesthetic. Dashed lines show the single Lorentzian curve fits to the control spectra in each example. Solid lines and data points are the spectra recorded in the presence of agonist plus anaesthetic.

and baseline current noise was low (standard deviation of Gaussian curve fit 0.4–0.45 pA). Application of low concentrations of carbachol (1–2 μM) evoked nicotinic AChR channel openings at a low frequency (0.5–13 events s^{-1}; open probability 0.4–6.6% in control). At the holding potential of −100 mV used for these experiments, the signal to noise ratio, coupled to the low channel open probability, allowed detection of single channel openings as described in the Methods.

Under these conditions, stable levels of channel activity could be recorded for long periods, allowing the sequential application to individual cells, of carbachol, and carbachol plus varying concentrations of anaesthetic, followed by a return to carbachol alone. Typically, cells were perfused with test solution for 5–10 min with 3–4 min between successive applications.

When activated by carbachol in the absence of anaesthetic, brief closures within channel openings were very infrequent. Control closed time distributions were well fitted by two exponential components, with the brief (τ ~ 0.6 ms) component having a small relative area (~1/15 of the major component). Control open time distributions were fitted by two exponential components with time constants of ~1 and 9 ms and approximately equal areas. This bi-exponential distribution of open state durations is similar to that observed previously for the chromaffin cell nicotinic receptor (Cull-Candy *et al.*, 1988). Due to the rarity of intraburst closures, control burst distributions were very similar to open time distributions, though the slow time constant was slightly longer (~10.5 ms).

Methoxyflurane

Visual inspection of records revealed that the predominant effect of methoxyflurane on nicotinic channel kinetics was to reduce the duration of individual openings, an effect which became more marked with increasing anaesthetic concentration (IC_{50} for halving mean open time: 370 μM). This was revealed in open state duration histograms as a progressive shift towards shorter duration events with increasing concentrations of methoxyflurane. An example is shown in Figure 2c.

Methoxyflurane, at any concentration studied, did not appear to induce brief channel closures, characteristic of agents such as procaine (see Charlesworth *et al.*, 1992). Despite there being no apparent increase in burst activity, records were subjected to burst analysis using a value of T_{crit} calculated from individual closed time histograms. This was done both to confirm the absence of an anaesthetic related increase in brief channel closures and to allow formal comparison with other agents. Table 2A shows that the mean number of brief closures (gaps per burst) did not increase with increasing concentrations of methoxyflurane. Bi-exponential curve fits to burst time distributions showed that the time constant of the brief events (τ ~ 1 ms) was unaffected by methoxyflurane, while the time constant of the longer component decreased with increasing concentrations of methoxyflurane.

Channel open probability was reduced by methoxyflurane with an IC_{50} of 250 μM, similar to that for suppression of macroscopic current. The sensitivity of open probability was somewhat greater than channel open lifetime, since the frequency of channel activation was also reduced by methoxyflurane. This was reflected in the closed time distribution: the time constant of long (inter-burst) closures increased with methoxyflurane concentration, rising to 192% of paired control values in the presence of 500 μM methoxyflurane.

Single channel amplitude (the mean of the Gaussian curve fitted to a histogram of open state amplitudes) was unaffected by all concentrations of methoxyflurane. Perfusion with methoxyflurane alone (250 μM) had no detectable effect on the recorded current.

The effect of methoxyflurane (250 μM) on nicotinic AChR channels was also studied in four cell-attached patches. In

these experiments, carbachol (20 μM) was present in the patch pipette (filled with Locke's solution) to activate channels and the cell perfused with methoxyflurane. Applied in this way, methoxyflurane produced rapid effects on channel gating kinetics, the magnitude of which were similar to those observed in the whole cell experiments described above. Expressed as a percentage of control values, 250 μM methoxyflurane reduced mean open time to 51.0 ± 11.0%, channel open probability to 36.2 ± 9.9% and opening frequency to 69.5 ± 2.9%.

Methohexitone

Small chromaffin cells, were sequentially perfused with carbachol (2 μM), or carbachol plus 10, 20 or 50 μM methohexitone. Using the glutamate recording solution described in the methods methohexitone alone did not elicit resolvable channel activity.

At increasing concentrations of methohexitone, individual channel openings became progressively shorter (IC_{50} for reduction of mean open time 30 μM. Open state duration frequency histograms show a corresponding leftward shift with increasing methohexitone concentration. This is shown in the example of Figure 2b. Figure 3 shows representative single channel events activated by 2 μM carbachol in the absence and presence of 50 μM methohexitone.

An increasing number of brief channel closures was also observed, although the majority of openings were uninterrupted under all recording conditions. Figure 4 illustrates the way in which methohexitone altered the closed time distribution; in the presence of the drug an additional component appeared with a time constant of 4.9 ± 0.6 ms, $n = 17$ (mean for all drug applications; the time constant did not depend on methohexitone concentration). Burst analysis confirmed that a small but significant rise in the number of brief channel closures was induced in a dose dependent manner by methohexitone. The mean duration of these drug induced closures was however independent of methohexitone concentration (see Table 2B). Fitting of burst length distributions showed that methohexitone caused a reduction in the time constant of the longer population of bursts, while the fast time constant was unaffected.

As a consequence of the channel 'flicker' induced by methohexitone, mean burst length was significantly greater than mean open time at any given anaesthetic concentration. Burst length nevertheless progressively declined with increasing methohexitone concentration, although this reduction was not quite statistically significant with low (10 μM) concentrations of drug.

Methohexitone depressed channel open probability relatively weakly ($IC_{50} > 50$ μM cf.30 μM for macroscopic current inhibition). This may suggest that the action of methohexitone is dependent on agonist concentration. The weak inhibition of single channel open probability is partly due to a dose dependent *increase* in burst frequency. This rise was statistically significant in the presence of 50 μM methohexitone, when burst frequency was 167.7 ± 12.9% and the time constant of inter-burst closures was 61.6 ± 4.5% of paired control values (= 6).

Etomidate

The duration of individual channel openings was reduced in a dose dependent manner by etomidate (10–50 μM (see Table

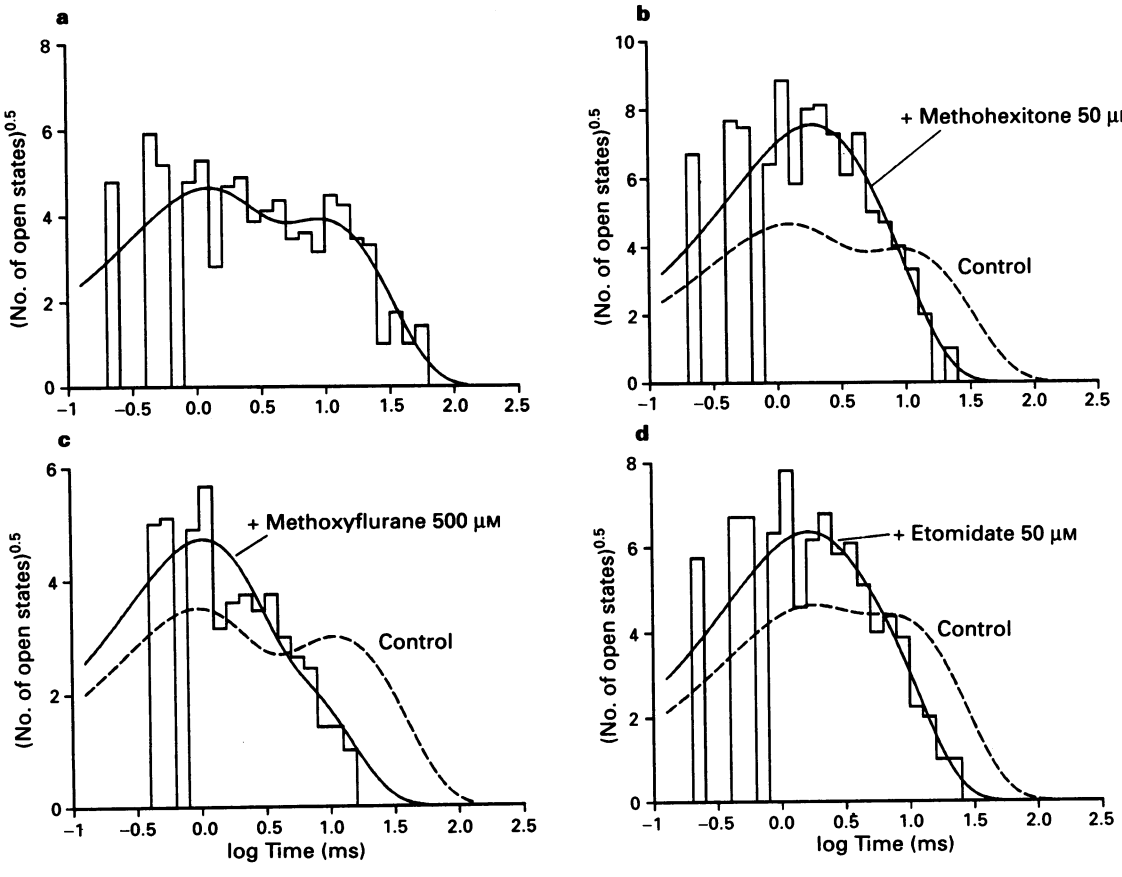


Figure 2 The effect of anaesthetic on the frequency distribution of nicotinic channel open times recorded in whole cell configuration. Histograms drawn on logarithmic abscissa and square root ordinate scales. Panel (a) is an example of a control distribution. In panels (b-d) columns and solid curve fits are the data obtained during co-application of anaesthetic plus agonist, dashed lines are the curve fits to the paired control histograms. Panels (a) and (b) show data from the same cell, (c) and (d) are separate cells. The total number of events represented in each panel is: (a) 344; (b) control 361, methohexitone 665; (c) control 213, methoxyflurane 245; (d) control 365, etomidate 487.

2C and Figure 5). An example of the change in the open state duration histogram brought about by 50 μM etomidate is shown in Figure 2d.

Similar to the situation with methohexitone, the reduction in channel open lifetime with increasing etomidate concentration is accompanied by a modest, but significant, rise in the number of brief channel closures. The duration of these anaesthetic-induced closures was exponentially distributed with a mean time constant of 2.3 ± 0.2 ms ($n = 14$) and did not vary significantly with etomidate concentration. As a result, burst length falls less steeply than open time as a function of etomidate concentration (see Table 2c). Curve fits to burst duration histograms showed a reduction in the longer time constant with increasing etomidate concentration. The mean duration of brief channel closures attributable to the action of etomidate was independent of anaesthetic concentration.

Etomidate inhibited channel open probability with an IC_{50} of 35 μM, equal to that for macroscopic current inhibition. Mean open time was reduced with an IC_{50} of 23 μM. The difference between these values is due to a rise in burst frequency in the presence of etomidate. As a percentage of paired control values, burst frequency was 147.8% (10 μM etomidate), 127.8% (25 μM etomidate) and 124.8% (50 μM etomidate). Corresponding values for the long closed time constant were: 71.9%, 81.1% and 81.8%.

Discussion

The clearest feature of the action of all three agents studied on nicotinic AChR channel gating was a dose-dependent reduction in mean open time. This could be described by the relation 1/(mean open time) = α + f.c. (see Figure 5),

Table 2 Parameters derived from the analysis of single channel currents in the presence and absence of anaesthetic: **A** methoxyflurane; **B** methohexitone and **C** etomidate

A Methoxyflurane

	Control (11)	*Methoxyflurane* (9) 100 μM	*Methoxyflurane* (8) 250 μM	*Methoxyflurane* (5) 500 μM
Apparent mean open time (ms)	5.26 ± 0.33	3.62 ± 0.18*	2.84 ± 0.18*	2.25 ± 0.19*
Open probability (%)	2.15 ± 0.53	1.79 ± 0.44*	0.82 ± 0.30*	0.64 ± 0.25*
Open time distribution τ fast	0.99 ± 0.10	0.89 ± 0.08	0.68 ± 0.11	0.71 ± 0.13
Open time distribution τ slow	9.04 ± 0.67	6.57 ± 1.01	4.47 ± 0.47*	4.01 ± 0.88*
$Area_{fast}/Area_{slow}$	1.18 ± 0.16	1.21 ± 0.28	1.26 ± 0.14	1.84 ± 0.35
Critical time contant (T_{crit})	1.63 ± 0.22	1.60 ± 0.21	2.00 ± 0.30	2.46 ± 0.73
Mean burst length (ms)	5.38 ± 0.27	3.85 ± 0.18*	3.11 ± 0.17*	2.47 ± 0.19*
Gaps per burst	0.05 ± 0.008	0.05 ± 0.007	0.06 ± 0.010	0.05 ± 0.014
Mean gap length (ms)	0.67 ± 0.07	0.63 ± 0.05	0.67 ± 0.07	0.99 ± 0.36
Burst length distribution τ fast	0.94 ± 0.08	0.93 ± 0.07	0.72 ± 0.08	0.81 ± 0.17
Burst length distribution τ slow	9.76 ± 0.61	7.38 ± 0.87*	5.18 ± 0.42*	4.40 ± 0.78*
$Area_{fast}/Area_{slow}$	1.23 ± 0.15	1.52 ± 0.26	1.23 ± 0.14	2.87 ± 0.66

B Methohexitone

	Control (9)	*Methohexitone* (3) 10 μM	*Methohexitone* (6) 20 μM	*Methohexitone* (7) 50 μM
Apparent mean open time (ms)	6.02 ± 0.33	4.29 ± 0.28*	3.55 ± 0.16*	2.32 ± 0.11*
Open probability (%)	2.89 ± 0.44	3.34 ± 0.73	3.58 ± 0.90	2.32 ± 0.11*
Open time distribution τ fast	1.10 ± 0.09	1.05 ± 0.32	0.68 ± 0.13	–
Open time distribution τ slow	11.87 ± 0.71	6.65 ± 0.90*	4.24 ± 0.22*	2.18 ± 0.12*
$Area_{fast}/Area_{slow}$	1.59 ± 0.20	0.96 ± 0.09	0.45 ± 0.06*	–
Critical time contant (T_{crit})	1.01 ± 0.31	5.68 ± 1.44*	7.42 ± 1.08*	6.83 ± 1.79*
Mean burst length (ms)	6.13 ± 0.32	5.06 ± 0.27	4.69 ± 0.24*	3.41 ± 0.36*
Gaps per burst	0.02 ± 0.003	0.11 ± 0.005*	0.18 ± 0.015*	0.19 ± 0.034*
Mean gap length (ms)	0.55 ± 0.10	2.27 ± 0.54*	2.77 ± 0.37*	2.53 ± 0.59*
Burst length distribution τ fast	1.10 ± 0.09	1.09 ± 0.23	0.81 ± 0.14	1.11 ± 0.09
Burst length distribution τ slow	12.16 ± 0.70	8.24 ± 0.41	6.49 ± 0.48*	5.14 ± 1.02*
$Area_{fast}/Area_{slow}$	1.57 ± 0.19	1.07 ± 0.05	0.73 ± 0.15*	1.35 ± 0.40

C Etomidate

	Control (6)	*Etomidate* (5) 10 μM	*Etomidate* (5) 25 μM	*Etomidate* (4) 50 μM
Apparent mean open time (ms)	6.44 ± 0.58	4.44 ± 0.22*	2.92 ± 0.11*	2.16 ± 0.11*
Open probability (%)	1.41 ± 0.36	1.33 ± 0.43	0.96 ± 0.20*	0.37 ± 0.12*
Open time distribution τ fast	1.33 ± 0.15	0.88 ± 0.15	0.93 ± 0.25	0.93 ± 0.21
Open time distribution τ slow	10.65 ± 0.87	6.12 ± 0.36*	4.16 ± 0.46*	2.56 ± 0.28*
$Area_{fast}/Area_{slow}$	1.06 ± 0.21	0.66 ± 0.07	1.10 ± 0.39	0.79 ± 0.22
Critical time contant (T_{crit})	1.42 ± 0.30	6.86 ± 0.52*	6.34 ± 0.85*	7.11 ± 1.15*
Mean burst length (ms)	6.91 ± 0.65	5.90 ± 0.33	4.24 ± 0.28*	3.16 ± 0.24*
Gaps per burst	0.05 ± 0.013	0.23 ± 0.013*	0.27 ± 0.034*	0.24 ± 0.033*
Mean gap length (ms)	0.49 ± 0.09	1.78 ± 0.06*	1.83 ± 0.15*	1.90 ± 0.22*
Burst length distribution τ fast	1.50 ± 0.17	1.26 ± 0.14	1.22 ± 0.20	0.91 ± 0.20
Burst length distribution τ slow	12.35 ± 1.29	10.21 ± 1.04	7.28 ± 0.51	4.93 ± 0.77*
$Area_{fast}/Area_{slow}$	1.24 ± 0.25	1.18 ± 0.20	1.42 ± 0.26	1.23 ± 0.55

Asterisk (*) denotes statistical significance at the 5% level for control-anaesthetic differences. Values for n are given in parentheses.

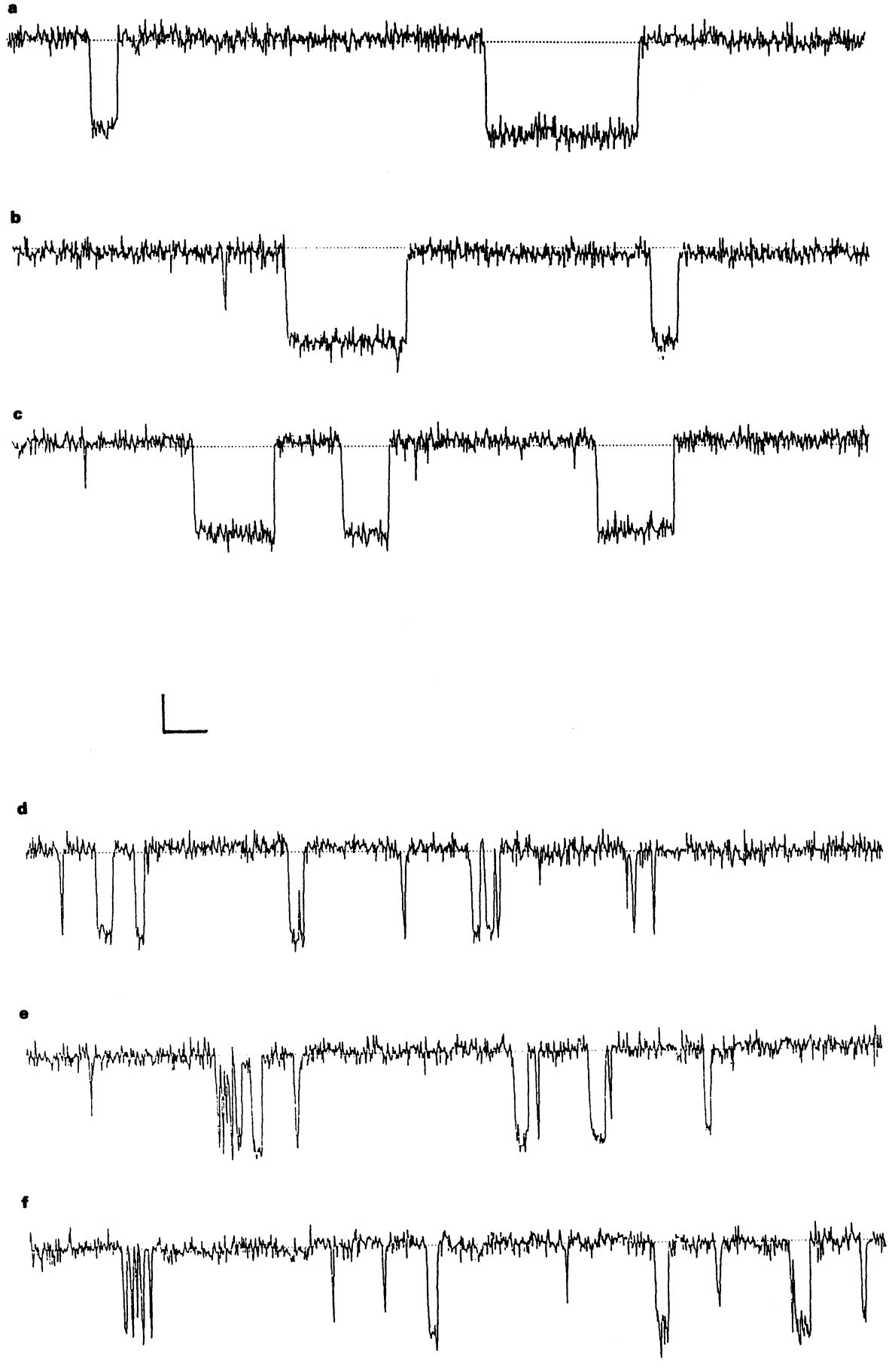


Figure 3 Examples of single channel currents recorded from chromaffin cells in whole cell mode. The upper three traces (a,b,c) show channel openings (downward deflections) activated by carbachol 2 μM alone, the lower three traces (d,e,f) show openings, and bursts of openings, recorded in the presence of methohexitone 50 μM plus carbachol 2 μM. Data from a single cell. Calibration bar, 2 pA vertical, 20 ms horizontal.

suggesting the applicability of some form of open channel block model, e.g.

$$C \underset{\alpha}{\overset{\beta}{\rightleftarrows}} O^* \underset{b}{\overset{f.c.}{\rightleftarrows}} O\text{-}B \ (\rightarrow C\text{-}B) \qquad \alpha'$$

* = conducting state

f = forward blocking rate constant. b = channel unblocking rate constant and c = concentration of anesthetic.

In addition, methohexitone and etomidate caused channel 'flicker', qualitatively consistent with this simple three state sequential blocking model. However, the model fails to account quantitatively for the actions of these anaesthetics, principally because burst length falls with increasing concentrations of each agent, whilst the model predicts that this parameter must rise. A similar finding was reached by Dilger *et al.* (1992) and Murrell *et al.* (1991), studying the action of isoflurane and n-alcohols respectively. These authors found that an extended blocking model, allowing the closure of blocked channels (shown in parentheses above), provided a satisfactory explanation of the data. Certain findings of the present study are also consistent with this model. The extension to the simple model, allowing the termination of bursts by a transition from the open-blocked to a closed block state is consistent with the observed reduction in mean burst length. Also in support of the extended model is the independence of blocked time on anaesthetic concentration. In this respect the action of the general anaesthetics differs from that of procaine (Charlesworth *et al.*, 1992), since mean blocked time is proportional to procaine concentration.

The number of anaesthetic-induced gaps within burst was low (virtually nil for methoxyflurane). This makes derivation of certain parameters of the model difficult. If the model were applicable, the value of b/α' (which is proportional to the number of gaps per burst, see Dilger *et al.*, 1992) must be low for methohexitone and etomidate and almost zero for methoxyflurane. Therefore, the most likely pathway for blocked channels is to a long-lived closed-blocked state, since the rate of release of anaesthetic from blocked channels (b) is relatively slow. By comparison with the results of Dilger *et al.* (1991) and Murrell *et al.* (1991) this finding is not unexpected. Both groups found a strong correlation between the unblocking rate and partition coefficient for a series of n-alcohols and volatile anaesthetics. The agents used in this study have high partition coefficients and would therefore be expected to bind strongly to a hypothesized hydrophobic

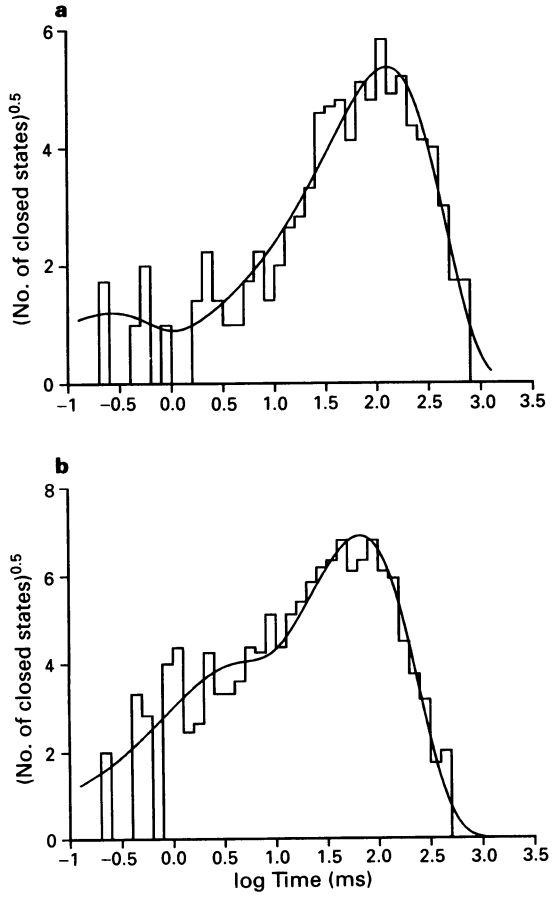


Figure 4 An example of the closed state duration frequency histogram in the presence (b) and and absence (a) of methohexitone 50 μM. Data are fitted to the following equation (sum of two exponentials): $P_1.\exp(t/\tau_1) + P_2.\exp(t/\tau_2)$.

Control ($n = 344$)	Methohexitone 50 μM ($n = 665$)
P_1 0.044	P_1 0.173
τ_1 0.24 ms	τ_1 2.54 ms
P_2 0.956	P_2 0.827
τ_2 126.9 ms	τ_2 66.8 ms

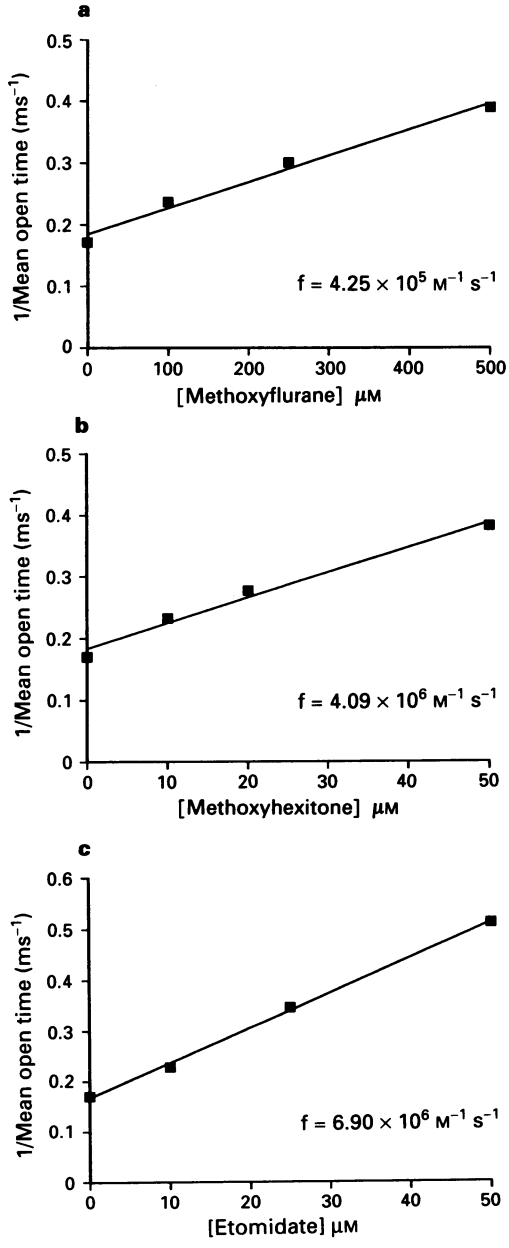


Figure 5 Plots of the reciprocal of average mean open time against anaesthetic concentration for (a) methoxyflurane (b) methohexitone and (c) etomidate. The gradient of the fitted regression line (equal to the forward rate constant of channel block) is shown in each case. Values of *n* for data points are shown in Table 2A, B and C.

binding site on the channel protein (see Dilger *et al.*, 1992).

Changes in burst frequency are not easily accommodated by the model. The rise observed with methohexitone and etomidate implies that a closed state must be modified so as to be destabilized with respect to an open state (have a higher channel opening rate than the unmodulated receptor). Alternatively, it may be due to closed-blocked channels returning to the open state via the open-blocked state, rather than releasing anaesthetic to return to the resting state (C). Unfortunately, slow transitions between non-conducting states (C-B, O-B, C) are not identifiable from the current record, and hence we are not able to distinguish between these possibilites. The fall in frequency seen with methoxyflurane is explicable if it is assumed that the release of methoxyflurane from the closed blocked channel is slow relative to the channel opening rate, β. The number of receptor-channels capable of being activated by agonist would then fall as a significant proportion of the receptor population will be in the inactive closed-blocked state at any time.

Models based upon anaesthetic modulation of closed states appear able to explain the reduction in mean open time seen with each agent:

$$O'^* \underset{\alpha'}{\overset{\beta'}{\rightleftarrows}} C' \underset{k.c}{\overset{}{\rightleftarrows}} C \underset{\alpha}{\overset{\beta}{\rightleftarrows}} O^*$$

k = rate constant of anaesthetic modulation

However, such a model predicts that fast and slow time constants of burst lifetime distributions should be independent of anaesthetic concentration, while the relative proportions of the two populations changes. This was not found to be true for any agent: in each case the ratio A_{fast}/A_{slow} was not altered consistently, while τ_s fell in a concentration-dependent manner, consistent with the extended blocking model.

Spectral analysis of the action of the anaesthetics on agonist-induced current fluctuations demonstrated reductions in open channel lifetime consistent with the decrease observed in single channel studies. It has previously been shown (Ruff, 1977; Neher & Steinbach, 1978) that agents which cause a flickering open channel block introduce a second, high frequency component into the power density spectrum. Given the absence of such oscillations in the presence of methoxyflurane the finding that this agent increased the corner frequency and depressed the intercept without introducing a second spectral component was expected. Considering the appearance of single channel currents recorded in the presence of etomidate and methohexitone, it was somewhat surprising that a high frequency component was not seen in the power spectra. This was most probably due to the relatively low frequency of inter burst closures, which would result in any additional spectral component having a low amplitude and even lower power (power being proportional to amplitude^2).

The agents studied all inhibited macroscopic currents with IC_{50}s close to those for the inhibition of carbachol-evoked catecholamine secretion. These concentrations are similar to those found in the blood during anaesthesia (methoxyflurane 250 μM (Pocock & Richards 1988), methohexitone 22 μM, etomidate 35 μM (Pocock & Richards, unpublished data)). With the exception of methohexitone, channel open probability was equally sensitive. The present study shows that the principal reason for the reduction in carbachol-evoked release is due to a decrease in the duration of nicotinic channel openings.

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References

ADAMS, P.R. (1976). Drug blockade of open end-plate channels. *J. Physiol.*, **260**, 531–552.

ANDERSON, C.R. & STEVENS, C.F. (1973). Voltage clamp analysis of acetylcholine produced endplate current fluctuations at frog neuromuscular junction. *J. Physiol.*, **235**, 655–691.

CHARLESWORTH, P., JACOBSON, I., POCOCK, G. & RICHARDS, C.D. (1992). The mechanism by which procaine inhibits catecholamine secretion from bovine chromaffin cells. *Br. J. Pharmacol.*, **106**, 802–812.

CLAPHAM, D.E. & NEHER, E. (1984). Substance P reduces acetylcholine-induced currents in isolated bovine chromaffin cells. *J. Physiol.*, **347**, 255–277.

COLQUHOUN, D. & B. SAKMANN. (1983). Bursts of openings in transmitter-activated ion channels. In Single-Channel Recording. ed. Sakmann, B. & Neher, E. pp. 345–363. New York: Plenum.

CULL-CANDY, S.G., MATHIE, A. & POWIS, D.A. (1988). Acetylcholine receptor channels and their block by clonidine in cultured bovine chromaffin cells. *J. Physiol.*, **402**, 255–278.

DILGER, J.P. & BRETT, R.S. (1991). Actions of volatile anesthetics and alcohols on cholinergic receptor channels. *Ann. N.Y. Acad. Sci.*, **625**, 616–27.

DILGER, J.P., BRETT, R.S. & LESKO, L.A. (1992). Effects of isoflurane on acetylcholine receptor channels. 1. Single channel currents. *Mol. Pharmacol.*, **41**, 127–133.

GAGE, P.W. & HAMILL, O.P. (1976). Effects of several inhalation anaesthetics on the kinetics of postsynaptic conductance changes in mouse diaphragm. *Br. J. Pharmacol.*, **57**, 263–272.

GAGE, P.W. & MCKINNON, D. (1985). Effects of pentobarbitone on acetylcholine-activated channels in mammalian muscle. *Br. J. Pharmacol.*, **85**, 229–235.

HAMILL, O.P., MARTY, A., NEHER, E., SACKMANN, B. & SIGWORTH, F.J. (1981). Improved patchclamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HIGGINS, L.S. & BERG, D.K. (1987). Immunological identification of a nicotinic acetylcholine receptor on bovine chromaffin cells. *J. Neurosci.*, **7**, 1792–1798.

KORDAS, M. (1970). The effect of procaine on neuromuscular transmission. *J. Physiol.*, **209**, 689–699.

KRISHTAL, O.A. & PIDOPLICHKO, V.I. (1980). A receptor for protons in the nerve cell membrane. *Neuroscience*, **5**, 2325–2327.

MCLARNON, J.G., PENNEFATHER, P. & QUASTEL, D.M.J. (1986). Mechanism of nicotinic channel blockade by anaesthetics. In *Molecular and Cellular Mechanisms of Anesthetics*. ed. Roth, S.H. & Miller, K.W. pp. 155–164. New York: Plenum.

MURRELL, R.D., BRAUN, M.S. & HAYDON, D.A. (1991). Actions of n-alcohols on nicotinic acetylcholine receptor channels in cultured rat myotubes. *J. Physiol.*, **437**, 431–448.

NEHER, E. (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J. Physiol.*, **339**, 663–678.

NEHER, E. & STEINBACH, J.H. (1978). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **277**, 153–176.

NOONEY, J.M., PETERS, J.A. & LAMBERT, J.J. (1992). A patch clamp study of the nicotinic acetylcholine receptor of bovine adrenomedullary chromaffin cells in culture. *J. Physiol.*, **455**, 503–527.

POCOCK, G. & RICHARDS, C.D. (1987). The action of pentobarbitone on stimulus-secretion coupling in adrenal Chromaffin cells. *Br. J. Pharmacol.*, **90**, 71–80.

POCOCK, G. & RICHARDS, C.D. (1988). The action of volatile anaesthetics on stimulus-secretion coupling in bovine adrenal chromaffin cells. *Br. J. Pharmacol.*, **95**, 209–217.

RUFF, R.L. (1977). A quantitative analysis of local anaesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol.*, **264**, 89–124.

SOKOLL, M.D., DAVIES, L.R., BHATTACHARYYA, B. & ZWAGERMAN, D.Q. (1989). Halothane and isoflurane alter acetylcholine activated ion channel kinetics. *Eur. J. Pharmacol.*, **173**, 27–34.

TAKARA, H., WADA, M., ARITA, K., SUMIKAWA, K. & IZUMI, F. (1986). Ketamine inhibits ^{45}Ca influx and catecholamine secretion by inhibiting ^{22}Na influx in cultured bovine adrenal medullary cells. *Eur. J. Pharmacol.*, **125**, 217–224.

TORDA, T.A. & GAGE, P.W. (1977). Postsynaptic effect of i.v. anaesthetic agents at the neuromuscular junction. *Br. J. Anaesth.*, **49**, 771–776.

WACHTEL, R.E. & WEGRZYNOWICZ, E.S. (1992). Kinetics of nicotinic acetylcholine ion channels in the presence of intravenous anaesthetics and induction agents. *Br. J. Pharmacol.*, **106**, 623–627.

WAYMIRE, J.C., BENNETT, W.F., BOEHME, R., HANKINS, L., GILMER WAYMIRE, K. & HAYCOCK, J.W. (1983). Bovine adrenal chromaffin cells: high-yield purification and viability in suspension culture. *J. Neurosci. Methods*, **7**, 329–351.

YASHIMA, N., WADA, A. & IZUMI, F. (1986). Halothane inhibits the cholinergic-receptor-mediated influx of calcium in primary culture of bovine adrenal medulla cells. *Anesthesiology*, **64**, 466–472.

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NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors*, as published in the *British Journal of Pharmacology, Br. J. Pharmacol.*, 1995, **114,** 253–255.

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