



SPECIAL REPORT

Identification of novel polymorphisms within the promoter region of the human β_2 adrenergic receptor gene

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By screening the 1470 bp 5' to the start codon of the human β_2 adrenergic receptor gene, we have identified a total of eight polymorphisms (–20 T→C, –47 T→C, –367 T→C, –468 C→G, –654 G→A, –1023 G→A, –1343 A→G and –1429 T→A *c.f.* β_2 adrenergic receptor start codon). Transient transfection of 5' flanking deletion luciferase reporter constructs demonstrated the majority of activity of the human β_2 adrenergic gene 5' flanking region to be present within a 549 bp fragment immediately upstream from the start codon. Because of linkage disequilibrium, some combinations of polymorphisms were particularly frequent. We transiently transfected COS-7 cells with luciferase constructs under the control of the 549 bp of 5' flanking DNA containing the two most frequent extended haplotypes in this region. Luciferase activity was significantly reduced in cells transfected with the 'mutant' construct (–20C, –47C, –367C, –468G) *c.f.* the 'wild-type' construct (–20T, –47T, –367T, –468C). These data suggest that polymorphisms have the potential to alter human β_2 adrenergic receptor gene expression.

Keywords: Human β_2 adrenergic receptor; human β_2 adrenergic receptor gene; polymorphisms; promoter; 5' flanking region; transfection; luciferase; COS-7 cells

Abbreviations: bp, base pairs; BUP, beta upstream peptide; kb, kilobases; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; sORF, short open reading frame

Introduction The human β_2 adrenergic receptor gene is situated on chromosome 5q (5q 31/32). This intronless gene is known to contain nine different point mutations within the coding region, of which four are non-degenerate resulting in amino acid changes (Arg→Gly 16, Gln→Glu 27, Val→Met 34, Thr→Ile 164). Three of these mutations (codon 16, 27 and 164) are functionally relevant, the first two altering agonist induced downregulation of the receptor and the third altering ligand binding (Green *et al.*, 1993; 1995; Reishaus *et al.*, 1993; Turki *et al.*, 1995b). Control of human β_2 adrenergic receptor gene expression is regulated in part by the 5' flanking region immediately upstream from the gene. This region contains a number of putative controlling elements including a short 5' leader peptide (beta upstream peptide or BUP), a cyclic AMP response element (CRE), two NF-IL6 sites, four AP-2 sites and a steroid binding hexamer.

We have screened the 5' flanking region of the human β_2 adrenergic receptor gene for the presence of polymorphisms, using a combination of restriction fragment length polymorphism (RFLP) and direct sequencing of polymerase chain reaction (PCR) products using genomic DNA as template. We describe here the presence of a number of novel polymorphisms within the 5' regulatory region of the β_2 adrenergic receptor gene, which in preliminary studies appear to alter expression of a luciferase based reporter plasmid in COS-7 cells.

Methods *Mutation screening* Genomic DNA from a panel of asthmatic and non-asthmatic individuals was initially screened for a possible mutation at base pair –367 (relative to the ATG start codon) identified by comparison of sequence information present in the human DNA database entries containing relevant sequences. Following confirmation of the

presence of this mutation by BSu36 I restriction digest, we subcloned various 5' flanking deletion fragments into the PCR cloning vector pGEM-T-Easy (Promega). Dideoxy sequencing of these clones revealed the presence of the –367 T→C mutation but in addition also revealed a number of other base changes (see Table 1). Subsequently, PCR products from genomic DNA from individual donors were sequenced directly. All of the mutations present were identified in at least two separate individual donors.

Construction of luciferase reporter constructs In order to assess the activity of the various promoter fragments in COS-7 cells, 5' deletion fragments were excised from pGEM-T-Easy constructs using restriction sites introduced on PCR primers and subcloned, using standard techniques, into the promoterless firefly luciferase vector pGL3 Enhancer (Promega). Further deletion constructs were made using convenient restriction sites within the 5' regulatory region of the gene. A total of seven reporter constructs were made containing 274, 379, 549, 744, 1018, 1244 and 1486 bp of 5' flanking sequence. Confirmation of sequence was again obtained by dideoxy sequencing.

Transfection of COS-7 cells COS-7 cells were seeded into 24-well plates at a density of 30,000 cells per well in DMEM, 10% foetal calf serum and glutamine (2 mM) the day prior to transfection. The following day cells were transfected using liposomal mediated DNA transfer (Transfast, Promega) with 250 ng of β_2 adrenergic receptor gene 5' deletion constructs and 12.5 ng of pRL.CMV (a *Renilla* based luciferase construct, Promega) per well. Forty-eight hours post transfection the medium was aspirated, cells were washed with PBS once, and lysed in passive lysis buffer (Promega). Luciferase activity in cell lysates was measured using a Turner luminometer (model 20e) with a commercially available kit (Dual-Luciferase Reporter Assay System, Promega). Cells were also transfected with pGL3 Control (Promega) and pGL3

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Enhancer which served as positive and negative controls respectively. Firefly luciferase activity was normalized to *Renilla* luciferase activity to correct for variations in transfection efficiency.

Results *Identification of β_2 adrenergic receptor promoter polymorphisms* Table 1 shows the polymorphisms identified from genomic DNA in PCR products from at least two different individuals. The mutations at -47 and -367 bp create and ablate restriction enzyme sites (Mspa I, BSu36 I) respectively.

Table 1 Polymorphisms identified within the 5' flanking region of the human β_2 adrenergic receptor gene

bp upstream from ATG	Base change	Putative regulatory site
-20	T→C	—
-47	T→C	Non-conservative amino acid change in 5' leader peptide (BUP: Cys→Arg)
-367	T→C	Creates 7/8 bases of a consensus AP2 site and is 7 bp 3' of an overlapping AP2/Sp1 site
-468	C→G	—
-654	G→A	p53 site
-1023	G→A	2 bp 3' of a NF-IL6 site
-1343	A→G	—
-1429	T→A	3 bp 3' of steroid binding hexamer

The table indicates location of polymorphisms relative to the translational start codon of the gene, the base change and the putative regulatory site the polymorphisms lie in or near.

Regulatory activity of the human β_2 adrenergic receptor promoter Initial studies were designed to identify the regions of the human β_2 adrenergic receptor promoter containing important transcriptional activity by examining the ability of a range of constructs to drive luciferase expression in COS-7 cells (Figure 1). These studies demonstrate that the majority of transcriptional activity is contained within the region immediately 5' to the start codon.

Preliminary characterization of β_2 adrenergic receptor promoter polymorphisms Because of strong linkage disequilibrium between the different polymorphisms identified, for preliminary studies we examined the two most frequent extended haplotypes in the region containing the majority of regulatory activity (-1 to -549 bp *c.f.* the ATG start codon). These plasmids (p0.55wt β_2 AR-LUC and p0.55mut β_2 AR-LUC) were transfected into COS-7 cells and luciferase activity assayed 48 h post transfection. The haplotype of these constructs is p0.55wt β_2 AR-LUC: -20 T, -47 T, -367 T, -468 C and p0.55mut β_2 AR-LUC: -20 C, -47 C, -367 C, -468 G. The response of p0.55mut β_2 AR-LUC was $83 \pm 1.7\%$ of the response seen with the 'wild-type' construct ($n = 15$, $P < 0.0001$).

Discussion In this study, we identified the presence of a number of polymorphisms within the 5' regulatory region of the human β_2 adrenergic receptor gene. We were able to find a total of eight polymorphisms in the 1470 bp immediately upstream of the start codon. Five of these polymorphisms have been reported in a recent brief report (Timmermann *et al.*, 1998). However, three of the polymorphisms (-367 , -468 and -1429) have not been previously described. Two of these (-367 and -468) can be deduced from information already

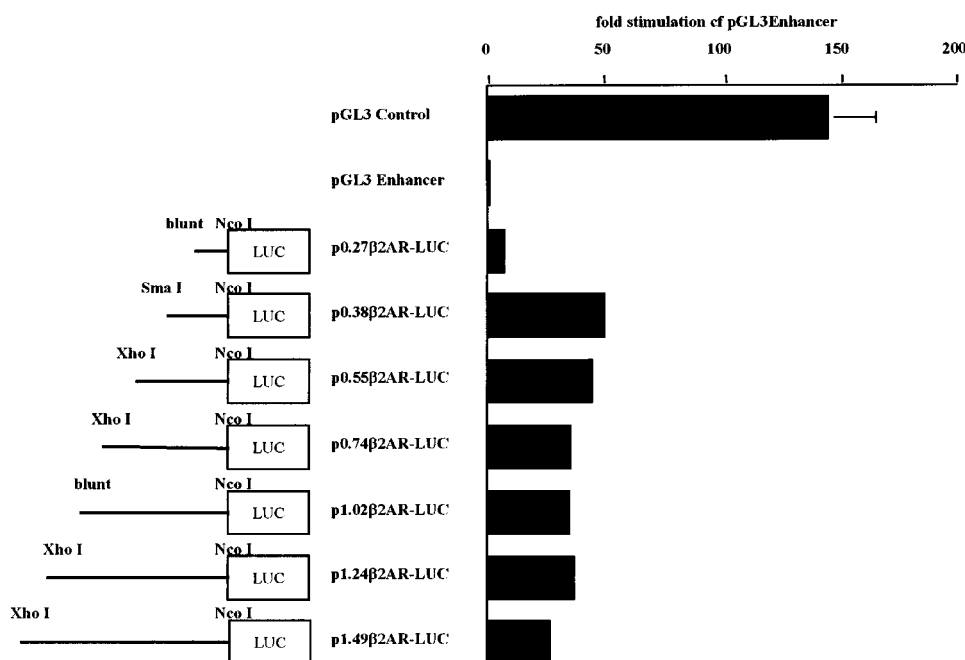


Figure 1 Relative promoter activity of the human β_2 adrenergic receptor gene in COS-7 cells. Schematic drawings of the constructs, indicating the restriction enzyme sites used for subcloning into pGL3 Enhancer, are shown on the left. Nomenclature of the constructs refers to the number of nucleotides (in kbp) of 5' flanking region relative to the translational start codon. Firefly luciferase activity was normalized to *Renilla* luciferase activity of the co-transfected plasmid pRL.CMV to correct for variation in transfection efficiency. The results are expressed relative to the backbone vector pGL3 Enhancer. pGL3 Control, which contains the luciferase cDNA under the control of both SV40 promoter and enhancer elements, was used as a positive control. Each bar represents the mean $\pm$ s.e. mean from triplicate determinations in individual experiments, $n = 7-19$. Where error bars are not shown they lie within the bar.

present in the databases by discrepancies in the available DNA sequence banks (accession numbers J02960 (Emorine *et al.*, 1987) and M15169 (Kobilka *et al.*, 1987)). These polymorphisms occur commonly in the general population: Timmermann *et al.* (1998) report the prevalence of the polymorphisms at -20, -47, -654, -1023, -1343 to lie between 33 and 67%, whilst in a random population we found the polymorphisms at -47 (T→C) and -367 (T→C) to have allelic frequencies of 25 and 28% ($n=80$) respectively. As would be expected from polymorphisms which are, in genetic terms, close together, these polymorphisms were found to be in strong linkage disequilibrium: that is, certain combinations of polymorphisms at the different sites occur more frequently than would be predicted by chance. Thus, 92% of individuals were concordant for genotypes at -47 and -367. This results in the presence of extended haplotypes across this region. It is interesting to speculate on the explanation for this region being a mutational hot spot: the accepted rate of polymorphism in coding DNA is around 1 in 1000 and in non coding DNA, 1 in 500. However, in the 5' flanking and coding region of the β_2 adrenergic receptor there are a total of 17 single nucleotide polymorphisms within approximately 3 kb. This is in marked contrast to other G protein coupled receptor genes, which in general show very little polymorphic variation. For example, in a recent study examining the histamine H1 receptor gene, we detected only one degenerate polymorphism (codon 356) (Dewar & Hall, 1998) in a gene of comparable size.

Given the known frequency of polymorphic variation within the human genome it is critical to assess the potential function of identified polymorphisms because the majority of polymorphisms will be functionally silent. An initial screen of the polymorphisms noted in the β_2 adrenergic receptor gene 5' flanking region suggested that whilst some of these polymorphisms might be expected to be non-functional others occur in known regulatory regions. In particular the polymorphisms at -47 and -367 introduce a non conservative amino acid change (Cys→Arg) in the upstream leader peptide (BUP) and create 7/8 bp of a consensus AP-2 site respectively. The leader peptide is believed to inhibit translation of the β_2 adrenergic receptor gene itself (Parola & Kobilka, 1994) and hence a non conservative amino acid change within the leader peptide might be expected to be functionally relevant. Evidence in favour for a role for BUP in inhibiting translation comes from studies in which site directed mutagenesis was used to mutate the start codon for BUP: this produced a marked increase in the level of expression of the

murine β_2 adrenergic receptor gene (Parola & Kobilka, 1994). Using a similar approach we found that a short open reading frame (sORF) inactivation *via* mutation of the human BUP start codon produced a 54% increase in COS-7 cells transfected with the relevant constructs (data not shown). It is also interesting to speculate that the introduction of 7/8 bp of a consensus AP-2 site 7 bp downstream of an overlapping Sp1/AP2 site may be able to alter gene expression through differences in transcription factor transactivation. Further investigation of these possibilities is currently being undertaken. Other potential regulatory motifs in the region of identified polymorphisms are shown in Table 1.

In order to examine the potential functional relevance of the identified polymorphisms we have performed preliminary experiments transfecting reporter constructs containing polymorphic variants of the β_2 adrenergic receptor gene promoter into COS-7 cells. As would be expected from commonly occurring polymorphisms very large differences in the reporter activity induced by constructs containing the commonest haplotypes of the β_2 promoter were not observed. However, we saw a small but consistent and significant reduction in transcriptional activity of the construct p0.55mut β_2 AR-LUC (*c.f.* 'wild-type') containing one common polymorphic variant haplotype, suggesting that individuals with these polymorphisms may have reduced receptor expression. This may be relevant given the known clinical correlates of coding region polymorphisms within the β_2 adrenoceptor gene: in particular the demonstration that the glycine 16 variant of the receptor is associated with increased receptor downregulation both *in vitro* and *in vivo* and with bronchodilator subsensitivity (Tan *et al.*, 1997; Turki *et al.*, 1995a). However, it is important to realize that changes in levels of gene expression may not relate directly to changes in the level of receptor expression at the cell surface. Preliminary evidence suggests that the coding region polymorphisms are also in linkage disequilibrium with the promoter polymorphisms and hence the physiological effects ascribed to the glycine 16 variant could possibly be related to altered gene expression. In summary, therefore, we have identified the presence of eight polymorphisms within the 1470 bp upstream flanking region of the β_2 adrenergic receptor gene. Preliminary data suggest that at least some of these polymorphisms have the potential to alter expression of the β_2 adrenergic receptor gene.

This work was funded in part by the National Asthma Campaign.

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(Received October 27, 1998

Revised November 18, 1998

Accepted November 23, 1998)



SPECIAL REPORT

Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta

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The stable prostacyclin analogue, iloprost relaxes a variety of blood vessels and increases cyclic AMP, although the relationship between adenosine 3':5'-cyclic monophosphate (cyclic AMP) and vasorelaxation remains unclear. We therefore investigated the effect of the adenylyl cyclase inhibitor, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) on iloprost-mediated relaxation and cyclic AMP elevation in endothelium-denuded aortic strips. Iloprost (1–1000 nM) caused a concentration-dependent inhibition of phenylephrine (1–6 μ M) contractions, the responses being unaffected by pre-incubation with SQ22536 (100 μ M) for 30 min. In other experiments 60 nM iloprost caused a 64% inhibition of phenylephrine contractions concomitant with a 3 fold rise in cyclic AMP. SQ22536 completely abolished the iloprost-induced elevation in cyclic AMP while having no significant effect on relaxation. Our results therefore strongly suggest that cyclic AMP-independent pathways are responsible for the vasorelaxant effects of iloprost in guinea-pig aorta.

Keywords: Vascular smooth muscle; iloprost; guinea-pig aorta; cyclic AMP measurement; adenylyl cyclase inhibitor; SQ22536; vasorelaxation

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PSS, physiological saline solution; PGI₂, prostacyclin; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine

Introduction Iloprost is a synthetic carbacyclin derivative of prostacyclin (PGI₂), mediating its effects through activation of cell surface prostanoid (IP) receptors which are known to be coupled to adenylyl cyclase through the G-protein, G_s (Coleman *et al.*, 1994). Like PGI₂, iloprost is a potent inhibitor of platelet aggregation and produces profound vasodilation *in vivo* as evidenced by its ability to relax a variety of isolated arterial preparations *in vitro* (Grant & Goa, 1992). However, the cellular mechanism(s) underlying the vascular action of iloprost remains to be elucidated. Because IP agonists increase cyclic AMP in many cell types, including smooth muscle (Coleman *et al.*, 1994), and responses to these agents can be potentiated by phosphodiesterase inhibitors (Holzmann *et al.*, 1980; Riva *et al.*, 1990), it has been proposed that cyclic AMP is the mediator of iloprost-induced vasorelaxation. This is consistent with the observation that iloprost causes concentration-dependent increases in cyclic AMP in arteries denuded of endothelium (Schroder *et al.*, 1987; Ozaki *et al.*, 1996) and that vasodilation in rat tail artery can be reduced by inhibitors of cyclic AMP-dependent protein kinase A (Schubert *et al.*, 1997). In contrast, low concentrations of PGI₂ have been reported to induce relaxation that is either associated with no detectable change or even a fall in cyclic AMP (see Frolich, 1990). Furthermore, in rabbit aorta PGI₂ increases cyclic AMP causing contraction, not relaxation (Vegesna & Diamond, 1986). Thus the link between cyclic AMP and tissue relaxation remains controversial (Grant & Goa, 1992; Wise & Jones, 1996). Surprisingly, there have been no studies in vascular muscle using IP agonists where tension and cyclic AMP levels have been measured either in the same tissue or with adenylyl cyclase inhibitors. Therefore, the aim of the present study was to

investigate the effect of a direct inhibitor of adenylyl cyclase, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536; Haslam *et al.*, 1978) on iloprost-induced relaxation and cyclic AMP levels in isolated guinea-pig aorta.

Methods *Organ bath studies* Male guinea-pigs (Duncan Harver, 250–300 g) were killed by cervical dislocation and the upper thoracic aorta removed and placed in physiological saline solution (PSS) containing in mM; NaCl 112, KCl 5, CaCl₂ 1.8, MgCl₂ 1, NaHCO₃ 25, KH₂PO₄ 0.5, NaH₂PO₄ 0.5, glucose 10 and 0.03 phenol red (pH 7.4 with 95% O₂/5% CO₂). The tissue was cut into rings (~2 mm wide, and cut longitudinally) or helical strips (~2 mm wide, 10 mm long). The endothelium was denuded by gently rubbing the lumen of the muscle with filter paper. Tissues were mounted in an organ bath (0.3 ml) and tension measured isometrically. Muscle strips were subjected to a basal tension of 1.25 g and continuously perfused with PSS (1.0 ml min⁻¹ at 37°C) for 30 min before being precontracted with phenylephrine (1 or 6 μ M). Removal of the endothelium was confirmed by lack of relaxation to acetylcholine (10 μ M for 2 min). Concentration-response curves were obtained by cumulatively applying iloprost (1–1000 nM) in the absence or presence of the adenylyl cyclase inhibitor, SQ22536 (100 μ M). SQ22536 was given 30 min before the addition of iloprost and remained throughout. A 1 h wash-out period was allowed between concentration-response curves.

Determination of cyclic AMP In other experiments, helical strips were precontracted with phenylephrine (1 μ M) for 40 min before being exposed to iloprost (60 nM) for 5 min in the presence or absence of SQ22536 (100 μ M). SQ22536 was applied for 30 min beforehand. At the end of the protocol, tissues were snap frozen in liquid N₂ and stored at

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–80°C for up to 2 weeks. To extract cyclic AMP, tissues were placed in acidified ethanol (~pH 3) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), homogenized and sonicated for 15 min. Homogenates were stored at 4°C for 18 h, and then centrifuged (12,000 r.p.m.) for 3 min. The supernatant was collected and the pellet resuspended in 66% ethanol followed by centrifugation. The total supernatant was evaporated to dryness at 55°C under a flow of N₂ gas and the residue resuspended in Tris buffer containing 0.4 mM ethylene-diaminetetracetic acid (EDTA). The cyclic AMP content was measured using a radioreceptor assay kit supplied by Amersham (Buckinghamshire, U.K.). Tissue pellets were dissolved in 0.5 mM NaOH and protein content determined using the Lowry method.

Drugs and statistics Phenylephrine and IBMX were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), SQ22536 from Alexis Corporation (Bingham, Nottingham, U.K.) and iloprost was kindly donated by Schering AG (Berlin, Germany). All data are expressed as mean $\pm$ s.e. mean of *n* observations and significance determined using Student's *t*-test or ANOVA with correction for pairwise comparisons of groups (Student-Newman-Keuls Method). *P* values <0.05 were considered significant. The concentration of iloprost causing 50% relaxation of phenylephrine contractions is expressed as the log EC₅₀ value.

Results Aortic rings precontracted with 6 μ M phenylephrine were relaxed by iloprost (1–1000 nM) in a concentration-dependent manner with an log EC₅₀ value for inhibition of phenylephrine-induced tone of -7.54 ± 0.03 (*n*=17; Figure 1). Under these conditions, the maximal relaxation produced by iloprost was $70.9 \pm 5.3\%$ (*n*=17). Pre-incubation for 30 min with the adenylyl cyclase inhibitor, SQ22536 (100 μ M) produced no significant changes in tone, nor did it affect the log EC₅₀ (-7.45 ± 0.1 ; *n*=11) or the maximal relaxation ($71.4 \pm 5.4\%$; *n*=11) elicited by iloprost (*P*>0.30, two tailed *t*-test; Figure 1). In order to ascertain that SQ22536 was effectively inhibiting any rise in cyclic AMP induced by iloprost, cyclic AMP levels were measured in tissues that had been contracted with phenylephrine and exposed to a single concentration of iloprost (60 nM) for 5 min (Figure 2a). Under these conditions, iloprost relaxed phenylephrine (1 μ M) contractions on average by $63.9 \pm 5.0\%$ (*n*=9) and produced a 3 fold rise in cyclic AMP, increasing it from

3.64 ± 0.80 – 10.48 ± 1.46 pmol mg⁻¹ protein (Figure 2c). This rise in cyclic AMP could be completely inhibited by a 30 min pre-incubation with 100 μ M SQ22536 (Figure 2c), although it had no significant effect on the magnitude of relaxation ($61.2 \pm 4.7\%$; *n*=9) induced by iloprost (Figure 2b).

Discussion The data presented in this paper provides strong evidence that cyclic AMP does not play a major role in vasorelaxation induced by the IP agonist, iloprost in guinea-pig aortic smooth muscle. This conclusion is based on the findings that the adenylyl cyclase inhibitor SQ22536 failed to affect relaxation induced by iloprost at any concentration tested, and yet it was able to completely inhibit the rise in cyclic AMP induced by a near maximal concentration of

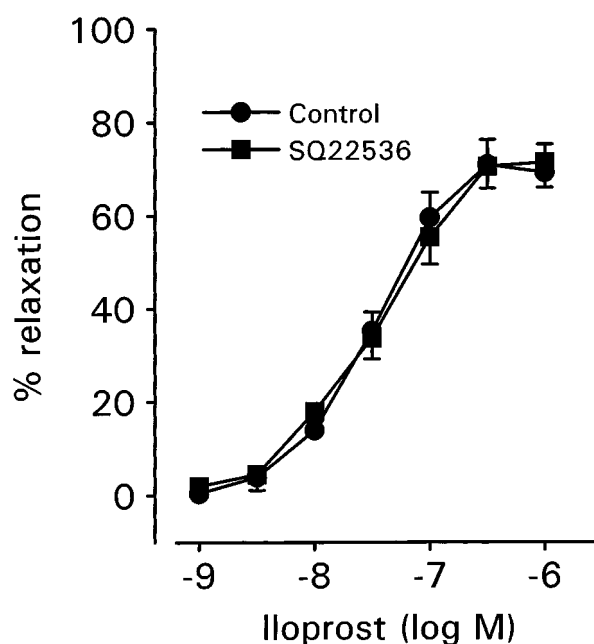


Figure 1 Cumulative concentration-response curves to iloprost (1–1000 nM) measured in the absence (control) and presence of the adenylyl cyclase inhibitor, SQ22536. Aortic rings, denuded of endothelium, were precontracted with 6 μ M phenylephrine and each concentration of iloprost applied for 5 min. SQ22536 (100 μ M) was applied 30 min before the first application of iloprost. Data are expressed as the mean $\pm$ s.e. mean of 11–17 experiments from nine animals.

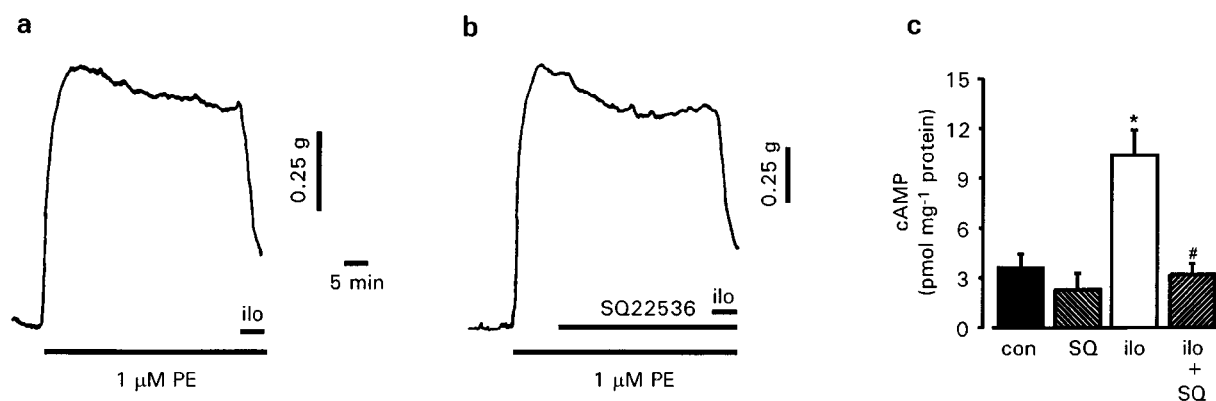


Figure 2 The effect of iloprost (ilo; 60 nM) on endothelium-denuded helical strips precontracted with 1 μ M phenylephrine under control conditions (a) or in the presence of 100 μ M SQ22536 (b). Determination of cyclic AMP was carried out at the end of the experiment and found to be 7.71 and 1.91 pmol mg⁻¹ protein in a and b, respectively. (c) Influence of SQ22536 (SQ) on cyclic AMP levels measured in the absence and presence of iloprost (ilo). Data are expressed as mean $\pm$ s.e. mean of nine experiments from nine animals. **P*<0.05 when compared to control value, #*P*<0.05 when compared to iloprost (one way ANOVA).

iloprost. To our knowledge this is the first study where the effects of IP agonists on tension and cyclic AMP levels have been measured in the same tissue. Moreover, unlike most studies to date, cyclic AMP was measured in tissues not exposed to phosphodiesterase inhibitors, thus allowing a direct comparison between tension and cyclic AMP under conditions where breakdown is not artificially prevented. This is important because not only do phosphodiesterase inhibitors elevate cyclic AMP to extremely high (and probably unphysiological) levels, they often cause substantial relaxation by themselves, and in many cases will also elevate cyclic GMP, thus complicating the interpretation of such data.

In this study we used SQ22536 in preference to other known adenylyl cyclase inhibitors (e.g. 2', 5'-dideoxyadenosine) since this was the only inhibitor out of several tested that we found could completely block the rise in cyclic AMP evoked by activators of adenylyl cyclase; all others reduced cyclic AMP levels by ~50% (Turcato & Clapp, unpublished observations). Whilst we have shown that SQ22536 was unable to block the functional effects of iloprost in this study, results from other studies demonstrate that this inhibitor at similar concentrations, does oppose the action of IP agonists in other cell types. For example, SQ22536 was reported to completely reverse the protective effects of iloprost against neutrophil-induced lung injury (Riva *et al.*, 1990) and also reduce the inhibitory action of IP agonists on platelet aggregation (Armstrong *et al.*, 1986). Thus, in some situations, cyclic AMP does appear to play an important role in mediating the biological effects of IP agonists.

Our previous observations in guinea-pig aorta showing that relaxations to iloprost were markedly inhibited by blockers of Ca^{2+} -activated K^{+} channels, whereas those to forskolin, a direct activator of adenylyl cyclase, were minimally affected (Clapp *et al.*, 1998), would also support the idea that cyclic AMP-independent pathways are involved in mediating relaxation to iloprost in this tissue. Consistent with this, we have observed that the cyclic AMP-dependent protein kinase

inhibitor, H-89 did not inhibit the effects of iloprost (Turcato & Clapp, 1998). Likewise, vasodilator responses to iloprost in the rabbit coronary artery were found to be sensitive to the K_{ATP} channel blocker glibenclamide but not those to forskolin or dibutyryl cyclic AMP (Jackson *et al.*, 1993). Although the above investigations are in direct contrast to studies implicating a role for cyclic AMP (Holzmann *et al.*, 1980; Schroder *et al.*, 1987; Schubert *et al.*, 1997), cellular mechanisms may alter depending on the presence of the endothelium, which is known to enhance the response to IP agonists (Schroder *et al.*, 1987). One possibility is that cyclic AMP plays an important role in controlling the release of endothelium-derived relaxing factors. Indeed iloprost-stimulated cyclic AMP levels have been observed to be significantly higher in endothelium intact vessels (Schroder *et al.*, 1987). Regardless of the underlying biochemical mechanisms associated with IP receptor activation, recent evidence suggests these receptors can couple to other G-protein signalling pathways, including G_i , G_p and possibly G_o (Wise & Jones, 1996). However, which, if any of these pathways mediates the vascular effects of iloprost clearly needs to be investigated, although we cannot at this stage rule out the possibility that G_s is involved as an intracellular mediator.

In conclusion, we suggest that cyclic AMP does not play a major role in mediating the vascular effects of iloprost in guinea-pig aorta. Moreover, in the light of recent evidence that IP receptors can couple to multiple signalling pathways, there needs to be a closer examination of the classical notion that cyclic AMP mediates the cardiovascular effects of IP receptor agonists.

S.T. is funded by a PhD studentship from the British Heart Foundation and L.H.C. is an MRC Senior Research Fellow. This work was supported by a grant from the British Heart Foundation (FS/96058). We thank Schering AG (Berlin, Germany) for the gift of iloprost.

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(Received September 29, 1998

Revised November 19, 1998

Accepted November 20, 1998)



Effects of the prostanoid EP₃-receptor agonists M&B 28767 and GR 63799X on infarct size caused by regional myocardial ischaemia in the anaesthetized rat

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1 This study investigates the effects of two agonists of the prostanoid EP₃-receptor (M&B 28767 and GR 63799X) on the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetized rat.

2 One hundred and sixty-seven, male Wistar rats were anaesthetized (thiopentone, 120 mg kg⁻¹, i.p.), ventilated (8–10 ml kg⁻¹, 70 strokes min⁻¹, inspiratory oxygen concentration: 30%; PEEP: 1–2 mmHg) and subjected to occlusion of the left anterior descending coronary artery (LAD, for 7.5, 15, 25, 35, 45 or 60 min) followed by reperfusion (2 h). Infarct size was determined by staining of viable myocardium with a tetrazolium stain (NBT), histological evaluation by light and electron microscopy and determination of the plasma levels of cardiac troponin T.

3 M&B 28767 (0.5 µg kg⁻¹ min⁻¹, i.v., *n* = 7) or GR 63799X (3 µg kg⁻¹ min⁻¹, i.v., *n* = 7) caused significant reductions in infarct size from 60 ± 3% (25 min ischaemia and 2 h reperfusion; saline-control, *n* = 8) to 39 ± 6 and 38 ± 4% of the area at risk, without causing a significant fall in blood pressure. Pretreatment of rats with 5-hydroxydecanoate (5-HD), an inhibitor of ATP-sensitive potassium channels, attenuated the cardioprotective effects of both EP₃-receptor agonists. The reduction in infarct size afforded by M&B 28767 was also abolished by glibenclamide and the protein kinase C (PKC) inhibitors staurosporine and chelerythrine.

4 Thus, M&B 28767 and GR 63799X reduce myocardial infarct size in the rat by a mechanism(s) which involves the activation of PKC and the opening of ATP-sensitive potassium channels.

Keywords: Cardioprotection; EP-receptors; E-type prostaglandins; GR 63799X; M&B 28767; myocardial ischaemia; myocardial infarction; protein kinase C; troponin T

Abbreviations: EP-receptor, E-type prostanoid receptor; HR, heart rate; LDH, lactate dehydrogenase; MAP, mean arterial blood pressure; PGE₁, prostaglandin E₁; PKC, protein kinase C; PRI, pressure-rate index; TnT, cardiac troponin T; PEEP, positive endexpiratory pressure

Introduction

E-type prostaglandins including prostaglandin E₁ (PGE₁) exert beneficial effects on biochemical, electrocardiographic and functional indices of myocardial ischaemia-reperfusion injury (Judgutt *et al.*, 1981; Schrör *et al.*, 1988; Simpson *et al.*, 1988) and reduce myocardial infarct size (Hide *et al.*, 1995). The effects of E-type prostaglandins are mediated by specific G-protein coupled receptors (EP-receptors) which have been classified into four subtypes, EP₁, EP₂, EP₃ and EP₄ (Coleman *et al.*, 1990). The cardioprotective effects of these eicosanoids may be secondary to a reduction in afterload, an increase in coronary blood flow, inhibition of platelet function and/or inhibition of the activation and extravasation of polymorphonuclear (PMNs) cells (Lucchesi & Mullane, 1986), all of which are secondary to the activation of EP₂-receptors, which activate G_s and cause an activation of adenylate cyclase (Coleman *et al.*, 1990). In addition, the protection of isolated cells or organs by prostaglandins has been attributed to an ill-defined 'cytoprotective' or 'cardioprotective' effect of these agents. The mechanism(s) or the prostanoid receptor(s) mediating this effect is unknown (Schrör, 1987).

In 1995/96, we have discovered that the cardioprotective effects of E-type prostaglandins are (at least in part) due to activation of EP₁ or EP₃-receptors which, in turn, leads to the opening of ATP-sensitive potassium (K_{ATP}) channels (Hide *et al.*, 1995; Hide & Thiemermann, 1996). This hypothesis is supported by the following findings: (1) The cardioprotective effects of PGE₁ (non-selective agonist for all EP-receptors) and sulprostone (selective agonist of EP₁ and EP₃-receptors) are abolished by inhibition of K_{ATP}-channels with glibenclamide or 5-hydroxydecanoate (Hide *et al.*, 1995; Hide & Thiemermann, 1996). (2) Sulprostone causes cardioprotection without having any haemodynamic (EP₂-mediated) effects (Hide & Thiemermann, 1996). (3) Activation of EP₁ and EP₃-receptors may result in activation of protein kinase C (PKC) (Coleman *et al.*, 1990; 1994). As EP₃-receptors are expressed on cardiomyocytes and are up-regulated following ischaemia of the heart (Hohlfeld, 1995; Hohlfeld *et al.*, 1997), we hypothesized that it is the activation of EP₃-receptors which accounts for the 'cardioprotective' and or 'cytoprotective' effects of E-type prostaglandins.

The overall aim of this study was to elucidate the effects of two prostanoid EP₃-receptor agonists, namely M&B 28767 and GR 63799X (see Coleman *et al.*, 1990), on the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetized rat. Having found that these agents do

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indeed reduce myocardial infarct size in the rat, we have subsequently investigated the role of protein kinase C (PKC) and of K_{ATP}-channels in the cardioprotective effects of these agents.

Methods

Coronary artery ligation in the rat in vivo

One hundred and sixty-seven male Wistar rats (240–350 g, Tucks, Reyleigh, Essex, U.K.) were anaesthetized with thiopentone sodium (120 mg kg⁻¹, i.p.). The rats were tracheotomized, intubated and ventilated with a Harvard ventilator (70 strokes min⁻¹, tidal volume: 8–10 ml kg⁻¹, inspiratory oxygen concentration: 30%; PEEP: 1–2 mmHg resulting in pCO₂ values of 36–44 mmHg and pO₂ values over 150 mmHg). Body temperature was maintained at 38 ± 1°C. The right carotid artery was cannulated and connected to a pressure transducer to monitor mean arterial blood pressure (MAP). The right jugular vein was cannulated for the administration of drugs. The chest was opened by a mid-sternal thoracotomy, the pericardium incised and an atraumatic needle was placed with an occluder around the left anterior descending coronary artery (LAD). Subdermal platinum electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). After completion of the surgical procedure the animals were allowed to stabilize for 30 min before infusion of drugs and LAD ligation. The coronary artery was occluded at time 0 by tightening of the occluder. This was associated with the typical electrocardiographic (ST-segment elevation and increase in R-wave amplitude) and haemodynamic changes (fall in MAP) of myocardial ischaemia. After 7.5, 15, 25, 35, 45 or 60 min of acute myocardial ischaemia, the occluder was re-opened to allow the reperfusion of the previously ischaemic myocardium for 2, 4 or 8 h. Heart rate (HR) and MAP were continuously recorded on a 4-channel Grass 7D polygraph recorder. The HR was automatically calculated from systolic pulse curves by means of a Grass 7P4H tachograph. The pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (Baller *et al.*, 1981), was calculated as the product of MAP and HR, and expressed in mmHg min⁻¹ 10³. After re-occluding the LAD and i.v. injection of Evans blue dye (1 ml of 2% w v⁻¹) to stain the area at risk (AR), the heart was removed and cut into four to five horizontal slices. The Evans blue solution stains the perfused myocardium, while the occluded vascular bed remains uncoloured. After removing the right ventricular wall, the AR and non-ischaemic myocardium were separated by following the line of demarcation between blue stained and unstained (pink/red) tissue. To distinguish between ischaemic and infarcted tissue, the AR was cut into small pieces and incubated with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml⁻¹, 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems (normal myocardium), NBT forms a dark blue formazan, while areas of necrosis lack dehydrogenase activity and therefore do not stain (Nachlas & Shnitka, 1963). The AR and infarct size were calculated and expressed as per cent of the AR.

Experimental design (in vivo studies)

Study I The following seven experimental groups were studied to elucidate the effects of varying periods of regional myocardial ischaemia on infarct size and on the plasma levels of cardiac troponin T: (1) No occlusion of the LAD (sham-

operation) plus infusion of vehicle (saline, 2.4 ml kg⁻¹ h⁻¹), starting 10 min prior to LAD-occlusion and maintained throughout the experiment (*n* = 3). (2) LAD-occlusion (7.5 min) and reperfusion (2 h) plus infusion of vehicle (*n* = 5). (3) LAD-occlusion (15 min) and reperfusion plus infusion of vehicle (*n* = 5). (4) LAD-occlusion (25 min) and reperfusion plus infusion of vehicle (*n* = 8). (5) LAD-occlusion (35 min) and reperfusion plus infusion of vehicle (*n* = 5). (6) LAD-occlusion (45 min) and reperfusion plus infusion of vehicle (*n* = 11). (7) LAD-occlusion (60 min) and reperfusion plus infusion of vehicle (*n* = 6).

Study II The following two experimental groups were studied to elucidate the effects of increasing periods of reperfusion after 25 min of myocardial ischaemia on infarct size: (1) LAD-occlusion (25 min) and reperfusion (4 h) plus infusion of vehicle (saline, 2.4 ml kg⁻¹ h⁻¹), starting 10 min prior to LAD-occlusion and maintained throughout the experiment (*n* = 5). (2) LAD-occlusion (25 min) and reperfusion (8 h) plus infusion of vehicle (*n* = 5).

Study III This study was designed to elucidate the effect of the EP₃-receptor agonists M&B 28767 and GR 63799X on the infarct size caused by regional myocardial ischaemia (25 min) and reperfusion (2 h). We have subsequently investigated the role of the activation of K_{ATP}-channels and/or PKC in the cardioprotective effects of M&B 28767 and GR 63799X. To do this, the following further 19 experimental groups were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle (identical to group 1 in study I, *n* = 3). (2) LAD-occlusion (25 min) and reperfusion (2 h) plus infusion of vehicle (identical to group 4 in study I, *n* = 8). (3) LAD-occlusion and reperfusion plus infusion of M&B 28767 (0.5 µg kg⁻¹ min⁻¹, i.v. starting 10 min prior to coronary artery occlusion, *n* = 7). (4) No occlusion of the LAD (sham-operation) plus infusion of M&B 28767 (*n* = 3). (5) LAD-occlusion and reperfusion plus infusion of GR 63799X (3 µg kg⁻¹ min⁻¹, i.v. starting 10 min prior to coronary artery occlusion, *n* = 7). (6) No occlusion of the LAD (sham-operation) plus infusion of GR 63799X (*n* = 3). (7) LAD-occlusion and reperfusion plus injection of 5-HD (5 mg kg⁻¹, i.v. at 10 min prior to coronary artery occlusion, *n* = 6). (8) No occlusion of the LAD (sham-operation) plus injection of 5-HD (*n* = 3). (9) LAD-occlusion and reperfusion plus administration of 5-HD 10 min prior the infusion of M&B 28767 (*n* = 6). (10) LAD-occlusion and reperfusion plus administration of 5-HD 10 min prior the infusion of GR 63799X (*n* = 6). (11) LAD-occlusion and reperfusion plus injection of glibenclamide (0.3 mg kg⁻¹, i.v. at 10 min prior to coronary artery occlusion, *n* = 6). (12) No occlusion of the LAD (sham-operation) plus injection of glibenclamide (*n* = 3). (13) LAD-occlusion and reperfusion plus administration of glibenclamide 10 min prior the infusion of M&B 28767 (*n* = 4). (14) LAD-occlusion and reperfusion plus injection of staurosporine (1 µg kg⁻¹, i.v. 10 min prior to LAD-occlusion, *n* = 6). (15) No occlusion of the LAD (sham-operation) and injection of staurosporine (*n* = 3). (16) LAD-occlusion and reperfusion plus administration of staurosporine 10 min prior the infusion of M&B 28767 (*n* = 6). (17) LAD-occlusion and reperfusion plus injection of chelerythrine (0.7 mg kg⁻¹, i.v. 10 min prior to LAD-occlusion, *n* = 6). (18) No occlusion of the LAD (sham-operation) and injection of chelerythrine (*n* = 3). (19) LAD-occlusion and reperfusion plus administration of chelerythrine 10 min prior the infusion of M&B 28767 (*n* = 6).

The *n*-numbers in the above experimental groups refer to animals, which survived until the end of the experiment. The

average mortality was approximately 13%. The number of animals which died in the individual groups of animals studied were the following: *Study I*: group 1, 0; group 2, 0; group 3, 0; group 4, 1; group 5, 1; group 6, 2; group 7, 2; *Study II*: group 1, 0; groups 2, 0; *Study III*: group 1, 0; group 2, 1 (identical to group 4, study I); group 3, 1; group 4, 0; group 5, 1; group 6, 0; group 7, 1; group 8, 0; group 9, 1; group 10, 1; group 11, 2; group 12, 0; group 13, 5; group 14, 1; group 15, 0; group 16, 1; group 17, 1; group 18, 0; group 19, 0.

Measurement of the plasma levels of cardiac troponin T in the rat

At the end of the experiment a blood sample (1 ml) was obtained from the carotid cannula and centrifuged to obtain plasma. To do this the following experimental groups were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle ($n=3$). (2) LAD-occlusion (7.5 min) and reperfusion (2 h) plus infusion of vehicle (identical to group 2 in study I, $n=3$). (3) LAD-occlusion (15 min) and reperfusion plus infusion of vehicle (identical to group 3 in study I, $n=3$). (4) LAD-occlusion (25 min) and reperfusion plus infusion of vehicle ($n=6$). (5) LAD-occlusion (35 min) and reperfusion plus infusion of vehicle (identical to group 5 in study I, $n=3$). The concentration of cardiac troponin T was determined by the STAT (short-turn-around-time) assay (provided by Boehringer Mannheim, Germany) using an Elecixs[®] System 2010.

Determination of the degree of myocardial tissue injury by lightmicroscopy

Biopsies of all sections of the heart (non-ischaemic, ischaemic and infarcted) were fixed in paraformaldehyde (4% w v⁻¹), embedded in paraffin, cut into section (4 μ m), de-waxed and stained with Fuchsin and Luxol-Fast-Blue according to Bancroft & Stevens (1995).

Determination of the degree of myocardial tissue injury by electronmicroscopy

Biopsies of all sections of the heart (non-ischaemic, ischaemic and infarcted) were fixed in paraformaldehyde (4% w v⁻¹), embedded in paraffin. All biopsies were de-paraffinized (washed twice for 5 min in xylol and then in decreasing concentrations of ethanol, 100–50%). The sample was subsequently fixed again in osmium tetroxid (1% w v⁻¹) and incubated in increasing concentrations of ethanol (50–100%) to remove any excess water. The biopsies were then embedded in Agar 100 Resin (Plano, Marburg, Germany) and cut into section of 1 μ m (Ultracut R, Leica, Cologne, Germany) and stained according to Richardson. Selected samples were then cut into ultrathin slices (90 nm) (Ultracut R, Leica, Cologne, Germany). The samples were then stained with lead acetate and analysed with an EM410 electronmicroscope (Phillips, Germany). All tissue biopsies were evaluated by an experienced cardiovascular pathologist in double-blind fashion (M.O.).

Drugs and materials

Unless otherwise stated all compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Thiopentone sodium (Intraval[®]) was obtained from May & Baker Ltd. (Dagenham, U.K.). Chelerythrine and staurosporine were from Calbiochem (Nottingham, U.K.). We thank Dr Simon

Lister (GlaxoWellcome, Research and Development, Stevenage, Herts, U.K.) for the generous supply of GR 63799X and Dr Jean Hough (Rhône-Poulenc Rorer, Research and Development, Dagenham, U.K.) for the generous supply of M&B 28767.

Statistical analysis

All values in the text, figures and tables are expressed as the means $\pm$ s.e. mean of n observations. Statistical analysis was performed (on absolute values) by one-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni's test for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Effects of varying lengths of regional myocardial ischaemia followed by reperfusion (2 h) on myocardial infarct size and plasma levels of cardiac troponin T in the rat

In rats which were subjected to the surgical procedure, but not to LAD-occlusion (sham-operation), there was no significant degree of myocardial necrosis as determined by NBT staining (Figure 1a). Occlusion of the LAD for 7.5 min followed by reperfusion for 2 h resulted in an infarct size of $\sim 33\%$ of the area at risk. Increasing the length of the period of regional ischaemia resulted in a time-related increase in infarct size (Figure 1a). A maximal degree of infarction of ~ 70 – 75% of the area at risk was observed after 35 min.

In rats which were subjected to the surgical procedure, but not to LAD-occlusion (sham-operation), there was no significant increase in the plasma levels of the cardiac-specific marker troponin T (Figure 1b). Occlusion of the LAD for 7.5 min followed by reperfusion for 2 h resulted in a significant increase in the plasma levels of cardiac troponin T (to ~ 14 ng ml⁻¹). Increasing the length of the period of regional ischaemia resulted in a time-related, further increase in the plasma levels of cardiac troponin T (Figure 1b).

Morphological alterations caused by regional myocardial ischaemia (25 min) and reperfusion (2 h) in the anaesthetized rat

Histological evaluation (by light microscopy) of biopsies of the area at risk (which failed to stain with NBT) of hearts subjected to regional ischaemia (25 min) and reperfusion (2 h) demonstrated the occurrence of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, which demonstrates a strong staining with Fuchsin (Figure 2a) as well as submaximal staining with Luxol-Fast Blue (Figure 2b) in the peripheral myocytes, whereas the central zone is negative. All nuclear details have disappeared. The section also demonstrates a substantial degree of extravasation of red blood cells.

Evaluation by electron microscopy of biopsies of the area at risk (which failed to stain with NBT) of hearts subjected to regional ischaemia (25 min) and reperfusion (2 h) demonstrated nuclei with margination and clumping of chromatin with intervening areas of pale nucleoplasm. A granular disruption of the I-bands and Z-lines compared to a normal A-band was also demonstrated (Figure 3a). The mitochondria

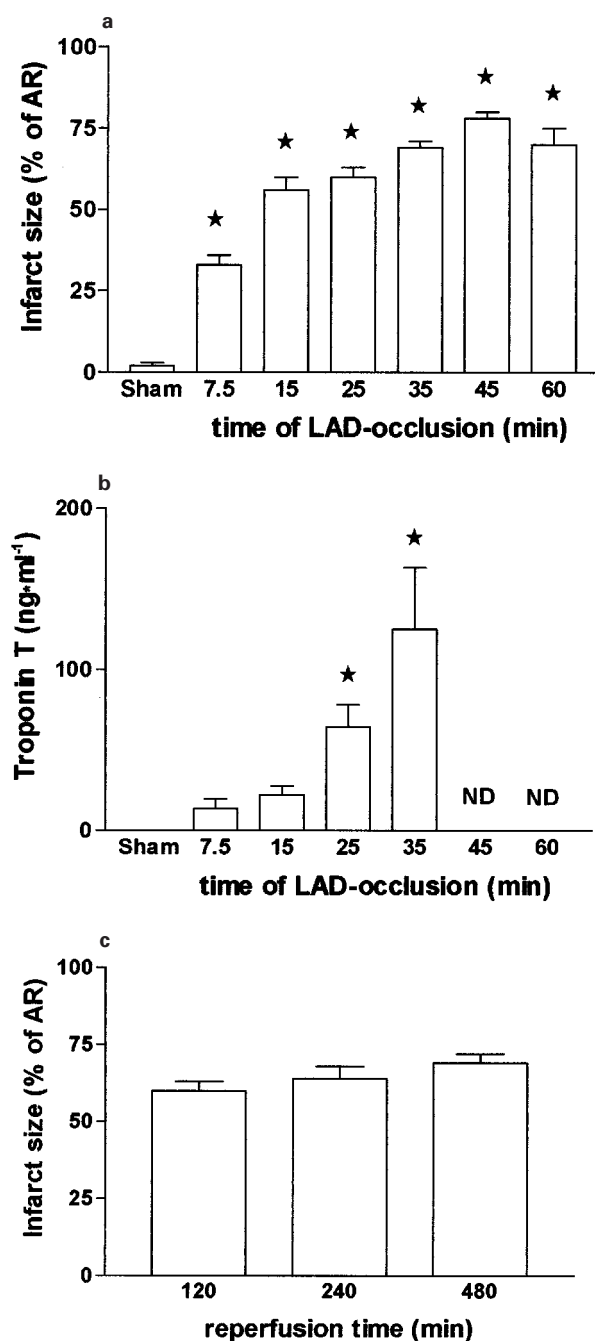


Figure 1 (a) Infarct size (expressed as per cent of the area at risk, AR) caused by occlusion 7.5, 15, 25, 35, 45 or 60 min and followed by reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. (b) Alterations in the plasma levels of cardiac troponin T caused by occlusion for 7.5, 15, 25 or 35 min and followed by reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. * $P < 0.05$ when compared to sham-operated control. ND, not determined. (c) Infarct size (expressed as per cent of the area at risk, AR) caused by occlusion for 25 min and reperfusion for 2, 4 or 8 h of the left anterior descending coronary artery (LAD) in the anaesthetized rat.

were markedly swollen, the inner compartment was electron translucent and expanded. Contraction bands (Figure 3b and c) as well as oedema was widely present. In several places, osmophilic degeneration (Figure 3b) of mitochondria as well as flocculent densities (Figure 3c) and intramitochondrial calcifications (Figure 3b and c) were present. In some places, the T tubules were swollen and partially disrupted.

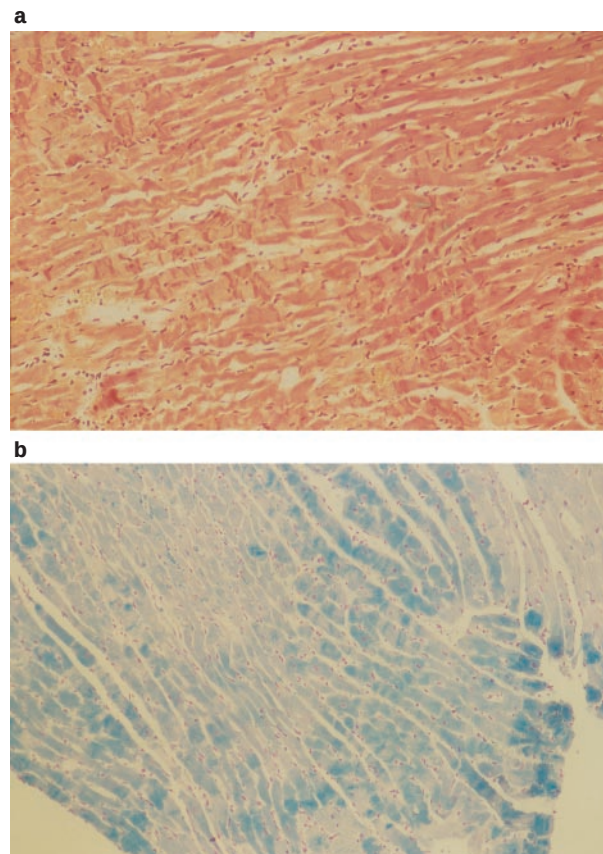


Figure 2 (a) Histological section after Fuchsin staining of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. Depicted is an example of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, which demonstrates a strong staining with Fuchsin. (b) Histological section after Luxol-Fast-Blue staining of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. Depicted is a further example of complete coagulation necrosis with eosinophilic cytoplasm of myocytes which demonstrates a submaximal staining with Luxol-Fast-Blue in the peripheral myocytes.

Effects of varying lengths of reperfusion following 25 min of regional myocardial ischaemia on myocardial infarct size in the rat

Occlusion of the LAD for 25 min followed by reperfusion for 2 h resulted in an infarct size of ~60% of the area at risk. Increasing the length of the reperfusion period to either 4 or 8 h did not result in a further, significant increase in infarct size (Figure 1c).

Effects of M&B 28767 or GR 63799X on the infarct size caused by regional myocardial ischaemia (25 min) and reperfusion (2 h) in the rat in vivo

The mean values for the areas at risk ranged from 41 ± 4 , to $52 \pm 3\%$ and, hence, were similar in all animal groups studied ($P > 0.05$, data not shown). In rats which had received an infusion of the vehicle (saline) for the EP₃-receptor agonists, occlusion of the LAD (for 25 min) followed by reperfusion (for 2 h) resulted in an infarct size of $60 \pm 3\%$ of the area at risk (control, $n = 8$). When compared to vehicle, infusion of either M&B 28767 ($n = 7$) or GR 63799X ($n = 7$) caused a significant reduction in infarct size of approximately 35% (Figure 4).

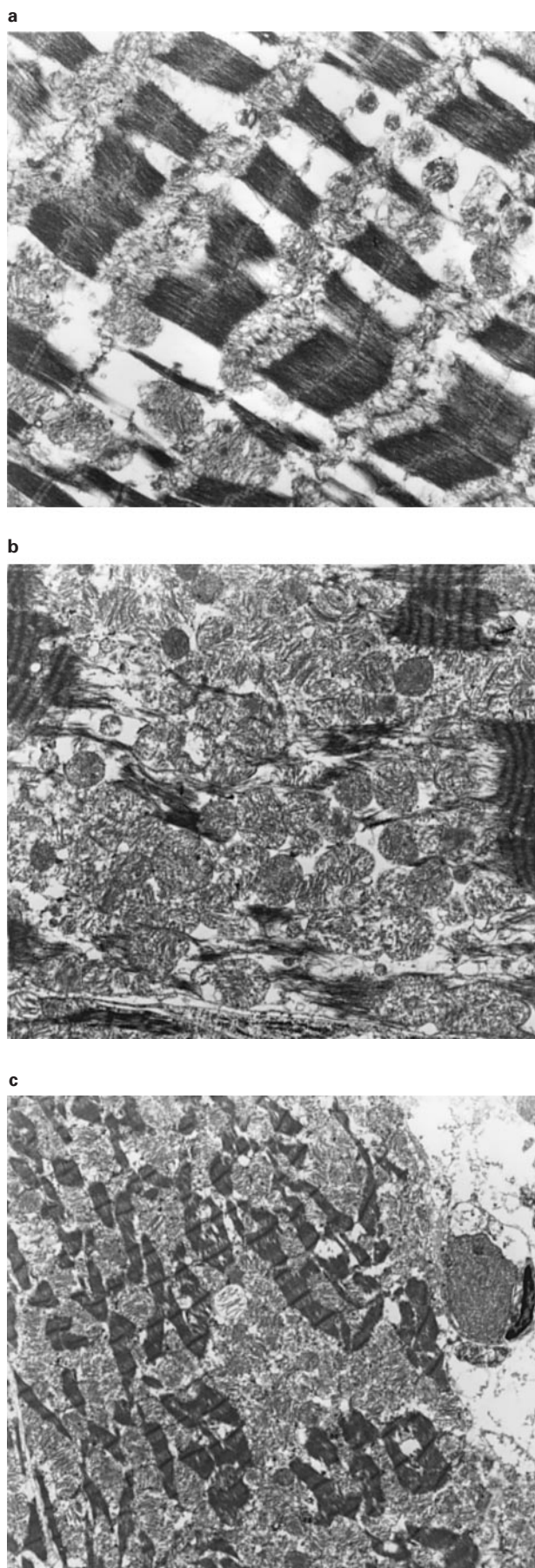


Figure 3 Electron microscopical section of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. (a) Depicted are typical examples of granular disruption of the I-bands and the Z-lines compared to a normal A-band. (b)

Pretreatment of rats with the K_{ATP}-channel blocker 5-HD abolished the reduction in infarct size afforded by the subsequent infusion of either M&B 28767 (Figure 4a, $n=6$) or GR 63799X (Figure 4b, $n=6$, $P>0.05$ when compared to control). However, pretreatment of rats with 5-HD prior to commencing the infusion of vehicle for the EP₃-receptor agonists (saline) did not result in a significant reduction in myocardial infarct size ($P>0.05$ when compared to vehicle-control) (Figure 4). Pretreatment of rats with glibenclamide also abolished the reduction in infarct size afforded by M&B 28767 (to $68 \pm 2\%$, $n=4$), while glibenclamide did not affect infarct size when given to rats treated with vehicle for M&B 28767 (infarct size: $64 \pm 6\%$, $n=6$). Similarly, pretreatment of rats with the PKC inhibitor staurosporine also abolished the reduction in infarct size afforded by M&B 28767 (Figure 5a), while staurosporine did not affect infarct size when given to rats treated with vehicle for M&B 28767 (Figure 5a). Pretreatment of rats with the PKC inhibitor chelerythrine also abolished the reduction in infarct size afforded by M&B 28767

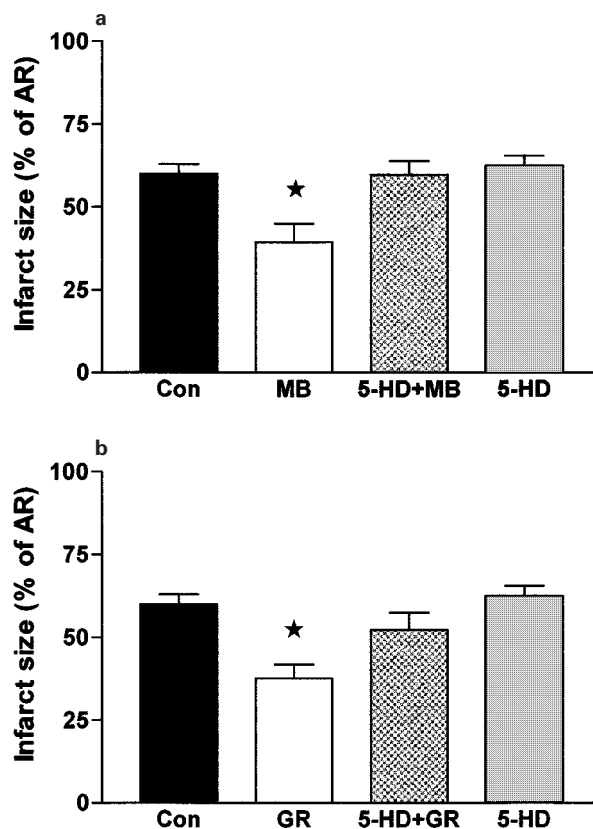


Figure 4 Infarct size caused by occlusion (25 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. Different groups of animals were treated with (a) vehicle (control, $n=8$), M&B 28767 ($0.5 \mu\text{g kg}^{-1} \text{ h}^{-1}$, i.v., $n=7$), 5-hydroxydecanoate (5-HD, 5 mg kg^{-1} , i.v., $n=6$), 5-HD plus M&B 28767 ($n=6$) and (b) vehicle (control, $n=8$), GR 63799X ($3.0 \mu\text{g kg}^{-1} \text{ h}^{-1}$, i.v., $n=7$), 5-HD (5 mg kg^{-1} , i.v., $n=6$), 5-HD plus GR 63799X ($n=6$). * $P<0.05$ when compared to control.

Depicted is an example of early contraction bands, osmophilic degradation of mitochondria and intramitochondrial calcifications. (c) Depicted are typical examples of swollen or osmophilic degraded mitochondria as well as vacuolization of the wall of small vessels. These findings are similar to those exhibited by sections of human heart at 12 h after transmural myocardial infarction.

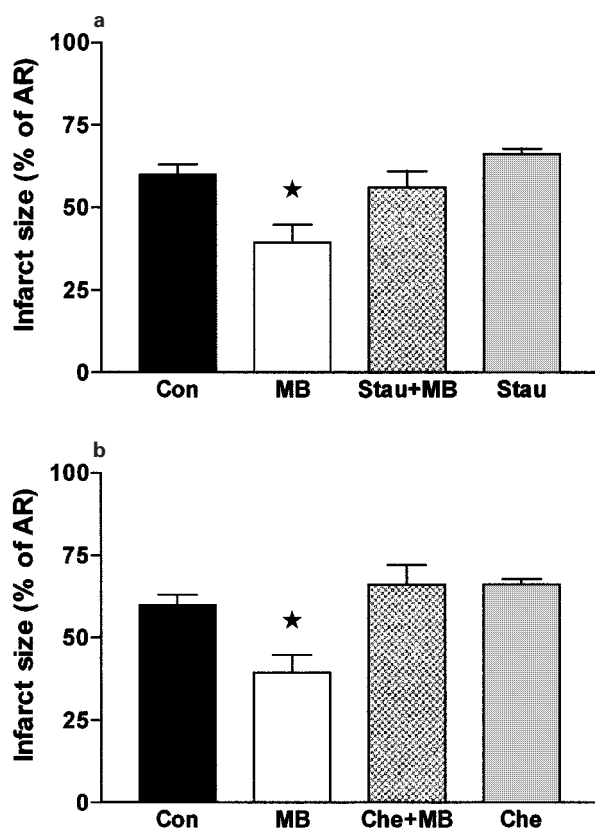


Figure 5 Infarct size caused by occlusion (25 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. Different groups of animals were treated with (a) vehicle (control, $n=8$), M&B 28767 ($0.5 \mu\text{g kg}^{-1} \text{h}^{-1}$, i.v., $n=7$), staurosporine ($1 \mu\text{g kg}^{-1}$, i.v., $n=6$), staurosporine plus M&B 28767 ($n=6$) and (b) vehicle (control, $n=8$), M&B 28767 ($0.5 \mu\text{g kg}^{-1} \text{h}^{-1}$, i.v., $n=7$), chelerythrine (0.7mg kg^{-1} , i.v., $n=6$), chelerythrine plus M&B 28767 ($n=6$). * $P<0.05$ when compared to control.

(Figure 5b), while chelerythrine did not affect infarct size when given to rats treated with vehicle for M&B 28767 (Figure 5b).

Sham-operation did not result in a significant degree of infarction in any of the groups studied (less than 3% of the AR, data not shown).

Haemodynamic effects of the EP₃-receptor agonists, M&B 28767 or GR 63799X, in rats subjected to regional myocardial ischaemia and reperfusion

Values for MAP, HR and PRI measured during the course of the experiments are given in Tables 1 and 2. Baseline haemodynamic data (e.g. MAP, HR and PRI) were similar ($P>0.05$) in all groups studied. In sham-operated rats (no LAD occlusion), infusion of vehicle (saline), 5-HD, M&B 28767 or GR 63799X, staurosporine or chelerythrine did not (within 60 min) cause any significant effects on any of the parameters measured. In some of the groups studied, the mean values for heart rate were, however, lower than in the control group (Tables 1 and 2). In rats subjected to LAD-occlusion and reperfusion which received an infusion of saline (control, $n=8$), mean values for MAP and PRI fell throughout the experimental period, but there was no alteration in heart rate (Table 1). In rats subjected to LAD-occlusion and reperfusion and treated with GR 63799X, the heart rate was lower than in the respective control group ($P<0.05$ at times -10 to 85 min), while there was no difference in either MAP or PRI ($P>0.05$). In rats subjected to LAD-occlusion and reperfusion and

treated with M&B 28767, the heart rate was lower than in the respective control group at one time point in the reperfusion period ($P<0.05$ at 85 min). However, M&B 28767 did not affect MAP or PRI in rats subjected to LAD-occlusion and reperfusion (Table 1). Pretreatment of rats subjected to LAD-occlusion and reperfusion with 5-HD, staurosporine or chelerythrine did also not result in any significant haemodynamic effects (Table 1). In rats subjected to LAD-occlusion and reperfusion and treated with 5-HD and GR 63799X, the heart rate was lower than in the respective control group at times 0 and 15 min ($P<0.05$). In rats subjected to LAD-occlusion and reperfusion and treated with staurosporine plus M&B 28767, the heart rate was lower than in the respective control group ($P<0.05$ at times -10 to 145 min). Similarly, in rats subjected to LAD-occlusion and reperfusion and treated with chelerythrine plus M&B 28767, the heart rate was lower than in the respective control group ($P<0.05$ at time points 0 , 15 , 85 and 145 min) (Tables 1 and 2). None of the above interventions, however, had any effect on MAP or PRI.

Discussion

This study demonstrates that two agonists of the prostanoid EP₃-receptor (M&B 28767 and GR 63799X) reduce the myocardial infarct size caused by coronary artery occlusion and reperfusion in the anaesthetized rat. As in previous studies (Hide *et al.*, 1995; Hide & Thiemermann, 1996), we have determined the degree of necrosis caused by regional ischaemia and reperfusion of the heart by staining of viable tissue within the area at risk (non-perfused myocardium) with the tetrazolium dye NBT. In the presence of intact dehydrogenase enzyme systems (viable myocardium), NBT forms a dark blue formazan, whilst areas of necrosis lack dehydrogenase activity and therefore do not stain (Nachlas & Shnitka, 1963). To ensure that those sections of the area at risk which failed to stain with NBT do indeed represent 'necrotic' myocardium, we have subsequently subjected biopsies of these sections to a histological analysis. Evaluation of these sections by light microscopy demonstrated the occurrence of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, the absence of any nuclear details as well as a substantial degree of extravasation of erythrocytes. The additional evaluation by electron microscopy of these biopsies also demonstrated (i) nuclei with margination and clumping of chromatin with intervening areas of pale nucleoplasm, (ii) granular disruption of the I-bands and Z-lines compared to a normal A-band, (iii) swollen mitochondria with intra-mitochondrial calcifications, (iv) contraction band necrosis (Reichenbach & Bendit, 1968; Baroldi, 1975) as well as (v) oedema. These data confirm that regional ischaemia (for 25 min) followed by reperfusion (for 2 h) does indeed lead to the development of a myocardial infarction, which can be detected by light microscopy, electron microscopy and the absence of staining with NBT. In order to further characterize the time-course of the development of myocardial infarction in the rat, we have elucidated the effects of increasing periods of regional myocardial ischaemia (from 7.5 – 60 min) followed by 2 h of reperfusion on infarct size. Clearly, there was a time-dependent increase in myocardial infarct size, which was maximal after 45 min. In subsequent studies, we have documented that an increase of the reperfusion period from 2 to 8 h does not lead to a further, significant increase in infarct size in the rat. This is

Table 1 Mean arterial pressure (MAP), heart rate (HR) and pressure rate index (PRI) in rats subjected to 25 min of LAD-occlusion and 2 h reperfusion

Group	Time (min)						
	-20	-10	0	Occlusion		Reperfusion	
				15	25	85	145
<i>Sham vehicle</i> (n=3)							
MAP (mmHg)	127±29	120±23	117±20	115±17	103±13	98±5	102±9
HR (beats min ⁻¹)	420±0	410±10	400±10	400±10	380±20	340±20*	370±26
PRI (mmHg min ⁻¹ 10 ⁻³)	53±12	49±10	46±7	46±6	39±4	33±1	37±1
<i>Vehicle control</i> (n=8)							
MAP (mmHg)	123±9	122±7	122±8	122±8	116±6	93±6	87±4
HR (beats min ⁻¹)	458±11	458±11	454±11	454±11	450±11	454±12	445±14
PRI (mmHg min ⁻¹ 10 ⁻³)	56±5	56±4	56±4	56±5	52±4	42±2	39±3
<i>MB 28767</i> (n=7)							
MAP (mmHg)	125±7	120±5	112±6	110±8	109±6	111±7	101±8
HR (beats min ⁻¹)	420±19	411±14	403±11	390±15	386±18	373±16*	394±21
PRI (mmHg min ⁻¹ 10 ⁻³)	53±4	49±3	45±3	43±3	42±2	41±3	40±4
<i>Sham MB 28767</i> (n=3)							
MAP (mmHg)	135±10	120±6	113±9	109±6	108±7	100±6	95±3
HR (beats min ⁻¹)	450±0	440±10	440±10	420±17	410±10	400±20	390±17
PRI (mmHg min ⁻¹ 10 ⁻³)	61±5	53±4	50±4	46±1	44±2	40±1	37±2
<i>GR 63799X</i> (n=7)							
MAP (mmHg)	134±2	126±4	113±8	109±6	119±6	124±8	115±9
HR (beats min ⁻¹)	399±9	386±10*	364±13*	351±19*	351±19*	356±10*	356±11
PRI (mmHg min ⁻¹ 10 ⁻³)	53±1	49±3	41±4	38±4	42±3	44±3	41±4
<i>Sham GR 63799X</i> (n=3)							
MAP (mmHg)	124±8	117±9	105±8	113±12	113±12	123±12	122±16
HR (beats min ⁻¹)	410±26	400±20	400±20	370±36	370±36	350±56*	370±70
PRI (mmHg min ⁻¹ 10 ⁻³)	51±2	47±2	42±3	42±6	42±6	43±6	45±7
<i>5-HD</i> (n=6)							
MAP (mmHg)	127±10	124±10	119±9	112±9	102±10	98±8	96±5
HR (beats min ⁻¹)	425±20	425±20	425±20	430±23	430±20	415±20	435±24
PRI (mmHg min ⁻¹ 10 ⁻³)	54±5	53±6	51±5	48±3	43±4	41±4	41±2
<i>Sham 5-HD</i> (n=3)							
MAP (mmHg)	120±12	113±9	113±9	99±6	93±3	95±3	97±4
HR (beats min ⁻¹)	450±17	450±17	460±10	440±20	430±10	410±10	410±10
PRI (mmHg min ⁻¹ 10 ⁻³)	54±7	51±6	52±5	44±5	40±2	39±1	40±2
<i>5-HD + MB 28767</i> (n=6)							
MAP (mmHg)	141±7	139±8	134±8	129±7	121±7	113±4	113±3
HR (beats min ⁻¹)	415±12	410±13	385±20	400±13	385±20	385±12	405±13
PRI (mmHg min ⁻¹ 10 ⁻³)	59±3	57±3	52±4	52±3	47±4	44±1	46±0
<i>5-HD + GR 63799X</i> (n=6)							
MAP (mmHg)	133±10	132±11	119±10	116±4	110±5	120±10	113±10
HR (beats min ⁻¹)	395±27	395±22	360±22*	370±24*	365±26	405±19	385±20
PRI (mmHg min ⁻¹ 10 ⁻³)	53±5	52±5	43±2	43±3	40±3	49±4	44±3

M&B 28767 or GR 63799X were administered as a continuous infusion (0.5 µg kg⁻¹ h⁻¹, i.v. or 3.0 µg kg⁻¹ h⁻¹, i.v.) starting 10 min prior to coronary artery occlusion and continued until the end of reperfusion. 5-hydroxydecanoate (5-HD) was administered as an i.v. bolus (5 mg kg⁻¹, i.v.) 10 min before onset of the M&B 28767 or GR 63799X infusion. In addition, sham-operation (no coronary artery-occlusion) and administration of saline, M&B 28767, GR 63799X or 5-HD was carried out. **P*<0.05 when compared to vehicle control.

not entirely surprising, as in rabbits subjected to regional myocardial ischaemia, an increase in the length of the reperfusion period from 2 to 8 h does not result in a significant further increase in infarct size in the rat. This is not entirely surprising as in rabbits subjected to regional myocardial ischaemia, an increase in the length of the reperfusion period from 2 to 8 h does also not result in a significant further increase in infarct size (McMurdo *et al.*, 1994). In addition to measuring myocardial cell necrosis by NBT-staining or histology, the determination of the plasma levels of enzymes which are released by cardiac myocytes may also be used as a reliable indicator of tissue necrosis. The most specific, biochemical marker of cardiac myocyte necrosis (which can be measured in plasma) is cardiac troponin T (Adams *et al.*, 1993). Cardiac troponin T is a structural, regulatory protein which is only present in

cardiac myocytes so that an increase in the plasma levels of this marker is synonymous with myocardial cell necrosis (Adams *et al.*, 1993). Unlike plasma levels of creatine phosphokinase or lactate dehydrogenase, which are relatively non-specific and, hence, are elevated in open-chest models of myocardial infarction due to the surgical procedure (Adams *et al.*, 1993; Zacharowski & Thiemermann, unpublished data), the thoracotomy employed here did not result in a detectable rise in the plasma levels of cardiac troponin T. Increasing periods of regional myocardial ischaemia, however, resulted in time-dependent increases in the plasma levels of cardiac troponin T. This finding also supports our conclusion (see above) that 25 min of regional myocardial ischaemia followed by 2 h of reperfusion leads to a significant degree of myocardial necrosis.

Table 2 Mean arterial pressure (MAP), heart rate (HR) and pressure rate index (PRI) in rats subjected to 25 min of LAD-occlusion and 2 h reperfusion

Group	Time (min)			Occlusion		Reperfusion	
	-20	-10	0	15	25	85	145
<i>Sham vehicle (n=3)</i>							
MAP (mmHg)	127 ± 29	120 ± 23	117 ± 20	115 ± 17	103 ± 13	98 ± 5	102 ± 9
HR (beats min ⁻¹)	420 ± 0	410 ± 10	400 ± 10	400 ± 10	380 ± 20	340 ± 20*	370 ± 26
PRI (mmHg min ⁻¹ 10 ⁻³)	53 ± 12	49 ± 10	46 ± 7	46 ± 6	39 ± 4	33 ± 1	37 ± 1
<i>Vehicle control (n=8)</i>							
MAP (mmHg)	123 ± 9	122 ± 7	122 ± 8	122 ± 8	116 ± 6	93 ± 6	87 ± 4
HR (beats min ⁻¹)	458 ± 11	458 ± 11	454 ± 11	454 ± 11	450 ± 11	454 ± 12	445 ± 14
PRI (mmHg min ⁻¹ 10 ⁻³)	56 ± 5	56 ± 4	56 ± 4	56 ± 5	52 ± 4	42 ± 2	39 ± 3
<i>Staurosporin (n=6)</i>							
MAP (mmHg)	103 ± 6	99 ± 6	97 ± 5	94 ± 4	88 ± 2	89 ± 5	91 ± 4
HR (beats min ⁻¹)	425 ± 12	420 ± 13	425 ± 12	430 ± 17	430 ± 17	420 ± 20	470 ± 10
PRI (mmHg min ⁻¹ 10 ⁻³)	44 ± 2	42 ± 3	41 ± 3	40 ± 2	38 ± 2	38 ± 3	42 ± 1
<i>Sham Stau (n=3)</i>							
MAP (mmHg)	103 ± 7	101 ± 10	98 ± 8	91 ± 5	93 ± 9	87 ± 3	90 ± 0
HR (beats min ⁻¹)	410 ± 36	410 ± 36	410 ± 36	400 ± 40	420 ± 35	410 ± 36	410 ± 36
PRI (mmHg min ⁻¹ 10 ⁻³)	43 ± 6	42 ± 8	41 ± 7	37 ± 6	39 ± 4	35 ± 2	37 ± 3
<i>Stau + MB 28767 (n=6)</i>							
MAP (mmHg)	111 ± 3	114 ± 5	113 ± 4	107 ± 8	104 ± 8	94 ± 7	86 ± 5
HR (beats min ⁻¹)	395 ± 14	385 ± 18*	380 ± 17*	375 ± 13*	370 ± 10*	365 ± 8*	370 ± 13*
PRI (mmHg min ⁻¹ 10 ⁻³)	44 ± 3	44 ± 3	43 ± 4	40 ± 3	38 ± 4	34 ± 1	32 ± 0
<i>Chelerythrine (n=6)</i>							
MAP (mmHg)	101 ± 7	102 ± 8	102 ± 8	89 ± 9	83 ± 7	88 ± 5	83 ± 4
HR (beats min ⁻¹)	435 ± 17	430 ± 13	430 ± 13	435 ± 10	430 ± 13	440 ± 6	435 ± 7
PRI (mmHg min ⁻¹ 10 ⁻³)	44 ± 2	44 ± 3	44 ± 3	39 ± 3	36 ± 3	39 ± 2	36 ± 2
<i>Sham Chel (n=3)</i>							
MAP (mmHg)	138 ± 15	133 ± 12	133 ± 12	127 ± 13	121 ± 12	108 ± 15	101 ± 12
HR (beats min ⁻¹)	440 ± 26	430 ± 36	430 ± 36	420 ± 35	420 ± 35	420 ± 17	420 ± 30
PRI (mmHg min ⁻¹ 10 ⁻³)	61 ± 10	57 ± 10	57 ± 10	53 ± 10	51 ± 9	45 ± 8	42 ± 8
<i>Chel + MB 28767 (n=6)</i>							
MAP (mmHg)	121 ± 9	119 ± 10	117 ± 11	114 ± 12	108 ± 11	93 ± 11	89 ± 11
HR (beats min ⁻¹)	440 ± 13	420 ± 11	380 ± 15*	383 ± 13*	385 ± 12	365 ± 9*	375 ± 13*
PRI (mmHg min ⁻¹ 10 ⁻³)	53 ± 3	50 ± 3	44 ± 4	44 ± 4	42 ± 2	34 ± 3	33 ± 2

M&B 28767 was administered as a continuous infusion (0.5 µg kg⁻¹ h⁻¹, i.v. or 3.0 µg kg⁻¹ h⁻¹, i.v.) starting 10 min prior to coronary artery occlusion and continued until the end of reperfusion. The PKC inhibitors staurosporine (1 µg kg⁻¹) or chelerythrine (0.7 mg kg⁻¹) were administered as an i.v. bolus 10 min before onset of the M&B 28767 infusion. In addition, sham-operation (no coronary artery-occlusion) and administration of staurosporine or chelerythrine was carried out. **P* < 0.05 when compared to vehicle control.

The observed (moderate) reduction in infarct size amounted to 35% and, hence, was similar to the reductions in infarct size caused by either PGE₁ (non-selective EP₃-receptor agonist) or sulprostone (selective EP₁/EP₃-receptor agonist) in the anaesthetized rabbit (~40%, see Hide *et al.*, 1995; Hide & Thiemermann, 1996). A reduction in infarct size of this magnitude may well be biologically important, as the reduction in left ventricular function (Field *et al.*, 1972) as well as the incidence of arrhythmias (Roberts *et al.*, 1975) occurring as a consequence of myocardial ischaemia are directly proportional to the size of the infarcted myocardium.

Mechanism(s) of the cardioprotective effect of EP₃-receptor agonists in the rat

What, then, is the mechanism(s) by which agonists of the EP₃-receptor reduce myocardial infarct size? Clearly, a reduction in blood pressure and, hence, myocardial oxygen consumption does not account for the cardioprotective effects of either M&B 28767 or GR 63799X, as neither of these EP₃-receptor agonists caused a significant reduction in blood pressure or pressure-rate index. There is a strong positive correlation between myocardial oxygen consumption and pressure-rate index (Baller *et al.*, 1981) and, hence, the observed

cardioprotective effects of M&B 28767 or GR 63799X are not likely to be secondary to a reduction in myocardial oxygen demand. Although the heart rate of rats treated with M&B 28767 or GR 63799X was (at some time points) lower than in the respective control group, it is unlikely that a moderate reduction in heart rate (which was not sufficient to cause a significant reduction in PRI) accounts for the cardioprotective effects of these EP₃-receptor agonists. This conclusion is also supported by our finding that a similar reduction in heart rate in rats subjected to myocardial ischaemia-reperfusion injury and treated with staurosporine and M&B 28767 was not associated with a significant reduction in myocardial infarct size.

Our finding that neither M&B 28767 nor GR 63799X reduced blood pressure in the rat also confirms that (at the doses used) neither M&B 28767 nor GR 63799X activate EP₂-receptors *in vivo*, activation which results in stimulation of G_s, an increase in cyclic AMP and vasodilatation. Thus, it is very unlikely that the doses of M&B 28767 or GR 63799X used in this study are sufficient to activate EP₂-receptors *in vivo*. We, therefore, propose that the observed cardioprotective effects are due to the activation of EP₃-receptors. In order to gain a better insight into the mechanism(s) by which the EP₃-receptor agonists M&B 28767 or GR 63799X reduce myocardial infarct

size in the rat, we carried out a number of further experiments to elucidate the signal transduction events underlying the observed cardioprotective effects.

Role of ATP-sensitive potassium channels in the cardioprotective effects of M&B 28767 or GR 63799X

The reduction in infarct size caused by either PGE₁ or by the EP₃/EP₃-receptor agonist sulprostone is (at least in part) due to the activation and opening of K_{ATP}-channels (Hide *et al.*, 1995; Hide & Thiemermann, 1996). Here we report that the cardioprotective effect of M&B 28767 and GR 63799X are abolished by pretreatment of the animals with 5-HD, a selective blocker of K_{ATP}-channels (Garlid *et al.*, 1997). Although glibenclamide is not a specific inhibitor of cardiac K_{ATP}-channels, both glibenclamide and 5-HD have been used to document that the cardioprotective effects of ischaemic preconditioning involve the activation of K_{ATP}-channels (Hide & Thiemermann, 1996). We confirm here that the reduction in infarct size afforded by M&B 28767 is also abolished by a dose of glibenclamide, which has previously been shown to abolish the cardioprotective effects of 'ischaemic preconditioning' (Hide & Thiemermann, 1996). These findings suggest that M&B 28767 and GR 63799X (and presumably the activation of EP₃-receptors) leads to opening of K_{ATP}-channels which, in turn, results in cardioprotection. The mechanism by which opening of K_{ATP}-channels protects the myocardium against ischaemic injury is not clear.

Role of protein kinase C in the cardioprotective effects of M&B 28767

The signal transduction events involved in the cardioprotective effects of M&B 28767 or GR 63799X are reminiscent of those that mediate the potent anti-ischaemic effects of 'ischaemic preconditioning' (Downey & Cohen, 1995). There is evidence that 'preconditioning of the myocardium' which ischaemia results in the release of adenosine and other mediators which

activate G-protein coupled receptors resulting in activation of protein kinase C, opening of K_{ATP}-channels and ultimately cardioprotection (Downey & Cohen, 1995; Millar *et al.*, 1996). Having demonstrated that the cardioprotective effects of M&B 28767 are abolished by glibenclamide and 5-HD, we have investigated the potential role of PKC in the observed cardioprotective effects of this EP₃-receptor agonist. We demonstrate here that the reduction in infarct size afforded by M&B 28767 in the rat is abolished by two inhibitors of PKC, namely staurosporine and chelerythrine. It should be noted that staurosporine and chelerythrine inhibit the activation of PKC *in vivo* and *ex vivo* (Speechly-Dick *et al.*, 1994; Kaye *et al.*, 1995; Kozak *et al.*, 1997) and also abolish the cardioprotective effects of 'ischaemic preconditioning' in rodents (Speechly-Dick *et al.*, 1994; Yoshida *et al.*, 1997). These findings suggest that the signal transduction events leading to a reduction in infarct size caused by M&B 28767 involve the activation of PKC.

Conclusion

In conclusion, this study demonstrates that two groups of the prostanoid EP₃-receptor (M&B 28767 and GR 63799X) reduce infarct size in rats subjected to regional myocardial ischaemia and reperfusion. The mechanism(s) of the cardioprotective effects of these agents are not entirely clear, but may involve the activation of PKC and the opening of ATP-sensitive potassium channels. We also propose that selective agonists of the prostanoid EP₃-receptor may be useful to protect the heart against ischaemia-reperfusion injury without causing significant haemodynamic (side-) effects.

K.Z. is supported by the Deutsche Gesellschaft für Kardiologie-Herz- und Kreislaufforschung A.O. is supported by the Deutsche Forschungsgemeinschaft (OL 109/1-1). C.T. is a Senior Fellow of the British Heart Foundation (BHF FS 96/018).

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(Received September 1, 1998

Revised October 22, 1998

Accepted November 11, 1998)



Prostanoid receptors involved in the relaxation of human pulmonary vessels

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1 To characterize the prostanoid receptors on human pulmonary smooth muscle involved in vasodilatations, isolated arteries and veins were contracted with norepinephrine (10 μ M) and vessels were subsequently challenged with different prostanoid-receptor agonists in the absence or presence of selective antagonists.

2 Prostaglandin D₂ (PGD₂) and the selective DP-receptor agonist, BW245C, induced relaxations in the contracted human pulmonary venous preparations. The pD₂ values were: 6.88 ± 0.11 ($n=17$) and 7.31 ± 0.12 ($n=5$), respectively. The relaxant responses induced by PGD₂ were reduced by the selective DP-receptor antagonist, BWA868C, and the estimated pA₂ value was 7.84 ± 0.16 ($n=4$). PGD₂ and BW245C did not relax contracted human pulmonary arteries.

3 The selective IP-receptor agonists, iloprost and cicaprost, both induced relaxations in the contracted human vascular preparations. The pD₂ values for iloprost were: 7.84 ± 0.08 ($n=6$) and 8.25 ± 0.06 ($n=4$) and for cicaprost: 8.06 ± 0.12 ($n=5$) and 8.11 ± 0.09 ($n=5$) in arteries and veins respectively.

4 Prostaglandin E₂ (PGE₂) and the EP₂/EP₃-receptor agonist, misoprostol, partially relaxed the contracted venous preparations and the pD₂ values were: 8.10 ± 0.15 ($n=15$) and 6.24 ± 0.33 ($n=3$), respectively. These relaxations suggest the presence of an EP receptor in the human pulmonary veins. The contracted human pulmonary arteries did not relax when challenged with PGE₂.

5 In human pulmonary venous preparations, the PGE₂-induced relaxations were neither modified by treatment with TP/EP₄-receptor antagonist, AH23848B (10 and 30 μ M, $n=6$), nor by the DP/EP₁/EP₂-receptor antagonist, AH6809 (3 μ M, $n=6$).

6 These data suggest that the relaxation induced by prostanoids involved DP-, IP-receptors and to a lesser extent an EP-receptor on human pulmonary venous smooth muscle. In contrast, only the IP-receptor is involved in the prostanoid induced relaxations on human pulmonary arterial smooth muscle.

Keywords: Human pulmonary vessels; relaxation; prostanoid receptors; prostaglandin; cicaprost; iloprost; AH6809; AH23848B; BW245C; BWA868C

Abbreviations: E_{max}, maximal relaxation; K_B value, equilibrium dissociation constant for the antagonist; L-NOARG, N^G-nitro-L-arginine; NC, not calculable; PG, prostaglandin

Introduction

Prostanoids may contract or relax smooth muscle by activating different prostanoid receptors (Coleman *et al.*, 1994b). Results derived from isolated tissue show that the activation of TP-, FP-, EP₁- or EP₃-receptor subtypes produce smooth muscle contraction by increasing Ca²⁺ or reducing cyclic AMP intracellular levels (Negishi *et al.*, 1995). TP- and EP₃-receptors have already been implicated in the vasoconstriction of human pulmonary vessels (Maddox *et al.*, 1985; Lumley *et al.*, 1989; Norel *et al.*, 1991; Quian *et al.*, 1994). When prostanoids activate the EP₂-, EP₄-, DP- or IP-receptor subtypes, present on smooth muscle, they induce relaxation by elevating intracellular cyclic AMP levels (Negishi *et al.*, 1995). Receptors present on the endothelial layer have only a small effect on the vasorelaxant response induced by the prostanoids. In fact, the relaxant responses provoked by these mediators were slightly or not modified after removal of endothelium in human isolated hand veins or pulmonary arteries (Arner *et al.*, 1994; Jones *et al.*, 1997).

In whole human lung samples, using the techniques of molecular biology, the prostanoid receptors associated with smooth muscle relaxation have been demonstrated, namely, the EP₂- (Regan *et al.*, 1994), EP₄- (An *et al.*, 1993; Bastien *et al.*, 1994) and IP-receptor (Nakagawa *et al.*, 1994; Boie *et al.*, 1994). However, the DP-receptor (Boie *et al.*, 1995) has not been detected. In human pulmonary vessels and specifically in pulmonary veins, the prostanoid receptors implicated in smooth muscle relaxation have not been determined. The aim of the present study was to characterize the prostanoid receptors present on human pulmonary vascular smooth muscle which are associated with vasodilatation.

Methods

Isolated preparations

Human lung tissues were obtained from patients (43 male and 3 female) who had undergone surgery for lung carcinoma. The mean age was 62 ± 1 years. Pulmonary arterial and venous

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preparations were removed, dissected free from adjoining connective tissue and lung parenchyma, placed in Tyrode's solution (concentration mM): NaCl 139.2, KCl 2.7, CaCl₂ 1.8, MgCl₂ 0.49, NaHCO₃ 11.9, NaH₂PO₄ 0.4 and glucose 5.5; pH 7.4 and maintained at 4°C. All tissues were used within 1–12 h postsurgery. Vascular preparations were cut as rings (3–6 mm internal diameter, 3–5 mm in length). The rings were then set up in 10-ml organ baths containing Tyrode's solution, gassed with 95% O₂/5% CO₂ and maintained at 37°C. An optimal load (1.5 g), which ensured maximal physiological responses to the agonists, was applied to each ring.

Sprague-Dawley rat tracheal preparations were used to confirm the efficacy of the antagonist AH23848B. These tissues were set up as rings under an initial load of 2 g.

Changes in force were recorded by isometric force displacement transducers (Narco F-60) and physiographs (Linseis). Subsequently, preparations were allowed to equilibrate for 90 min with bath fluid changes taking place every 10 min.

Experimental protocol

After the equilibration period, the vascular preparations were incubated 30 min with BAY u3405 (1 µM), indomethacin (1.7 µM) and 15 min with N^G-nitro-L-arginine (L-NOARG; 0.1 mM). These agents were used to avoid any physiological effect induced by the activation of the TP-receptor or by the release of endogenous prostanoids and nitric oxide. The preparations were then contracted with norepinephrine (10 µM), when the contraction reached a plateau, increasing concentrations of prostanoids or selective agonists were applied in a cumulative fashion. In some experiments different antagonists: AH23848B (10 or 30 µM), AH6809 (3 µM) or BWA868C (0.1–30 µM) were added to the tissue bath for 30 min before the norepinephrine-induced contraction to determine their affinity values. The maximal relaxation of each preparation with papaverine (0.1 mM) was obtained at the end of the experimental protocols.

In experiments using rat tissues, subsequent to the equilibration period, four rat tracheal rings were incubated (30 min) with BAY u3405 (1 µM), indomethacin (1.7 µM), atropine (1 µM) and (last 15 min) with L-NOARG (0.1 mM). Concomitantly, two of these rings were also incubated (30 min) with AH23848B (30 µM). Preparations were then contracted with carbachol (10 µM), when a plateau was reached, cumulative concentration-effect curves were induced with PGE₂.

Data analysis

The changes in force were measured from isometric recordings and expressed in grams (g). The relaxations produced with the different agonists were expressed as per cent of the relaxation induced with papaverine. The maximal relaxation ($E_{\max}$ value) produced with the highest concentration of an agonist and the half-maximum effective concentration value (EC_{50} value) were interpolated from the individual concentration-effect curves. The pD_2 values were calculated as the negative log of EC_{50} values. When the pD_2 values obtained in the presence and absence of antagonist were significantly different, the equilibrium dissociation constant for the antagonist (K_B value) was calculated. The following equation was used: $K_B = [B]/(DR - 1)$, where $[B]$ is the concentration of the antagonist and DR (dose ratio) is the ratio of EC_{50} of agonist in the presence and absence of antagonist. The pK_B values were calculated as the negative log of K_B values. In the relaxation study with

PGD₂ on the veins, different concentrations of BWA868C were used to determine the pA_2 value according to the method of Arunlakshana & Schild (1959). For each lung sample, Schild plot analysis was performed, the slope and pA_2 value was determined by least square fitting of a regression line to the points. All results are expressed as means $\pm$ s.e.mean and were derived from different lung samples (n). Statistical analysis was performed using ANOVA with a confidence level of 95%.

Compounds

PGE₂, PGE₁, PGD₂ and misoprostol (($\pm$)-11, 16-dihydroxy-16-methyl-9-oxoprost-13-en-1-oic acid methyl ester) were purchased from Cayman Chemical Company, Ann Arbor, MI, U.S.A. Iloprost (5-[(E)-(1S,5S,6R,7R)-7-hydroxy-6-[(E)-(3S,4RS)-3-hydroxy-4-methyl-1-octen-6-ynyl]bicyclo[3.3.0]octan-3-ylidene]pentanoic acid) and cicaprost ([2-[hexahydro-5-hydroxy-4-(3-hydroxy-4-methyl-1,6-nonadynyl)-2-(1H)-pentalenylidene]ethoxy] acetic acid) were a gift from Schering AG, Berlin, Germany. AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) and AH23848B ([1 α (z),2 β ,5 α)-(±)-7-[5-[[[1,1'-biphenyl]-4-yl]methoxy]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid) were a gift from Glaxo Wellcome, U.K. BAY u3405 (3(R)-3-(4-fluorophenylsulphonamido)-1,2,3,4-tetrahydro-9-carbazole propanoic acid) and butaprost were a gift from Bayer, Stokes Poges, U.K. BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl) hydantoin) and BWA868C (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino) hydantoin) were a gift from Wellcome Research Laboratories, Beckenham, U.K. Norepinephrine, atropine sulphate, carbamylcholine chloride (carbachol), L-NOARG (N^G-nitro-L-arginine) and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Papaverine was obtained from Meram Laboratories (77020 Melun, France).

Results

Arterial and venous preparations were obtained from 24 and 40 different human lungs, respectively. The cocktail of inhibitors and antagonists (indomethacin, L-NOARG and BAY u3405) with which the vascular preparations were first incubated have a slight contractile effect on the basal tone of these preparations (artery: 0.39 ± 0.09 g and vein: 0.11 ± 0.04 g). Arterial and venous preparations were contracted with norepinephrine (10 µM: 1.7 ± 0.17 g and 2.21 ± 0.15 g, respectively). At the end of the protocols, preparations relaxed with papaverine (0.1 mM: 2.05 ± 0.18 g and 2.59 ± 0.18 g, respectively).

Concentration-dependent relaxations of human pulmonary venous preparations produced by PGD₂ and BW245C are shown in Figure 1a and Table 1. BWA868C (0.1–30 µM) caused a concentration-related rightward shift of the PGD₂ concentration-effect curves. A mean pA_2 value of 7.84 ± 0.16 ($n = 4$) and a mean slope of 0.67 ± 0.07 , which was significantly less than unity, were derived from Schild Plots (Figure 2). In additional lung samples pK_B values were calculated for BWA868C against PGD₂ or BW245C and are presented in Table 1. PGE₁ and the stable PGI₂ analogues (iloprost and cicaprost) produced concentration-dependent relaxations (Figure 3a and Table 2) in human pulmonary venous preparations. PGE₂ and misoprostol partially reversed the norepinephrine-induced contraction in the human pulmonary veins (Figure 4a and Table 1). The PGE₂-induced relaxations were not modified

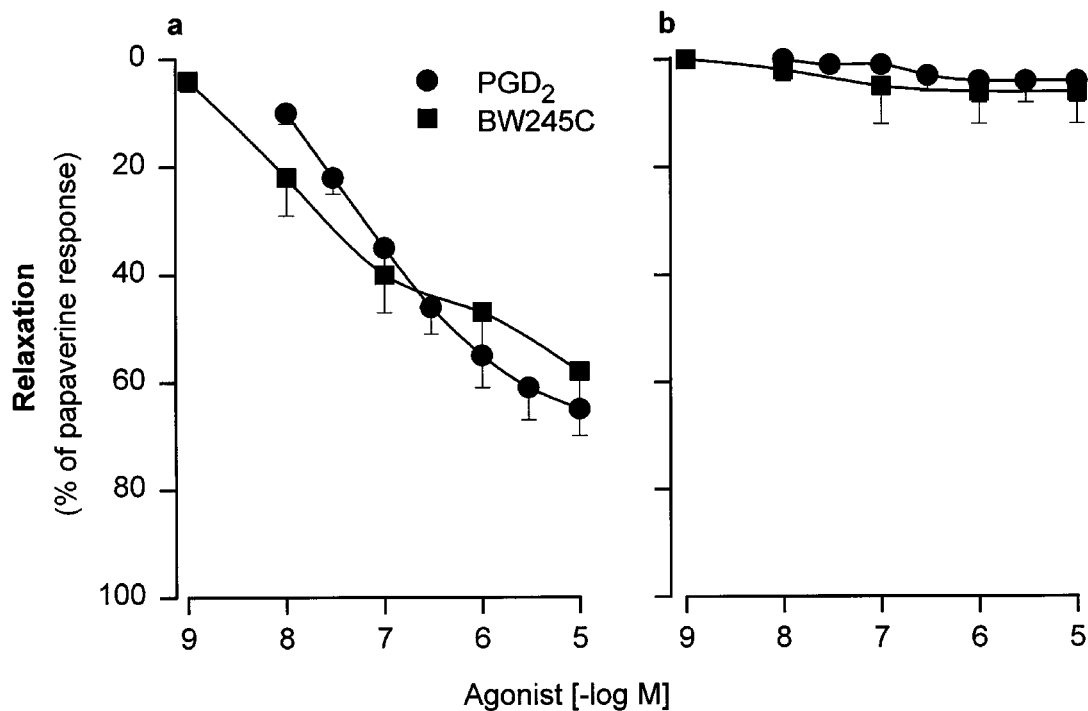


Figure 1 Relaxation of human isolated pulmonary veins (a) and arteries (b) induced by PGD_2 or BW245C. Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and number of lung samples used are indicated in Table 1 and 3.

Table 1 Relaxation of human isolated pulmonary venous preparations: effect of DP- and EP-receptor agonists or antagonists

Treatment	Concentration (μM)	n	E_{max} (%)	Agonist	pD_2 value	pK_B value
Tyrode BWA868C	0.1 0.3 1 3	18	65 $\pm$ 05	PGD_2	6.89 $\pm$ 0.10	
		6	47 $\pm$ 11		6.10 $\pm$ 0.17*	7.23 $\pm$ 0.25
		5	51 $\pm$ 09		5.76 $\pm$ 0.27*	7.54 $\pm$ 0.14
		3	22 $\pm$ 03		5.33 $\pm$ 0.47*	7.37 $\pm$ 0.32
		4	42 $\pm$ 18		5.38 $\pm$ 0.30*	7.11 $\pm$ 0.16
Tyrode BWA868C	1	5	58 $\pm$ 07	BW245C	7.31 $\pm$ 0.12	
		3	69 $\pm$ 23		6.16 $\pm$ 0.06*	7.18 $\pm$ 0.12
Tyrode		3	31 $\pm$ 09	Misoprostol	6.24 $\pm$ 0.33	
Tyrode AH6809 AH23848B AH23848B	3 10 30	15	43 $\pm$ 04	PGE_2	8.10 $\pm$ 0.15	
		6	38 $\pm$ 08		8.24 $\pm$ 0.39	NC
		6	39 $\pm$ 08		7.38 $\pm$ 0.24	NC
		6	51 $\pm$ 13		7.46 $\pm$ 0.33	NC

The maximal responses (E_{max}) were expressed as per cent of the relaxation induced by papaverine (0.1 mM). Values are means $\pm$ s.e.mean, (n) indicates the number of lung samples used. NC: not calculable. *Values significantly different when results from control (Tyrode) and treated tissues were compared by ANOVA.

in the presence of AH6809 (3 μM ; Figure 4a and Table 1) or AH23848B (10 or 30 μM ; Table 1). In paired tissues, comparison of pD_2 values obtained in presence of AH23848B were not statistically different from the control values (pD_2 values: 7.87 ± 0.17) obtained in venous preparations derived from the same human lung sample. However, this antagonist at 30 μM inhibited the relaxation curves induced by PGE_2 in the rat tracheal preparations (data not shown). Butaprost, a PGE_1 analogue, provoked concentration-dependent relaxations of human pulmonary venous preparations (pD_2 value: 5.00 ± 0.11 and E_{max} value: $62 \pm 15\%$ of papaverine response, $n = 4$; Figure 5a). However, AH6809 (3 μM) did not modify the butaprost-induced vasodilatations in this tissue (pD_2 value: $4.60 - 5.22$ and E_{max} value: $38 - 89\%$ of papaverine response,

$n = 2$; Figure 5a). BWA868C, AH6809 and AH23848B at the concentrations used had no effect on the basal tone of the human pulmonary veins.

The norepinephrine induced contractions in the human pulmonary arteries were reversed by PGE_1 , iloprost and cicaprost (Figure 3b and Table 2). Butaprost produced also concentrations-dependent relaxations in this tissue (pD_2 value: 4.40 ± 0.07 and E_{max} value: $59 \pm 17\%$ of papaverine response; $n = 3$; Figure 5b). In addition, the butaprost-induced responses were not altered when arterial preparations were pre-treated with AH6809 (3 μM ; pD_2 value: 4.57 ± 0.17 and E_{max} value: $37 \pm 15\%$ of papaverine response $n = 4$; Figure 5b). AH6809 (3 μM) did not modify the basal tone of the human pulmonary arteries. PGE_2 , PGD_2 and BW245C were without effect on the

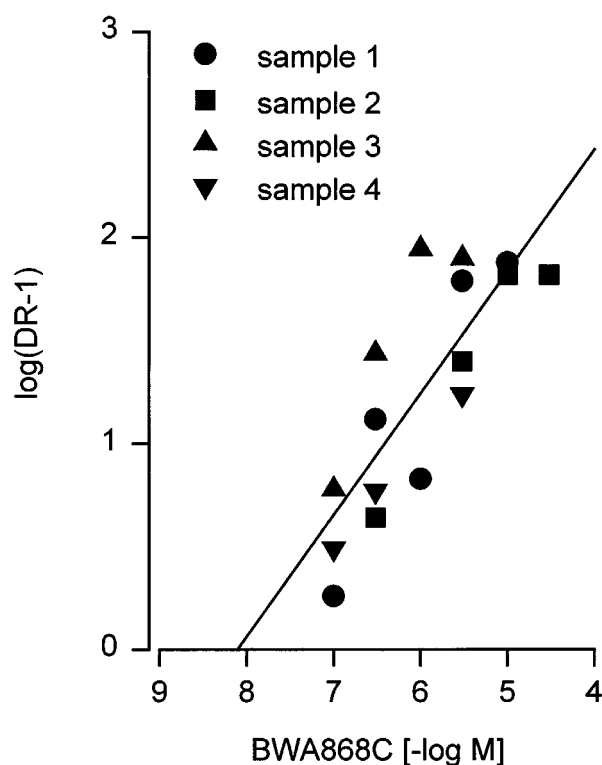


Figure 2 Schild-plot analysis of the antagonism by BWA868C of relaxant concentration-effect curves to PGD_2 in veins derived from four human lung samples. Analysis was based on pD_2 values calculated in treated preparations which were significantly different from control values. The linear regression presented was performed by use of all data points. However, regressions ($r=0.95 \pm 0.02$) were also performed through data derived from each lung sample. The calculated average of pA_2 values and slopes are indicated in Results section.

Table 2 Relaxant effects of IP-receptor agonists on human isolated pulmonary vascular preparations

Agonist	n	Artery		n	Vein	
		$E_{\max}$ (%)	pD_2 value		$E_{\max}$ (%)	pD_2 value
Cicaprost	5	83 ± 02	8.06 ± 0.12	5	71 ± 07	8.11 ± 0.09
Iloprost	6	91 ± 03	7.84 ± 0.08	4	91 ± 03	8.25 ± 0.06
PGE_1	4	78 ± 04	6.34 ± 0.20	4	62 ± 14	7.86 ± 0.31

The maximal responses ($E_{\max}$) were expressed as per cent of the relaxation induced by papaverine (0.1 mM). Values are means $\pm$ s.e.mean and (n) indicates the number of lung samples used. The relaxation induced by PGE_1 or iloprost were performed in presence of AH6809 ($3 \mu\text{M}$).

norepinephrine-induced arterial contractions (Figures 1b, 4b and Table 3).

Discussion

IP-receptors appear to be the only prostanoid receptors implicated in the vasodilatation of the human pulmonary arteries. In contrast, the present report suggest the involvement of DP-, IP- and to a lesser extent EP-receptors in the relaxation of human pulmonary venous preparations. The subtype(s) of these latter receptors remain uncharacterized since the responses induced by PGE_2 were not modified by AH6809 and AH23848B.

The relaxations observed with iloprost and cicaprost, two IP-receptor selective agonists (Schrör *et al.*, 1981; Stürzebecher *et al.*, 1985), indicate the presence of IP-receptor in human pulmonary arterial preparations. These results are in agreement with the data previously reported by Haye-Legrand *et al.* (1987). In addition, results (present report) suggest that PGE_1

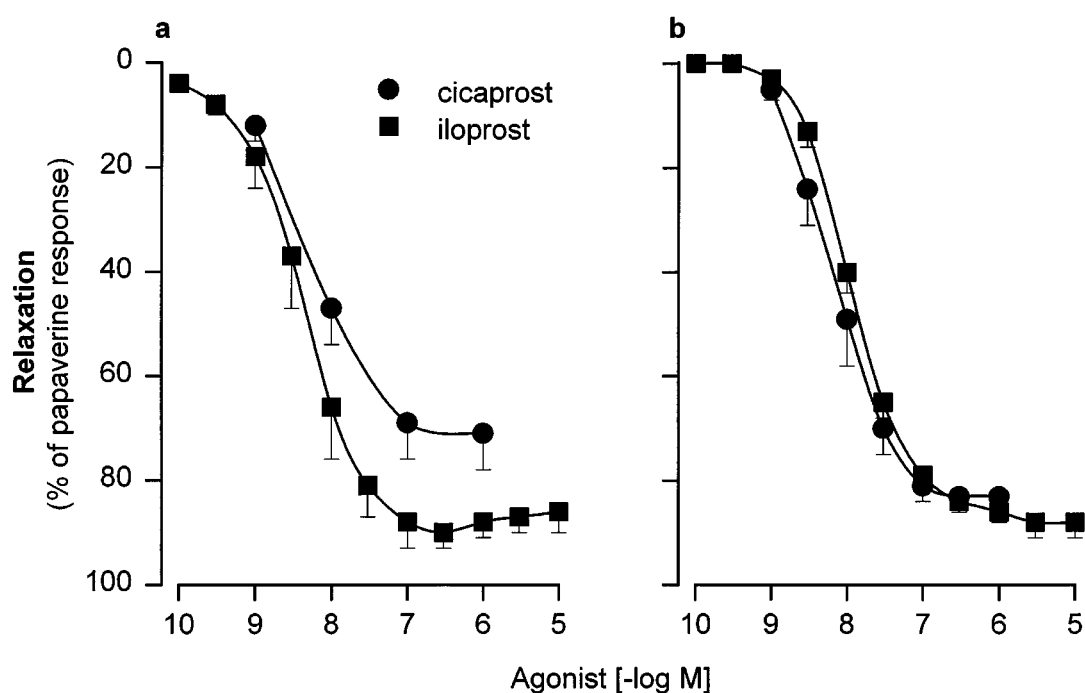


Figure 3 Relaxation of human isolated pulmonary veins (a) and arteries (b) induced by cicaprost or iloprost. Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and number of lung samples used are indicated in Table 2.

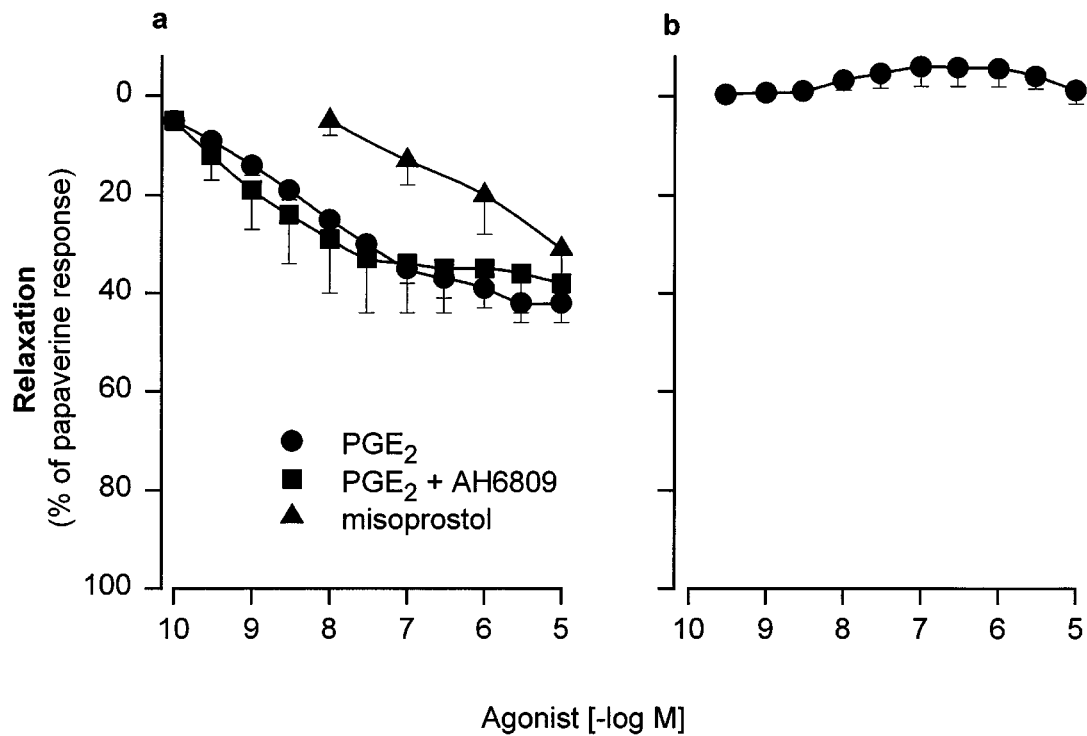


Figure 4 Relaxations of human isolated pulmonary veins (a) and arteries (b) induced by PGE₂ or misoprostol. Some venous preparations were treated 30 min with AH6809 (3 μ M). Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and number of lung samples used are indicated in Table 1 and 3.

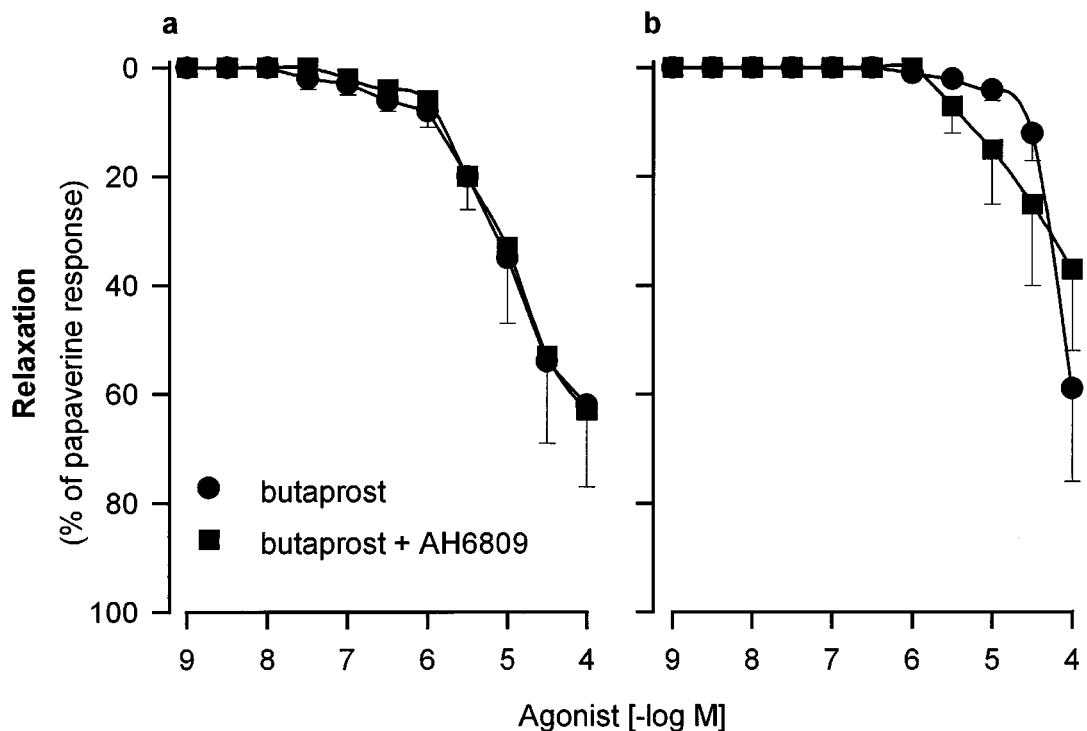


Figure 5 Relaxation of human isolated pulmonary veins (a) and arteries (b) produced by butaprost. Some preparations were treated 30 min with AH6809 (3 μ M). Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and number of lung samples used are indicated in text.

a preferential agonist for IP-receptors in both binding and physiological studies (Blair & McDermot, 1981; Corsini *et al.*, 1987; Adie *et al.*, 1992) relaxed human pulmonary arterial preparations. IP-receptors appear to be the only prostanoid

receptors involved in the prostanoid induced relaxations of human pulmonary arteries since EP- (except butaprost, see below) and DP-receptor agonists did not provoke vasodilatation.

Table 3 Relaxation of human isolated pulmonary arterial preparations: effect of EP- and DP-receptor agonists

Agonist	n	E_{max} (%)	pD_2 value
PGD ₂	5	04 ± 04	NC
BW245C	6	06 ± 06	NC
PGE ₂	5	00 ± 00	NC
Misoprostol	1	05	NC

The maximal responses (E_{max}) were expressed as per cent of the relaxation induced by paraverine (0.1 mM). Values are means ± s.e.mean and (*n*) indicates the number of lung samples used, NC, not calculable.

Butaprost, a PGE₁ analogue, was shown to be a selective agonist for EP₂-receptors (Gardiner, 1986). More recently, Matthews & Jones (1993) suggested that this compound may interact with IP-receptors in human platelets. These latter results were confirmed (present report) since butaprost but not PGE₂ or misoprostol provoked concentration-dependent relaxations in human pulmonary arteries. AH6809 was classically described as a DP/EP₁-antagonist (Coleman *et al.*, 1985; Eglen & Whiting, 1988; Keery & Lumley, 1988), but Woodward *et al.* (1995) have recently suggested that this compound has an antagonistic effect on a recombinant human EP₂-receptor. This latter finding was confirmed in physiological studies involving isolated tissues: human myometrium (Brown *et al.*, 1996) and human bronchial preparations (Norel *et al.*, 1999). The butaprost-induced relaxations in human pulmonary arterial preparations were resistant to treatment with AH6809, suggesting that EP₂-receptors are not implicated in this response.

The data obtained with PGD₂ and BW245C, two DP-receptor selective agonists, also suggested the presence of DP-receptors in the human pulmonary venous preparations. Narumiya & Toda (1985) as well as Eglen & Whiting (1989) showed that BW245C does not activate the EP₂-receptor in the guinea-pig trachea. In addition, Town *et al.* (1983) and Whittle *et al.* (1983) demonstrated that this agonist does not stimulate the IP-receptor present on the rat platelets. These results together with data (present report) suggest that relaxations induced by BW245C in the human pulmonary venous preparations may not be attributed to activation of the IP- or EP₂-receptors. Furthermore, in the human pulmonary venous preparations, the relaxations induced by PGD₂ were significantly inhibited in presence of the selective DP-receptor antagonist BWA868C (Giles *et al.*, 1989). This effect of BWA868C was not associated with an inhibitory effect on EP₂- or IP-receptors since Giles *et al.* (1989), Chen & Woodward (1992) and Bhattacharjee *et al.* (1993) have demonstrated that BWA868C does not block these receptors. These results suggest that prostanoid relaxation of pulmonary veins is *via* a heterogeneous group of receptors and is in contrast with observations in pulmonary arteries.

In human pulmonary veins, cicaprost and iloprost induced relaxations suggesting the presence of IP-receptors in this tissue. While the activation of the IP- or DP-receptors by their respective agonists produced a complete reversal of the norepinephrine contraction, PGE₂ and misoprostol, a selective agonist for the EP₂- and EP₃-receptors (Coleman *et al.*, 1988; Reeves *et al.*, 1988; Lydford & McKechnie, 1994), induced only a partial relaxation. In the human pulmonary artery, PGE₂ and misoprostol did not produce any relaxation suggesting that these agonists are ineffective on the IP-receptor. The inhibition of guinea-pig platelet aggregation is mediated by IP- and DP-receptors, Eglen & Whiting (1988) demonstrated that PGE₂ was ineffective in blocking this aggregation while misoprostol produced only a slight inhibition (pD_2 value = 4.71). These

results together with data (present report) suggest that the partial relaxation induced by PGE₂ or misoprostol in the human pulmonary venous preparations could not be attributed to the activation of IP- or DP-receptors. These reasons and the marked sensitivity of the venous preparations to misoprostol and PGE₂ suggest the presence of an EP-receptor.

EP₂-receptors do not seem to be involved in the human pulmonary venous relaxations as suggested by the two following observations. First, AH6809 did not modify the PGE₂- or the butaprost-induced responses; indicating that butaprost activated IP-receptors in venous preparations as in human pulmonary arteries. Actually, the sensitivity of human pulmonary veins (pD_2 value: 5) to this agonist was closer to the sensitivity of human pulmonary arteries (pD_2 value: 4.40) than to those of guinea-pig and cat trachea in which EP₂-receptors are present (pD_2 values: 6.30–6.35, respectively; Gardiner, 1986). Secondly, misoprostol was 70 fold less potent than PGE₂ to induce relaxation in venous preparations. This ratio is not in agreement with the activation of an EP₂-receptor since Wise & Jones (1994) have shown that misoprostol is 10 fold less potent than PGE₂ to induce inhibition of intracellular free calcium in rat neutrophils (IP- and EP₂-receptors).

Results (present report) were contradictory concerning the involvement of EP₄-receptors in human pulmonary venous relaxations. The presence of EP₄-receptors may be suggested by data obtained with the antagonist BWA868C. In fact, the pK_B and the pA_2 values (7.18–7.84) determined for BWA868C in human venous preparations were lower than the predicted values in preparations where this antagonist acts only on DP-receptor (pA_2 value = 9.26 in human washed platelets; Giles *et al.*, 1989). Furthermore, the mean slope value derived from Schild-plot analysis of the antagonism by BWA868C was significantly less than unity, suggesting that this antagonist interacts with an heterogeneous population of receptors. A similar *in vitro* result has been described in rabbit saphenous veins by Lydford *et al.* (1996), where BWA868C acts on DP- and EP₄-receptors. However, the presence of EP₄-receptors was not confirmed by results obtained with AH23848B, the TP/EP₄-antagonist (Coleman *et al.*, 1994a), which failed to inhibit the relaxation induced by PGE₂ in the human venous preparations. The efficacy of this antagonist was confirmed using rat isolated trachea which is a standard model for preparations containing EP₄-receptors (Lydford & McKechnie, 1994).

Finally, the EP-receptor subtype(s) implicated in the prostanoid induced relaxation of human pulmonary veins could not be characterized in the present study. Similarly, data obtained by Milne *et al.* (1995) in rabbit jugular veins did not differentiate between EP₄- and EP₂-receptors. In this latter study, while data derived from experiments using AH6809 and two EP₂-receptor agonists (AH13205 and butaprost) suggested the presence of EP₄-receptors, this was not confirmed when AH23848B was used as the antagonist. Similar observations were also reported by Meja *et al.* (1997) in human blood monocytes where AH6809 and AH23848B failed to antagonize the inhibitory response induced by PGE₂.

In conclusion, the DP- and IP-receptors are the dominant prostanoid receptors implicated in the relaxation produced by prostaglandins in human pulmonary venous smooth muscle and there is a small relaxant effect due to stimulation of EP-receptors. In the human pulmonary arterial smooth muscle only the IP-receptor is responsible for the prostanoid induced relaxations.

The authors would like to thank Yvette Le Treut and Dominique Gusmini for excellent technical assistance.

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(Received August 27, 1998
Revised November 18, 1998
Accepted November 26, 1998)



Prostanoid receptors involved in the relaxation of human bronchial preparations

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1 Iloprost and cicaprost (IP-receptor agonists) induced relaxations in the histamine- (50 μ M) contracted human bronchial preparations (pD_2 values, 6.63 ± 0.12 and 6.86 ± 0.08 ; E_{max} values, 90 ± 04 and $65 \pm 08\%$ of the papaverine response for iloprost ($n=6$) and cicaprost ($n=3$), respectively).

2 Prostaglandin E_2 (PGE_2) and misoprostol (EP-receptor agonist) relaxed the histamine-contracted human bronchial preparations (pD_2 values, 7.13 ± 0.07 and 6.33 ± 0.28 ; E_{max} values, 67 ± 04 and $57 \pm 08\%$ of the papaverine response for PGE_2 ($n=14$) and misoprostol ($n=4$), respectively). In addition, both relaxations were inhibited by AH6809 (DP/EP₁/EP₂-receptor antagonist; 3 μ M; $n=5-6$).

3 The PGE_2 -induced relaxations of human bronchial preparations were not modified by treatment with AH23848B (TP/EP₄-receptor antagonist; 30 μ M; $n=4$).

4 The contracted human bronchial preparations were significantly relaxed by prostaglandin D_2 (PGD_2) or by BW245C a DP-receptor agonist. However, these responses did not exceed 40% of the relaxation induced by papaverine. In addition, the relaxations induced by PGD_2 were significantly inhibited by treatment with a DP-receptor antagonist BWA868C (0.1 μ M; $n=3$).

5 These data suggest that the relaxation of human isolated bronchial preparations induced by prostanoids involved IP-, EP₂- and to a lesser extent DP-receptors but not EP₄-receptor.

Keywords: Human bronchial preparations; relaxation; prostanoid receptors; prostaglandin; misoprostol; cicaprost; AH6809; BW245C; BWA868C; AH23848B

Introduction

In asthmatic patients, pretreatment with oral prostaglandin E_1 (PGE_1) has been shown to prevent the bronchoconstriction to both inhaled histamine and methacholine (Manning *et al.*, 1989). In addition, PGE_1 or prostaglandin E_2 (PGE_2) may attenuate allergen-induced early and late asthmatic response (Pavord *et al.*, 1993; Pasargiklian *et al.*, 1976). These clinical results suggest a role for the EP-receptors in the relaxation of the human airway smooth muscle tone. Most of the pharmacological studies performed in airways derived from animals have demonstrated that the EP-receptors are involved in the prostanoid-induced relaxations. The EP₂-receptor has been characterized in the cat trachea (Gardiner & Collier, 1980), while the EP₄-receptor has been detected in the rat trachea (Lydford & McKechnie, 1994). Prostacyclin (PGI_2) analogues are ineffective as airway muscle relaxants on isolated trachea derived from cat, guinea-pig (Dong *et al.*, 1986) and rat (Lydford & McKechnie, 1994), suggesting no role for the IP-receptor in the relaxation of large airways. In contrast, these PGI_2 analogues relax human bronchial preparations (Haye-Legrand *et al.*, 1987). However, the effect of inhaled PGI_2 does not alter airway calibre in normal or asthmatic subjects (Hardy *et al.*, 1985; Bianco *et al.*, 1979). Together, the results obtained in these studies suggest that the subtypes of prostanoid-receptors involved in the relaxation of airway

smooth muscle vary between species. The DP-receptor and the EP-subtypes have not been systematically investigated in human airways. The aim of the present study was to characterize the different prostanoid receptors involved in relaxation of human bronchial preparations.

Methods

Isolated preparations

Human lung tissues were obtained from patients (26 male and 2 female) who had undergone surgery for lung carcinoma. The mean age was 65 ± 2 years. Bronchial preparations were removed, dissected free from adjoining connective tissue and lung parenchyma, placed in Tyrode's solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5; pH 7.4 and maintained at 4°C. All tissues were used within 1–12 h postsurgery. Bronchial preparations were cut as rings (3–6 mm internal diameter, 3–5 mm in length). The rings were then set up in 10-ml organ baths containing Tyrode's solution, gassed with 95% $O_2/5\%$ CO_2 and maintained at 37°C. An optimal load (2 g) which ensured maximal physiological responses to the agonists used was applied to each ring. Changes in force were recorded by isometric force displacement transducers (Narco F-60) and physiographs (Linseis). Subsequently, preparations were allowed to equilibrate for 90 min with bath fluid changes taking place every 10 min.

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Experimental protocol

After the equilibration period, the bronchial preparations were incubated 30 min with BAY u3405 (1 μ M), atropine (1 μ M), indomethacin (1.7 μ M) and 15 min with L-NOARG (0.1 mM). These agents were used to avoid any physiological effects induced by the activation of TP- or muscarinic receptors and by the release of endogenous prostanoids or nitric oxide. When PGE₁ or iloprost were used as the relaxant agonist, AH6809 (3 μ M) was added to the previous drug combination (30 min) to avoid any physiological effects induced by the activation of EP₁-receptors. After incubation, the preparations were contracted with histamine (50 μ M), when the response reached a plateau, increasing concentrations of prostanoid receptor agonists (PGE₂, PGE₁, PGD₂, cicaprost, iloprost, BW245C or misoprostol) were applied in a cumulative fashion. The maximal relaxation was obtained for each preparation with papaverine (0.1 mM) at the end of the experiment.

The same protocol was performed to determine the affinity values of prostanoid receptor antagonists (AH23848B, AH6809 or BWA868C) which were added simultaneously with the drug combination during 30 min before the histamine-induced contraction.

Data analysis

The changes in force were measured from isometric recordings and expressed in grams (g). The relaxations produced with the different agonists were expressed as per cent of the relaxations induced with papaverine. The $E_{\max}$ value was the maximal relaxation produced with the highest agonist-concentration used and EC_{50} value was the concentration which produced $E_{\max}/2$. These values were interpolated from the individual agonist concentration-effect curves. The pD_2 values were calculated as the negative log of EC_{50} values. When the pD_2 values obtained in the presence and absence of an antagonist were significantly different, the equilibrium dissociation constant for the antagonist (K_B value) was calculated. The following equation was used: $K_B = [B]/(DR - 1)$, where $[B]$ is the concentration of the antagonist and DR (dose ratio) is the ratio of EC_{50} values of agonist in the presence and absence of antagonist. The pK_B values were calculated as the negative log of the K_B values. All results were expressed as means $\pm$ s.e.mean of data derived from n different lung samples. Statistical analysis was performed using ANOVA with a confidence level of 95% and taking into account the preparations derived from the same or different lung samples (covariate).

Compounds

PGE₂, PGE₁, PGD₂ and misoprostol (($\pm$)-11, 16-dihydroxy-16-methyl-9-oxoprost-13-en-1-oic acid methyl ester) were purchased from Cayman Chemical Company, Ann Arbor, MI, U.S.A. Iloprost (5-[(E)-(1S,5S,6R,7R)-7-hydroxy-6-[(E)-(3S,4RS)-3-hydroxy-4-methyl-1-octen-6-ynyl]bicyclo[3.3.0]octan-3-ylidene]pentanoic acid) and cicaprost ([2-[hexahydro-5-hydroxy-4-(3-hydroxy-4-methyl-1,6-nonadynyl)-2-(1H)-pentalenylidene]ethoxy]acetic acid) were a gift from Schering AG, Berlin, Germany. AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) and AH23848B ([1 α (z),2 β ,5 α]-(($\pm$)-7-[5-[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid) were a gift from Glaxo Wellcome, U.K. BAY u3405 (3(R)-3-(4-fluorophenylsulphonamido)-1,2,3,4-tetrahydro-9-carbazole propanoic acid) was a gift from Bayer, Stokes Poges, U.K. BW245C (5-(6-carboxyhexyl)-1-

(3-cyclohexyl-3-hydroxypropyl) hydantoin) and BWA868C (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino) hydantoin) were a gift from Wellcome Research Laboratories, Beckenham, U.K. Histamine dihydrochloride, L-NOARG (N^G-nitro-L-arginine), indomethacin and atropine sulphate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Papaverine was obtained from Meram Laboratories (77020 Melun, France).

Results

In each experiment, the human bronchial preparations contracted with histamine (50 μ M: 2.37 ± 0.16 g, $n=28$) and at the end of the protocols the preparations were relaxed with papaverine (0.1 mM: 2.90 ± 0.18 g, $n=28$). The combination of inhibitors and antagonists (indomethacin, L-NOARG, BAY u3405 and atropine) with which the bronchial preparations were incubated, had no significant relaxant effect on the basal tone of these preparations (-0.06 ± 0.06 g; $n=28$).

PGE₁ as well as two stable PGI₂ analogues, iloprost and cicaprost, produced concentration-dependent relaxations (Figure 1 and Table 1) in human bronchial preparations. PGE₂ and misoprostol also relaxed the histamine-contracted human bronchial preparations (Figure 2 and Table 2). Concentration-dependent relaxations produced by PGE₂ and misoprostol were significantly shifted in presence of AH6809 (3 μ M; Figure 2). The pK_B value for this antagonist against PGE₂ is presented in Table 2. On the contrary, no significant displacement of the relaxation curves induced by PGE₂ was observed after an incubation with AH23848B (30 μ M, Figure 3 and Table 2). In paired bronchial preparations, derived from the same lung sample, the pD_2 values obtained in presence of AH23848B were not statistically different from the control values (6.97 ± 0.11 , $n=4$).

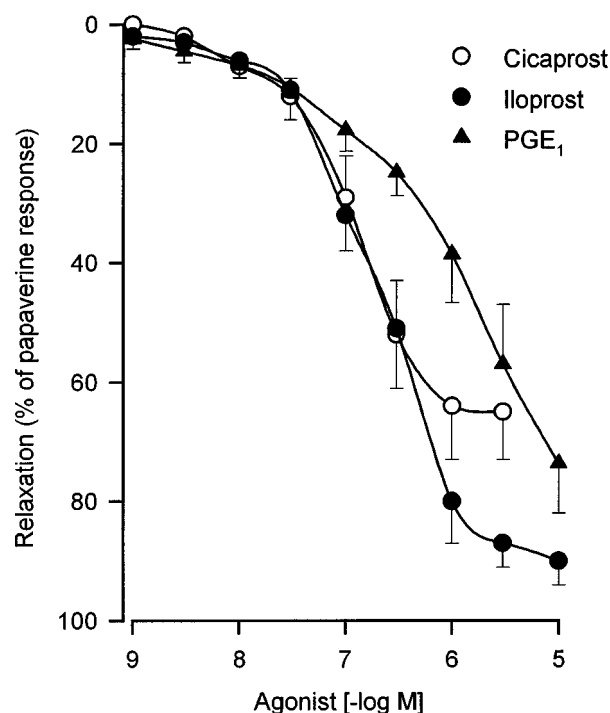


Figure 1 Relaxation of human isolated bronchial preparations induced by cicaprost, iloprost or PGE₁. Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and the number of lung samples used are indicated in Table 1.

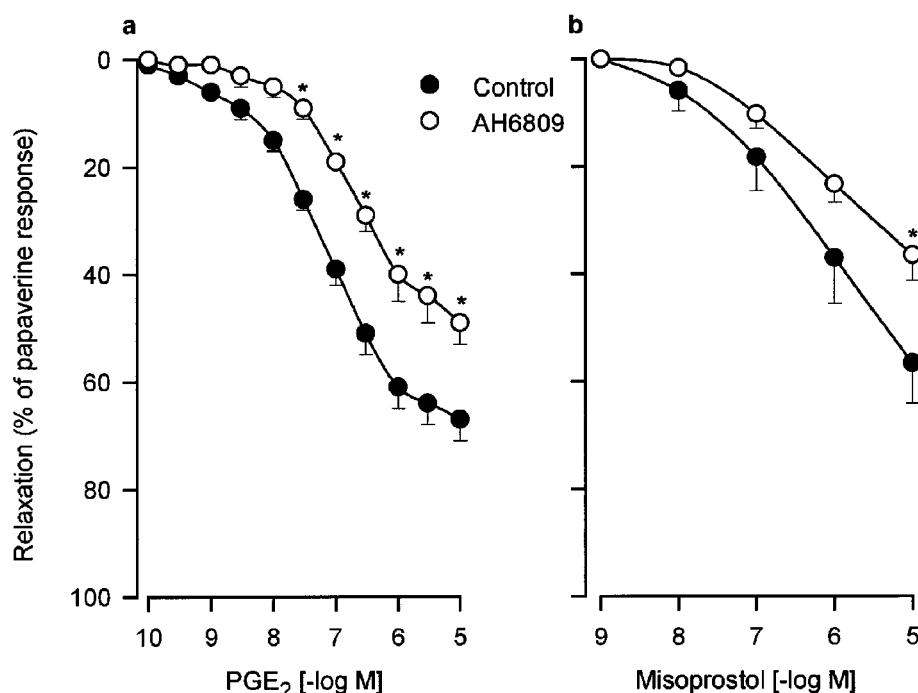


Figure 2 Relaxation of human isolated bronchial preparations induced by PGE₂ (a) or misoprostol (b). Some bronchial preparations were treated 30 min with AH6809 (3 μ M). Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. In each panel, values are means $\pm$ s.e.mean and the number of lung samples used are indicated in Table 2. *Values significantly different when results from control (Tyrode) and treated tissues were compared.

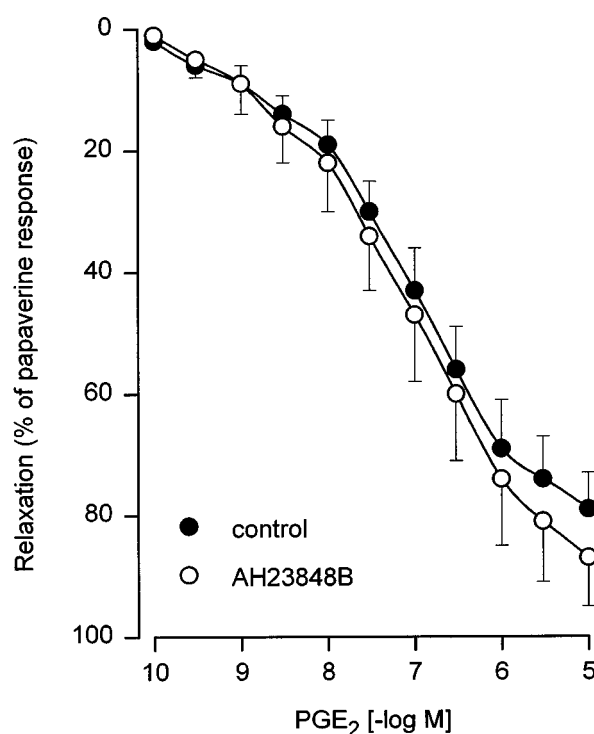


Figure 3 Relaxation of human isolated bronchial preparations induced by PGE₂ in absence or in presence of AH23848B (30 μ M). Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean derived from four lung samples.

While PGE₂, misoprostol, PGE₁ and PGI₂ analogues totally reversed the histamine contraction, PGD₂ and BW245C induced only a partial reversal of this contraction. Concentration-dependent relaxations of human bronchial preparations

Table 1 Relaxant effects of IP-receptor agonists on the isolated human bronchial preparations

Agonist	n	E _{max} (%)	pD ₂ value
Cicaprost	3	65 $\pm$ 08	6.86 $\pm$ 0.08
Iloprost	6	90 $\pm$ 04	6.63 $\pm$ 0.12
PGE ₁	4	74 $\pm$ 08	6.05 $\pm$ 0.09

The maximal response (E_{max}) were expressed as per cent of the relaxation induced by papaverine (0.1 mM). Values are means $\pm$ s.e.mean, (n) indicates the number of lung samples used. The relaxations induced by PGE₁ or iloprost were performed in presence of AH6809 (3 μ M).

produced by PGD₂ and BW245C are shown in Figure 4 and Table 2. In addition, BWA868C (0.1 μ M) significantly reduced the relaxation induced by PGD₂ (Table 2).

AH6809, AH23848B and BWA868C at the concentrations used had no significant effect on the basal tone.

Discussion

These data suggest the involvement of IP- and EP₂-receptors and to a lesser extent DP-receptor in the relaxant response produced by prostanoids in human airways.

Gardiner & Collier (1980) and Lydford & McKechnie (1994) have demonstrated that in the guinea-pig and in the rat trachea, relaxations induced by the prostanoids are attributed to the activation of EP-receptors. Data (present report) are in contrast to the classical description of prostanoid receptors in the airways derived from animals. Actually, the relaxations induced by cicaprost (IP-receptor agonist; Stürzebecher *et al.*, 1985), indicate the presence of IP receptor in human bronchial preparations. Similar results were obtained with iloprost (EP₁/IP-receptor agonist; Schrör *et al.*, 1981; Sheldrick *et al.*, 1988) when the EP₁-receptors were blocked by AH6809 (DP/EP₁-

Table 2 Relaxations of isolated human bronchial preparations: effect of EP- and DP-receptor agonists or antagonists

<i>Treatment</i>	<i>Concentration</i>	n	<i>E_{max}</i> (%)	<i>Agonist</i>	<i>pD₂</i> value	<i>pK_B</i> value
<i>Misoprostol</i>						
Tyrode	3 μM	4	57 ± 08		6.33 ± 0.28	NC
AH6809		5	36 ± 05*		6.39 ± 0.18	
<i>PGE₂</i>						
Tyrode	3 μM	14	67 ± 04		7.13 ± 0.07	5.78
AH6809		6	49 ± 04*		6.68 ± 0.08*	
AH23848B		30 μM	4	87 ± 08		
<i>PGD₂</i>						
Tyrode	0.1 μM	4	25 ± 08		6.12 ± 0.18	NC
BWA868C		3	11 ± 01*		NC	
<i>BW245C</i>						
Tyrode		5	36 ± 11		7.28 ± 0.53	

The maximal responses (E_{max}) were expressed as per cent of the relaxation induced by papaverine (0.1 mM). Values are means $\pm$ s.e.mean, (*n*) indicates the number of lung samples used, NC: not calculable. *Values significantly different when results from control (Tyrode) and treated tissues were compared.

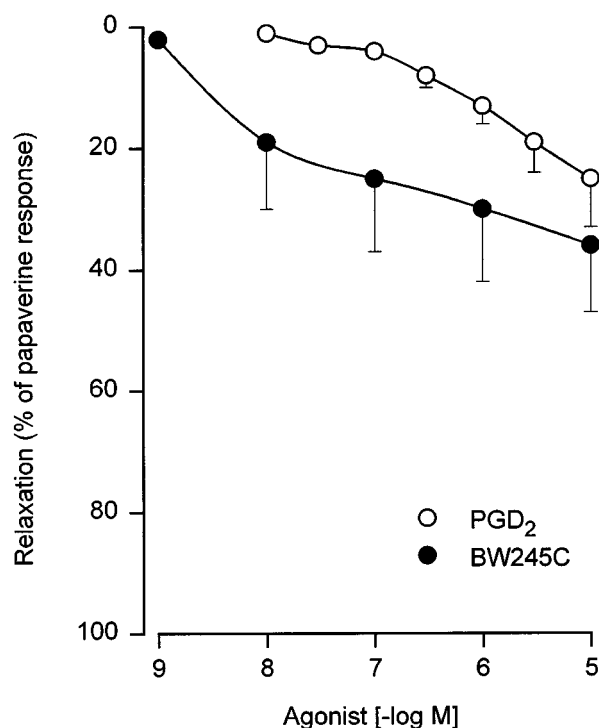


Figure 4 Relaxation of human isolated bronchial preparations induced by PGD₂ and BW245C. Responses were expressed as per cent of the papaverine (0.1 mM) relaxation. Values are means $\pm$ s.e.mean and the number of lung samples used are indicated in Table 2.

receptor antagonist; Coleman *et al.*, 1985; Eglen & Whiting, 1988; Keery & Lumley, 1988). These results (present report) are in agreement with data obtained by Haye-Legrand *et al.* (1987) describing relaxations induced by iloprost, cicaprost (ZK 96480) and PGI₂ in the human isolated airways. These authors demonstrated that PGI₂, the natural agonist activating IP-receptor, induced quite variable relaxations of the human bronchial preparations. These variations may be due to the short half life of this prostaglandin. Blair & McDermot (1981), Corsini *et al.* (1987) and Adie *et al.* (1992) have shown that PGE₁, a more stable endogenous prostanoid, is a potent agonist for the IP-receptor in both binding and physiological studies. The effective relaxations of the human bronchi

observed with PGE₁ (present report) suggest that, this prostaglandin, may be the preferential natural activator for IP-receptor in human airways *in vivo*.

Kennedy *et al.* (1982) and Gardiner (1986) have demonstrated that the EP₂-receptor is involved in the relaxation of guinea-pig and cat tracheal preparations. These studies were based on the effects induced by butaprost, a PGE₁ analogue. In human bronchial preparations, butaprost induced concentration-dependent relaxations (Gardiner, 1986; Norel *et al.*, 1991), these results suggest the presence of EP₂-receptor on human airways. Additional evidence consistent with the involvement of the EP₂-receptor in the relaxation of human bronchial preparations is suggested by the following observations. First, PGE₂ and misoprostol, two preferential agonists for the EP-receptors, were potent airway muscle relaxants. These relaxations cannot be attributed to the activation of the IP-receptor, since these agonists are totally ineffective on the IP-receptor as in human pulmonary arteries (Walch *et al.*, 1999). Misoprostol is a preferential agonist for EP₂- and EP₃-receptors (Coleman *et al.*, 1988; Reeves *et al.*, 1988; Lydford & McKechnie, 1994). Wise & Jones (1994) showed that this agonist was 10 fold less potent than PGE₂ in producing an inhibition of intracellular free calcium in the rat neutrophils (EP₂- and IP-receptors). In contrast, Smith *et al.* (1994) demonstrated that misoprostol was 145 fold less potent than PGE₂ for inducing dilatation of the foetal rabbit ductus arteriosus (EP₄- and IP-receptors). In human bronchial preparations (present report), misoprostol was only 6 fold less potent than PGE₂ in provoking relaxations. Such a ratio is in agreement with the activation of an EP₂-receptor when the IP-receptor is present in the same preparation. Secondly, the TP/EP₄-receptor antagonist (AH23848B) failed to inhibit the relaxation induced by PGE₂, these data suggest that EP₄-receptor is probably not involved in the relaxations produced by either PGE₂ or misoprostol in human bronchial preparations. Finally, evidence in support of the presence of EP₂-receptor is derived from a new effect of AH6809 reported recently (Woodward *et al.*, 1995; Brown *et al.*, 1997) which indicates AH6809 as an EP₂ antagonist. The concentration-dependent relaxations induced by both EP agonists (PGE₂ or misoprostol) were significantly shifted in presence of this antagonist (present report) suggesting the presence of EP₂-receptor.

The human bronchial preparations relaxed to PGD₂ and BW245C (present report). These compounds have been

described as DP-receptor agonists. Actually, Narumiya & Toda (1985) and Eglen & Whiting (1989) have shown that BW245C is ineffective on the EP₂-receptor in the guinea-pig trachea. In a similar fashion, the relaxation induced by PGD₂ may not be attributed to the activation of IP-receptors since PGD₂ was totally ineffective on human pulmonary arteries (Walch *et al.*, 1999). The involvement of DP-receptors in human bronchial relaxation, is suggested by the 15 fold greater potency of BW245C in comparison with PGD₂. This result is in agreement with those obtained by Narumiya & Toda (1985) and Giles *et al.* (1989) in human washed platelets. Furthermore, the relaxation induced by PGD₂ (present report) is significantly reduced in presence of BWA868C a DP-antagonist (Giles *et al.*, 1989). This antagonist does not block the EP₂- or IP-receptors as demonstrated by Giles *et al.* (1989), Chen & Woodward (1992) and Bhattacharjee *et al.* (1993). Taken together, these results (present report) suggest that PGD₂ and BW245C induced relaxations by the activation of DP-receptors. However, these agonists produced relaxations which were less than 50% of the papaverine response. These results suggest a lower density of the DP-receptor or a less effective coupling of this receptor with adenylate cyclase when compared with EP₂- or IP-receptors in human airways.

The venous preparations exhibited a similar or a greater sensitivity to the prostanoid-receptor agonists (Walch *et al.*, 1999) than the bronchial preparations (present report). A

marked difference was observed with the prostacyclin analogues in bronchial versus pulmonary vascular preparations even though the E_{max} were the same. These results suggest that there is a difference at the receptorial level between the IP-receptor present in human pulmonary vessels and that in human airways. These data are in agreement with previous reports (Corsini *et al.*, 1987; Armstrong *et al.*, 1989; Merritt *et al.*, 1991; Wise *et al.*, 1995; Takechi *et al.*, 1996) suggesting a heterogeneity of the IP-receptor in various tissues or cells. A comparison of the relaxation induced by PGE₂ in the airways (present report) and in the human pulmonary veins (Walch *et al.*, 1999) demonstrates a difference in sensitivity and in maximal relaxations. These differences are consistent with the presence of two different subtypes of EP-receptor in these tissues. While an EP₂-receptor is involved in human bronchial preparations, the subtype of EP-receptor involved in venous preparations remains to be characterized.

In conclusion, the results (present report) suggest a major involvement of IP- and EP₂-receptors and a minor role for the DP-receptor in the bronchial relaxation induced by the prostanoids in the human lung.

The authors would like to thank Yvette Le Treut and Dominique Gusmini for excellent technical assistance.

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(Received January 14, 1998

Revised March 8, 1998

Accepted November 26, 1998)



Effects of varying the expression level of recombinant human mGlu1 α receptors on the pharmacological properties of agonists and antagonists

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1 Different expression levels of the human type 1 α metabotropic glutamate (mGlu1 α) receptor were obtained in transfected Chinese hamster ovary cells using an isopropyl β -D-thiogalactopyranoside (IPTG) inducible system. Expression of mGlu1 α receptors could not be detected using immunoblotting or immunocytochemical approaches in non-induced cells, however, controlled expression could be induced following IPTG addition in a time- and concentration-dependent manner.

2 In induced cells (100 μ M IPTG, 20 h) the agonists L-quisqualate or 1-aminocyclopentane-1S,3R-dicarboxylic acid stimulated large increases in [³H]-inositol (poly)phosphate (in the presence of Li⁺) and inositol, 1,4,5-trisphosphate levels.

3 Induction with 1–100 μ M IPTG allowed the receptor density to be increased incrementally and this not only resulted in an increase in the maximum response to L-quisqualate, 1-aminocyclopentane-1S,3R-dicarboxylic acid and (S)-3,5-dihydroxy-phenylglycine, but also in an increase in the respective potencies of each agent to activate phosphoinositide hydrolysis.

4 The intrinsic activity of the partial agonist 1-aminocyclopentane-1S,3R-dicarboxylic acid dramatically increased with increasing receptor expression.

5 The activities of the competitive mGlu1 α receptor antagonists (S)- α -methyl-4-carboxyphenylglycine and (S)-4-carboxy-3-hydroxyphenylglycine for inhibition of the effects of L-quisqualate or (S)-3,5-dihydroxyphenylglycine were found to be independent of the receptor expression level.

6 When the mGlu1 α receptor was expressed at very high levels, no evidence for receptor constitutive activity could be detected, and none of the antagonists tested revealed either any intrinsic activity or negative efficacy.

7 These data demonstrate that both the potency and efficacy of mGlu1 α receptor agonists are influenced by expression level, whilst mGlu1 α receptor antagonist activities are independent of expression level.

Keywords: Type 1 α metabotropic glutamate receptor; phosphoinositide turnover; inositol 1,4,5-trisphosphate; LacSwitch; receptor induction; IPTG; inducible expression

Abbreviations: 1S,3R-ACPD, 1-aminocyclopentane-1S,3R-dicarboxylic acid; AIDA, (RS)-1-aminoindan-1,5-dicarboxylic acid; CHO, Chinese hamster ovary; (S)-4-C3HPG, (S)-4-carboxy-3-hydroxyphenylglycine; (S)-4CPG, (S)-4-carboxy-phenylglycine; (S)-3,5-DHPG, (S)-3,5-dihydroxyphenylglycine; GPT, glutamic-pyruvic transaminase; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; [³H]-InsP, [³H]-inositol (poly)phosphate; IPTG, isopropyl β -D-thiogalactopyranoside; KHB, Krebs-Henseleit buffer; (S)-MCPG, (S)- α -methyl-4-carboxyphenylglycine; mGlu receptor, metabotropic glutamate receptor; PBS, phosphate buffer saline

Introduction

The original observation that glutamate not only triggers the opening of ion channels, but also activates phospholipase C (Sladeczek *et al.*, 1985) led to the further identification of glutamate receptors coupled to G proteins (Sugiyama *et al.*, 1987). Molecular cloning revealed the existence of a large family of glutamate metabotropic receptor (mGlu receptors) containing at least eight different subtypes that can be classified on the basis of their biochemical and pharmacological properties into three different groups. Group I receptors (mGlu1 and mGlu5) are preferentially coupled to the activation of phospholipase C through functional coupling to G_{q/11}, although mGlu1 α has also been reported to activate adenylyl cyclase and to mediate arachidonic acid release.

Group II (mGlu2 and mGlu3) and group III (mGlu4 and mGlu6–8) are coupled to the inhibition of adenylyl cyclase through pertussis toxin-sensitive G (G_i) proteins (see Pin & Duvoisin, 1995; Conn & Pin, 1997). Despite the large number of compounds investigated, mainly in the family of phenylglycine derivatives, the pharmacological distinction of each subtype within a group is hampered by the lack of high specific ligands. As a consequence, most studies concerning the specific interaction of putative metabotropic agonists or antagonists are performed with transfected cells expressing cloned mGlu receptors (Akam *et al.*, 1997; Pickering *et al.*, 1993; Thomsen *et al.*, 1994a; Hayashi *et al.*, 1994; Joly *et al.*, 1995; Lin *et al.*, 1997). Unfortunately, because of the absence of high-affinity radioligands for most of the mGlu receptors, quantitative determination of the level of expression of the receptor in these transfected cells is not possible, and interaction of compounds with expressed receptors needs to

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be investigated at the function level. In addition, stable and maintained expression of functional mGlu receptors in transfected cells has been shown to be problematic, perhaps as a consequence of regulatory processes related to the presence of glutamate in the culture medium of most cell lines (Gabellini *et al.*, 1994; Desai *et al.*, 1995; Lin *et al.*, 1997; Carruthers *et al.*, 1997).

We have previously reported a stably transfected CHO cell line in which the expression of the mGlu1 α receptor is under control of an IPTG-inducible promoter (Hermans *et al.*, 1998a). The use of this inducible promoter not only confers the possibility of maintaining the receptor density at very low levels during the growth of the cells and to induce its expression when required, but also allows us to manipulate the expression level of the receptor by means of varying the concentration of inducer added to the culture medium or the time of induction. In the present study, this model was used in order to study the consequences of modulating the expression level of the mGlu1 α receptor on some properties of agonists and antagonists at this prototypic mGlu receptor subtype. Such an inducible system provides an appropriate model for such studies. It avoids the need to compare responses measured in different cell clones expressing different densities of receptors and constitutes an advantage in the case of this mGlu receptor, as its precise quantitation is complicated by a lack of high-affinity (antagonist) radioligands. A preliminary report of some of these data has been presented to the British Pharmacological Society (Hermans *et al.*, 1998b).

Methods

Cell culture and induction

The establishment of the stably transfected Chinese hamster ovary (CHO) cell line in which the expression of the human mGlu1 α receptor is controlled by the activity of a Lac-repressible promoter has been previously reported (Hermans *et al.*, 1998a). These cells (CHO-Lac-hmGlu1 α) were routinely cultured in Modified Eagle's medium containing glutamax, supplemented with 10% foetal calf serum, 100 $\mu\text{g ml}^{-1}$ streptomycin and 10⁵ u ml⁻¹ penicillin in 175-cm² flasks in a humidified 5% CO₂ incubator maintained at 37°C. Cells were used between passage 8 and 30. The cells were seeded onto 24-well plates in 0.75 ml well⁻¹ culture medium 48 h prior to signal transduction experiments. When required, IPTG was added 20 h before the experiment directly to the culture medium at the concentration indicated.

[³H]-InsP determination

For [³H]-inositol (poly)phosphates ([³H]-InsP) determination, the cells were seeded in medium containing 1 $\mu\text{Ci ml}^{-1}$ [³H]-inositol. Medium was replaced with 0.5 ml of the same medium supplemented with the indicated concentration of IPTG 18–20 h before the experiment. Cells were washed three times with Krebs-Henseleit buffer (KHB) (in mM) NaCl 118, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, CaCl₂ 1.3, MgSO₄ 1.2, HEPES 5 and D-Glucose (10) (pH 7.4). When glutamic-pyruvic transaminase (GPT) and pyruvate were used, these were dissolved in KHB buffer and added to the incubation buffer 15 min before any other manipulation and 30 min before agonist challenge. When antagonists were tested, these were added 15 min before agonist addition. During the last 15 min before the agonist challenge, LiCl (10 mM) was added to the incubation medium. Experiments were performed at

37°C in a final volume of 0.5 ml well⁻¹ and were terminated by the addition of 0.5 ml ice-cold 1 M trichloroacetic acid. After extraction on ice for 20 min, samples (0.8 ml) were collected from the well, mixed with 200 μl EDTA (10 mM, pH 7.0) and extracted with 1 ml of a 1:1 (v v⁻¹) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. An 800 μl sample of the aqueous extract was mixed with 50 μl NaHCO₃ (62.5 mM) and [³H]-InsP recovered by ion-exchange chromatography on Dowex AG1-X8 (formate form) columns as previously described (Challiss *et al.*, 1993).

Ins(1,4,5)P₃ determination

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) mass was determined as described previously (Challiss *et al.*, 1993). The preparation of samples for Ins(1,4,5)P₃ determination was similar to the protocol described above for [³H]-InsP except that [³H]-inositol was omitted from the culture medium and no LiCl was added at the incubation stage.

Immunofluorescent labelling and imaging

Cells grown for 20 h on coverslips in the absence or in the presence of IPTG (3–300 μM) were fixed for 10 min at room temperature in 2% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized for 30 min with 0.5% Triton X-100 in PBS containing 10% goat serum. After three washes with PBS, the cells were incubated for 2 h with a specific mGlu1 α receptor antibody diluted 1:250 in PBS/goat serum. After washing, the cells were incubated for 90 min with a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody diluted 1:500 in PBS/goat serum. After washing, the cells were mounted on slides and examined using a Bio-Rad 600 laser scanning confocal microscope equipped with a 60 $\times$ objective.

Immunoblot analysis

Cells were plated in 24-well multidishes and maintained in culture medium for 48 h before the experiment. When required, IPTG (1–100 μM) was added to the medium for the final 20 h. Plates were placed on ice and after two washes with 0.5 ml KHB, the cells were solubilized in 400 μl (Tris/HCl (125 mM), dithiothreitol (50 mM), 4% sodium dodecylsulphate, 20% glycerol, 0.01% bromophenol blue, pH 6.8). The samples were collected in centrifuge tubes and combined with a further 400 μl H₂O which was used to rinse the well. Immunoblotting was performed as previously described, using a mGlu1 α receptor antiserum (100 ng ml⁻¹) and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected with enhanced chemiluminescence (ECL) reagents.

Data analysis

EC₅₀ values were determined by non-linear regression analysis using the software Prism II (GraphPad, San Diego, CA, U.S.A.). Data were fitted as sigmoidal dose-response curves with variable slope and analysed by a four parameter logistic equation. Antagonist equilibrium dissociation constants (expressed as pK_B) were calculated using the following:

$$K_B = A/(CR - 1)$$

where A is the concentration of antagonist used and CR is the ratio of EC₅₀ values for the agonist measured in the presence or in the absence of the antagonist, respectively.

Materials

L-Quisqualic acid, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), (*S*)-3,5-dihydroxyphenylglycine ((*S*)-3,5-DHPG), (*S*)- α -methyl-4-carboxyphenylglycine ((*S*)-MCPG), (*S*)-4-carboxyphenylglycine ((*S*)-4-CPG), (*RS*)-1-aminoinidan-1,5-dicarboxylic acid (AIDA) and (*S*)-4-carboxy-3-hydroxyphenylglycine ((*S*)-4-C3-HPG) were from Tocris-Cookson (Bristol, U.K.); IPTG, dithiothreitol and glutamic-pyruvic transaminase (GPT), Triton X-100, fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody, paraformaldehyde and goat serum were from Sigma Chemical Co. (Poole, U.K.); *myo*-[2-³H]-inositol (70–120 Ci mmol⁻¹) and ECL reagents were from Amersham (Little Chalfort, U.K.). The antiserum raised against the C-terminus of the rat mGlu1 α was from Chemicon. All cell culture media and reagents were from Gibco Life Technologies (Paisley, U.K.). LY367385 and (Clark *et al.*, 1997) was kindly provided by Dr J.R. Harris (Lilly Research Laboratories, Windlesham, U.K.). All other reagents were of analytical grade and were from Fisons (Loughborough, U.K.).

Results

Immunocytochemical localization of mGlu1 α receptor expression

We have previously shown that treatment of transfected CHO-Lac-hmGlu1 α cells with IPTG caused a concentration- and time-dependent increase in the expression of functional mGlu1 α receptors, as detected by immunoblot, using a specific mGlu1 α antiserum or by measuring agonist-induced phosphoinositide hydrolysis. In the present experiments, the time of exposure of the cell to IPTG was maintained constant (20 h)

whereas different levels of expression were obtained by varying the concentration of the inducer added to the culture medium. Figure 1 shows the expression of the receptor detected by immunofluorescence using a specific antiserum raised against the intracellular C-terminus of the mGlu1 α receptor. When cells were grown in the absence of IPTG, no immunoreactivity could be detected. Increasing the concentration of IPTG in the culture medium from 0–300 μ M resulted in a progressive increase in immunofluorescence. No specific labelling of the cells could be detected in non-transfected cells or when incubation with the specific mGlu1 α receptor antiserum was omitted from the protocol. Analysis of cells induced with IPTG revealed diffuse immunoreactivity throughout the cytoplasm. Intense labelling was also observed close to the nucleus, especially in cells induced with 100 or 300 μ M IPTG suggesting that a significant amount of newly synthesized receptors may be present after 20 h of induction (Figure 1). Increasing the expression of the mGlu1 α receptor also resulted in marked changes in the morphology (increase in length-width ratio, data not shown) of the CHO cells, as recently reported by Kubo and colleagues (1998).

Effects of mGlu1 α receptor on basal and agonist-stimulated [³H]-InsP responses

Induction of mGlu1 α receptor expression in the transfected CHO cells resulted in a significant increase in the basal level of [³H]-InsP accumulation in the presence of Li⁺ (Figure 2, inset). However, this increase most likely results from receptor activation by endogenously released glutamate, as it could be completely suppressed by addition of the glutamate metabolizing enzyme glutamic-pyruvic transaminase (GPT) and its co-substrate pyruvate. Experiments performed in the absence or presence of GPT/pyruvate indicated that the increase in the basal level [³H]-InsP did not alter the response to L-quisqualate (Figure 2).

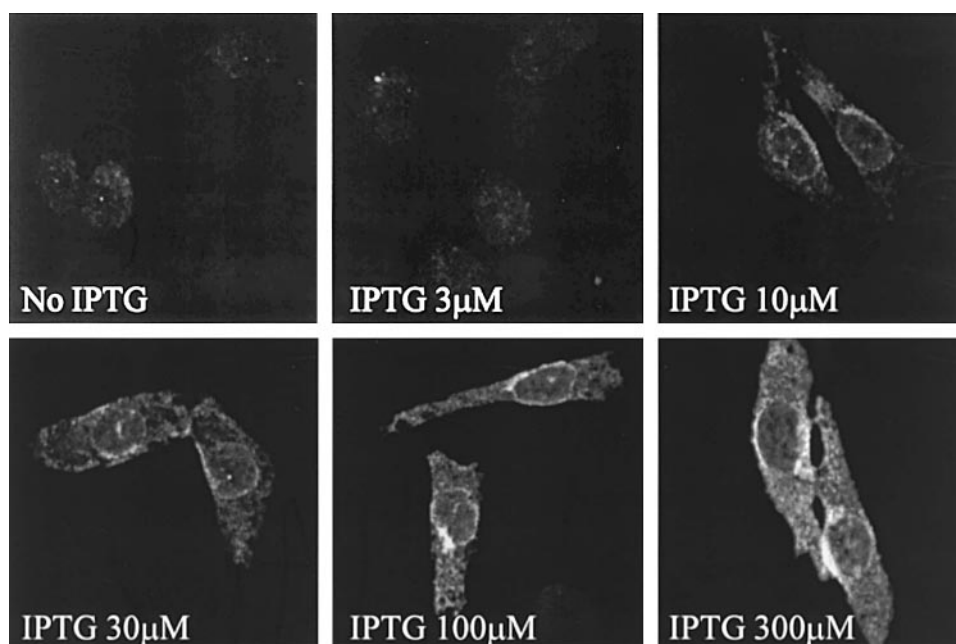


Figure 1 Fluorescence imaging of mGlu1 α receptor expressed in CHO-Lac-hmGlu1 α cells pre-incubated for 20 h in the absence or presence of IPTG (3, 10, 30, 100, 300 μ M). Immunofluorescence staining was performed as indicated under Methods using a specific antiserum raised against the mGlu1 α receptor and revealed by a fluorescein isothiocyanate-conjugated secondary antibody. Representative images corresponding to horizontal sections through the middle of the cell are shown.

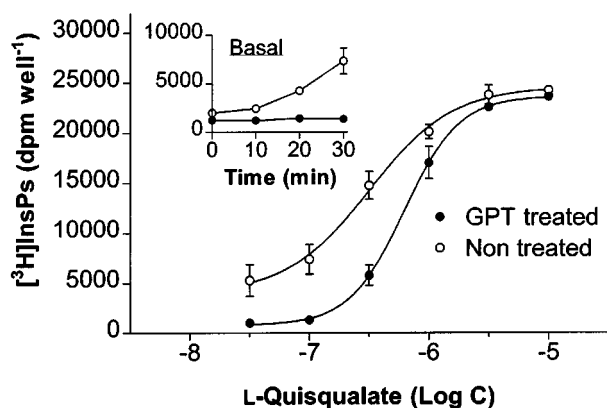


Figure 2 Effect of GPT/pyruvate on the basal and L-quisqualate-induced accumulations of [^{3} H]-InsP. After induction with IPTG (100 μ M) for 20 h, CHO-Lac-hmGlu1 α cells were washed and preincubated for 15 min in the presence (closed symbols) or absence (open symbols) of GPT/pyruvate before the addition of Li $^+$ and L-quisqualate (10 μ M) (closed symbols) at the indicated concentrations. Inset: time-course of basal accumulation of [^{3} H]-InsP after addition of Li $^+$ alone in the presence (closed symbols) or absence (open symbols) of GPT/pyruvate. Data are means $\pm$ s.e. mean from representative experiments performed three times in duplicate.

Effects of varying mGlu1 α receptor expression on agonist activity

The consequence of varying mGlu1 α receptor density on the properties of agonists was studied by measuring agonist-induced phosphoinositide hydrolysis in cells induced for 20 h with different concentration of IPTG. Immunoblot analysis of total cell extracts with a mGlu1 α antiserum indicated that maximal expression of the receptor was obtained after induction with 30–100 μ M IPTG (Figure 3C). As reported in our previous publication (Hermans *et al.*, 1998a), when analysed by immunoblotting of low percentage gels, the mGlu1 α receptor migrates as two different bands ($\sim$ 145 and $\sim$ 160 kDa). Although both bands are induced by IPTG application, and undoubtedly represent mGlu1 α receptor protein, the relative expression level of each of the bands differs depending on the level of induction. At present the biochemical difference(s) between these two bands has not been characterized and we do not know whether both represent active (i.e. cell-surface located, G protein-coupled) mGlu1 α receptors. This complicates the quantitation of the level of receptor expression from immunoblot analyses and therefore the variation in functional responses measured in cells expressing different densities of receptors has always been referred to the concentration of IPTG added to the culture medium.

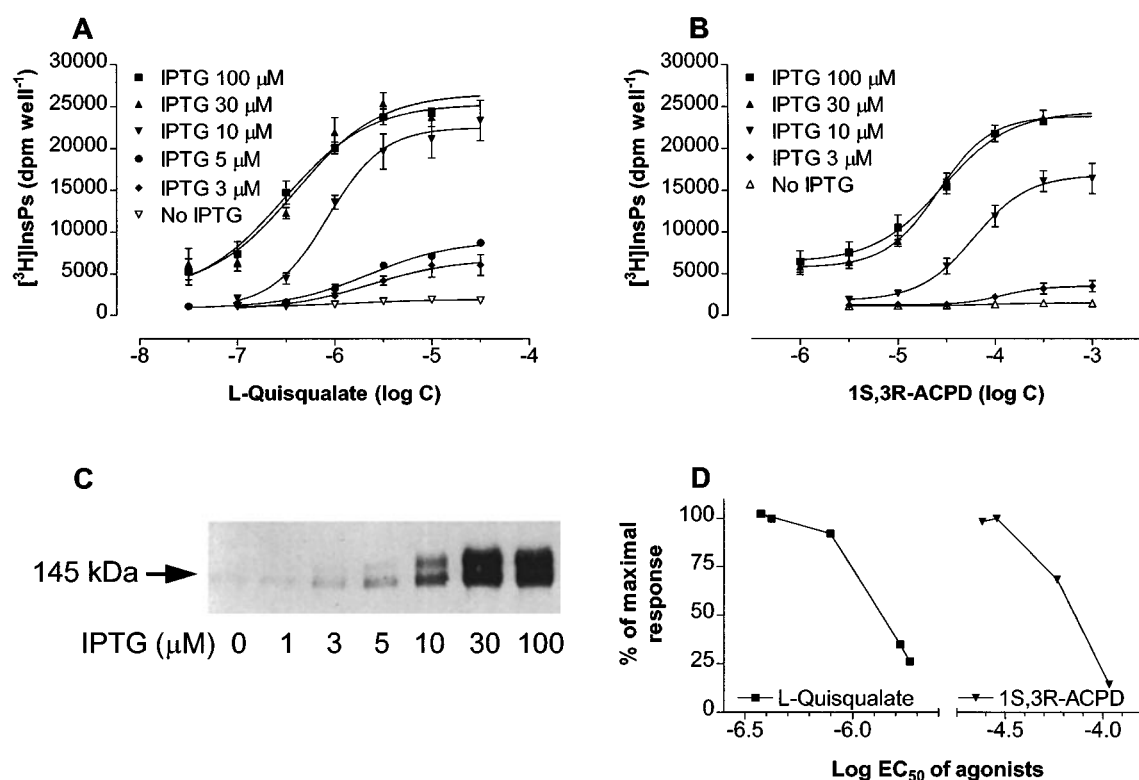


Figure 3 (A) L-quisqualate- and (B) 1S,3R-ACPD-induced accumulations of [^{3} H]-InsP in CHO-Lac-hmGlu1 α cells expressing the mGlu1 α receptor at different densities (obtained after pre-incubation for 20 h in the absence or presence of IPTG at different concentrations). Cells were stimulated with the agonists for 30 min in the presence of LiCl (10 mM). Data are means $\pm$ s.e. mean from at least three different experiments performed in duplicate. Log EC $_{50}$ values calculated from these curves are shown in Table 1. (C) Immunoblot analysis of total cell extracts using a specific mGlu1 α receptor antiserum showing the increase in receptor expression obtained after incubation of the cells for 20 h in the presence of increasing concentrations of IPTG. (D) Shows the relationship between the potency and the efficacy of L-quisqualate and 1S,3R-ACPD measured on the cells expressing mGlu1 α receptors at different densities.

Figure 3A and B show concentration-response curves to the agonists L-quisqualate and 1S,3R-ACPD in cells incubated in the absence or presence of increasing concentrations of IPTG. In non-induced cells, no significant response was detected to either agonist. Increasing the receptor density not only resulted in an increase in the maximal response to each agonist, but also in significant changes in the EC_{50} values (Table 1). For both agonists, increasing the concentration of IPTG from 3–30 μ M resulted in a 4–5 fold decrease in the EC_{50} value for stimulation of [^{3} H]-InsP accumulation. Figure 3D illustrates the relationships between the increase in the maximal response and the increase in the potency for each agonist. Increasing receptor expression was found simultaneously to increase the potency and the magnitude of the response until a maximum response was obtained. As shown in the case of L-quisqualate, further increases in receptor density tended only to increase the potency of the agonist without changing the maximum response. As indicated in Table 1, Hill coefficients for agonist-induced accumulation of [^{3} H]-InsP were found to be greater than unity, possibly revealing the complex nature of the activation of phospholipase C by G-protein coupled receptors in CHO cells. Positive cooperativity may result from the amplification of the signal through the signalling cascade as well as from the positive contribution of intracellular [Ca^{2+}] in the activation of phospholipase C (Simpson *et al.*, 1995).

When the concentration-response curves to L-quisqualate and 1S,3R-ACPD were evaluated for cells induced with a low concentration of IPTG (3 and 10 μ M), the intrinsic activity of 1S,3R-ACPD in activating the mGlu1 α receptor was significantly lower than that of L-quisqualate (being approximately 52 and 75% of the responses to L-quisqualate, respectively), confirming the partial agonist activity of 1S,3R-ACPD at this receptor subtype. However, when the receptor was expressed at a high density, the intrinsic activity of 1S,3R-ACPD approached that of L-quisqualate. The consequence of modulating the density of mGlu1 α receptor on the maximal responses to L-quisqualate and 1S,3R-ACPD was further studied by measuring the accumulation of [^{3} H]-InsP induced by maximal concentrations of these two agonists (30 μ M and 1 mM, respectively) on cells previously incubated with increasing concentrations of IPTG. As shown on Figure 4, although the maximal response to 1S,3R-ACPD was always lower than that for L-quisqualate, the difference between these agonist responses decreased as the concentration of IPTG was increased above 10 μ M.

The pharmacological properties of L-quisqualate and 1S,3R-ACPD on the mGlu1 α receptor were further investigated by measuring changes in Ins(1,4,5) P_3 , the immediate second messenger product of phospholipase C activity.

Contrasting with the dramatic increase in [^{3} H]-InsP accumulations observed in this cell-line, agonist-induced increases in Ins(1,4,5) P_3 were relatively small. As shown on Figure 5, L-quisqualate caused a 2–3 fold increase in Ins(1,4,5) P_3 levels to a peak value at 30 s, followed by a decrease to a lower, but still elevated plateau level. 1S,3R-ACPD elicited a lower response that consisted of an essentially monophasic increase in Ins(1,4,5) P_3 to a sustained elevated plateau level (Figure 5A). Interestingly, the levels of Ins(1,4,5) P_3 obtained during the late plateau phase of the response were comparable for both agonists. Analysis of concentration-response curves for L-quisqualate and 1S,3R-ACPD performed at 30 s generated log EC_{50} (M) values of -6.11 ± 0.11 ($n=4$) and -4.49 ± 0.20 ($n=3$), respectively (Figure 5B), in broad agreement with potency estimated obtained from [^{3} H]-InsP measurements (see Table 1). The maximal increase in Ins(1,4,5) P_3 induced by each agonist were measured in cells incubated with different concentrations of IPTG (Figure 5C). A significant Ins(1,4,5) P_3 response to L-quisqualate was only obtained in cells induced with ≥ 5 μ M IPTG. Maximal responses obtained with 1S,3R-ACPD were found to be small as compared to the response obtained with L-quisqualate; even when tested on cells induced with high concentrations of IPTG (100–300 μ M) these never achieved >45% of the responses to the full agonist. Unfortunately, the magnitude of the responses to either

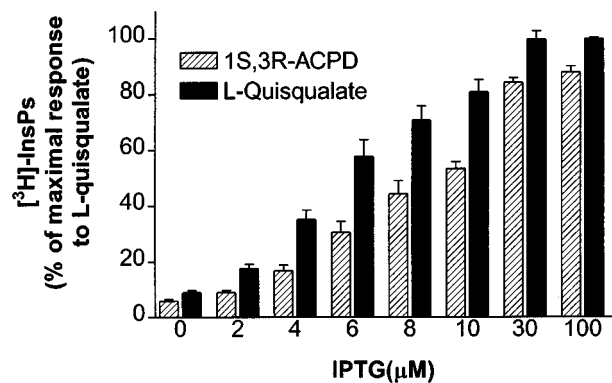


Figure 4 Maximal [^{3} H]-InsP responses to L-quisqualate (30 μ M, filled bars) and 1S,3R-ACPD (1 mM, hatched bars) in CHO-Lac-hmGlu1 α cells. Cells were induced for 20 h with the indicated concentrations of IPTG and the accumulations of [^{3} H]-InsP induced by the agonists were measured over a 30 min incubation in the presence of LiCl (10 mM). Results are expressed as a percentage of the response to L-quisqualate measured in cells induced with IPTG (100 μ M) and are shown as means $\pm$ s.e.mean from four different experiments performed in duplicate.

Table 1 Potencies of L-quisqualate and 1S,3R-ACPD for induction for phosphoinositide hydrolysis in cells expressing the mGlu1 α receptor at different densities

[IPTG] (μ M)	EC_{50} (log M) (n)	L-Quisqualate		EC_{50} (log M) (n)	1S,3R-ACPD	
		Slope	E_{max} (d.p.m. $\times 10^3$)		Slope	E_{max} (d.p.m. $\times 10^3$)
100	-6.38 ± 0.10 (16)	1.66 ± 0.35	24.5 ± 1.9	-4.54 ± 0.10 (4)	1.61 ± 0.31	24.5 ± 1.1
30	-6.43 ± 0.05 (4)	1.82 ± 0.21	24.6 ± 1.1	-4.62 ± 0.06 (4)	1.47 ± 0.09	23.9 ± 0.6
10	-6.10 ± 0.12 (4)	1.52 ± 0.30	22.5 ± 2.5	-4.23 ± 0.03 (4)	1.55 ± 0.21	16.9 ± 1.1
5	-5.78 ± 0.06 (4)	1.81 ± 0.34	8.6 ± 0.3	N.D.	N.D.	N.D.
3	-5.73 ± 0.11 (5)	1.58 ± 0.35	6.3 ± 0.7	-3.97 ± 0.12 (4)	1.96 ± 0.45	3.5 ± 0.3

CHO-Lac-hmGlu1 α cells were incubated for 20 h with different concentrations of IPTG and concentration-response curves were performed by measuring the accumulations of [^{3} H]-InsP after exposure to agonist for 30 min in the presence of LiCl (10 mM). Log EC_{50} values were estimated by non-linear regression and data are shown as means $\pm$ s.e.mean from 'n' different experiments performed in duplicate. N.D., not determined.

agonist precluded further study in cells induced with sub-optimal concentrations of IPTG.

Effects of varying mGlu1 α receptor expression on antagonist activity

In addition to studying the consequence of modulating the expression of mGlu1 α receptors on the properties of agonists, the effects on antagonist activities were also investigated. In order to study the effects of antagonists on the exogenously added agonists, GPT and pyruvate were added to the

incubation medium of the cells. All of the mGlu1 α receptor antagonists studied competitively inhibited the [^{3} H]-InsP responses to the agonists L-quisqualate, 1S,3R-ACPD and (S)-3,5-DHPG. (S)-MCPG, (S)-4-CPG, AIDA, (S)-4-C3HPG and LY367385 were found to cause parallel rightward shifts of the concentration-response curves for agonist-stimulated [^{3} H]-InsP accumulations (as shown for L-quisqualate in Figure 6). The concentration-ratios for EC₅₀ values obtained in the presence compared to the absence of antagonists were used to calculate pK_B values for each antagonist. Table 2A shows that the ranking for antagonist activities was LY367385 > (S)-4-

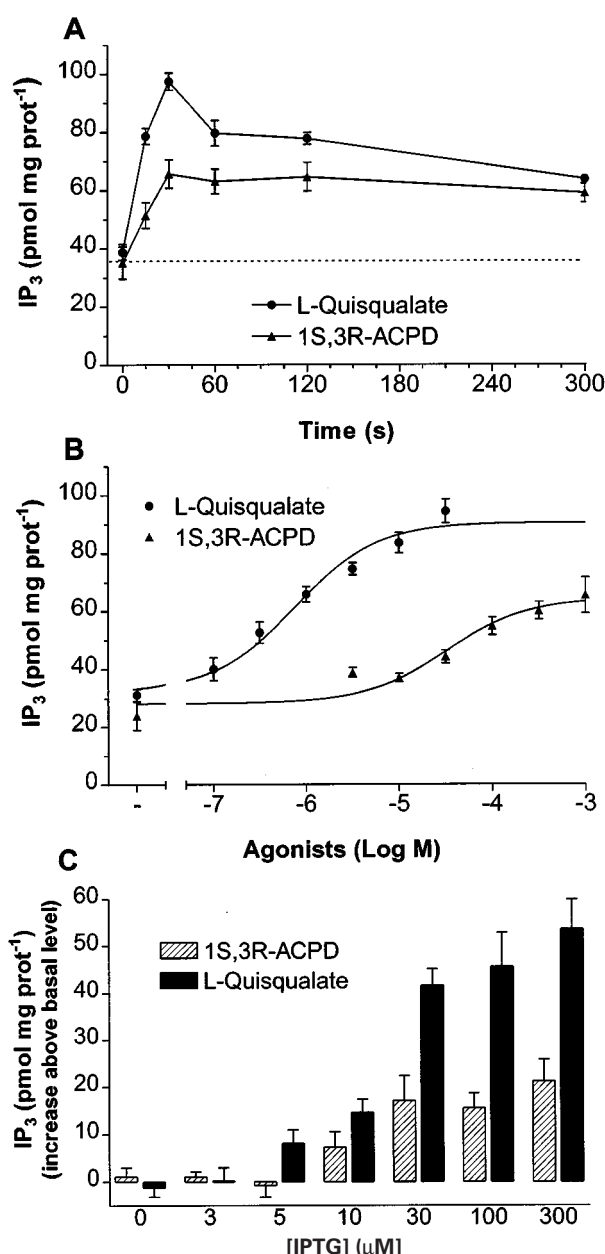


Figure 5 L-quisqualate- and 1S,3R-ACPD-induced increases in Ins(1,4,5)P₃ in CHO-Lac-hmGlu1 α cells. Time-course (A) and concentration-response (B) curves for L-quisqualate- (filled circles) and 1S,3R-ACPD- (filled triangles) induced increases in Ins(1,4,5)P₃ measured in cells pre-incubated with IPTG (100 μ M) for 20 h. (C) Responses to both agonists were measured on cells previously induced for 20 h with different concentrations of IPTG. In (A) and (C), concentrations of L-quisqualate and 1S,3R-ACPD were 30 μ M and 1 mM, respectively. In (B) and (C), Ins(1,4,5)P₃ levels were measured after 30 s stimulation. Data are presented as means $\pm$ s.e.mean from three (A, B) and four (C) experiments performed in duplicate.

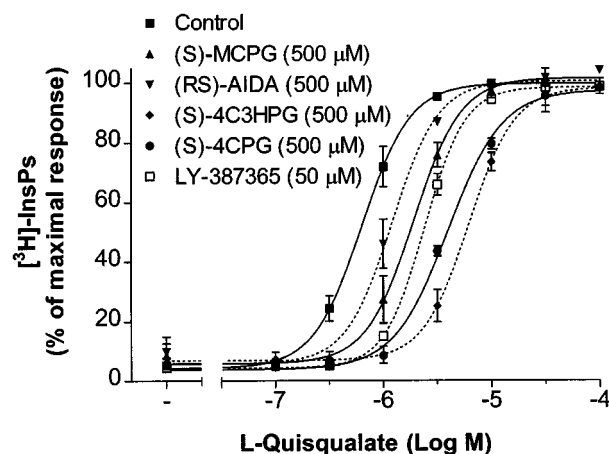


Figure 6 Antagonism of L-quisqualate-induced accumulation of [^{3} H]-InsP in CHO-Lac-hmGlu1 α cells by (S)-MCPG, (S)-4-CPG, AIDA, (S)-4-C3HPG and LY367385. Concentration-response curves for L-quisqualate were performed in the absence or presence of each antagonist used at 500 μ M, except LY367385 (50 μ M) in cells previously induced by 20 h with IPTG (100 μ M). Accumulations of [^{3} H]-InsP induced by L-quisqualate were measured for a 30 min incubation period in the presence of LiCl (10 mM). GPT (3 μ l ml⁻¹) and pyruvate (3 mM) were added to the incubation medium 30 min, and antagonists 15 min, before agonist challenge. Results are expressed as a percentage of the maximal response to L-quisqualate in the absence of antagonist. Data shown are means $\pm$ s.e.mean from at least three different experiments performed in duplicate.

Table 2 Analysis of antagonist activities at the mGlu1 α receptor in CHO-Lac-hmGlu1 α cells (pK_B values)

Antagonist	L-quisqualate	pK _B values (S)-3,5-DHPG	1S,3R-ACPD
(A) IPTG (100 μ M)			
(S)-MCPG	3.73 $\pm$ 0.05 (3)	3.87 $\pm$ 0.10 (3)	3.76 $\pm$ 0.16 (4)
(S)-4-C3HPG	4.32 $\pm$ 0.07 (3)	4.48 $\pm$ 0.14 (3)	4.51 $\pm$ 0.12 (3)
(RS)-AIDA	3.40 $\pm$ 0.12 (3)	N.D.	N.D.
(S)-4CPG	4.08 $\pm$ 0.14 (3)	N.D.	N.D.
LY367385	4.92 $\pm$ 0.12 (4)	N.D.	N.D.
(B) IPTG (5 μ M)			
(S)-MCPG	3.75 $\pm$ 0.05 (3)	3.66 $\pm$ 0.17 (3)	
(S)-4-C3HPG	4.40 $\pm$ 0.13 (3)	4.42 $\pm$ 0.15 (3)	

(A) Cells were incubated for 20 h with IPTG (100 μ M) and the effects of different agonists were tested on the responses induced by L-quisqualate, (S)-3,5-DHPG or 1S,3R-ACPD. (B) Comparison of pK_B values for (S)-MCPG and (S)-4-C3HPG in inhibiting L-quisqualate- and (S)-3,5-DHPG-induced phosphoinositide hydrolysis in cells incubated for 20 h with IPTG (5 or 100 μ M). Data shown are pK_B values calculated on the basis EC₅₀ values for agonist-induced accumulations of [^{3} H]-InsP determined from concentration-response curves in the absence or presence of different antagonists used at a single concentration (means $\pm$ s.e.mean from 'n' different experiments performed in duplicate). N.D., not determined.

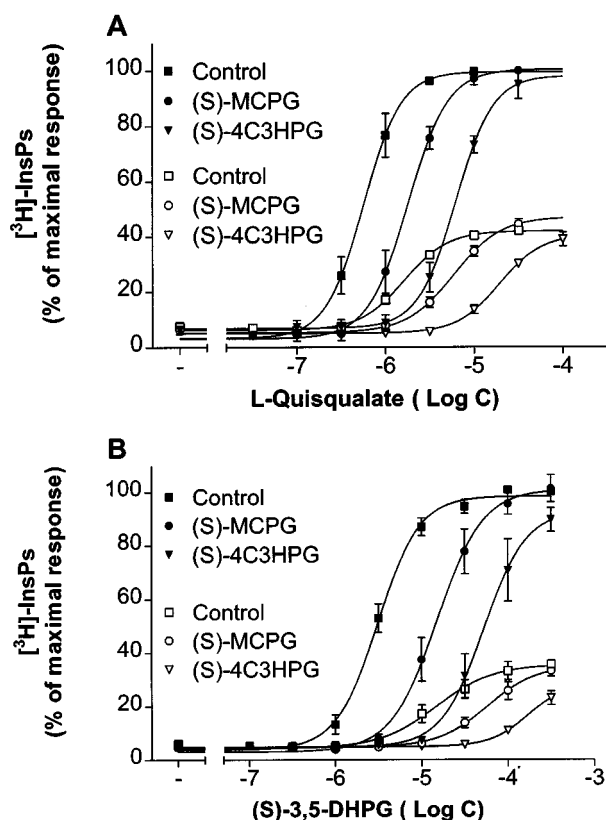


Figure 7 Antagonism of (A) L-quisqualate and (B) (S)-3,5-DHPG-induced accumulations of $[^3\text{H}]\text{-InsP}$ in CHO-Lac-hmGlu1 α cells by (S)-MCPG and (S)-4-C3HPG in cells pre-incubated for 20 h in the presence of IPTG (5 or 100 μM). Concentration-response curves for the agonists were performed in the absence or presence of antagonist (500 μM). Accumulations of $[^3\text{H}]\text{-InsP}$ induced by the agonists were measured for a 30 min time-period in the presence of LiCl (10 mM), GPT (3 u ml^{-1}) and pyruvate (3 mM) were added to the incubation medium 30 min, and antagonists 15 min, before agonist challenge. Results are expressed as a percentages of the maximal responses to L-quisqualate or (S)-3,5-DHPG in the absence of antagonist on cells induced for 20 h with IPTG (100 μM). Data shown are means $\pm$ s.e. mean from at least three different experiments performed in duplicate. In these experiments, the log EC_{50} (M) values measured for L-quisqualate and (S)-3,5-DHPG in the absence of antagonist were -6.29 ± 0.11 and -5.48 ± 0.11 , respectively in cells induced with IPTG (100 μM); and -5.79 ± 0.14 and -4.73 ± 0.30 , respectively in cells induced with IPTG (5 μM). These values were used in the estimation of pK_B values for the antagonists shown in Table 2.

C3HPG > (S)-4-CPG > (S)-MCPG > AIDA. The pK_B values calculated for a given antagonist tested against the three agonists used were not significantly different, indicating that the activities of these antagonists were independent of the agonist utilized (shown for (S)-MCPG and (S)-4-C3HPG, see Table 2A). In the presence of GPT/pyruvate, it was not possible to characterize the effect of antagonists on the response induced by exogenously added L-glutamate, however, preliminary experiments performed in the absence of GPT/pyruvate indicated that all the antagonists tested exhibited approximately similar activities for inhibition of the L-glutamate-mediated response (data not shown).

It is important to note that when used alone, none of these antagonists were found to increase basal $[^3\text{H}]\text{-InsP}$ accumulations, indicating the absence of any positive partial agonist activity, even when the receptor was expressed at the highest levels attainable in this model cell system. Likewise, no inverse agonism could be detected for any of the antagonists examined

as no decrease in basal $[^3\text{H}]\text{-InsP}$ levels was observed when the activity of endogenous glutamate was prevented by the presence of GPT/pyruvate. The consequences of varying the receptor expression level on the activities of antagonists were studied by measuring the inhibition of the $[^3\text{H}]\text{-InsP}$ responses (stimulated by L-quisqualate or (S)-3,5-DHPG) by (S)-MCPG or (S)-4-C3HPG in cells expressing the mGlu1 α receptor at two different levels (Figure 7). As shown above, the potencies of L-quisqualate or (S)-3,5-DHPG were significantly different when measured on cells previously induced with 5 or 100 μM IPTG (see legend of Figure 7). However, when this was corrected in the calculated of the pK_B values for (S)-MCPG and (S)-4-C3HPG, no significant differences were found (Table 2B). This clearly demonstrates that the activity of an antagonist does not depend upon the expression level of the mGlu1 α receptor.

Discussion

A particular feature of the amino acid glutamate is that it plays a dual role as a metabolic intermediate and a neurotransmitter. As most cell-lines release significant quantities of glutamate into their culture medium, the maintenance of a stable expression of glutamate receptors in transfected cells can be problematic (Gabellini *et al.*, 1994; Desai *et al.*, 1995; Lin *et al.*, 1997). In order to overcome this problem, we have used an IPTG-inducible expression system to develop a model of transfected CHO cells stably expressing the human mGlu1 α receptor (Hermans *et al.*, 1998a). A similar approach has been used previously by the group in Minneman in order to express adrenoceptors in transfected cells (Esbenshade *et al.*, 1995; Zhong *et al.*, 1996). Such a model of metabotropic receptor expression has proved to be useful, not only because the receptor is only expressed after induction, but also because receptor expression can be tightly controlled. In the present study, this model was used to investigate the consequences of varying the density of mGlu1 α receptors expressed in CHO cells on the activities of various agonists and antagonists at this important CNS receptor subtype.

Altering the concentration of IPTG added to the culture medium allowed us to modulate the expression level in the transfected CHO-Lac-hmGlu1 α cells, as shown by immunofluorescence analysis using a specific mGlu1 α receptor antiserum, or by analysis of total cell extracts by immunoblots experiments. Considering the high expression level obtained despite the relatively limited duration of the induction (20 h), it was not surprising to detect the receptor widely distributed throughout the cell. When a high concentration of inducer was used, staining was particularly intense close to the nucleus, perhaps associated with newly synthesized receptors at the Golgi apparatus. Interestingly, increasing the receptor density was also found to result in changes in cell shape. Similar observations have been made recently in CHO cells stably transfected to express mGlu1 α receptors and this effect was found to be dependent on the functional activation of the receptor by extracellular Ca^{2+} (Kubo *et al.*, 1998). Our own experiments would suggest that mGlu1 α receptor activation is enhanced in the presence of normal extracellular (1.3 mM) Ca^{2+} concentration, but also requires endogenous extracellular glutamate (Saunders *et al.*, 1998).

Inducing the expression of the mGlu1 α receptor in CHO cells was found to result in an increase in the basal level of phosphoinositide hydrolysis. However, this was found to be caused by glutamate release by the cells as it did not occur in the presence of the glutamate degrading enzyme GPT and its

co-substrate pyruvate. Thus, when precautions were taken to exclude the possibility of receptor activation by endogenous glutamate, the human mGlu1 α receptor appeared to be devoid of any constitutive activity, even when the receptor was expressed at very high levels. This observation contrasts with previous reports indicating that the mGlu1 α receptor exhibits significant constitutive activity in LLC-PK1 cells which could not be reserved by either GPT/pyruvate pre-treatment, or specific nGlu1 α receptor antagonists (Brabet *et al.*, 1995; Pr  zeau *et al.*, 1996). Whether this difference reflects the cell-type and the stoichiometry of transmembrane signalling components, or a species-related (human vs rat) difference in the mGlu1 α receptor, remains to be established. It is however noteworthy that previous studies have reported on the probable activation of metabotropic receptors by glutamate present in incubation medium, and the endogenous glutamate has been postulated as a possible cause of receptor desensitization or down-regulation (Thomsen *et al.*, 1994b; Desai *et al.*, 1995; Pr  zeau *et al.*, 1996; Moroni *et al.*, 1997; Carruthers *et al.*, 1997).

When the functional coupling of the receptor was investigated by measuring agonist-induced accumulations of [^{3} H]-InsP (in the presence of Li $^+$), increasing the receptor expression level not only resulted in an increase in the maximal response, but also in a significant leftward shift of the concentration-response curves for L-quisqualate, (1S,3R)-ACPD and (S)-3,5-DHPG. Such an increase in agonist potency as a result of an increase in the receptor number is consistent with the involvement of a receptor reserve (see Kenakin, 1993). Classical receptor theory predicts that increasing the number of receptors will first result in an increase in the maximal responsiveness to agonists, without a change in potency until the maximal response which can be achieved in the system is reached. Thereafter, further increases in the functional receptor density should result in the development of a receptor reserve, leading to increases in the potencies of agonists without a further increase in the maximal responsiveness. As summarized in Figure 3D, although both potency and maximal response appeared to change simultaneously when the receptor level was increased, there was a direct relationship between the level of expression and the potency of agonists. Using a similar approach of controlling receptor expression, Esbenshade *et al.* (1995) and Zhong *et al.* (1996) have described changes in concentration-response relationships for agonists in cells expressing varying levels of β_1 -, β_2 - or α_{1B} -adrenoceptors that closely followed classical receptor theory.

Comparison of the maximal [^{3} H]-InsP responses obtained after stimulation with L-quisqualate and (1S,3R)-ACPD revealed that the relative intrinsic activity of the latter agonist was dependent on the level of receptor expression. Thus, comparable [^{3} H]-InsP responses were observed with both agonists at very high receptor density, whereas the partial agonist activity of (1S,3R)-ACPD was evident at lower receptor expression levels. This observation is in accordance with classical receptor theory that predicts that intrinsic activity of an agonist is dependent upon the extent of the receptor reserve (Hoyer & Boddeke, 1993). The relationship between receptor density and the intrinsic activity of agonists was further investigated by measuring agonist-induced Ins(1,4,5)P $_3$ accumulation. When the peak Ins(1,4,5)P $_3$ response was measured after 30 s stimulation (1S,3R)-ACPD was found to behave as a partial agonist as compared to L-quisqualate at all receptor expression levels. However, analysis of the time-course of Ins(1,4,5)P $_3$ accumulation CHO-LacmGlu1 α cells induced with IPTG (100 μ M) revealed marked

differences in the responses to L-quisqualate and (1S,3R)-ACPD. Although the magnitude of the peak response measured after 30 s was significantly greater with the full agonist L-quisqualate, the level of Ins(1,4,5)P $_3$ accumulation measured during the plateau phase was similar for both agonists. Thus, depending upon whether Ins(1,4,5)P $_3$ was assessed at the peak or during the plateau phase of second messenger accumulation, (1S,3R)-ACPD could be shown to behave as either a partial or full agonist, respectively. As the plateau phase is approached within 1 min following agonist addition it is not surprising that the agonist activity of (1S,3R)-ACPD on this phase of the response closely resembles that observed for the [^{3} H]-InsP accumulation measured over a 30 min period in the presence of Li $^+$. The relatively small increase in Ins(1,4,5)P $_3$ levels over basal values during the plateau phase precluded further investigation of the difference in response obtained with L-quisqualate and (1S,3R)-ACPD at different levels of induction. However, it might be anticipated that low receptor expression levels would result in substantial differences in the magnitude of the plateau level of Ins(1,4,5)P $_3$ obtained with L-quisqualate and (1S,3R)-ACPD, again revealing the partial activity of the latter in activating phospholipase C through the mGlu1 α receptor. Taken together, these results indicate that differences in the intrinsic activities of mGlu receptor agonists can be observed depending on the index of receptor-stimulated phosphoinositide turnover assessed. Virtually all other studies concerning the pharmacological characterization of agonists interacting with mGlu receptors coupled to the activation of phospholipase C (Group I) are based on the measurements of the accumulation of total inositol phosphates fraction in the presence of Li $^+$ (Hayashi *et al.*, 1994; Brabet *et al.*, 1995; Birse *et al.*, 1993; Bal  zs *et al.*, 1997; Joly *et al.*, 1995). In the light of the present observations regarding the partial activity of (1S,3R)-ACPD, it appears that caution should be exercised when considering data obtained by measuring different indices of phosphoinositide turnover. This would support previously reported differences in the abilities of mGlu agonists to induce Ins(1,4,5)P $_3$ production compared to [^{3} H]-InsP accumulation in rat brain slices (Mistry & Challiss, 1996).

The activities of mGlu antagonists in this model were in accordance with the values previously reported for these compounds at the recombinant mGlu1 α receptor (Conn & Pin, 1997; Moroni *et al.*, 1997; Baker *et al.*, 1997). However, in contrast to the results obtained with agonists, varying the density of the mGlu1 α receptor in the transfected cells did not affect the activities of competitive antagonists for inhibition of agonist-induced phosphoinositide hydrolysis. This observation is consistent with classical receptor theory (Kenakin, 1993) and would further support the proposal that these competitive antagonists do not display positive or negative (inverse) agonist activity.

The identification of selective agonists and antagonists which discriminate between mGlu receptors subtypes is still a limiting step in elucidating the physiological, and possible pathophysiological roles of these receptors in glutamatergic transmission in the CNS. Some important insights have already come from the investigation of mGlu receptors in heterologous expression systems (Pickering *et al.*, 1993; Hayashi *et al.*, 1994; Thomsen *et al.*, 1994a; Brabet *et al.*, 1995; Moroni *et al.*, 1997; Doherty *et al.*, 1997). However, in the absence of appropriate radioligands, and therefore knowledge of relative receptor expression levels, any extrapolation of such information to native mGlu receptors with unknown receptor reserves and the potential promiscuous coupling to a variety of effectors, remains potentially hazardous. The current

study has highlighted major changes in the potency and intrinsic activity of mGlu agonists in cell expressing different levels of the mGlu1 α receptor using an inducible system. This reinforces our view that any selective effects of agonists (or indeed inverse agonists) between receptor subtypes should be investigated in cells exhibiting a range of receptor expression levels and furthermore, that the inducible system described here provides a highly suitable model for such studies.

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We would like to thank Dr Blair Grubb and Mr Chris D'Lacey for their help with the immunocytochemistry. We are grateful to Dr J.R. Harris (Lilly Research Laboratories, Windlesham, U.K.) for providing us with LY367385. This work was supported by Programme Grant 16895/96 from the Wellcome Trust to SRN. E.H. is a Visiting Research Fellow of the Wellcome Trust (Grant No. 048460/96).

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(Received July 21, 1998
Revised November 6, 1998
Accepted November 11, 1998)



Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO

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1 The rat colonic circular muscle displays cyclic episodes of myenteric potential oscillations (MPOs), each of them associated with a spontaneous contraction. Nifedipine 1 μM abolished both MPOs and their associated contractions. TTX (1 μM) increased the amplitude and frequency of spontaneous contractions.

2 Electrical field stimulation (EFS) induced a non-adrenergic non-cholinergic (NANC) inhibitory junction potential (IJP), with two phases: an initial fast hyperpolarization (characterized by IJP amplitude) and a sustained hyperpolarization (characterized by IJP duration).

3 Sodium nitroprusside (10 μM) hyperpolarized and abolished spontaneous contractions even in presence of TTX or 1 μM apamin. ATP (100 μM) also hyperpolarized and abolished spontaneous contractions but its effects were decreased by TTX and abolished by apamin.

4 Suramin (100 μM) or apamin reduced the amplitude of the IJPs, but did not affect their duration. Incubation with L-NOARG (1 mM) reduced the duration but not the amplitude of the IJPs. In presence of L-NOARG plus suramin or L-NOARG plus apamin, both duration and amplitude of the IJPs were reduced but a residual IJP could still be recorded.

5 We conclude that the mechanical and electrical cyclic activity of the rat colonic circular muscle is modulated but not originated by the enteric nervous system and involves L-type calcium channel activity. EFS induces release of NANC inhibitory neurotransmitters which hyperpolarize and relax smooth muscle cells. Both ATP and NO are involved in IJP generation: ATP is responsible for the first phase of the IJPs involving activation of apamin-sensitive potassium channels, whereas NO initiates the second phase which is independent of the activation of such channels.

Keywords: Myenteric potential oscillations (MPOs); non-adrenergic non-cholinergic (NANC); inhibitory junction potential (IJP); ATP; NO; apamin; rat colon

Introduction

Intracellular recordings from colonic smooth muscle cells have revealed a spontaneous cyclic electrical activity. In several species, such as human and cat, rapid oscillations (20–40 cycles min^{-1}) that might trigger action potentials at their peaks, have been described. This electrical activity has been designated as myenteric potential oscillations (MPOs) (Duthie & Kirk, 1978; Sanders & Smith, 1989). In the mouse colon, successive depolarizations take place and might allow action potentials during them. These 'myoelectric complexes' alternate with quiescence periods (Lyser *et al.*, 1993; 1995). Thus, MPOs are very similar to depolarizations recorded in the myoelectric complexes.

Electrical field stimulation (EFS) of isolated gastrointestinal preparations causes a neurogenic transient hyperpolarization, known as the inhibitory junction potential (IJP), which results in relaxation of the muscle. IJPs are mediated by inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitters (Bywater & Taylor, 1986). The identity of these neurotransmitters has been extensively investigated. First ATP and then VIP, were regarded as putative neurotransmitters in the gut (Burnstock *et al.*, 1978; Manzini *et al.*, 1986; Grider & Makhlof, 1988). More recently, nitric oxide (NO) has been viewed as the main NANC inhibitory neurotransmitter in the gastrointestinal tract of many species (Boeckxstaens *et al.*, 1991; Burleigh, 1992; Suthamnatpong *et al.*, 1994; Serio *et al.*, 1995; Takeuchi *et al.*, 1996). However, it has become evident

that IJPs may often show two phases, a fast and a slow hyperpolarization. Thus, this profile has been described for the IJP elicited by EFS of mouse colon (Shuttleworth *et al.*, 1997), mouse ileum (Ward *et al.*, 1994), guinea-pig taenia caeci (Bridgewater *et al.*, 1995), guinea-pig ileum (Crist *et al.*, 1992), human colon (Keef *et al.*, 1993) and human jejunum (Stark *et al.*, 1993). This biphasic shape of the IJP may suggest the release of more than one neurotransmitter. In other species such as dog and pig, IJPs are monophasic with a triggered slow wave afterwards (Stark *et al.*, 1993; Borderies *et al.*, 1997).

In the rat colon, co-localization of ATP and NO has been described in neurons of the myenteric plexus using the quinacrine fluorescence technique to identify purinergic nerves and NADPH-diaphorase histochemistry to localize nitrergic neurons. This co-localization suggests a putative role for both substances in NANC neurotransmission (Belai & Burnstock, 1994). In fact, a role for NO in the IJP has been proposed from results obtained with the sucrose-gap technique (Serio *et al.*, 1995). However, NO is not fully responsible for the hyperpolarization induced by EFS since, in the presence of NO synthase (NOS) inhibitors, EFS does still cause a residual IJP. This may suggest a co-transmission in this tissue (Serio *et al.*, 1995). In fact, in the rabbit stomach, NO, ATP and VIP have all been described as NANC mediators (Baccari *et al.*, 1994).

Accordingly, the aims of this study have been: (1) to investigate the spontaneous electrical and mechanical activity of the isolated circular muscle of the rat colon; (2) to study the putative neurotransmitter(s) that mediate the inhibitory NANC response to EFS.

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Methods

Tissue preparation

Male Sprague-Dawley rats (300–350 g), 8–10-weeks-old, were kept at a constant temperature ($21\text{--}23^{\circ}\text{C}$), with lighting cycle of 12 h light/12 h dark, fasted overnight (18 h) but allowed *ad libitum* access to water. Rats were killed by decapitation and bled. This procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona. The colon was quickly removed, placed in Krebs solution on a dissection dish, and the mucosal and submucosal layers removed. Circular muscle strips were cut 1 cm long and 0.3 cm wide. The composition of the Krebs solution was (in mM) glucose, 10.10; NaCl, 115.48; NaHCO_3 , 21.90; KCl, 4.61; NaH_2PO_4 , 1.14; CaCl_2 , 2.50 and MgSO_4 , 1.16 bubbled with a mixture of 5% CO_2 –95% O_2 (pH 7.4).

Recordings of spontaneous mechanical activity

Muscle strips were attached with silk threads to a stable mount in the bottom of a 10 ml organ bath filled with carbogenated Krebs solution at $37\pm 1^{\circ}\text{C}$. The upper end was tied to an isometric force transducer (Harvard VF-1) connected to an amplifier and then to a computer. Data were digitalized (25 Hz) and simultaneously displayed and collected using Datawin1 software (Panlab-Barcelona) coupled to an ISC-16 A/D card installed in a PC Pentium computer. A tension of 1 g was applied and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity.

To estimate the responses to drugs, the amplitude, duration and frequency of the contractions were measured before and after drug addition.

Simultaneous recordings of electrical and mechanical activities

Muscle strips were placed (circular muscle side up) in a Sylgard coated chamber and continuously perfused with carbogenated Krebs solution at $37\pm 1^{\circ}\text{C}$. One end was pinned for intracellular recordings and the opposite end was attached to an isometric transducer and preloaded with 1 g. Preparations were allowed to equilibrate for approximately 1 h before experiments started. Circular muscle cells were impaled with glass microelectrodes ($R=40\text{--}60\text{ M}\Omega$) filled with 3 M KCl. Membrane potential was measured using standard electrometer Duo773 (WPI Inc., FL, U.S.A.). Both electrical and mechanical activities were displayed on a digital storage oscilloscope 4026 (Racal-Dana Ltd., England), and simultaneously digitalized (100 Hz) and collected using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., CA, U.S.A.) installed in a 486 PC computer.

Electrophysiological response to electrical field stimulation (EFS)

Tissue samples were placed in a silgard coated chamber in the same conditions described above. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and

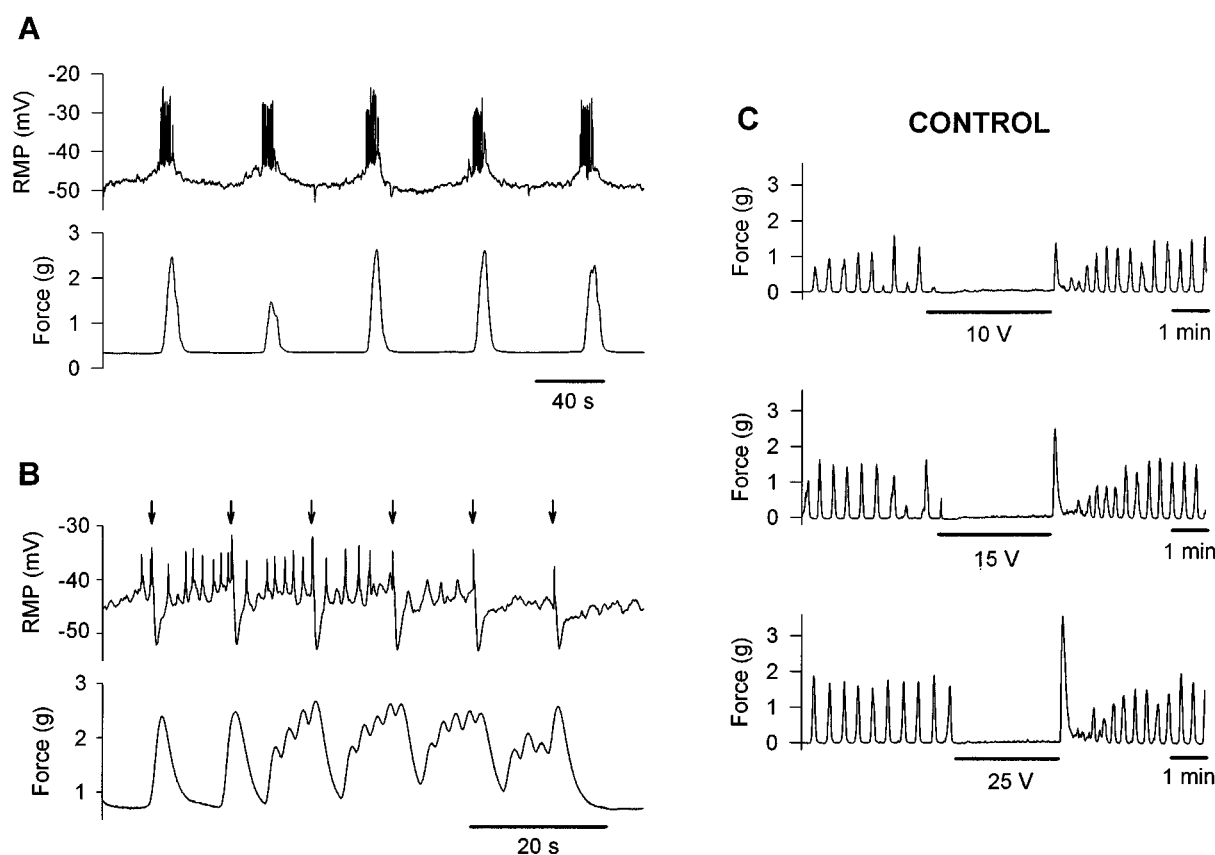


Figure 1 (A) Simultaneous intracellular microelectrode (top) and mechanical (bottom) recordings from the circular muscle layer of the rat colon showing cyclic episodes of MPOs and associated contractions. (B) Intracellular recording showing IJPs (top) evoked by EFS (12 V, 20 Hz, train duration 100 ms and pulse duration 0.3 ms) and the simultaneous associated relaxations on the mechanical activity (bottom). Arrows indicate the start of the stimuli. (C) Mechanical recordings showing the response to EFS elicited during 3 min (1 Hz, train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

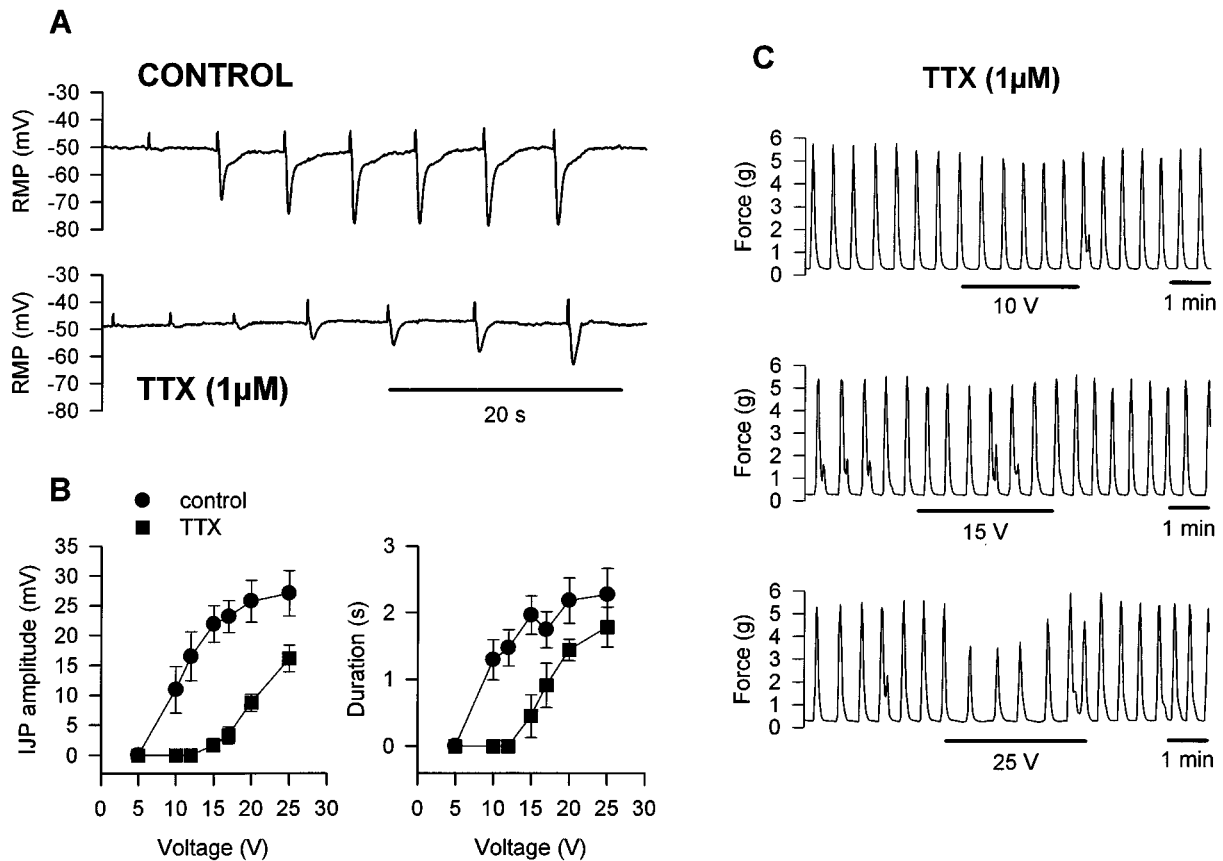


Figure 2 (A) Intracellular recordings showing IJPs elicited by EFS increasing the voltage of stimulation (5, 10, 12, 15, 17, 20 and 25 V) in control conditions (top), and in the presence of TTX (1 μ M) (bottom). Both recordings were obtained in the presence of nifedipine (1 μ M) to abolish mechanical activity and to increase the duration of the impalement. (B) Effect of TTX (1 μ M) on the amplitude (left) and duration (right) of the IJPs. (C) Mechanical recording, in the presence of TTX (1 μ M), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

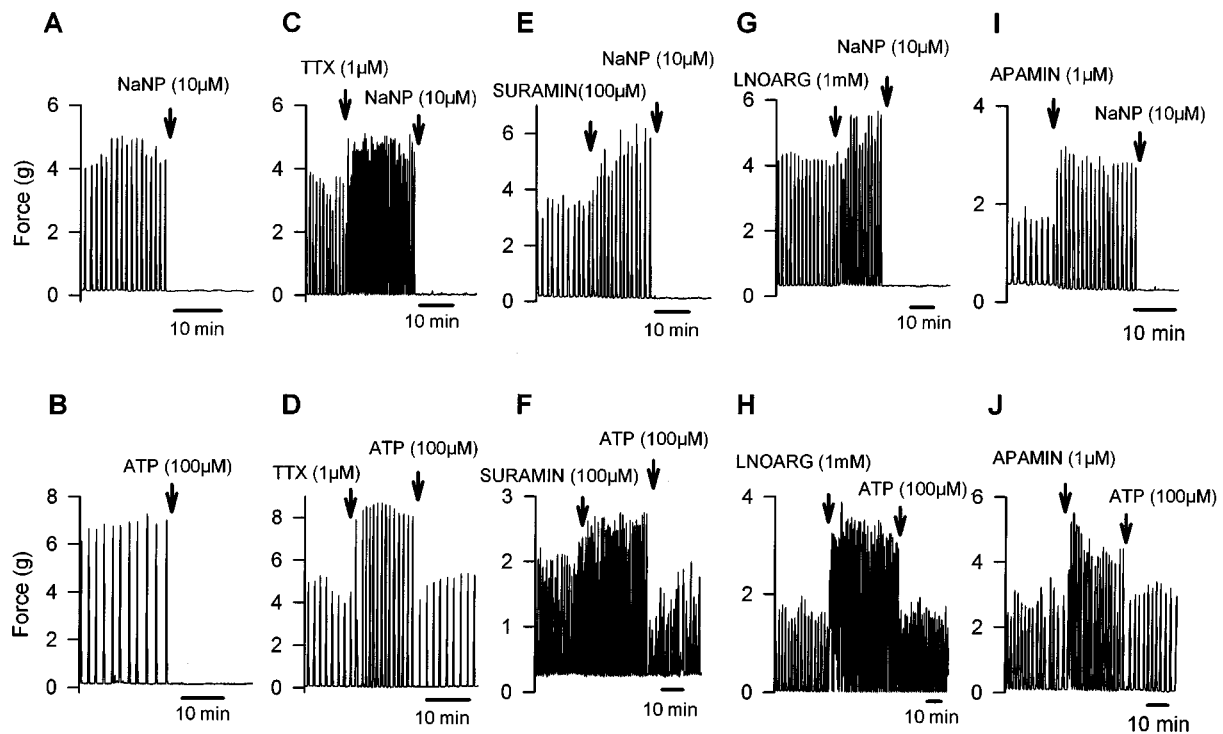


Figure 3 Recording showing the effects of NaNP (10 μ M) and ATP (100 μ M) on the spontaneous mechanical activity under control conditions (A and B); in the presence of TTX (1 μ M) (C and D); in the presence of suramin (100 μ M) (E and F); in the presence of L-NOARG (1 mM) (G and H); and in the presence of apamin (1 μ M) (I and J, respectively).

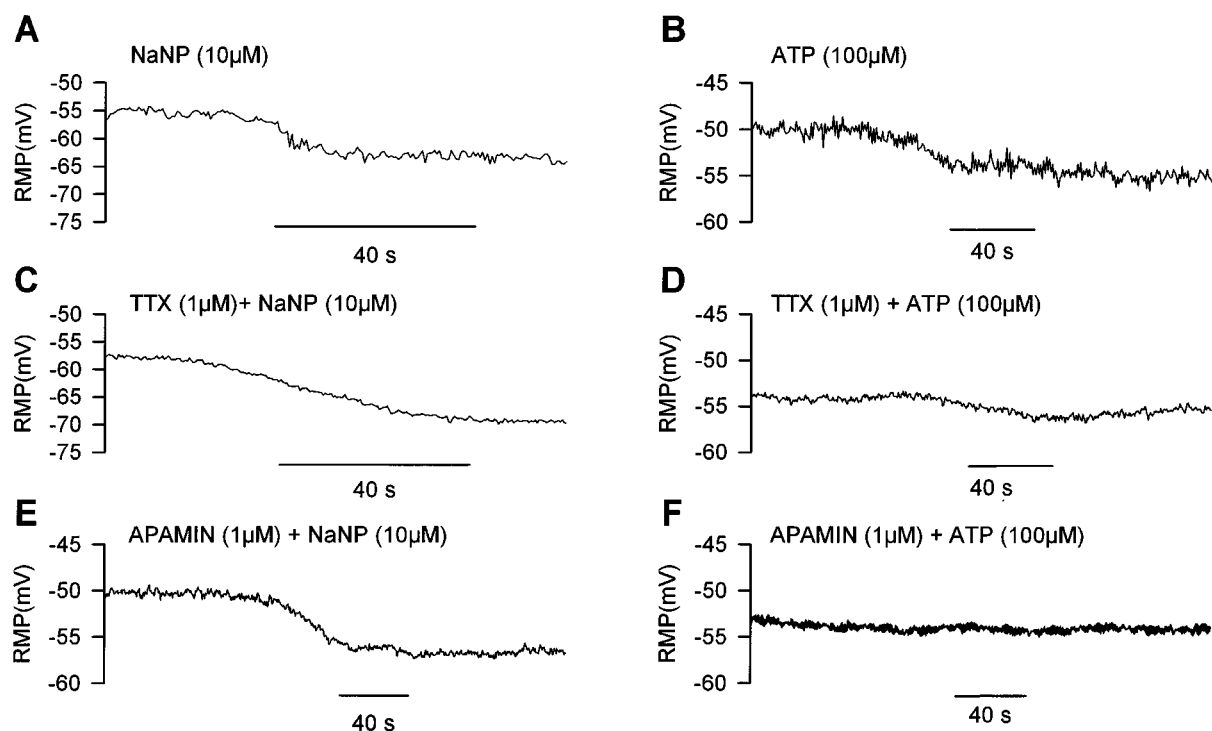


Figure 4 Intracellular microelectrode recordings showing the effects of NaNP (10 μ M) (A) and ATP (100 μ M) (B) on the resting membrane potential under control conditions. The same substances were tested in the presence of TTX 1 μ M (C and D, respectively) and in the presence of apamin (1 μ M) (E and F, respectively). All intracellular recordings were obtained in the presence of nifedipine (1 μ M).

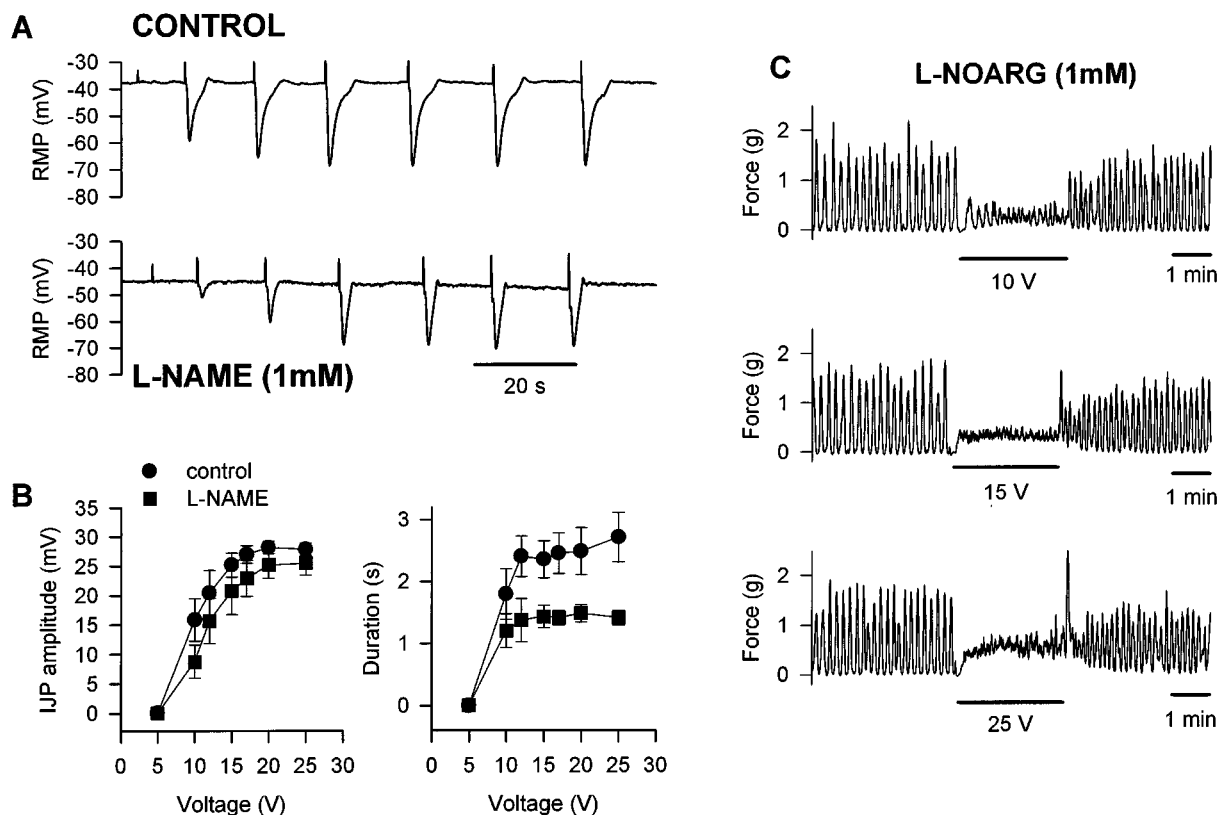


Figure 5 (A) Intracellular recording showing the effect of L-NAME (1 mM) on the IJPs obtained by EFS (same procedure as in Figure 2). All recordings were obtained in the presence of 1 μ M nifedipine (B) Effect of L-NAME (1 mM) on the amplitude (left) and duration (right) of the IJPs. (C) Mechanical recording, in the presence of L-NOARG (1 mM), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

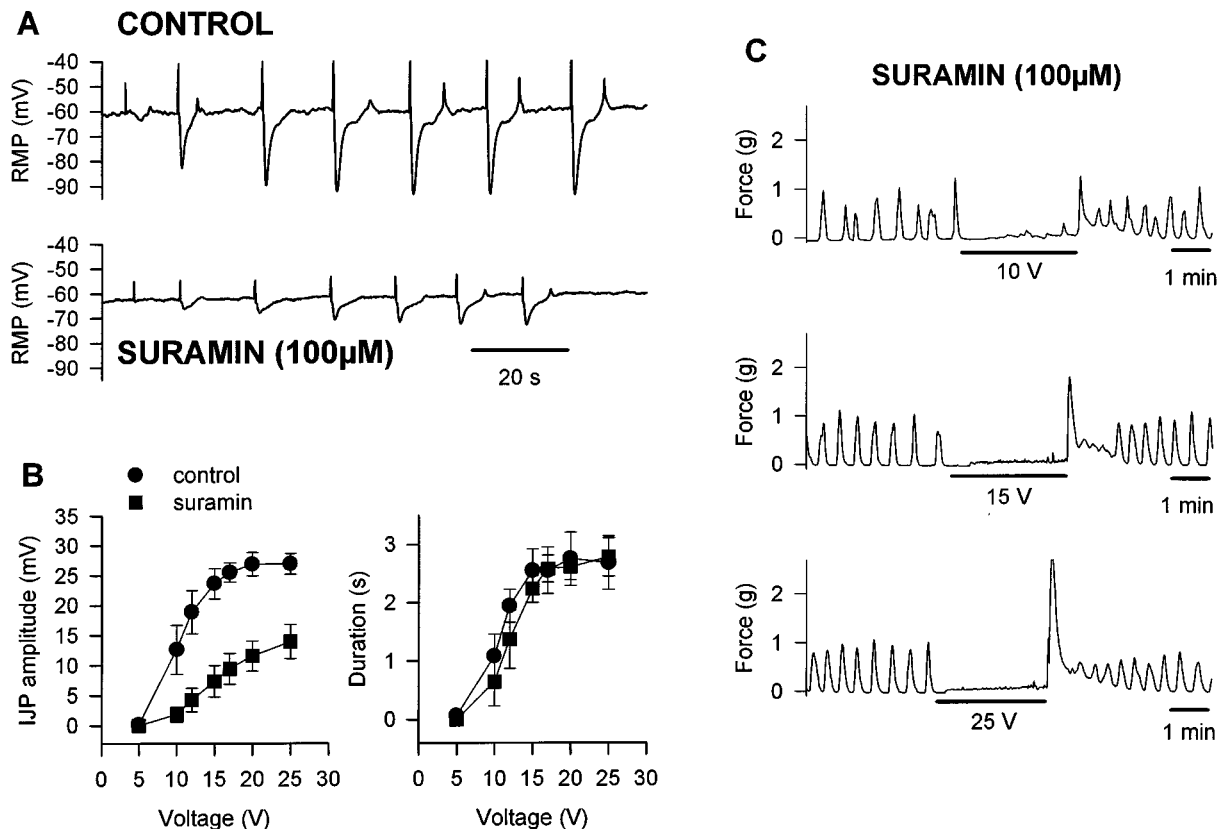


Figure 6 (A) Microelectrode recording showing the IJPs obtained by EFS in control conditions (top) and in the presence of suramin ($100 \mu\text{M}$), (same procedure as in Figure 2). All recordings were obtained in the presence of nifedipine ($1 \mu\text{M}$). (B) Effect of suramin ($100 \mu\text{M}$) on the amplitude (left) and duration (right) of the IJPs obtained at different stimulus strength. (C) Mechanical recording, in the presence of suramin ($100 \mu\text{M}$), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

1.5 cm apart. Train stimulation had the following parameters: total duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms and increasing amplitude strengths (5, 10, 12, 15, 17, 20 and 25 V). The amplitude and duration of IJPs were measured under control conditions and after infusion of each drug. In order to obtain stable impalements, nifedipine ($1 \mu\text{M}$) was perfused to abolish mechanical activity.

Mechanical response to electrical field stimulation (EFS)

In separate experiments the mechanical responses to electrical field stimulation were recorded using the same set up in the absence of nifedipine. EFS was applied during 3 min, 1 train s^{-1} (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V). To estimate the responses to EFS, the amplitude, duration and frequency of the contractions were measured before and during EFS. When a drug qualitatively modified the response, a description of the effect is reported.

Solutions and drugs

The following drugs were used: nifedipine, N-nitro-L-arginine (L-NOARG), N-methyl-L-arginine ester (L-NAME), adenosine 5'-triphosphate (ATP), phentolamine (Sigma Chemicals, St. Louis, U.S.A.); tetrodotoxin (TTX), atropine sulphate, propranolol, suramin, apamin, sodium nitroprusside (NaNP) (Research Biochemicals International, Natick, U.S.A.). Stock

solutions were made by dissolving drugs in distilled water except for nifedipine which was dissolved in ethanol.

Data analysis and statistics

Data are expressed as mean $\pm$ s.e.mean. Paired Student's *t*-test was used to compare mechanical activity in the absence and in the presence of drugs or in the absence and presence of EFS. The differences between amplitude or duration of the IJPs before and after drug infusion were compared by analysis of variance for repeated measurements followed by pairwise comparisons using Student-Newman-Keuls method. A *P* value <0.05 was considered as statistically significant.

Results

Spontaneous electrical and mechanical activity

The resting membrane potential of circular muscle cells from the rat colon was $-50.2 \pm 1.3 \text{ mV}$ ($n=41$). This membrane potential was spontaneously unstable showing cyclic episodes of depolarization (5–10 mV) that trigger action potentials which were considered myenteric potential oscillations (MPOs) (Figure 1A). This cyclic activity occurred at a frequency of $0.78 \pm 0.17 \text{ episodes min}^{-1}$. Each episode of MPOs is associated with a spontaneous muscle contraction which averaged $3.3 \pm 0.1 \text{ g}$ amplitude and $22.6 \pm 1.4 \text{ s}$ duration ($n=29$). The cyclic electrical activity (depolarizations and

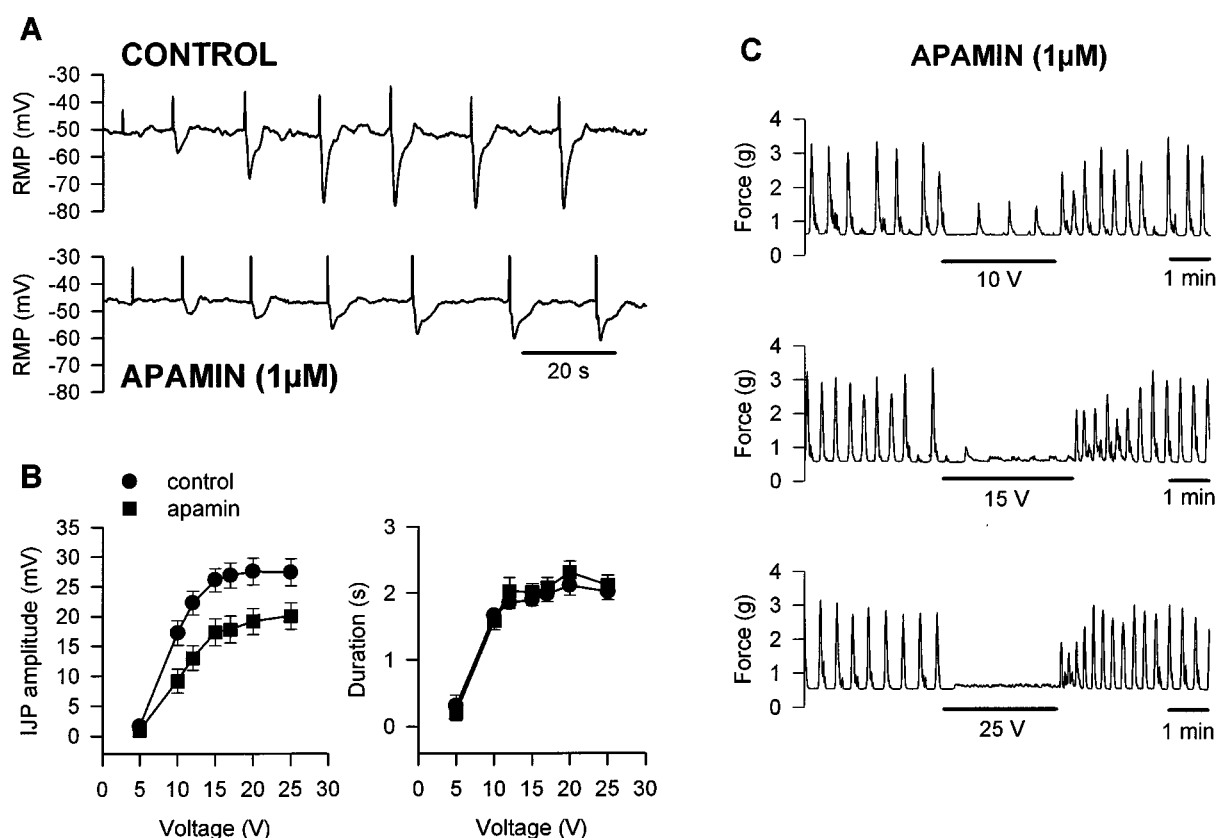


Figure 7 (A) Effect of apamin ($1 \mu\text{M}$) on the IJPs obtained at different stimulus strengths (same procedure as in Figure 2). All recordings were performed in the presence of nifedipine ($1 \mu\text{M}$). (B) Effect of apamin ($1 \mu\text{M}$) on the amplitude (left) and duration (right) of the IJP. (C) Mechanical recording, in the presence of apamin ($1 \mu\text{M}$), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

action potentials) and associated contractions were abolished by $1 \mu\text{M}$ nifedipine ($n=5$).

Relationship between electrical field stimulation and the mechanical activity

EFS induced a membrane hyperpolarization considered as an inhibitory junction potential (IJP) (see next paragraph). IJPs could be elicited both during the resting state and during the episode of depolarization. When an IJP was triggered during the resting period the mechanical activity was not modified. However, when the IJP was elicited during the spontaneous contraction, a transient relaxation was immediately recorded (Figure 1B). Moreover, when repetitive IJPs (1 Hz) were elicited (10, 15, 25 V) for 3 min, spontaneous contractions were abolished. An off-response was observed at the end of the stimulation (Figure 1C).

Characterization of the inhibitory junction potential

EFS evoked a transient membrane hyperpolarization which showed two phases: a fast hyperpolarization followed by a slow one (Figure 2A). These phases were characterized by the amplitude and the duration of the transient hyperpolarization respectively. Both parameters increased when increasing voltages were applied (Figure 2B). The maximum response ($-26.6 \pm 0.6 \text{ mV}$ in amplitude and $2.4 \pm 0.1 \text{ s}$ in duration) was reached at EFS of 25 V ($n=60$).

Nifedipine ($1 \mu\text{M}$, $n=5$) abolished MPOs and spontaneous contractions but did not modify the resting membrane

potential. The amplitude and duration of IJPs remained unchanged (not shown). After this fact was evidenced, all intracellular recordings were made in the presence of nifedipine, as the absence of spontaneous contractions increased significantly the duration of impalements. TTX ($1 \mu\text{M}$, $n=6$) abolished the hyperpolarizations evoked by EFS at stimuli ranging from 5–12 V. At higher voltages, a residual hyperpolarization was still present (Figures 2A and B) which could be due to a direct stimulation of nerve endings. Thus, transient hyperpolarizations evoked by train stimuli were (IJPs). Atropine, phentolamine and propranolol (each at $1 \mu\text{M}$, $n=6$) did not change IJP characteristics regardless of the strength of the stimulus used (not shown). The spontaneous mechanical activity displayed by the tissue in control conditions was abolished when EFS was applied (Figure 1C). TTX $1 \mu\text{M}$ ($n=4$) decreased but did not block the inhibitory effects of stimuli of higher voltages (Figure 2C).

Effect of NaNP and ATP on the mechanical activity

Sodium nitroprusside (NaNP) ($10 \mu\text{M}$, $n=4$) and ATP ($100 \mu\text{M}$, $n=4$) abolished spontaneous contractions (Figures 3A and B). TTX ($1 \mu\text{M}$, $n=8$) increased the amplitude (4.0 ± 0.6 vs $7.5 \pm 1.1 \text{ g}$) and the frequency (0.66 ± 0.10 vs $0.83 \pm 0.13 \text{ c.p.m.}$) of phasic contractions ($P<0.01$ and $P<0.05$, respectively) (Figures 3C and D). The duration of the contractions was not affected. NaNP ($10 \mu\text{M}$, $n=4$) caused a TTX resistant abolition of the spontaneous mechanical activity (Figure 3C). In the presence of TTX ($1 \mu\text{M}$, $n=4$), ATP reduced the amplitude (9.4 ± 1.3 vs $5.3 \pm 0.7 \text{ g}$; $P<0.05$)

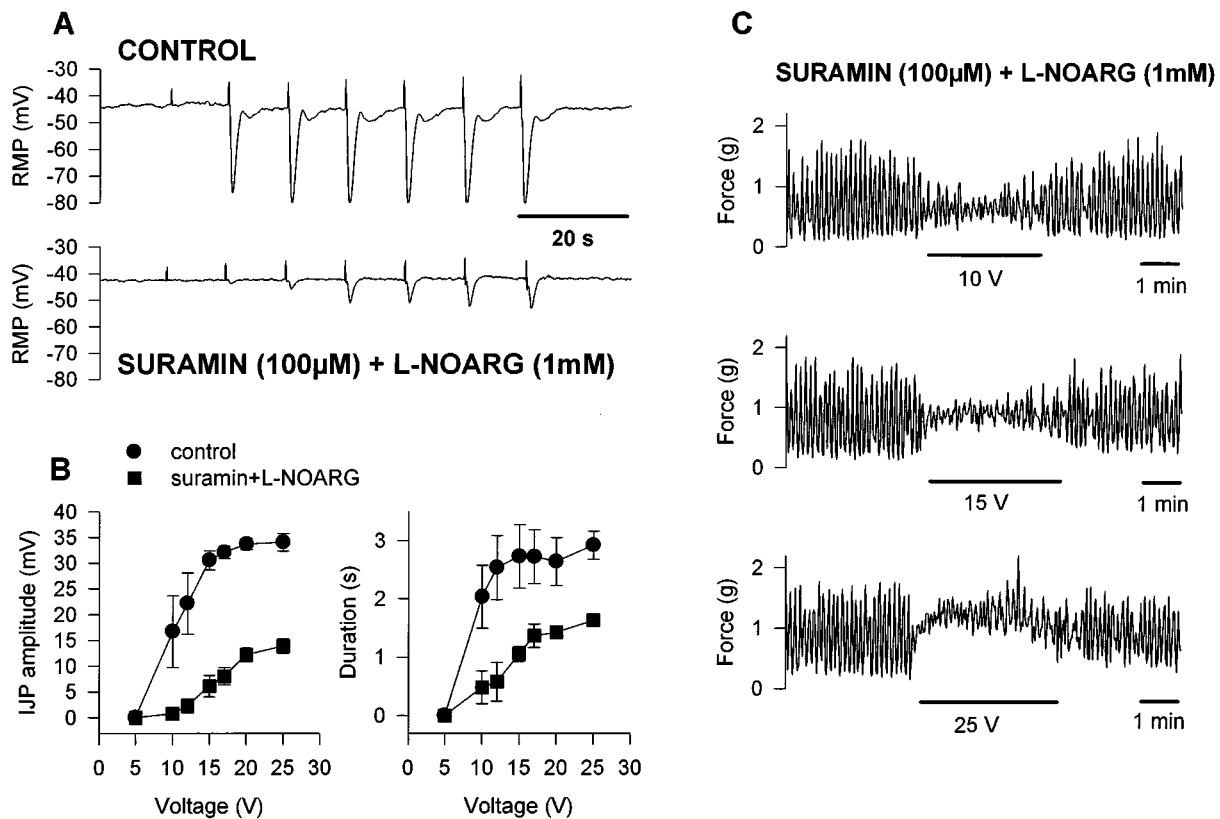


Figure 8 (A) Microelectrode recording showing the IJPs obtained by EFS (same procedure as in Figure 2), in control conditions (top) and in the presence of suramin (100 μ M) plus L-NOARG (1 mM). All recordings were obtained in the presence of nifedipine (1 μ M). (B) Effect of suramin (100 μ M) plus L-NOARG (1 mM) on the amplitude (left) and duration (right) of the IJP. (C) Mechanical recording, in the presence of suramin (100 μ M) plus L-NOARG (1 mM), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

and frequency of the contractions (0.88 ± 0.10 vs 0.51 ± 0.12 c.p.m.; $P < 0.01$), but a complete blockade of the mechanical activity was not achieved (Figure 3D).

In the presence of suramin (100 μ M, $n = 8$), the amplitude (3.4 ± 0.6 vs 4.5 ± 0.5 g; $P < 0.01$) and frequency (0.55 ± 0.06 vs 0.74 ± 0.09 c.p.m.; $P < 0.05$) of the contractions increased (Figures 3E and F) whereas the duration of the contractions was not affected. NaNP (10 μ M, $n = 4$) abolished the cyclic mechanical activity in the presence of suramin (100 μ M) (Figure 3E). In contrast, ATP (100 μ M, $n = 4$) reduced but did not abolish the amplitude of spontaneous mechanical activity (4.3 ± 0.9 vs 1.9 ± 0.9 g; $P < 0.05$) (Figure 3F).

L-NOARG (1 mM, $n = 5$) increased the amplitude (3.1 ± 1.0 vs 5.0 ± 1.4 g; $P < 0.01$), duration (22.0 ± 1.1 vs 33.2 ± 1.7 s; $P < 0.01$) and frequency (0.55 ± 0.08 vs 0.80 ± 0.03 c.p.m.; $P < 0.05$) of the contractions (Figures 3G and H). L-Arg (10 mM, $n = 6$) partly reversed this effect (not shown). In the presence of L-NOARG (1 mM), NaNP (10 μ M, $n = 4$) abolished the mechanical activity (Figure 3G), whereas ATP (100 μ M, $n = 6$) reduced the amplitude (4.6 ± 1.2 vs 2.3 ± 0.7 g; $P < 0.01$) and frequency (0.98 ± 0.18 vs 0.67 ± 0.14 ; $P < 0.01$) of cyclic contractions (Figure 3H).

Apamin (1 μ M, $n = 6$) increased the amplitude (3.3 ± 0.4 vs 4.7 ± 0.6 g; $P < 0.01$) and frequency (0.66 ± 0.16 vs 0.92 ± 0.13 c.p.m.; $P < 0.05$) of contractions, but their duration was unchanged (Figure 3I and J). In the presence of apamin (1 μ M), NaNP (10 μ M, $n = 4$) was able to abolish the spontaneous mechanical activity (Figure 3I) whereas ATP

(100 μ M, $n = 4$) did not abolish but reduced the amplitude of contractions (5.5 ± 0.6 vs 3.6 ± 0.7 g; $P < 0.05$) (Figure 3J).

Effects of NaNP and ATP on the membrane potential

The NO donor, sodium nitroprusside (10 μ M, $n = 5$; $P < 0.001$), caused a hyperpolarization of 13 ± 1 mV of the muscle cells (Figure 4A). This hyperpolarization was resistant to TTX (1 μ M) (9 ± 2 mV, $n = 4$, $P < 0.01$) (Figure 4C), and apamin (1 μ M) (10 ± 2 mV, $n = 4$, $P < 0.01$) (Figure 4E).

Perfusion of ATP (100 μ M, $n = 6$; $P < 0.001$) caused a hyperpolarization of 12 ± 2 mV of the muscle cells (Figure 4B). In the presence of TTX (1 μ M, $n = 5$), the ATP-induced hyperpolarization was also observed (7 ± 2 mV; $P < 0.05$) (Figure 4D). In the presence of apamin (1 μ M), ATP (100 μ M) did not change the resting membrane potential ($n = 4$) (Figure 4F).

Effect of NO synthase inhibitors, suramin and apamin on the IJP and spontaneous contractions

NO synthase inhibitors, L-NOARG ($n = 5$) and L-NAME ($n = 6$) (1 mM), reduced the duration of the IJPs ($P < 0.001$) but did not affect their amplitude (Figures 5A and B). In the presence of L-NOARG (1 mM, $n = 4$) EFS still abolished the cyclic mechanical activity but a sustained tone was reached (Figure 5C).

The purinoceptor blocker suramin (100 μ M, $n = 6$; $P < 0.001$) reduced the amplitude of IJPs at all stimulus

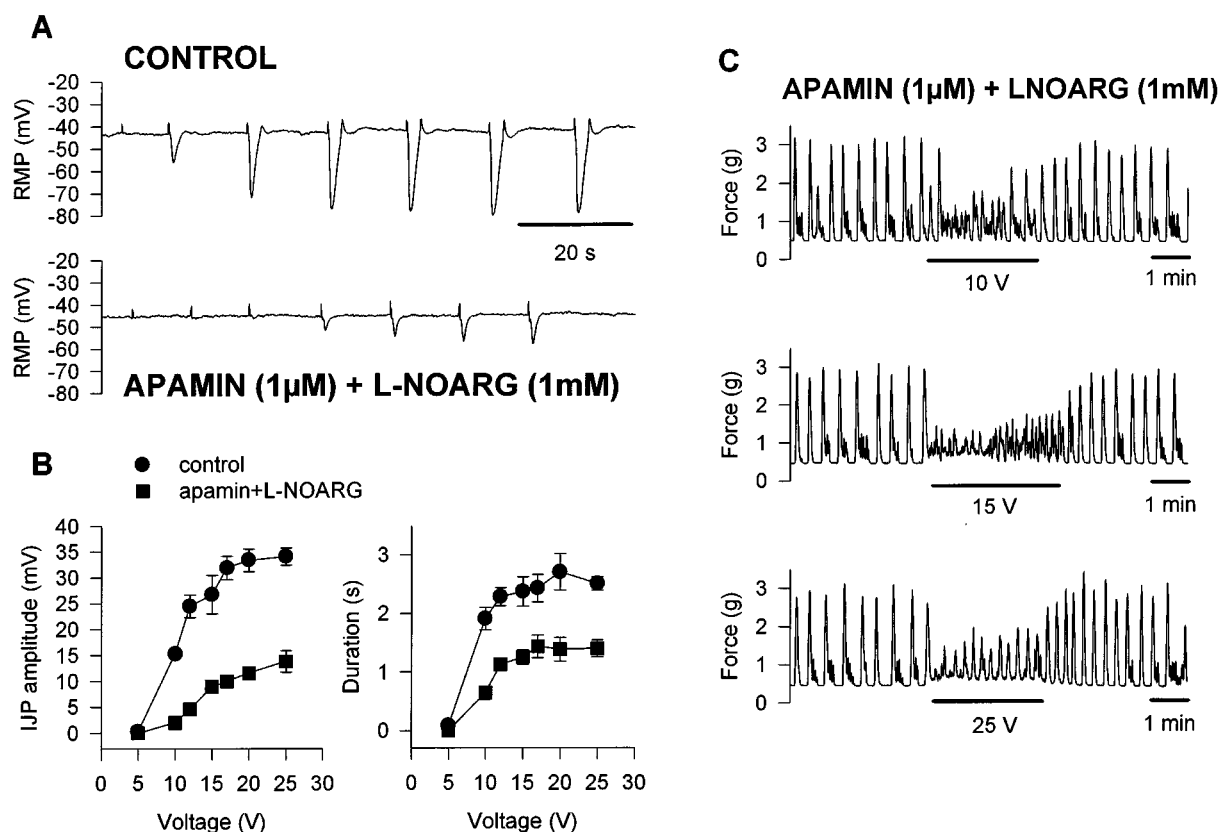


Figure 9 (A) Intracellular recordings showing the effect of apamin ($1 \mu\text{M}$) plus L-NOARG (1 mM) on the IJPs obtained by EFS (same procedure as in Figure 2). All recordings were obtained in the presence of nifedipine ($1 \mu\text{M}$). (B) Effect of apamin ($1 \mu\text{M}$) plus L-NOARG (1 mM) on the amplitude (left) and duration (right) of the IJP. (C) Mechanical recording, in the presence of apamin ($1 \mu\text{M}$) plus L-NOARG (1 mM), showing the response to EFS elicited during 3 min at 1 Hz (train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms) and increasing amplitude strengths (10, 15 and 25 V).

strengths. The duration of the IJPs was not affected (Figures 6A and B). In the presence of suramin ($100 \mu\text{M}$, $n=4$), repetitive EFS (1 Hz) still caused an abolition of the spontaneous mechanical activity (Figure 6C).

Apamin ($1 \mu\text{M}$, $n=12$) reduced the amplitude but not the duration of the IJPs ($P<0.001$; Figures 7A and B). However, in the presence of apamin ($1 \mu\text{M}$, $n=4$) repetitive EFS (1 Hz) was still able to abolish the spontaneous mechanical activity at 15 and 25 V. (Figure 7C)

In the presence of L-NOARG (1 mM) plus suramin ($100 \mu\text{M}$) ($n=4$) the amplitude and duration of IJPs were reduced ($P<0.01$) (Figures 8A and B). However, a residual IJP was still recorded (Figure 8A). In the presence of both drugs, EFS was still able to decrease phasic contractions (Figure 8C).

In the presence of apamin ($1 \mu\text{M}$) plus L-NOARG (1 mM) ($n=4$), the amplitude and duration of IJPs were decreased ($P<0.01$) (Figures 9A and B) but a residual IJP could be recorded. In the presence of both drugs, EFS (1 Hz) was still able to reduce the spontaneous mechanical activity but phasic contractions were observed during EFS.

Discussion

The electrical activity pattern of rat colonic circular smooth muscle consists of cyclic episodes of slow depolarizations that trigger smooth muscle action potentials which induce a contraction. Accordingly, cyclic depolarizations are related to cyclic contractions of smooth muscle. In the mouse colon, neurogenic cyclic depolarizations have been reported and

designated as 'myoelectric complexes' (Lyster *et al.*, 1993; 1995). Smooth muscle action potentials are similar to those described in the human colon (Duthie & Kirk, 1978). In our study TTX increased the amplitude and frequency of cyclic contractions which could be taken as evidence against the concept that the cyclic activity is dependent on a neural input. These results suggest that the rat colonic circular muscle is neurally inhibited. NO and ATP are putative neurotransmitters involved in this tonic neural inhibition because NO synthase inhibitors and suramin increased the frequency and amplitude of contractions. Accordingly, our data suggest that although the cycle is neurally modulated, myogenic mechanisms may originate this cyclic activity. Our results do also suggest that in the rat colon, the cyclic electrical and mechanical activities are mainly due to calcium influx through L-type calcium channels because in the presence of nifedipine, slow depolarizations, action potentials and the corresponding mechanical activity are abolished.

Inhibitory junction potentials (IJPs) could be elicited during the whole cycle. Between contractions when the muscle was relaxed, IJPs caused hyperpolarization without any change in mechanical activity. When a contraction occurred, IJPs induced a relaxation. The fact that repetitive IJPs inhibited cyclic activity shows that inhibitory mediators could be released during the whole cycle. IJPs and relaxations elicited by EFS were sensitive to TTX when a low voltage was applied. However, at higher voltages a residual IJP and relaxation was still observed. TTX-resistant IJPs and relaxations might be due to a direct stimulation of nerve endings. This has also been described in the canine ileum (Cayabyab *et al.*, 1996) and

(using the same set-up) in the porcine ileum (Borderies *et al.*, 1997). In this last case, N-type calcium channels allow the calcium influx that triggers the release of inhibitory transmitters. However, in the rat colon, N-type calcium channels are not fully responsible for neurotransmitter release from nerve endings (Borderies *et al.*, 1996) and further investigations are needed to characterize the voltage gated calcium channels involved.

IJPs did not change under NANC conditions, which shows that they are due to the release of NANC inhibitory mediator(s). IJPs recorded from the rat colon showed two phases: a sharp decrease of the membrane potential (fast component) followed by a sustained hyperpolarization (slow component). This biphasic IJP is similar to those described in other preparations such as mouse colon (Shuttleworth *et al.*, 1997), mouse ileum (Ward *et al.*, 1994), guinea-pig taenia caeci (Bridgewater *et al.*, 1995), guinea-pig ileum (Crist *et al.*, 1992), human colon (Keef *et al.*, 1993) and human jejunum (Stark *et al.*, 1993), whereas a monophasic IJP has been recorded from the pig (Borderies *et al.*, 1997) and dog (Stark *et al.*, 1993) ileum. The presence of two components in the IJP may suggest a cotransmission. The amplitude of the IJP was used to define the fast component and the duration as a feature of the second one.

We tested the ability of several putative inhibitory transmitters to hyperpolarize the smooth muscle cell and to decrease the mechanical activity. Exogenous addition of both ATP and the NO donor NaNP, hyperpolarized and relaxed the smooth muscle. Since the effects of NaNP were unchanged in the presence of TTX, NO may be considered as a putative final inhibitory NANC transmitter in this preparation. The inhibitory effect of ATP on the mechanical activity was partially decreased by TTX, suggesting that both muscle cells and neurons may have purinoceptors. In the presence of suramin, NaNP but not ATP abolished the mechanical activity. The same result was found when apamin was added to the bath. Moreover, apamin blocked the hyperpolarization induced by ATP but did not modify the hyperpolarization induced by NaNP. Altogether, these results show that both suramin and apamin might be pharmacological tools to discriminate between ATP and NO effects in this tissue. An interesting finding was that although NaNP was able to abolish cyclic contractions in the presence of L-NOARG, ATP did not abolish the cyclic mechanical activity when the preparation was incubated with a NO synthase inhibitor. This result is consistent with the fact that suramin and TTX only partially blocked the effects of ATP and suggests that ATP might also be releasing NO from neural and/or non neural sources.

When EFS was applied, L-NOARG or L-NAME reduced the slow component and suramin reduced the fast component of the IJP. These effects were selective for each phase suggesting that ATP might be involved in the sharp hyperpolarization and NO might be related to the sustained phase. Morphological studies have shown that ATP and NO colocalize in neurons of the myenteric plexus of the rat colon (Belai & Burnstock, 1994), providing structural support to a presumptive cotransmission. A mechanism of cotransmission between NO and another neurotransmitter, perhaps ATP, has been proposed in the circular muscle of human (Boeckstaens *et al.*, 1993) and guinea-pig (Zagorodnyuk & Maggi 1994; Selemidis *et al.*, 1997) colon.

In the presence of L-NOARG or suramin, a residual IJP could be recorded. This is consistent with results related to spontaneous mechanical activity where an inhibitory effect was still present when L-NOARG or suramin were perfused.

However, the increase in tone recorded in the presence of L-NOARG might be explained in terms of reduction of the IJP duration. In this case EFS at 1 Hz is not able to keep the smooth muscle relaxed because the sustained hyperpolarization is clearly reduced (total duration of the IJP 1s). Apamin selectively reduced the amplitude of the IJP suggesting that the fast component, probably ATP mediated, involves small conductance calcium activated potassium channels. As we found in the case of suramin, a residual IJP (mainly the slow component) is recorded in the presence of apamin and this residual IJP might keep the smooth muscle relaxed when EFS was applied. Apamin-sensitive IJPs elicited in the rat colon have been reported (Suthamnatpong *et al.*, 1994). As we found in our study the fast hyperpolarization is not NO-mediated (Suthamnatpong *et al.*, 1994). In this study, the slow component of the IJP could not be recorded probably due to differences in EFS parameters or location of stimulating electrodes. In contrast to what we found in the rat, in the human colon apamin seems to affect both the fast and slow components of the IJP (Keef *et al.*, 1993). In the canine small intestine, NO has been reported to be the only neurotransmitter responsible for the IJPs by acting partially through apamin-sensitive potassium channels (Christinck *et al.*, 1991; Stark *et al.*, 1993). On the other hand, ATP-mediated IJPs, are apamin-sensitive in the porcine intestine (Fernandez *et al.*, 1998). These discrepancies might be explained in terms of different mechanism of action of inhibitory mediators in different species.

When both suramin and L-NOARG were perfused together into the bath, the amplitude and duration of the IJP were reduced, showing an additive effect. The same result was found when apamin plus L-NOARG were perfused. However, a residual IJP was still present in both situations. Two hypothesis might explain these results: (1) Concentrations of antagonists used in this study are not sufficient to completely abolish the effects of neural release of ATP and NO. (2) Other neurotransmitters might also be involved in the inhibitory response. In relation to this hypothesis, our results do not rule out the possibility that VIP and PACAP might act as putative inhibitory neurotransmitters too.

In conclusion, the present findings show that the electrical activity of the circular muscle of the rat colon displays cyclic episodes of depolarizations that trigger MPOs which in turn cause contractions. This cyclic activity is not of neural origin but is neurally modulated. Both electrical and mechanical activities are dependent on calcium influx through L-type calcium channels. EFS induces the release of inhibitory NANC neurotransmitters which hyperpolarize and relax the smooth muscle cell. In the rat colon the hyperpolarization shows two components: a fast hyperpolarization followed by a sustained one. Our results suggest that the neurotransmitter responsible for the first phase may be ATP, acting through purinoceptors sensitive to suramin and probably located on the muscle cell. The fast hyperpolarization is due to the activation of apamin-sensitive potassium channels. Finally, the release of NO may initiate the sustained hyperpolarization. The ionic basis of such sustained hyperpolarization does not involve apamin-sensitive potassium channels and awaits further investigation. In contrast to what has been found in other species (where IJPs are monophasic and due to NO release), the circular muscle of the rat colon provides a model of cotransmission very similar to that found in the human colon.

Grant support: PM95-0121. Dirección General de Investigación Científica y Técnica (DGICYT). Personal grant from Universitat Autònoma de Barcelona (Lidia Plujà).

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(Received April 23, 1998)

Revised November 11, 1998

Accepted November 13, 1998)



Selective activation of excitation-contraction coupling pathways by ET_A and ET_B receptors in guinea-pig tracheal smooth muscle

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1 Signalling events responsible for endothelin_A (ET_A) and ET_B receptor-induced contraction were examined in epithelium-denuded guinea-pig tracheal smooth muscle strips. Selective stimulation of each subtype was achieved by a combination of ET-1 (100 nM) and ET_A and ET_B receptor-selective antagonists, BQ-123 (10 μ M) and BQ-788 (3 μ M), respectively.

2 Both ET_A and ET_B receptors induced long-lasting contraction that was totally dependent on Ca²⁺ influx. Stimulation of ET_A receptor induced both transient and sustained (Ca²⁺)_i increases whereas that of ET_B receptor induced only a sustained increase. Suppression of the transient (Ca²⁺)_i increase by U73122 (3 μ M) did not affect the ET_A-induced sustained (Ca²⁺)_i increase and tension development. Stimulation of ET_A receptor, but not ET_B, induced phosphoinositide breakdown and protein kinase C (PKC). The activated PKC contributed to the contraction by increasing the Ca²⁺-sensitivity of the contractile apparatus.

3 Thus, ET_A receptor is coupled both with phospholipase C/Ca²⁺/PKC signalling and Ca²⁺ influx pathways whereas ET_B receptor was coupled only with the latter.

4 Stimulation of ET_B receptor, but not ET_A, caused membrane depolarization measured with a fluorescent indicator, bis-(1,3 dibutylbarbituric acid)-trimethine oxonol. Both nifedipine (1 μ M) and verapamil (10 μ M) abolished ET_B-induced Ca²⁺ influx and contraction, while they barely affected ET_A-induced responses.

5 Therefore, the Ca²⁺ influx pathways activated by each subtype appeared to be completely different; ET_A and ET_B receptors opens voltage-independent Ca²⁺ channels and L-type voltage-dependent Ca²⁺ channels, respectively.

Keywords: Endothelin-1; guinea-pig trachea; contraction; endothelin receptor subtypes; ET_A receptor; ET_B receptor; Ca²⁺ channels; membrane depolarization; Ca²⁺-sensitivity

Abbreviations: 4 β -PDBu, 4 β -phorbol dibutyrate; [Ca²⁺]_i, intracellular Ca²⁺ concentration; CCh, carbachol; DG, diacylglycerol; [DiBAC₄(3)], bis-(1,3 dibutylbarbituric acid)-trimethine oxonol; E/C coupling, excitation-contraction coupling; ET-1, endothelin-1; fura-2-AM, fura-2-acetoxymethyl ester; IAA-94, indanyloxyacetic acid; IP₃, inositol 1,4,5-triphosphate; IPs, inositol phosphates; L-VDCC, L-type voltage-dependent Ca²⁺ channel; NMG-C1, N-methylglucamine chloride; PI, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; T_{max}, maximum contraction; TPA, 12-*o*-tetradecanoylphorbol-13-acetate

Introduction

Endothelins (ETs) are a family composed of three isopeptides, designated as ET-1, ET-2 and ET-3, (Inoue *et al.*, 1989; Yanagisawa *et al.*, 1988). A variety of biological effects of ETs are mediated by at least two distinct subtypes of receptors, ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990), both of which belong to a superfamily of G protein-coupled receptors. ET_A receptor has higher affinities for ET-1 and ET-2 than for ET-3 (Lin *et al.*, 1991), whereas ET_B receptor shows almost equal affinities for the three isopeptides (Hosoda *et al.*,

1991). In other words, ET-1 is a non-selective agonist and ET-3 is an ET_B receptor-selective agonist.

As for the vascular smooth muscle, it is generally accepted that ET-1-induced contraction is ET_A receptor dependent, because of the selective expression of ET_A receptor by smooth muscle cells and also of the inhibition of the contraction by an ET_A receptor-selective antagonist (Hay *et al.*, 1993). ET_B receptor, expressed on endothelial cells and coupled with nitric oxide synthase, counteracts ET_A receptor-mediated signalling (deNucci *et al.*, 1988). This simple schema of ET_A receptor-mediated contraction versus ET_B receptor-mediated relaxation, however, is not applicable ubiquitously. Both pharmacological and histochemical evidence has accumulated recently that indicate the involvement of both ET_A and ET_B receptors in ET-1-induced contraction of smooth muscle tissues (Henry, 1993; Higashi *et al.*, 1997; Seo *et al.*, 1994). Tracheal smooth muscle is one of the typical examples. In the previous study (Inui *et al.*, 1994), we found that ET_A and ET_B receptors coexist in the major population of single smooth muscle cells isolated from guinea-pig trachea. A nonselective endothelin receptor antagonist, BQ-928, but not ET_A receptor-selective antagonist BQ-123, inhibited the ET-1-induced contraction of

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human bronchi (Fukuroda *et al.*, 1996). Physiological significance for the presence of the two receptor subtypes to transmit the effect of ET-1, however, is left unclear.

Smooth muscle contraction is triggered by an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the mechanism for ET_A receptor-induced increase in $[\text{Ca}^{2+}]_i$ has been extensively studied. In most, if not all, smooth muscle preparations, ET_A receptor increases $[\text{Ca}^{2+}]_i$ by inducing both the influx of Ca^{2+} from the extracellular space and the release of Ca^{2+} from intracellular store site (Gardner *et al.*, 1992; Little *et al.*, 1992; Nakamura *et al.*, 1992). ET_A receptor can activate diverse Ca^{2+} influx pathways including both voltage-dependent and -independent calcium channels (Komuro *et al.*, 1997; Minowa *et al.*, 1997). On the other hand, although it is clear that ET_B receptor can induce $[\text{Ca}^{2+}]_i$ increase in various cell lines expressing this receptor subtype, the mechanism for the ET_B receptor-induced increase in $[\text{Ca}^{2+}]_i$ in smooth muscle cells has been poorly clarified. Therefore, the primary purpose of the current study was to delineate the mechanisms for $[\text{Ca}^{2+}]_i$ increase elicited by ET_A and ET_B receptors in guinea-pig tracheal smooth muscle. By simultaneous measurements of $[\text{Ca}^{2+}]_i$ and tension, we also attempted to identify the $[\text{Ca}^{2+}]_i$ increase mechanism responsible for the ET_A - and ET_B -receptor-mediated contractions.

Methods

Materials

ET-1 and ET-3 were purchased from the Peptide Institute Inc. (Osaka, Japan), fura-2-acetoxymethyl ester was from Dojin (Kumamoto, Japan), cremophor EL was from Nacalai Tesque (Kyoto, Japan), BQ-123 (Ihara *et al.*, 1991) was from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.), calphostin C and 12-*o*-tetradecanoylphorbol-13-acetate (TPA) were from Funakoshi (Tokyo, Japan) and U73122 was from Sigma Chemical Company (St. Louis, MO, U.S.A.). BQ-788 (Ishikawa *et al.*, 1994) was synthesized in our laboratory. All other chemicals were of analytical grade.

Animals

Male Hartley guinea-pigs weighing 350–500 g were used in this study. The animals were housed in a temperature- ($25 \pm 2^\circ\text{C}$) and moisture- (50%) controlled room with a 12-h light/dark cycle (light on at 06.00 h and off at 18.00 h). They were given standard guinea-pig chow (CLEA Japan, Inc., Osaka, Japan) and tap water *ad libitum*.

Muscle preparations and contraction assay

The trachea was removed quickly and placed in oxygenated (95% O_2 –5% CO_2) Krebs-Henseleit solution (mM) (NaCl 113.0, KCl 4.8, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25.0 and glucose 5.5, EDTA 0.01, pH 7.4) at 37°C . After the removal of adherent fat and connective tissue, the trachea was cut into rings of approximately 2 mm width. The epithelium was removed by gently turning the rings on the shaft of a pair of watchmaker's forceps. The ring was equilibrated for at least 1 h in the oxygenated Krebs-Henseleit solution at 37°C , and placed in a 4.0 ml water-jacketed organ bath (Iwashiyu UFER, Kyoto, Japan) under a constant tension of 1 g. Responses were measured isometrically with a force-displacement transducer (Nihon Kohden TB-612T, Tokyo, Japan). After the equilibration period, each preparation was first stimulated with

carbachol (CCh) 10 μM and the resultant contraction was used as a reference standard. The tissues were washed three times at the interval of 15 min before the application of agonists. Concentration-dependence was tested by cumulative addition of the drugs in logarithmic increments.

We investigated contraction and $[\text{Ca}^{2+}]_i$ increases induced by 30 pM to 300 nM ET-1, and found that the transient increase in $[\text{Ca}^{2+}]_i$ was observed only above 10 nM (data not shown). After 20 min of the treatment with BQ-788 3 μM or BQ-123 10 μM , ET-1 100 nM was applied for selective stimulation of ET_A and ET_B receptors, respectively. The L-type voltage-dependent Ca^{2+} channel (L-VDCC) blockers, nifedipine (1 μM) and verapamil (10 μM) were applied 15 min after the application of ET-1 when the plateau tension level was achieved. For low Na^+ treatment, tissues were incubated at 37°C for 30 min in a low Na^+ Krebs solution [NaCl 10 mM and N-methylglucamine (NMG-Cl) 128 mM] before stimulation. A Cl^- channel blocker, indanyloxyacetic acid (IAA-94) of 30 μM , was applied 10 min before stimulation either in normal or low Na^+ conditions.

Simultaneous measurements of $[\text{Ca}^{2+}]_i$ and tension

Epithelium-denuded tracheal rings were opened into rectangle strips and loaded with fura-2-acetoxymethyl ester (fura-2-AM, 5 μM) by overnight incubation at 4°C . A noncytotoxic detergent, cremophor EL (0.02%) was included in the loading medium to increase solubility and cell-penetration of fura-2-AM. After the loading, the tracheal strip was rinsed with Krebs-Henseleit solution and held horizontally in a temperature-controlled (30°C), 5 ml organ bath which was attached to a fluorescence spectrometer (CAM-220, Japan Spectroscopic, Tokyo, Japan). Changes in tension were monitored by a force-displacement isometric transducer (Orientec, Tokyo, Japan). The central portion of strips was illuminated alternately at wavelengths of 340 and 380 nm, and the emitted fluorescence at 500 nm monitored. The contractile responses of fresh tissues and fura-2-loaded tissues induced by carbachol 10 μM were indistinguishable (data not shown). $[\text{Ca}^{2+}]_i$ was estimated from the ratio of fluorescence intensities for excitation at 340 and 380 nm (F_{340}/F_{380}) as described (Sato *et al.*, 1988).

In some experiments, extracellular Ca^{2+} was removed by washing the tissues with Ca^{2+} -free Krebs-Henseleit solution, and adding EGTA 20 μM to the organ bath. After agonist-stimulation, extracellular Ca^{2+} was substituted in a cumulating manner from 0.01–2.5 mM. The free calcium concentrations at each point of Ca^{2+} substitution were calculated with a BASIC program using the stability constants of Ca^{2+} , Mg^{2+} and H^+ for EGTA (Martell & Smith, 1974), and taking into consideration the purity of EGTA and the activity of H^+ (Oiki & Okada, 1987).

Measurement of membrane potentials

Membrane potentials of the epithelium-denuded tracheal strips were monitored using a fluorescent dye bis-(1,3 dibutylbarbituric acid)-trimethine oxonol [$\text{DiBAC}_4(3)$] (excitation–emission, 490–518 nm) according to the technique as described (Takenaka *et al.*, 1992). In brief, the strip was attached on quartz cover glass, and then transferred to a temperature-controlled (37°C) quartz cuvette in a fluorescence spectrometer. The strip was incubated in Krebs-Henseleit solution containing $\text{DiBAC}_4(3)$ 50 nM for 60–70 min. After the fluorescent trace reached the plateau level, BQ-788 (3 μM), BQ-123 (10 μM) or BQ-123 and IAA-94 (30 μM) was/were added and then after 20 min incubation, ET-1 was applied.

[³H]-inositol phosphates ([³H]-IPs) accumulation

The epithelium-denuded strips were cut into five pieces of equivalent size (approximately 10 mm width). Following preincubation for 45 min, each tissue was incubated in 1 ml of oxygenated (95% O₂–5% CO₂) Krebs-Henseleit solution containing 20 µCi of [³H]-myo-inositol for 4 h at 37°C. After washing with Krebs-Henseleit solution to remove excess [³H]-myo-inositol, the tissues were incubated for a further 15 min in Krebs-Henseleit solution containing LiCl 10 mM to inhibit the breakdown of IPs. The tissues were then stimulated for 20 min with drugs and the stimulation was terminated by freezing in liquid N₂ (–196°C). Each frozen tissue was exposed to 1 ml of distilled water and 3 ml of chloroform:methanol:hydrochloric acid (2:1:0.1, v/v) with vigorous shaking. The upper methanol/water phase was applied to an anion exchange chromatography column (0.6 ml of Dowex AG1-X8 in formate form). After washing with distilled water (10 ml), 5 mM sodium tetraborate (5 ml) and 60 mM sodium formate, [³H]-IPs were eluted in two 2.5 ml of 0.1 M formic acid with 0.75 M ammonium formate. The aliquots of the final fraction were mixed with 8 ml of Aquasol-2 liquid scintillation cocktail (Dupont, U.S.A.) and radioactivity counted in a liquid scintillation counter (Packard, Model 1900CA). Total [³H]-IPs accumulation was expressed as d.p.m. mg^{–1} wet weight tracheal smooth muscle.

Translocation of PKCα

The epithelium-denuded strips were cut into five pieces of equivalent size (approximately 10 mm width). The smooth muscle band of each tissue was carefully removed. Each muscle band was stimulated in Krebs-Henseleit solution for the time indicated with ET-1 100 nM in the presence either BQ-788 3 µM (ET_A receptor stimulation) or BQ-123 10 µM (ET_B receptor stimulation) and the reaction was halted by chilling in liquid nitrogen. Tissue was homogenized in Tris-buffered saline (Tris-HCl 10 mM pH 7.4, NaCl 150 mM) and centrifuged at 100,000 × *g* for 30 min at 4°C. PKCα in the membrane fraction was extracted from the pellet with 0.1% Triton X-100. Samples (5 µg of protein) were subjected to SDS-PAGE (10% acrylamide), Western transfer and immunoblotting with anti-PKCα antibody (GIBCO BRL). The blot was developed with ABC immunodetection kit (Vecstein) and Kodak HRP-1000.

Statistical and data analyses

All values are expressed as mean ± s.e.mean and statistical significance was assessed by Student's *t*-test. *P* < 0.05 was considered to be significant.

Comparison of cumulative concentration-dependent curves for contraction (Table 1) was performed by nonlinear regression analysis (Meddings *et al.*, 1989) based on a logistic equation (Randall *et al.*, 1989). The fitted curves were compared by the method of Dunnett's multiple comparison. *P* < 0.05 was considered to be significant.

Results

Both ET_A and ET_B receptors are involved in the ET-1-induced contraction of guinea-pig tracheal smooth muscle

To stimulate each receptor subtype selectively, we used a combination of ET-1 (30 pM to 300 nM) and the ET_A receptor-

and ET_B receptor-selective antagonists, BQ-123 and BQ-788. In the presence of BQ-123 10 µM (ET_B stimulation), the EC₅₀ value of ET-1-induced contraction was 3 fold higher and the maximum contraction value (T_{max}) was decreased by ~64% compared with those in the absence (simultaneous stimulation of ET_A and ET_B receptors). In contrast, BQ-788 (3 µM) affected neither EC₅₀ nor T_{max} values of the concentration-response curve for ET-1 (Table 1). The combination of BQ-123 10 µM and BQ-788 3 µM, however, completely inhibited the ET-1-induced contraction up to 10 nM, and also 3 µM BQ-788 completely abolished the contraction induced by ET-3 up to 300 nM (Table 1). Thus, neither BQ-123 nor BQ-788, when applied alone, did not abolish the ET-1-induced contraction, indicating that both ET_A and ET_B receptors were synergistically responsible for the ET-1-induced contraction of guinea-pig tracheal smooth muscle.

Dependence of ET_A and ET_B receptors-mediated contraction on Ca²⁺ influx

To delineate the mechanism of [Ca²⁺]_i increase responsible for contraction, we carried out simultaneous measurements of [Ca²⁺]_i and muscle tension. When stimulated in the presence of extracellular Ca²⁺ (2.5 mM), both ET_A and ET_B receptors induced a sustained increase in [Ca²⁺]_i and contraction of epithelium-denuded tracheal strips (Figure 1). Figure 1a shows the representative time courses of [Ca²⁺]_i and force traces of a strip of trachea induced by ET-1. When stimulated with ET-1 100 nM in the presence of extracellular Ca²⁺ (2.5 mM), there happened biphasic increases of [Ca²⁺]_i; an initial transient increase (from the basal level (100 ± 18 nM, mean ± s.e.mean, *n* = 3) to a peak (350 ± 45 nM) and a subsequent long lasting increases (175 ± 20 nM for more than 20 min). These increases in [Ca²⁺]_i were accompanied by long-lasting contraction that maintained a plateau level for more than 20 min. Selective stimulation of ET_A receptor induced both transient and sustained increases in [Ca²⁺]_i (364 ± 38, 184 ± 26 nM, respectively) and a contraction that was indistinguishable from that induced by simultaneous ET_A and ET_B receptors stimulation (Figure 1b). In contrast, selective stimulation of ET_B receptor induced only a sustained increase in [Ca²⁺]_i (174 ± 19 nM) accompanied by a sustained contraction (Figure 1c). These results indicated that ET_A receptor was coupled both with the Ca²⁺ release from intracellular stores and the Ca²⁺ influx from extracellular space, whereas ET_B receptor was linked only to the latter.

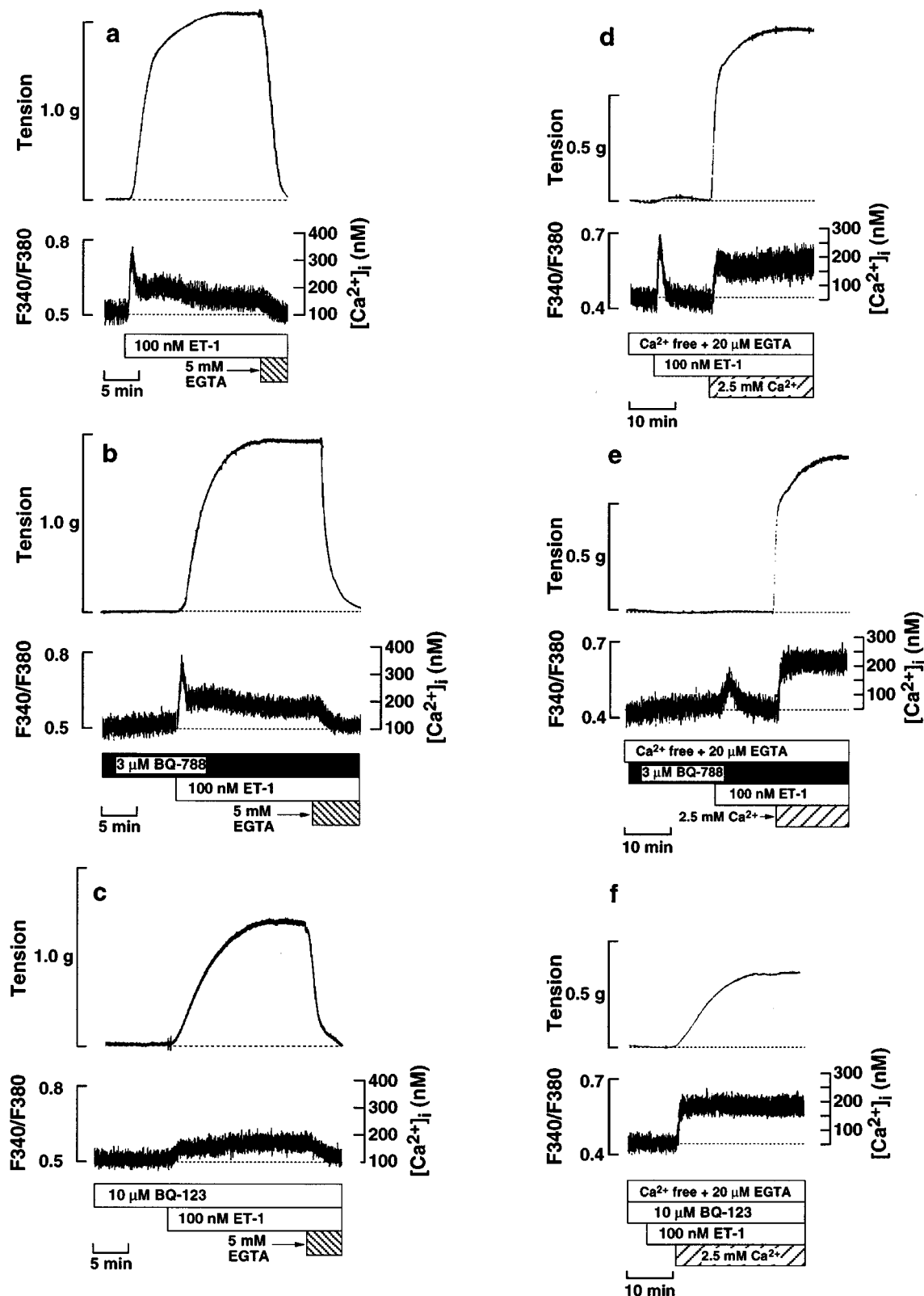
Table 1 Statistical comparison of the concentration-contraction response curves

Data set	T _{max} (% of 10 µM carbachol)	EC ₅₀ (nM)
ET-1	87 ± 2.0	1.1 ± 0.1
ET-3	68 ± 1.0†	6.3 ± 0.4*
ET-1 + 10 µM BQ-123	56 ± 1.7*§	3.6 ± 0.4†§
ET-1 + 3 µM BQ-788	77 ± 1.5	2.2 ± 0.2‡
ET-1 + 10 µM BQ-123 + 3 µM BQ-788	58 ± 2.1*§	126 ± 18.8*‡
ET-3 + 3 µM BQ-788	1.9 ± 1.0*‡	N.D.

Values are the estimates of parameters ± s.e.mean of more than four independent determinations based on nonlinear regression analysis using a logistic equation. T_{max}, maximum contraction, EC₅₀, half-maximally effective concentration. **P* < 0.01; †*P* < 0.05 compared with the data obtained with ET-1; ‡*P* < 0.01; §*P* < 0.05 compared with the data obtained with ET-3 (Dunnett's multiple comparison). N.D., not determined.

Both in the case of ET_A and ET_B receptors stimulation, chelation of extracellular Ca²⁺ by EGTA 5 mM promptly reversed both [Ca²⁺]_i and tension to the resting levels, suggesting the dependence of ET-1-induced contraction on Ca²⁺ influx (Figure 1a–c). This was more clearly demonstrated when extracellular Ca²⁺ was substituted after stimulation. When extracellular Ca²⁺ was removed by washing with Ca²⁺-free Krebs-Henseleit solution and adding EGTA

20 μM (the basal level of [Ca²⁺]_i decreased to 58 ± 5 nM, *n* = 8), ET-1 100 nM induced only a transient increase in [Ca²⁺]_i (252 ± 23 nM) and elicited no increase in tension (Figure 1d). The contraction and sustained increase in [Ca²⁺]_i were obtained by substituting the extracellular Ca²⁺. Both responses were indistinguishable from those obtained by the stimulation with ET-1 100 nM in the presence of extracellular Ca²⁺ (see Figure 1a). Selective stimulation of ET_A receptor



(Figure 1e) caused similar responses; transient increase in $[Ca^{2+}]_i$ (198 ± 27 nM) that was not accompanied with any increase in tension. Selective stimulation of ET_B receptor (Figure 1f) caused neither contraction nor $[Ca^{2+}]_i$ increase and again, the maximum contraction and sustained increase in $[Ca^{2+}]_i$ were obtained by subsequent substitution of Ca^{2+} . These results demonstrated that the Ca^{2+} influx from the extracellular space, not the release of Ca^{2+} from intracellular store site is responsible for ET-1-induced contraction both in the case of ET_A and ET_B receptors.

Histamine ($100 \mu M$, $n=4$) also induced a transient increase in $[Ca^{2+}]_i$ (380 ± 35 nM) followed by a sustained increase (174 ± 22 nM, Figure 1g). When extracellular Ca^{2+} was removed, $100 \mu M$ histamine induced a transient increase in $[Ca^{2+}]_i$ (271 ± 21 nM, $n=3$) that was accompanied by a transient contraction (Figure 1h). The maximum contraction and sustained increase in $[Ca^{2+}]_i$ were obtained by the supplementation of extracellular Ca^{2+} to 2.5 mM. Thus, the contractile profile of histamine was in marked contrast to that of ET-1, which did not induce a transient contraction accompanied with a transient increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} .

Further evidence for the independence from Ca^{2+} release of the ET_A receptor-induced tension development was provided with the use of a PLC inhibitor U73122 of $3 \mu M$ (Figure 2) (Smith *et al.*, 1990). This drug abolished the initial transient increase in $[Ca^{2+}]_i$ without affecting the sustained increase in $[Ca^{2+}]_i$ and tension development.

Distinct Ca^{2+} entry pathways activated by ET_A and ET_B receptors

The dependence of the ET-1-induced contraction on Ca^{2+} influx prompted us to examine the differences in the Ca^{2+} influx pathways activated by ET_A and ET_B receptors. We tested the effects of a series of Ca^{2+} channel blockers and found that L-VDCC blockers nifedipine ($1 \mu M$) and verapamil ($10 \mu M$) dramatically abolished the Ca^{2+} influx and tension

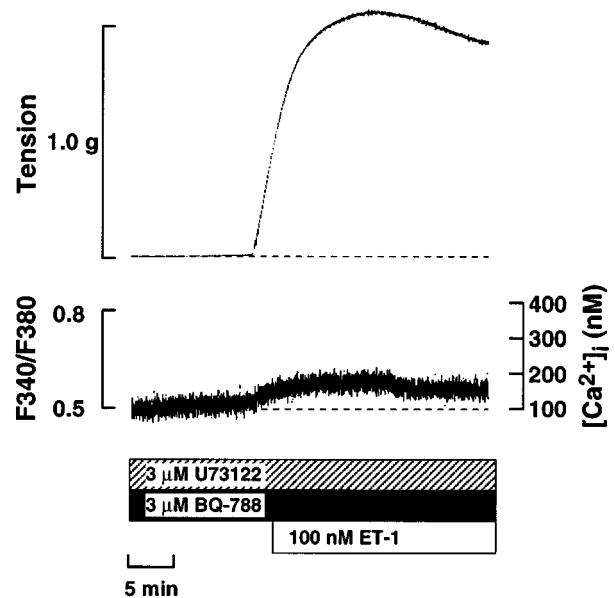


Figure 2 Effects of U73122 on ET_A receptor-mediated increase in $[Ca^{2+}]_i$ and tension. A tracheal strip was treated exactly as described in Figure 1b except for the presence of U73122. U73122 ($3 \mu M$) was added 30 min prior to addition of ET-1. Shown are the representative traces obtained in four independent experiments.

development mediated by ET_B receptor, while they barely affected those mediated by ET_A receptor (Figure 3 and Table 2). A non-selective Ca^{2+} channel blocker SK&F96365 ($3 \mu M$) (Merritt *et al.*, 1990) failed to inhibit either ET_A receptor- or ET_B receptor-induced Ca^{2+} influx (and tension development) although the divalent cation channel blocker Ni^{2+} (1 mM) (Hurwitz, 1986) totally abolished both the ET_A receptor- and ET_B receptor-induced responses (Table 2).

Because the selective effects of L-VDCC blockers suggested the involvement of membrane depolarization in the ET_B

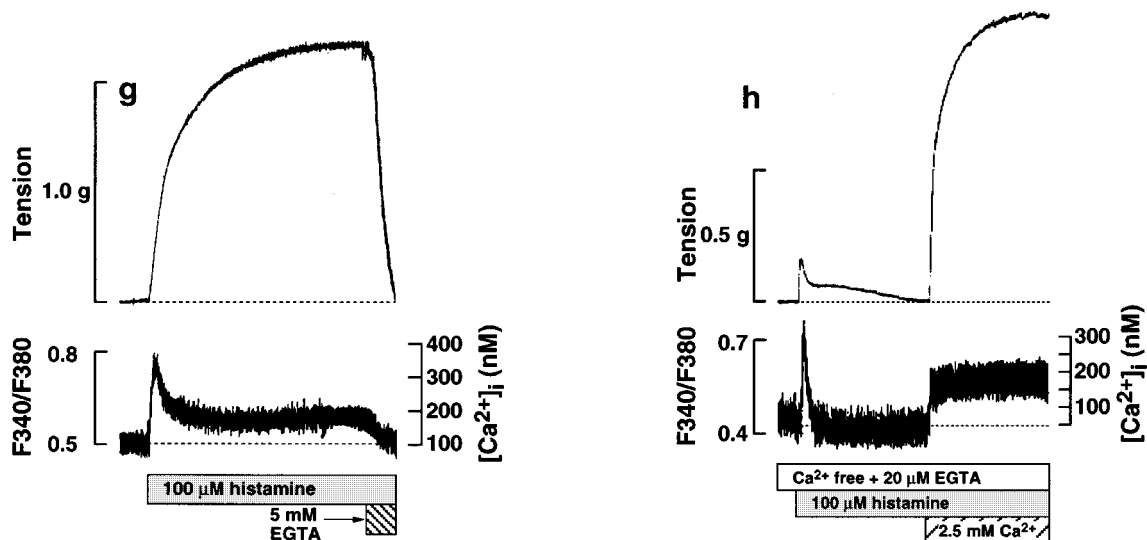


Figure 1 Representative traces of tension development (upper) and $[Ca^{2+}]_i$ (lower) of epithelium-denuded tracheal strips. Simultaneous measurements of $[Ca^{2+}]_i$ and tension were performed as described in Methods. The strips were stimulated with (a) and (d), ET-1 100 nM (stimulation of both ET_A and ET_B receptors), (b) and (e), ET-1 100 nM in the presence of BQ-788 $3 \mu M$ (selective stimulation of ET_A receptor), (c) and (f), ET-1 100 nM in the presence of BQ-123 $10 \mu M$ (selective stimulation of ET_B receptor), and (g) and (h), histamine $100 \mu M$. In (a)–(c) and (g), the concentration of extracellular Ca^{2+} was 2.5 mM and the end of the experiment, EGTA 5 mM was added to chelate the extracellular Ca^{2+} . In (d)–(f), the strips were stimulated with ET-1 in the absence of extracellular Ca^{2+} that was substituted subsequently. In (h), the strip was stimulated with histamine in the absence of extracellular Ca^{2+} that was substituted subsequently. Dotted lines indicate basal levels of tension and $[Ca^{2+}]_i$.

receptor-mediated response, we tested the effects of ET-1 on membrane potentials. Stimulation of ET_B receptor, but not ET_A receptor, caused depolarization of the membrane potentials which was comparable with that caused by KCl 30 mM (Figure 4). The maximum shifts in membrane potential caused by ET_B receptor-stimulation (>20 mV) were large enough to induce Ca²⁺ influx through VDCC to elicit smooth muscle contraction (Nelson *et al.*, 1990). Both influx of Na⁺ and efflux of Cl⁻ were found to be responsible for the depolarization. A Cl⁻ channel blocker, IAA-94 of 30 μ M (Takenaka *et al.*, 1992) partially suppressed the ET_B receptor-induced membrane depolarization and the inhibition was complete when extracellular Na⁺ was largely replaced with NMG-Cl (Figure 4). The same was the case for the ET_B receptor-induced tension development. Either application of IAA-94 or depletion of extracellular Na⁺ partially suppressed the ET_B receptor-induced tension development and the combination of these two procedures abolished it (Table 2). Neither procedure affected the ET_A receptor-induced tension development.

Increased Ca²⁺ sensitivity secondary to ET_A receptor-dependent PLC and PKC-signalling

The transient increase in [Ca²⁺]_i induced by ET_A receptor-stimulation (Figure 1) suggested the ET_A receptor-selective activation of PLC and PKC signalling pathway. This increase, however, was not accompanied with any increase in tension. To find out the physiological significance of the ET_A receptor-induced PLC and PKC signalling, we tested the possible modulation of Ca²⁺ sensitivity by ET-1. The ET_A receptor-selective activation of PI cascade was further verified when we examined the formation of [³H]-IPs in tissues prelabelled with [³H]-inositol and the translocation of PKC α from cytosol to membrane fractions by Western blotting. Both responses were specifically elicited by ET_A receptor (Figure 5a and b).

Cumulative substitution of extracellular Ca²⁺ to ET_A- or ET_B-receptor-stimulated strips resulted in corresponding increases both in [Ca²⁺]_i and tension and there was a linear relationship between the two parameters within the range of [Ca²⁺]_i from 100–300 nM (Figure 6). Both a PLC inhibitor U73122 (3 μ M) and a PKC inhibitor calphostin C (1 μ M, Shimamoto *et al.*, 1992) caused a rightward shift of the [Ca²⁺]_i-tension relationship in ET_A receptor-stimulated strips while neither of the drugs caused any change in the relationship in ET_B receptor-stimulated strips (Figure 6). These results

Table 2 Effects of L-VDCC blockers, IAA-94 and low Na²⁺ condition on ET-1-induced contraction of epithelium-denuded guinea-pig trachea

Treatment	Contraction (% of that induced by 66 mM KCl)	
	ET _A	ET _B
None	113 $\pm$ 7	89 $\pm$ 3
Nifedipine (1 μ M)	100 $\pm$ 3	0*
Verapamil (10 μ M)	87 $\pm$ 7	0*
Ni ²⁺ (1 mM)	0†	0*
IAA-94 (30 μ M)	101 $\pm$ 2	44 $\pm$ 2*
Low Na ²⁺ (10 mM)	106 $\pm$ 10	66 $\pm$ 2*
Low Na ²⁺ (10 mM) plus IAA-94 (30 μ M)	117 $\pm$ 13	2 $\pm$ 0*

After 20 min treatment with BQ-788 3 μ M or BQ-123 10 μ M, ET-1 100 nM was applied for selective stimulation of ET_A and ET_B receptors, respectively. The L-VDCC blockers, nifedipine (1 μ M) and verapamil (10 μ M), and the divalent cation channel blocker, Ni²⁺ (1 mM), were applied 15 min after stimulation with ET-1. The data shown are the relative values of the plateau tension levels achieved. Values are means $\pm$ s.e. mean of more than four independent determinations. †*P* < 0.01 and **P* < 0.01 compared with the control contraction mediated by ET_A receptor and ET_B receptor, respectively.

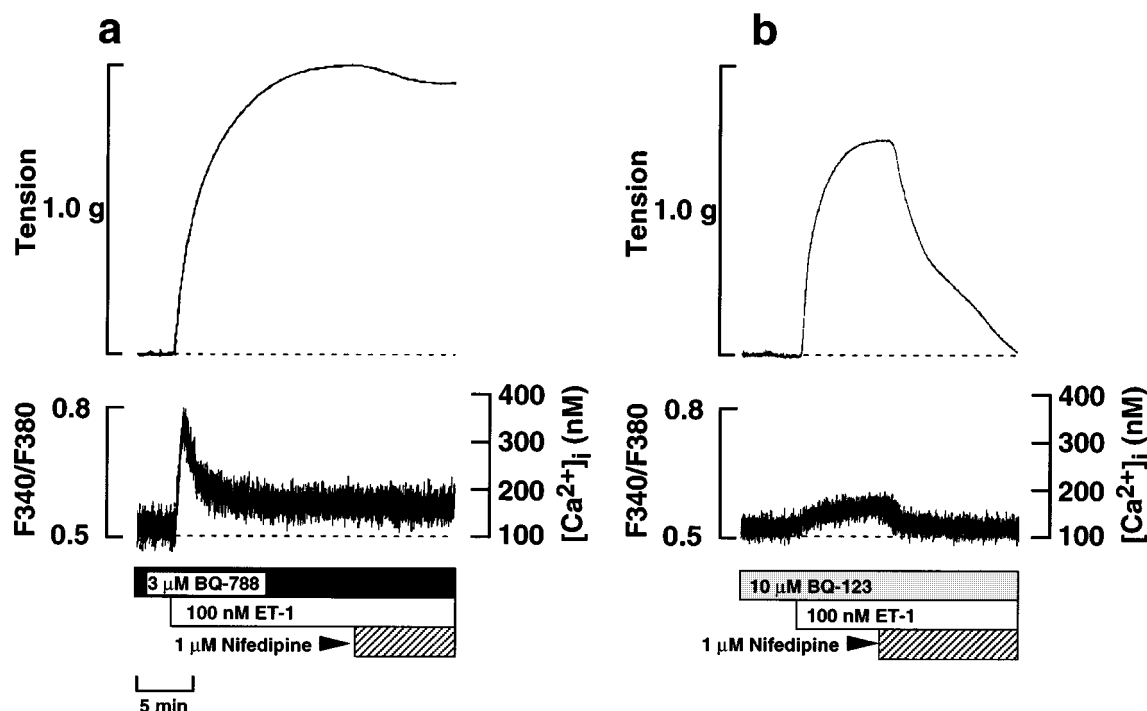


Figure 3 Effects of nifedipine on ET-1-induced increase in [Ca²⁺]_i and tension. Selective stimulation of ET_A and ET_B receptors in the presence of extracellular Ca²⁺ (2.5 mM) was done as described in the legend to Figure 1. Nifedipine (1 μ M) was applied 15 min later when the plateau tension (and [Ca²⁺]_i) level was achieved. Another L-VDCC blocker verapamil (10 μ M) gave essentially the same results (see Table 2). Shown are the representative traces obtained in more than three independent experiments.

indicated that the ET_A receptor-induced activation of PKC indirectly contributed to the contraction by increasing the Ca^{2+} -sensitivity of the contractile apparatus.

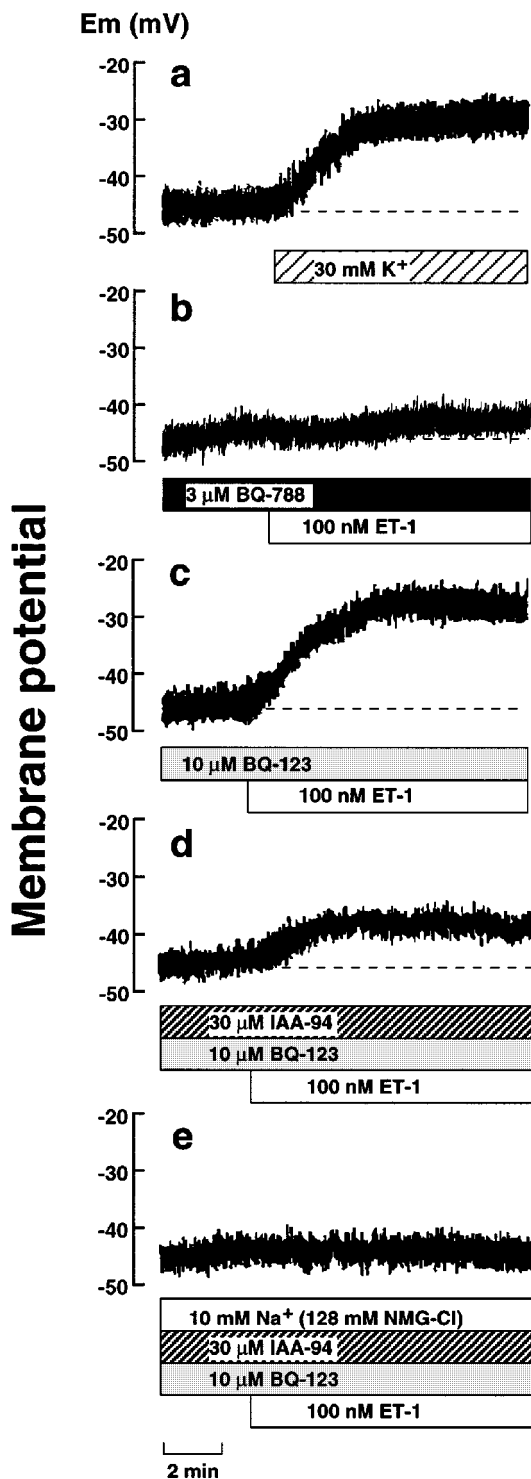


Figure 4 ET_B receptor-induced membrane depolarization of tracheal smooth muscle and effects of IAA-94 and depletion of Na^+ . Membrane depolarization was measured by the fluorescence method using DiBAC₄(3) (excitation - emission, 490–518 nm). Traces presented are representative of three experiments. (a) Membrane depolarization induced by KCl 30 mM. (b) ET_A receptor-selective stimulation did not induce any membrane depolarization. Membrane depolarization caused by ET_B receptor-selective stimulation (c) was partially blocked by 30 μ M IAA-94, a Cl^- channel blocker (d) and was completely blocked by the same drug under a low Na^+ condition (e). Shown are the representative traces reproduced at least twice for each experimental condition.

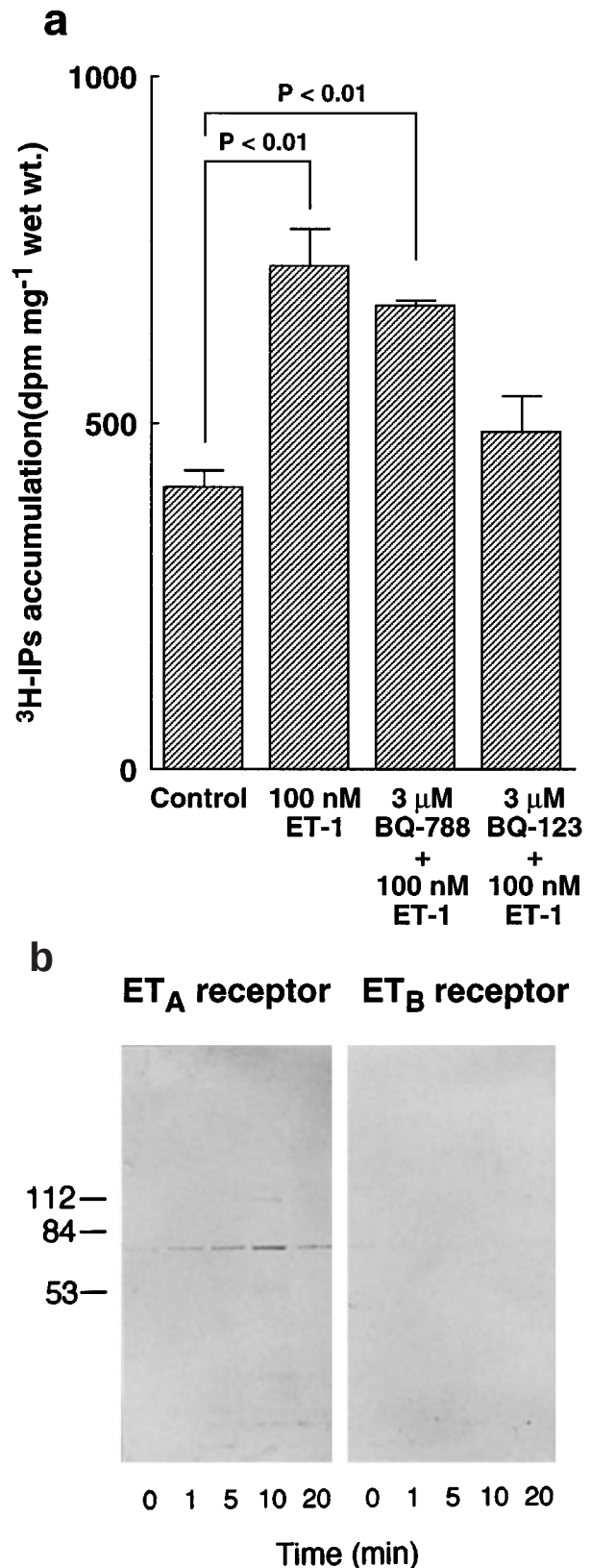


Figure 5 ET_A receptor-selective activation of PLC and PKC in tracheal smooth muscle. (a) IPs. [^3H]-IPs accumulation in [^3H]-myo-inositol-labelled strips was measured as described in Methods. For selective stimulation of ET_A or ET_B receptor, BQ-788 3 μ M or BQ-123 10 μ M was applied 20 min prior to stimulation with ET-1 100 nM, respectively. Each bar represents the mean $\pm$ s.e. mean of 3–5 independent experiments. Significant difference based on Student's *t*-test, $P < 0.01$. (b) Translocation of PKC α . ET_A or ET_B receptor on tracheal strips was selectively stimulated for the time indicated PKC α was extracted from the membrane preparations with Triton X-100 and detected by Western blotting.

Discussion

This paper reports the different signal transduction pathways activated by ET_A and ET_B receptors in guinea-pig tracheal

smooth muscle. The contraction induced by ET-1 was long-lasting and potent, and was synergistically mediated by ET_A and ET_B receptors. By simultaneous measurements of $[Ca^{2+}]_i$ and muscle tension, we demonstrated that ET_A receptor was

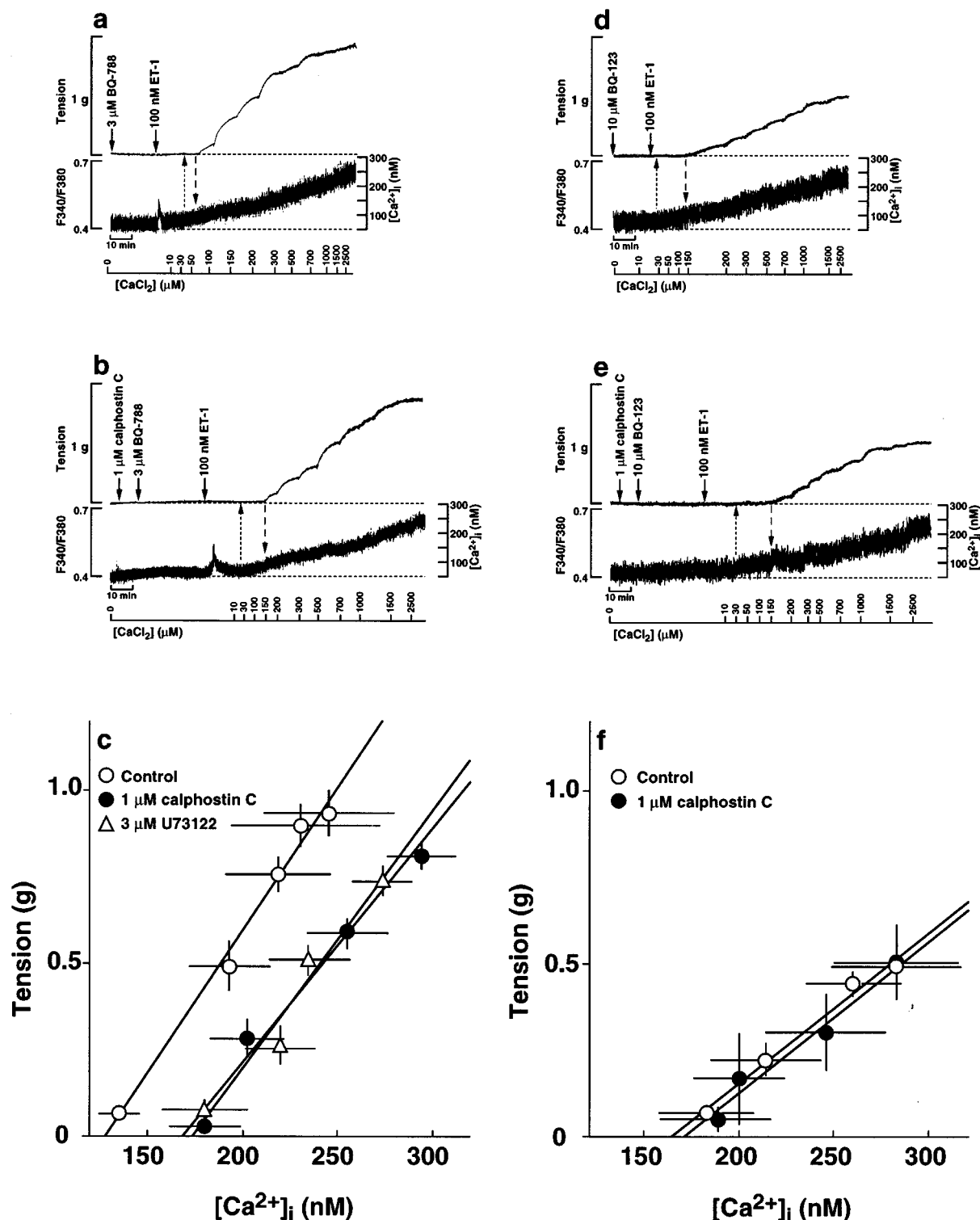


Figure 6 Quantitative relationship between $[Ca^{2+}]_i$ and tension. Fura-2-loaded tracheal strips (prepared as described in the legend to Figure 1) were stimulated in Ca^{2+} free Krebs-Henseleit solution including EGTA 20 μ M with ET-1 100 nM in the presence of either BQ-788 3 μ M (ET_A receptor-selective stimulation, (a)) or BQ-123 10 μ M (ET_B receptor-selective stimulation, (d)). Extracellular Ca^{2+} (10–2500 μ M) was then cumulatively added and the tension and $[Ca^{2+}]_i$ monitored simultaneously. The same experiments were done in the presence of calphostin C (b, e) or U73122. Calphostin C at 1 μ M completely inhibited the contractile response induced by TPA 1 μ M, whereas it did not affect 60 mM KCl-induced contraction. The tension development was plotted against $[Ca^{2+}]_i$ in (c) and (f) for ET_A and ET_B receptors selective stimulation, respectively. Strips were stimulated in the absence or presence of calphostin C or U73122. U73122 caused no change in the relationship in ET_B receptor-stimulated strips (data not shown). Shown are the mean $\pm$ s.e. mean of four experiments.

coupled to PLC and PKC activation and Ca^{2+} release from intracellular stores and also to Ca^{2+} influx, whereas ET_B receptor was linked only to the Ca^{2+} influx (Figures 1, 2 and 5). ET_A receptor-induced Ca^{2+} release was not accompanied with any increase in tension and both in the case of ET_A and ET_B receptors the contractile response was totally dependent on Ca^{2+} influx (Figures 1 and 2). The contractile profile of ET-1 is in marked contrast to that of histamine, which induced a transient contraction accompanied with a transient increase in $[\text{Ca}^{2+}]_i$ (Figure 1).

In guinea-pig trachea, ET-1-induced contraction was attenuated by nicardipine, a L-VDCC blocker (Uchida *et al.*, 1988). Similarly, in bovine bronchial smooth muscle, ET-3-induced contraction (ET_B receptor-selective stimulation) was abolished by 10 μM nifedipine (Nally *et al.*, 1994). In rat isolated trachea, both ET_A and ET_B receptors contribute to the ET-1-induced contraction through different second messenger pathways: induction of phosphatidylinositol turnover and activation of Ca^{2+} influx, respectively (Henry, 1993). In our present study, we have confirmed and further delineated the differential signalling events activated by ET_A and ET_B receptors. In guinea-pig trachea, the Ca^{2+} influx pathways activated by ET_A and ET_B receptors were completely different; the ET_A receptor-induced influx was due to the opening of voltage-independent Ca^{2+} channels whereas the ET_B receptor-induced influx was due to the opening of L-type voltage-dependent Ca^{2+} channels following membrane depolarization (Figures 3 and 4). According to the dependence on the activation of VDCCs secondary to membrane depolarization, the excitation-contraction (E/C) coupling pathways in smooth muscle cells can be divided into two categories; electromechanical and pharmacomechanical (Somlyo & Somlyo, 1994). Our results clearly indicate the selective activation of pharmacomechanical and electromechanical E/C pathways by ET_A and ET_B receptors, respectively. The mechanism of ET_B receptor-activated electromechanical coupling was the opening of L-VDCC secondary to membrane depolarization caused by the opening of Na^+ and Cl^- -permeable channels. The mechanism of ET_A receptor-activated pharmacomechanical coupling involved the opening of Ca^{2+} channels which did not require membrane depolarization.

PKC, a Ca^{2+} - and phospholipid-dependent enzyme, can be activated by DAG and phorbol esters (Nishizuka, 1989). Several studies indicate that activation of this enzyme is involved in ET-1-induced contraction. The combination of A23187 and phorbol 12-myristate 13-acetate (PMA) has a synergistic action to produce a sustained contraction in tracheal smooth muscle (Park & Rasmussen, 1985). Threshold concentrations of agents which increase cytosolic calcium shifted the cumulative 4β -phorbol dibutyrate (4β -PDBu) curve to the left in parenchymal strips (Obianime *et al.*, 1988). Furthermore, the inhibition of PKC abolished the effect of potentiation of the Ca^{2+} -induced contraction by 12-deox-

yphorbol 13-isobutyrate in vascular smooth muscle (Hori *et al.*, 1993). These reports suggested that the synergism between the calcium pathway and the PKC pathway in smooth muscle contraction, the PKC increasing the sensitivity of the contractile apparatus for calcium. The present data showed that PI breakdown and the transient increase in $[\text{Ca}^{2+}]_i$ elicited by the ET_A receptor, and that the PKC-inhibitor, calphostin C, suppressed Ca^{2+} -sensitivity to ET_A receptor-stimulated contraction. Therefore, the stimulation of ET_A receptor activates PKC, and elevates intracellular Ca^{2+} -sensitivity to muscle contraction, whereas the activation of ET_B receptor does not activate PKC, because PI turnover did not occur.

The functional distinction between ET_A and ET_B receptors may depend on the different G-proteins coupled to each receptor subtype. In smooth muscle cells of pig coronary artery, pertussis toxin ADP ribosylated a 41-kDa membrane protein and selectively attenuated ET-1-induced contraction and $^{45}\text{Ca}^{2+}$ uptake, but not PI hydrolysis (Kasuya *et al.*, 1992), suggesting that the various signalling pathways activated in parallel by ET-1 are coupled to different G-proteins in the same cells. In the ET_A receptor expressing rat vascular smooth muscle cells, ET-1 stimulated cyclic AMP formation in a concentration dependent manner (Eguchi *et al.*, 1992a), while in bovine aortic endothelial cells expressing the ET_B receptor, ET-3 concentration dependently inhibited forskolin-induced cyclic AMP formation which could be prevented by pertussis toxin (Eguchi *et al.*, 1992b). These reports suggest that in addition to coupling to PLC via Gq-protein, ET_A and ET_B receptors are coupled to adenylate cyclase, probably ET_A receptor coupled to Gs in smooth muscle cells and ET_B receptor coupled to Gi in endothelial cells. In our case, the functional distinction between ET_A and ET_B receptors may be caused by the contribution of different G-proteins coupled to each receptor subtype.

In conclusion, using guinea-pig tracheal smooth muscle cells, the present study revealed a total dependency of ET-1-induced contraction on Ca^{2+} influx and indirect role of Ca^{2+} release. The Ca^{2+} influx pathways activated by ET_A and ET_B receptors were completely different; the ET_A receptor-induced influx was due to the opening of voltage-independent Ca^{2+} channels whereas the ET_B receptor-induced influx was due to the opening of L-type voltage-dependent Ca^{2+} channels following membrane depolarization. These results revealed a unique mode of action of ET-1 among the smooth muscle constrictors which activate G protein-coupled receptors. Thus, ET-1 is the first example of a single ligand which activates both pharmacomechanical and electromechanical pathways, via two receptor subtypes specialized to each E/C coupling pathway.

We thank Dr A. F. James of King's College London for discussion and critical reading of the manuscript and H. Saiki for technical assistance.

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(Received July 14, 1998)

Revised November 13, 1998

Accepted November 17, 1998)



Inhibition of inflammatory actions of aminobisphosphonates by dichloromethylene bisphosphonate, a non-aminobisphosphonate

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1 When injected intraperitoneally into mice in doses larger than those used clinically, all the amino derivatives of bisphosphonates (aminoBPs) tested induce a variety of inflammatory reactions such as induction of histidine decarboxylase (HDC, the histamine-forming enzyme), hypertrophy of the spleen, atrophy of the thymus, hypoglycaemia, ascites and accumulation of exudate in the thorax, and an increase in the number of macrophages and/or granulocytes in the peritoneal cavity of blood. On the other hand, dichloromethylene bisphosphonate (Cl₂MBP) a typical non-aminoBP, has no such inflammatory actions. In the present study, we found that this agent can suppress the inflammatory actions of aminoBPs.

2 Cl₂MBP, when injected into mice before or after injection of 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP; a typical aminoBP), inhibited the induction of HDC activity by AHBuBP in a dose- and time-dependent manner. The increase in HDC activity induced by AHBuBP was largely suppressed by the injection of an equimolar dose of Cl₂MBP. Cl₂MBP also inhibited other AHBuBP-induced inflammatory reactions, as well as the inflammatory actions of two other aminoBPs. However, Cl₂MBP did not inhibit the increase in HDC activity induced by lipopolysaccharide (LPS).

3 We have previously reported that AHBuBP augments the elevation of HDC activity and the production of interleukin-1 β (IL-1 β) that are induced by LPS. These actions of AHBuBP were also inhibited by Cl₂MBP.

4 Based on these results and reported actions of bisphosphonates, the mechanisms underlying the contrasting effects of aminoBPs and Cl₂MBP, a non-aminoBP are discussed. The results suggest that combined administration of Cl₂MBP and an aminoBP in patients might be a useful way of suppressing the inflammatory side effects of aminoBPs.

Keywords: Bisphosphonates; aminobisphosphonates; histidine decarboxylase; histamine; lipopolysaccharide (LPS); interleukin-1 (IL-1)

Abbreviations: aminoBP, aminobisphosphonate; AHBuBP, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid; BP, bisphosphonate; CHAMP, cycloheptylaminoethylene bisphosphonate; Cl₂MBP, dichloromethylene bisphosphonate; HDC, histidine decarboxylase; IL, interleukin; LPS, lipopolysaccharide; HEBP, 1-hydroxyethylidene-1,1-bisphosphonate; MP-AHPBP, 3-(N-methyl-N-pentyl)amino-1-hydroxypropane-1,1-diphosphonic acid; TNF, tumour necrosis factor

Introduction

Many derivatives of bisphosphonates (BPs), inhibitors of bone resorption, have been developed as promising agents for the treatment of conditions in which there is enhanced bone resorption, such as Paget's disease, tumoral osteolysis, tumoral hypercalcaemia, osteoporosis and rheumatoid arthritis (Green *et al.*, 1994; Bonjour *et al.*, 1994; Geddes *et al.*, 1994). Among these derivatives, the aminobisphosphonates (aminoBPs) are particularly powerful (Geddes *et al.*, 1994). However, undesirable acute phase responses, including fever, occur in humans after administration of aminoBPs (Adami *et al.*, 1987). Recently, Sauty *et al.* (1996) suggested that an increase in the plasma levels of IL-6 and TNF may be responsible for the acute phase reactions induced by aminoBPs.

We found some time ago that injection of aminoBPs into mice, in doses larger than those used clinically, enhances the activity of histidine decarboxylase (HDC), the enzyme that forms histamine, in the bone marrow, spleen, lung and liver, resulting in an increase in histamine levels in these tissues

(Endo *et al.*, 1993). In addition, we have also found that aminoBPs induce hypertrophy of the spleen, atrophy of the thymus, ascites and accumulation of exudate in the thorax, and an increase in the number of granulocytes, macrophages and even osteoclasts (Endo *et al.*, 1993), although the activity of osteoclasts is impaired. The inflammatory actions of aminoBPs described above are assumed to affect immune responses. Indeed, we have demonstrated that aminoBPs exacerbate the arthritis induced in mice by co-injection of a type II collagen and adjuvant, and we proposed the idea that this effect might be related to the ability of aminoBPs to increase HDC activity and/or to increase macrophages and granulocytes (Nakamura *et al.*, 1996). Recently, we have shown that administration of an aminoBP augments both the production of interleukin-1 (IL-1) and the induction of HDC by a lipopolysaccharide (LPS) (Sugawara *et al.*, 1998).

On the other hand, dichloromethylene bisphosphonate (Cl₂MBP), a typical non-aminoBP with a weak bone-resorbing action, has none of the effects described above and has an anti-inflammatory action on collagen-induced arthritis (Nakamura *et al.*, 1996 and references cited therein). In the present study,

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we found that Cl_2MBP can inhibit or suppress the inflammatory actions of all three of the aminoBPs we tested.

Methods

Mice and reagents

Male BALB/c mice (6–7-weeks-old) were obtained from the facility for experimental animals in our university. Dichloromethylene bisphosphonate (Cl_2MBP) and 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP) were synthesized by ourselves. Cycloheptylaminoethylene bisphosphonate (CHAMBP) and 3-(N-methyl-N-pentyl)amino-1-hydroxypropane-1,1-diphosphonic acid (MP-AHPrBP) were provided by Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and Boehringer Mannheim (Mannheim, Germany), respectively. Bisphosphonates (BPs) were dissolved in sterile saline and adjusted to pH 7 with NaOH or HCl. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 prepared by Boivin's method was obtained from Difco Laboratories (Detroit, MI, U.S.A.). These solutions were injected intraperitoneally (i.p.) (0.1 ml per 10 g body weight) as indicated in the text. All experiments complied with the guidelines for care and use of laboratory animals in Tohoku University.

Assay of HDC activity

HDC activity was assayed by a previously described method (Endo *et al.*, 1992a). HDC activity in the liver is expressed as nmol of histamine formed in 1 h by the enzyme contained in 1 g of each tissue ($\text{nmol h}^{-1} \text{g}^{-1}$). HDC activity in the bone marrow is expressed as the activity in 1 g of tibia plus femur, because these bone tissues were themselves assayed for HDC activity (Endo *et al.*, 1992a).

Determination of cytokines in the serum

IL-1 β and TNF α were assayed using ELISA kits (Endogen, Cambridge, MA, U.S.A.), the assay procedures being performed exactly as described by the manufacturer. The blood was collected directly into test tubes following decapitation of the mice. Serum was recovered by centrifugation at $2000 \times g$ at 4°C , then stored at -80°C until used. The amount of each cytokine is expressed as pg per ml serum.

Determination of exudate in thorax

After the thorax had been opened with scissors, the exudate present in the thorax was absorbed using small pre-weighed pieces of filter paper and the amount of exudate was measured as the increase in the weight of the filter paper.

Determination of cell number of peritoneal cavity

Peritoneal exudate cells were obtained as follows. Sterile saline (10 ml) was injected into the peritoneal cavity of ether-anesthetized mice, and the cavity was massaged. Then, the suspension of cells in the saline (5 ml) was recovered using a syringe, and the number of cells in the suspension was counted after appropriate dilution.

Determination of serum glucose

Serum was prepared as described above, and serum glucose levels were measured by the glucose oxidase method using a

glucometer (Accutrend; Boehringer-Mannheim, Mannheim, Germany).

Data analysis

Experimental values are given as mean $\pm$ standard deviation. The statistical significance of differences was analysed by Dunnett's multiple comparison test after ANOVA, *P* values less than 0.05 being considered to indicate significance.

Results

Effects of Cl_2MBP and AHBuBP on HDC activity in mouse tissues

The effects of Cl_2MBP and AHBuBP (separately or in combination) on HDC activity in mouse tissues are shown in Figure 1. Three days after a single intraperitoneal injection of AHBuBP ($40 \mu\text{mol kg}^{-1}$) there was a marked elevation of HDC activity in the lung, liver, spleen and bone marrow. On the other hand, Cl_2MBP had no such effects, even at $800 \mu\text{mol kg}^{-1}$. The combined injection of these agents (injection of Cl_2MBP immediately after injection of AHBuBP) completely abolished the AHBuBP-induced elevation of HDC activity in all the tissues examined. Bone marrow is the tissue with the highest HDC activity in normal mice (Endo *et al.*, 1992a). It should be noted that Cl_2MBP did not decrease the normal level of HDC activity in the bone marrow or in the other tissues examined.

Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity

Injection of Cl_2MBP immediately after the injection of AHBuBP produced a dose-dependent inhibition of the AHBuBP-induced elevation of HDC activity in the lung, liver, spleen and bone marrow (Figure 2). Cl_2MBP at $32 \mu\text{mol kg}^{-1}$ strongly inhibited the HDC elevation induced by AHBuBP ($40 \mu\text{mol kg}^{-1}$). The inhibitory effect of Cl_2MBP in the spleen was significant even at $6.4 \mu\text{mol kg}^{-1}$.

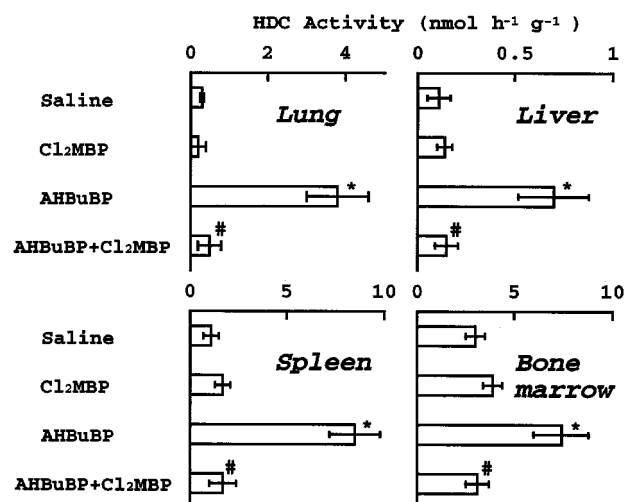


Figure 1 Effects of Cl_2MBP and AHBuBP on HDC activity in mouse tissues. Cl_2MBP ($800 \mu\text{mol kg}^{-1}$) was injected (i.p.) immediately after injection of AHBuBP ($40 \mu\text{mol kg}^{-1}$, i.p.), and the mice were sacrificed 3 days later. Each value is the mean $\pm$ s.d. from four mice. **P* < 0.001 vs saline. #*P* < 0.001 vs AHBuBP.

Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity

The magnitude of the inhibition of the AHBuBP ($40 \mu\text{mol kg}^{-1}$)-induced elevation of HDC activity was dependent on the time at which Cl_2MBP ($160 \mu\text{mol kg}^{-1}$) was injected (Figure 3). The inhibitory effect of Cl_2MBP was greatest when it was injected simultaneously with AHBuBP, and it declined as the interval between the injections was increased. In the liver and spleen, there was still a significant inhibition when Cl_2MBP was injected as much as 2 h after the injection of AHBuBP.

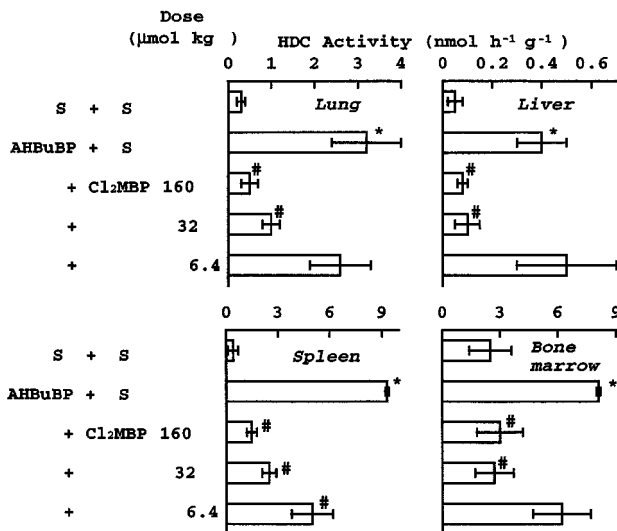


Figure 2 Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity. Saline (S) or Cl_2MBP (6.4 – $160 \mu\text{mol kg}^{-1}$) was injected (i.p.) immediately after injection of saline or AHBuBP ($40 \mu\text{mol kg}^{-1}$, i.p.). The mice were sacrificed 3 days later. Each value is the mean $\pm$ s.d. from four mice. * $P < 0.001$ vs S+S. # $P < 0.001$ vs AHBuBP+S.

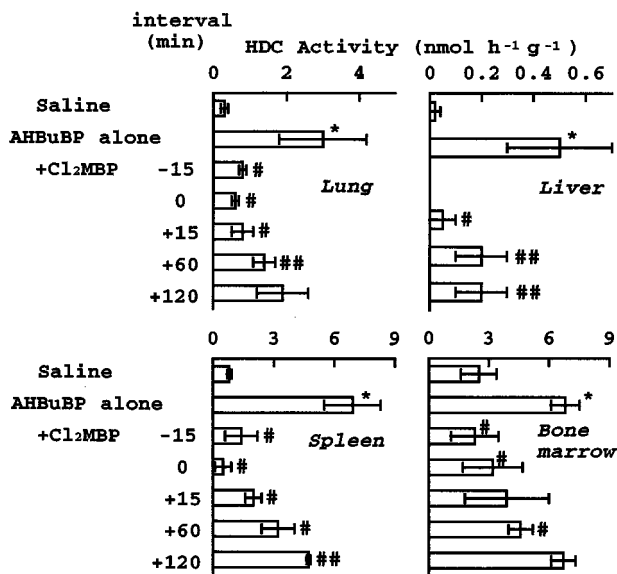


Figure 3 Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity. Cl_2MBP ($160 \mu\text{mol kg}^{-1}$) was injected (i.p.) into mice 15 min before (–), simultaneously with, or 15–120 min after (+) injection of AHBuBP ($40 \mu\text{mol kg}^{-1}$, i.p.). The mice were sacrificed 3 days later. Each value is the mean $\pm$ s.d. from four mice. * $P < 0.001$ vs normal control. # $P < 0.01$, ## $P < 0.05$ vs AHBuBP alone.

Effects of Cl_2MBP on other inflammatory reactions induced by AHBuBP

AHBuBP induces in mice a variety of inflammatory reactions, such as hypertrophy of the spleen, atrophy of the thymus, accumulation of exudate in the thorax and peritoneal cavity, and accumulation of macrophages and granulocytes in the peritoneal cavity. The AHBuBP-induced hypertrophy of the spleen was also inhibited (both dose-dependently and time-dependently) by Cl_2MBP (Figure 4). The atrophy of the thymus, the accumulation of exudate in the thorax, and the accumulation of cells in the peritoneal cavity were also suppressed by Cl_2MBP (Table 1). In the present study, it was found that AHBuBP induced hypoglycaemia, and this effect was also prevented by Cl_2MBP (Table 1).

Effects of Cl_2MBP on inflammatory reactions induced by other aminoBPs

Like AHBuBP, two other aminoBPs (CHAMBP and MP-AHPrBP) induced elevations of tissue HDC activity and other inflammatory reactions. Cl_2MBP ($160 \mu\text{mol kg}^{-1}$) also inhibited these reactions. (Figure 5 and Table 2).

Effect of Cl_2MBP on the induction of HDC by LPS

LPS is a potent inducer of HDC in various tissues, as well as in haematopoietic organs (Endo, 1982; 1983). Some cytokines have been implicated in the induction of HDC by LPS (see Discussion). In contrast to the HDC induction induced by AHBuBP, the induction by LPS is transient: HDC activity induced by an injection of LPS in mice peaks within 3–5 h of

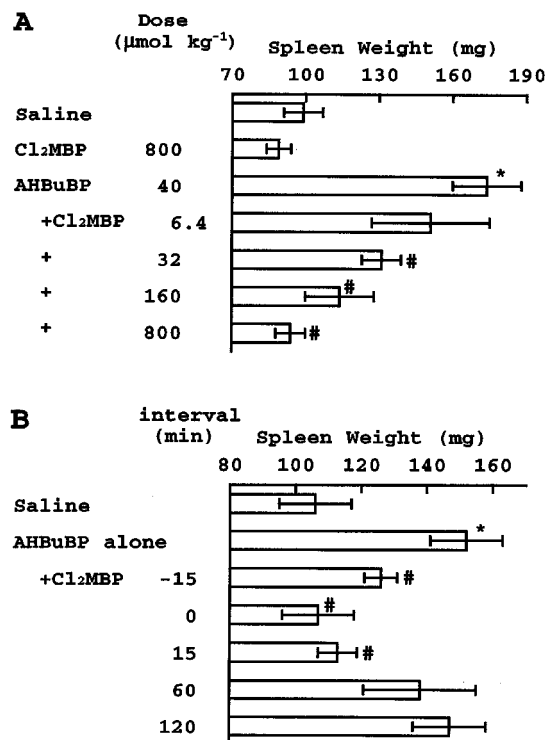


Figure 4 (A) Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced hypertrophy of spleen. Spleen weights in the experiments shown in Figures 1 and 2 are shown. * $P < 0.001$ vs Saline. # $P < 0.001$ vs AHBuBP. (B) Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced hypertrophy of spleen. Spleen weights in the experiment shown in Figure 3 are shown. * $P < 0.001$ vs Saline. # $P < 0.01$ vs AHBuBP alone.

Table 1 Inhibition of Cl₂MBP of inflammatory reactions induced by other AHBuBP

	Body (g)	Weight of Spleen (mg)	Thymus (mg)	Exudate in thorax (mg)	Cells in p. cavity (10 ⁶ /mouse)	Serum glucose (mg dl ⁻¹)
Control	24.8 ± 1.1	102 ± 5	85 ± 14	30 ± 10	1.1 ± 0.2	168 ± 13
Cl ₂ MBP	24.5 ± 0.6	100 ± 2	77 ± 4	25 ± 8	0.6 ± 0.1	180 ± 20
AHBuBP	21.9 ± 0.7	146 ± 9*	49 ± 7*	77 ± 25*	25.9 ± 1.3*	118 ± 17*
+ Cl ₂ MBP	25.1 ± 1.1	109 ± 6#	73 ± 10#	33 ± 15#	4.5 ± 0.6#	180 ± 20#

Cl₂MBP (160 µmol kg⁻¹) was injected into mice immediately after an injection of AHBuBP (40 µmol kg⁻¹, i.p.) and the mice were sacrificed 3 days later. Control mice were given saline. Each value is the mean ± s.d. from four mice. **P* < 0.01 vs control. #*P* < 0.01 vs AHBuBP.

Table 2 Inhibition by Cl₂MBP of inflammatory reactions induced by other AminoBPs

	Body (g)	Weight of Spleen (mg)	Thymus (mg)	Exudate in thorax (mg)	Cells in p. cavity (10 ⁶ /mouse)	Serum glucose (mg dl ⁻¹)
Control	24.5 ± 0.5	95 ± 12	74 ± 5	28 ± 9	2.0 ± 0.7	186 ± 16
CHAMBP	23.0 ± 1.2	132 ± 10*	57 ± 2*	455 ± 150*	14.3 ± 0.4*	107 ± 19*
+ Cl ₂ MBP	24.2 ± 1.1	101 ± 6#	72 ± 9#	95 ± 29#	5.9 ± 1.9#	156 ± 12#
MP-AHPrBP	24.2 ± 1.1	137 ± 12*	67 ± 9	195 ± 88*	20.1 ± 4.1*	141 ± 13*
+ Cl ₂ MBP	24.2 ± 0.4	106 ± 7†	70 ± 7	55 ± 16†	7.8 ± 2.1†	175 ± 11†

Cl₂MBP (160 µmol kg⁻¹) was injected into mice immediately after an injection of CHAMBP or MP-AHPrBP (each 40 µmol kg⁻¹, i.p.) and the mice were sacrificed 3 days later. Control mice were given saline. Each value is the mean ± s.d. from four mice. **P* < 0.01 vs control. †*P* < 0.01 vs CHAMBP. ‡*P* < 0.01 vs MP-AHPrBP.

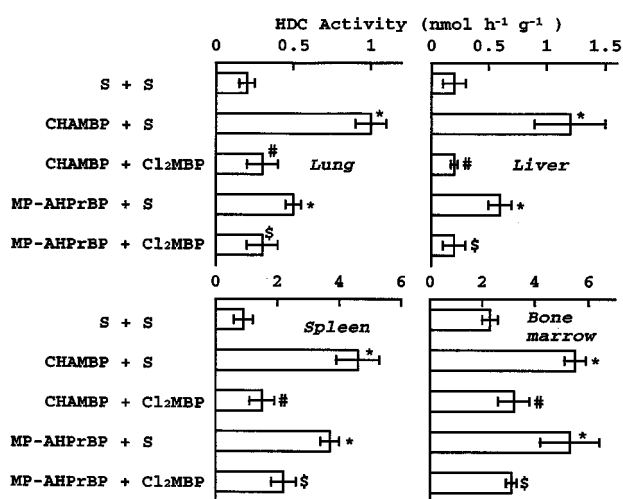


Figure 5 Effects of Cl₂MBP on the elevation of HDC activity induced by other aminoBPs. Saline (S) or Cl₂MBP (160 µmol kg⁻¹) was injected (i.p.) into mice immediately after injection of saline or of CHAMBP or MP-AHPrBP (each 40 µmol kg⁻¹, i.p.). The mice were sacrificed 3 days later. **P* < 0.001 vs saline + saline. #*P* < 0.01 vs CHAMBP + S. \$*P* < 0.01 vs MP-AHPrBP + S.

the injection and returns to the normal level within 20 h (Endo, 1982). Cl₂MBP (500 µmol kg⁻¹), when injected (i.p.) 10, 30 min or 3 days before LPS injection (0.1 mg kg⁻¹, i.p.), did not inhibit the LPS-induced production of IL-1β and elevation of HDC activity in the liver, spleen, lung and bone (Table 3).

Effects of Cl₂MBP on the augmentation by AHBuBP of the elevation of HDC activity and production of IL-1 induced by LPS

Recently, we have shown that AHBuBP pretreatment augments the LPS-induced elevation of HDC activity and production of IL-1 (both α and β), but suppresses the LPS-induced production of TNF (Sugawara *et al.*, 1998). As shown

in Figure 6, there were elevations of HDC activity in the lung, liver and spleen at 2 h after an i.v. injection of LPS. In mice treated with AHBuBP 3 days before the injection of LPS, there was a very considerable augmentation of the effect of LPS on HDC activity (note that the scale for HDC activity in Figure 6 is different from those in Figures 1, 2, 3 and 5). This augmentation was largely inhibited by Cl₂MBP. In the present experiment, the contrasting effects of AHBuBP on the production of IL-1 and TNF were confirmed (Figure 7). The AHBuBP-induced augmentation of IL-1 production was completely prevented by Cl₂MBP. Interestingly, Cl₂MBP showed a tendency to reverse the inhibition of TNFα production induced by AHBuBP, although this effect of Cl₂MBP was not statistically significant.

Discussion

Intraperitoneal injection of any of the aminoBPs we have tested so far induces inflammatory reactions. The doses used in the present study are higher than those used clinically. Therefore, our experimental system is indeed an animal model to induce inflammation by aminoBPs in mice. However, it is the fact that even at clinical doses, aminoBPs have inflammatory side effects. By contrast, the non-aminoBPs tested did not produce such inflammatory reactions. In the present study, we found that the development of the inflammatory reactions induced by these aminoBPs was largely prevented by Cl₂MBP, a typical non-aminoBP. Our findings highlight several interesting and important areas, all of which, however, will require more work before they can be clarified. We discuss these points in the following paragraphs.

Inhibition by Cl₂MBP of inflammatory actions of aminoBPs

AminoBPs and Cl₂MBP are structurally related analogues of pyrophosphate with a non-hydrolyzable P-C-P structure (Figure 8). It has been shown that Cl₂MBP injected i.v. into

Table 3 Effect of Cl₂MBP on the induction of IL-1 β and HDC by LPS

	<i>Serum IL-1</i> (pg mg ⁻¹)		<i>Liver</i>	<i>HDC activity</i> (nmol h ⁻¹ g ⁻¹)		
	<i>IL-1α</i>	<i>IL-1β</i>		<i>Lung</i>	<i>Spleen</i>	<i>Bone</i>
Control	5±5	6±4	0.1±0.1	0.6±0.3	0.6±0.4	3.8±0.8
LPS alone	36±17	25±7	3.3±0.3	10.6±2.3	18.2±2.3	14.2±1.7
Cl ₂ MBP + LPS						
10 min#	57±20	22±7	2.7±0.8	8.1±1.7	15.4±1.4	12.3±0.5
30 min	32±12	20±6	3.4±0.6	11.9±3.5	20.9±2.4	16.5±0.7
3 days	36±14	28±4	3.4±0.5	12.3±3.8	19.3±1.0	12.1±1.2

Cl₂MBP (500 μ mol kg⁻¹) was i.p. injected into mice 10, 30 min or 3 days before i.p. injection of LPS (0.1 mg kg⁻¹). For the assay of IL-1 α and IL-1 β , the mice were sacrificed 3 h after the injection of LPS or saline (control). For the assay of HDC activity, the mice were sacrificed 4 h after the injection of LPS or saline. #Pretreatment time before LPS injection. Each value is the mean $\pm$ s.d. from four mice.

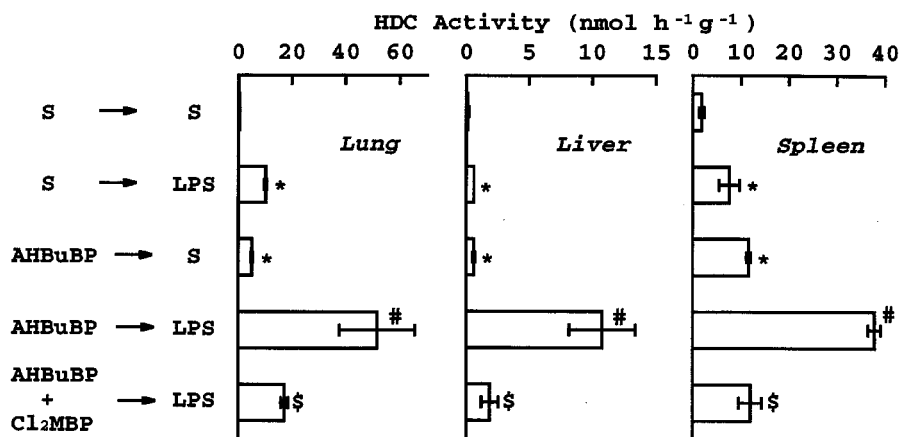


Figure 6 Effect of Cl₂MBP on the augmentation by AHBuBP of the LPS-induced elevation of HDC activity. Saline (S), AHBuBP (40 μ mol mg kg⁻¹) or AHBuBP + Cl₂MBP (160 μ mol kg⁻¹, immediately after AHBuBP) was injected intraperitoneally. Three days later, LPS (100 μ g kg⁻¹) or S was injected intravenously into the mice and tissues were removed 2 h later. Each value is the mean $\pm$ s.d. from four mice. **P* < 0.01 vs S + S. #*P* < 0.01 vs S + LPS and AHBuBP + S. \$*P* < 0.01 vs AHBuBP + LPS.

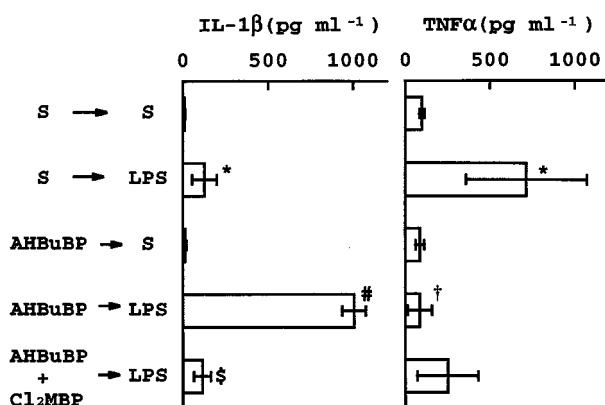


Figure 7 Effect of Cl₂MBP on the influence of AHBuBP on the LPS-induced production of IL-1 β and TNF α . The blood of mice in the experiment shown in Figure 6 was assayed for the cytokines. **P* < 0.01 vs S + S. #*P* < 0.01 vs S + LPS and AHBuBP + S. \$*P* < 0.01 vs AHBuBP + LPS. †*P* < 0.01 vs S + LPS.

mice (100 μ mol kg⁻¹) is cleared rapidly from the plasma, spleen and liver and disappears almost completely within 2 h of its injection (Mönkkönen *et al.*, 1989). In contrast, a high concentration of aminoBPs (AHPBP and AHBuBP) have been shown to be retained for a much longer time in the liver, spleen and lung of mice and rats (Mönkkönen *et al.*, 1989; Lin *et al.*, 1992). Therefore, the suppressive effect of Cl₂MBP may

depend on its concentration immediately after its injection. In addition, the effect of Cl₂MBP was greatest when it was injected simultaneously with AHBuBP and was markedly lowered when it was injected 2 h after injection of AHBuBP (Figures 3 and 4). Therefore, our findings are likely to suggest the following two possibilities. First, aminoBPs and Cl₂MBP may bind to common receptors involved in the induction of inflammatory reactions, and they may act on these receptors as an agonist or as an antagonist, respectively. Second, both aminoBPs and Cl₂MBP may positively or negatively affect a step in a biochemical pathway(s) in which a certain kind of phosphate may be involved. For example, Schmidt *et al.* (1996) reported that AHBuBP inhibits protein-tyrosine phosphatase, and they suggested that the activity of this enzyme may be important in the function and formation of osteoclasts. However, this pathway seems unlikely to be the common step on which aminoBPs act to induce inflammation, because 1-hydroxyethylidene-1,1-bisphosphonate (HEBP, a non-aminoBP) inhibits the activity of this enzyme with an IC₅₀ value similar to that of AHBuBP (Schmidt *et al.*, 1996), and yet HEBP does not induce inflammatory reactions (Endo *et al.*, 1993). However, the testing of these hypotheses will require further experiments.

On the other hand, Mönkkönen and his co-workers found that non-aminoBPs, clodronate (Cl₂MBP) and tiludronate, inhibit LPS-induced production of IL-1 β , IL-6 and TNF α (Pennanen *et al.*, 1995; Mönkkönen *et al.*, 1998) by a macrophage cell line (RAW 264) *in vitro*. They explained that

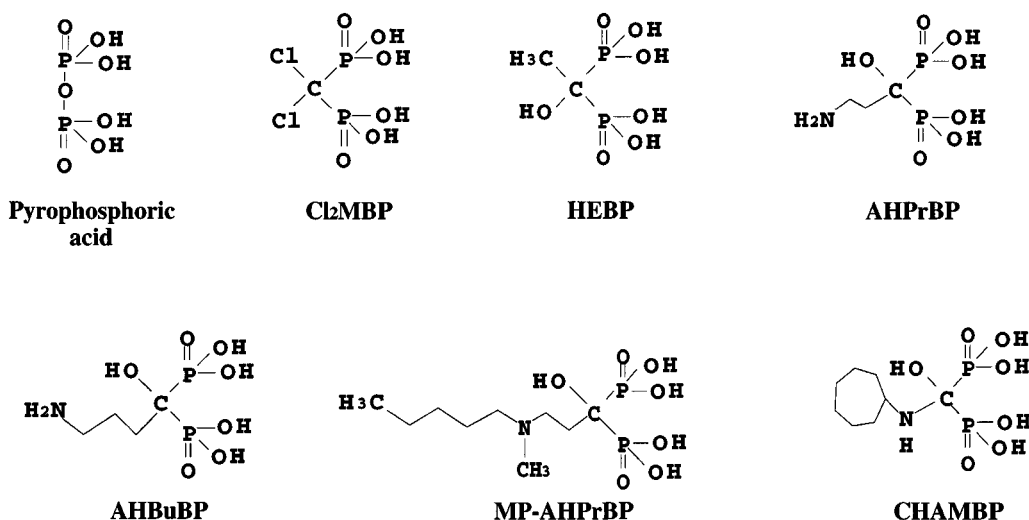


Figure 8 Structure of the bisphosphonates described in this paper.

this effect may be attributed to toxic metabolites formed after non-aminoBPs are ingested within cells (Frith *et al.*, 1997; Auriola *et al.*, 1997). We found that administration of Cl₂MBP-liposomes, which selectively deplete phagocytic macrophages *in vivo* (Van Rooijen & Sanders, 1994), largely abolishes the augmentation of LPS-induced production of IL-1 in AHBuBP-treated mice (Sugawara *et al.*, 1998). Therefore, the formation of the cytotoxic metabolite of Cl₂MBP may underlie the depletion of macrophages. However, it is not clear whether the toxic metabolite is formed when Cl₂MBP itself is given to mice as a free drug. As described above, Cl₂MBP is cleared rapidly from the plasma, spleen and liver (Mönkkönen *et al.*, 1989). Moreover, when free Cl₂MBP exerts its inhibitory effect, 10–20 times larger doses of Cl₂MBP are required than when it is encapsulated in liposomes (Pennanen *et al.*, 1995). Therefore, to clarify whether the toxic metabolite of Cl₂MBP is involved in its suppressive effect on the inflammatory actions of aminoBPs, its formation *in vivo* following the injection of Cl₂MBP itself, requires investigation.

Cl₂MBP does not inhibit the inflammatory actions of LPS

Cl₂MBP itself, when injected 10, 30 min or 3 days before LPS-injection, was entirely ineffective in suppressing both the LPS-induced production of IL-1 β and elevation of HDC activity. Although Cl₂MBP accumulates almost irreversibly in bone, it does not accumulate in soft tissues including liver and spleen of mice (Mönkkönen *et al.*, 1989). Therefore, the reasons why Cl₂MBP does not inhibit the actions of LPS in mice not treated with an aminoBP are likely that (i) Cl₂MBP may be not ingested in macrophages in a sufficient amount to induce its toxic effect and/or (ii) the major cells in the mice responsible for both the LPS-induced production of IL-1 β and elevation of HDC activity may be not phagocytic macrophages as suggested by previous studies (Salkowski *et al.*, 1995; Endo *et al.*, 1995).

Mechanisms underlying the inflammatory actions of aminoBPs

Among the cytokines capable of inducing HDC activity (including IL-1, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating

factor (G-CSF)), IL-1 is the most potent (Endo, 1989; Endo *et al.*, 1992b). In the present study, it was found that aminoBPs induce hypoglycaemia, and it is known that injection of a low dose of IL-1 is also capable of inducing hypoglycaemia (Endo *et al.*, 1985; Rey & Besedovsky, 1989; Endo, 1991). We found that AHBuBP greatly augments *in vivo* both the LPS-induced production of IL-1 (both α and β) and the LPS-induced elevation of HDC activity (Sugawara *et al.*, 1998). In contrast, the LPS-induced production of TNF α was completely inhibited in AHBuBP-treated mice (Sugawara *et al.*, 1998 and Figure 7 in the present study). Although IL-1 is barely detectable by current methods in the serum of mice injected with AHBuBP alone, we found that the cells of the peritoneal cavity, spleen and bone marrow in AHBuBP-injected mice spontaneously produced a higher level of IL-1 *in vitro* than the cells of normal mice (Sugawara *et al.*, 1998). In addition, Northern blot analysis showed that there is a large increase in IL-1 mRNA, but not TNF- and GM-CSF-mRNAs, in the spleen of AHBuBP-injected mice (unpublished data). Moreover, our recent study using IL-1 (α/β)-deficient mice prepared by gene targeting indicated that in these mice, the elevation of HDC activity and most of other inflammatory reactions induced by aminoBPs are very small (unpublished data). These results suggest that a very slight but prolonged production of IL-1 by macrophages and/or other cells capable of producing IL-1 (such as endothelial cells) might be an important cause of the inflammatory action of aminoBPs, although the underlying mechanisms remain to be clarified.

Mönkkönen's group reported that aminoBPs are not converted to the toxic metabolite (Frith *et al.*, 1997). However, they reported that pamidronate (AHPPrBP) at higher concentrations inhibited the LPS-induced production of IL-1 β *in vitro* by a macrophage cell line, RAW264, although this agent showed no significant effect at lower concentrations (Pennanen *et al.*, 1995). In their experiments, they showed that the production of IL-6 was enhanced by the agent at its lower concentrations. By contrast, using the same experimental system, they recently reported that ibandronate (MP-AHPPrBP) did not inhibit but did markedly enhance the LPS-induced secretion of IL-1 β (Mönkkönen *et al.*, 1998). Therefore, whether the effects of aminoBPs observed in these *in vitro* experiments can explain the *in vivo* inflammatory actions of aminoBPs is unclear. However, their finding that AHBuBP, but not non-aminoBPs, deposits in a large amount

in the liver and spleen of mice (Mönkkönen *et al.*, 1989) seems to be an important cause to induce its inflammatory actions.

Possible involvement of histamine in the inflammatory and haematopoietic actions of aminoBPs

In addition to the actions on the microcirculation, histamine has been shown to stimulate the proliferation of haematopoietic precursor cells *in vitro* (Byron, 1977; 1980; Gross & Worthington-White, 1984; Shounan & You-Heng, 1988; Nakaya & Tasaka, 1988; Schneider *et al.*, 1990; Dy *et al.*, 1993). Indeed, the HDC activity in the bone marrow is the highest found in the tissues of normal mice (Endo *et al.*, 1992a). Moreover, the haematopoietic cytokines, interleukin-3 (IL-3), GM-CSF and G-CSF, induce HDC activity *in vivo* only in the haematopoietic organs of the mouse (spleen and bone marrow) (Lebel *et al.*, 1990; Endo *et al.*, 1992b). It is known that haematopoiesis in the spleen is inactive in adult rats and, although LPS strongly induces HDC activity in the spleen of adult mice, it does not have this effect in the spleen of adult rats (Endo *et al.*, 1995). Moreover, histamine has been shown to exert various effects on the immune system (Beer *et al.*, 1983; Falus & Merétey, 1992). Collectively, these data suggest that histamine may be involved in the inflammatory and haematopoietic actions (increase in macrophages and granulocytes) of aminoBPs. However, further work is required to investigate this.

Relationship between inflammatory or anti-inflammatory actions of BPs and their inhibitory actions on bone-resorption

The potency with which Cl_2MBP inhibits bone resorption *in vivo* has been shown to be much less than those of aminoBPs (Mühlbauer *et al.*, 1991; Geddes *et al.*, 1994). Reitsma *et al.* (1982) have reported that the *in vitro* action on bone resorption of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPBP), an aminoBP which also induces inflammatory reactions (Endo *et al.*, 1993), is different from that of Cl_2MBP . Various mechanisms have been proposed to explain the inhibitory action of BPs on bone resorption. These include a physico-chemical stabilization of the bone resulting from the binding of BPs to hydroxyapatite, a cytotoxic action on osteoclasts, an inhibition of osteoclast formation or recruitment, a metabolic inhibition that is specific to osteoclasts (Geddes *et al.*, 1994; Rodan & Fleisch, 1996), an osteoblast-

mediated inhibition (Sahni *et al.*, 1993), apoptosis (Hughes *et al.*, 1995) and inhibition of a protein-tyrosine phosphatase (Schmidt *et al.*, 1996). In our present study, the inflammatory reactions induced by $40 \mu\text{mol kg}^{-1}$ of aminoBPs were all significantly inhibited by co-injection of an equimolar dose of Cl_2MBP (Figures 2 and 4). It could be of considerable interest to determine whether the mechanism by which aminoBPs inhibit bone resorption is independent of their inflammatory actions, and also whether the mechanism by which Cl_2MBP inhibits bone resorption is independent of its anti-inflammatory actions. It is also of interest to examine whether the suppressed production of TNF might be involved in the action of aminoBPs to inhibit bone resorption.

Possibility that other non-aminoBPs have anti-inflammatory actions

We have pointed out previously (Nakamura *et al.*, 1996) that while aminoBPs are not effective at suppressing experimental arthritis in rats and mice (in fact, they exacerbate the arthritis), non-aminoBPs are effective. Dunn *et al.* (1993a and b) and Nugent *et al.* (1993) have reported that pyrazoline BPs (non-aminoBPs) resemble Cl_2MBP in having anti-inflammatory and anti-arthritic activities. On this basis, it seems likely that other non-aminoBPs, including pyrazoline BPs, will prove, like Cl_2MBP , to suppress the inflammatory actions of aminoBPs.

Proposal of a combined clinical use of an aminoBP and non-aminoBP

Finally, it has been shown that administration of aminoBPs produces inflammatory responses, including fever, in 10–50% of treated patients (Adami *et al.*, 1987; Sauty *et al.*, 1996). In addition, inflammatory side effects have also been reported in the field of ophthalmology (Siris, 1993; Macarol & Frauenfelder, 1994). Our findings suggest that the combined administration of an aminoBP (a potent inhibitor of bone resorption) with Cl_2MBP (a less potent inhibitor of bone resorption) may be a useful regimen in that it might produce much weaker inflammatory side effects than use of an aminoBP alone, while possibly producing a stronger inhibitory effect on bone resorption than that obtained by the separate use of either agent.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan (No. 09470409).

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(Received July 7, 1998

Revised October 13, 1998

Accepted November 17, 1998)



Studies on the role of dopamine in the degeneration of 5-HT nerve endings in the brain of Dark Agouti rats following 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') administration

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1 We investigated whether dopamine plays a role in the neurodegeneration of 5-hydroxytryptamine (5-HT) nerve endings occurring in Dark Agouti rat brain after 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') administration.

2 Haloperidol (2 mg kg⁻¹ i.p.) injected 5 min prior and 55 min post MDMA (15 mg kg⁻¹ i.p.) abolished the acute MDMA-induced hyperthermia and attenuated the neurotoxic loss of 5-HT 7 days later. When the rectal temperature of MDMA + haloperidol treated rats was kept elevated, this protective effect was marginal.

3 MDMA (15 mg kg⁻¹) increased the dopamine concentration in the dialysate from a striatal microdialysis probe by 800%. L-DOPA (25 mg kg⁻¹ i.p., plus benserazide, 6.25 mg kg⁻¹ i.p.) injected 2 h after MDMA (15 mg kg⁻¹) enhanced the increase in dopamine in the dialysate, but subsequent neurodegeneration was unaltered. L-DOPA (25 mg kg⁻¹) injected before a sub-toxic dose of MDMA (5 mg kg⁻¹) failed to induce neurodegeneration.

4 The MDMA-induced increase in free radical formation in the hippocampus (indicated by increased 2,3- and 2,5-dihydroxybenzoic acid in a microdialysis probe perfused with salicylic acid) was unaltered by L-DOPA.

5 The neuroprotective drug clomethiazole (50 mg kg⁻¹ i.p.) did not influence the MDMA-induced increase in extracellular dopamine.

6 These data suggest that previous observations on the protective effect of haloperidol and potentiating effect of L-DOPA on MDMA-induced neurodegeneration may have resulted from effects on MDMA-induced hyperthermia.

7 The increased extracellular dopamine concentration following MDMA may result from effects of MDMA on dopamine re-uptake, monoamine oxidase and 5-HT release rather than an 'amphetamine-like' action on dopamine release, thus explaining why the drug does not induce degeneration of dopamine nerve endings.

Keywords: 3,4-methylenedioxymethamphetamine; ecstasy; dopamine; free radicals; neurodegeneration; clomethiazole; 5-hydroxytryptamine; L-DOPA, haloperidol

Abbreviations: ANOVA, analysis of variance; CMZ, clomethiazole; DA, dopamine; DHBA, dihydroxybenzoic acid; DOPAC, dihydroxyphenylacetic acid; Hal, haloperidol; 5-HIAA, 5-hydroxyindoleacetic acid; h.p.l.c., high performance liquid chromatography; 5-HT, 5-hydroxytryptamine; HVA, homovanillic acid; MAO, monoamine oxidase; MDMA, (±) 3,4-methylenedioxymethamphetamine; 6-OHDA, 6-hydroxydopamine; PCA, p-chloroamphetamine

Introduction

The commonly misused recreational drug 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') has two well established phases of action in experimental animals. The acute phase consists of a rapid release of 5-hydroxytryptamine (5-HT) from neuronal stores, thereby producing many of the acute behavioural changes, including behavioural excitation and hyperthermia, that are seen in both experimental animals and humans (see reviews of White *et al.*, 1996; Green *et al.*, 1995; Steele *et al.*, 1994).

The second action of MDMA in the brain of experimental animals is to produce a long lasting neurotoxic loss of 5-HT nerve terminals in several areas of the brain, an effect demonstrated both histologically (O'Hearn *et al.*, 1988; Molliver *et al.*, 1990) and biochemically (Battaglia *et al.*,

1987; Sharkey *et al.*, 1991; Hewitt & Green, 1994). The mechanisms involved in producing this neurodegeneration are not fully understood at present, but recent data have given substantial evidence to support the contention that increased free radical production is involved (see Colado *et al.*, 1997b).

Studies on both the pharmacology of MDMA and putative neuroprotective agents have also indicated a role for dopamine in the neurodegenerative process. For example Stone *et al.*, (1988) proposed a role for dopamine based on their studies which showed that the long term depletion of cerebral 5-HT which follows administration of MDMA was attenuated by the dopamine synthesis inhibitor α -methyl p-tyrosine or the monoamine depletor reserpine. Schmidt *et al.* (1990b) reported that injection into the striatum of the selective dopamine neurotoxin 6-hydroxydopamine (6-OHDA) blocked the neurotoxic effects of MDMA on 5-HT neurones not only in the striatum but also other forebrain regions such as the

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hippocampus and cortex. In addition Stone *et al.* (1988) reported that the dopamine uptake inhibitor GBR 12909 was neuroprotective. Finally, both Schmidt *et al.* (1990b) and Hewitt & Green (1994) have shown that acute administration of the dopamine antagonist haloperidol together with MDMA provides effective protection against neurotoxic damage. It is acknowledged that none of these studies demonstrate authoritatively the involvement of dopamine in the MDMA-induced neurodegeneration because none of the compounds used in these investigations (haloperidol, 6-OHDA, α -methyl p-tyrosine) selectively alter dopamine function. Nevertheless, when taken with other evidence (see below) that MDMA administration alters dopamine biochemistry in the brain, the interpretation that dopamine was involved in the mechanisms by which MDMA causes neurodegeneration of 5-HT nerve terminals appeared plausible.

Whilst MDMA has generally been reported to not produce neurotoxic degeneration of dopamine neurones (Stone *et al.*, 1986; Schmidt, 1987; Schmidt & Kehne, 1990; Colado *et al.*, 1997a, b), both *in vitro* and *in vivo* studies have found that MDMA enhances dopamine efflux from cerebral tissue. For example MDMA increases release of [^{3} H]-dopamine from striatal slices (Johnson *et al.*, 1986; Schmidt *et al.*, 1987) and the drug has also been reported to inhibit [^{3} H]-dopamine uptake into striatal synaptosomes (Steele *et al.*, 1987). Use of *in vivo* voltammetry (Yamamoto & Spanos, 1988) and *in vivo* microdialysis (Hiramatsu & Cho, 1990; Nash, 1990; Nash & Brodtkin, 1991) has demonstrated that MDMA increases dopamine release and these results are supported by the observation that MDMA produces an acute increase in striatal dopamine content and a decrease in the dihydroxyphenylacetic acid (DOPAC) content (Schmidt *et al.*, 1986; Johnson *et al.*, 1991; Colado & Green 1994).

These data led us to suggest that the reason that drugs which enhanced GABA function such as pentobarbitone (Colado & Green, 1994) and clomethiazole (Colado *et al.*, 1993) were neuroprotective was because their administration resulted in an inhibition of dopamine release (Colado & Green, 1994; Green *et al.*, 1995). Recently however it has been apparent that some re-evaluation is necessary of reports on the neuroprotective action of various compounds. This is because it is now clear that some drugs attenuate damage solely because they either induce hypothermia (Farfel & Seiden, 1995a, b; Malberg *et al.*, 1996) or merely prevent the MDMA-induced hyperthermia (Colado *et al.*, 1998b). In particular the report that α -methyl p-tyrosine is neuroprotective because of its hypothermic effect (Malberg *et al.*, 1996) and our recent observation that pentobarbitone is not protective when the animals are kept warm (Colado *et al.*, 1998a) has encouraged us both to re-examine our earlier data on haloperidol and also to try and clarify further the possible role of dopamine in the neurodegenerative changes which follow MDMA administration.

Methods

Animals, drugs and reagents

Adult male Dark Agouti rats (Interfauna, Barcelona, Spain) weighing 150–170 g were used. They were housed in groups of five, in conditions of constant temperature ($21^\circ\text{C} \pm 2^\circ\text{C}$) and a 12 h light/dark cycle (lights on: 07 h 00 min) and given free access to food and water.

The following drugs were used: ($\pm$) 3,4-methylenedioxymethamphetamine HCl (Ministry of Health, Spain), clo-

methiazole edisylate (Astra Arcus, Södertälje, Sweden), haloperidol (Sigma-Aldrich Química S.A., Spain), L-3,4-dihydroxyphenylalanine methyl ester (Methyl L-DOPA, Sigma-Aldrich Química S.A., Spain) and benserazide (Sigma-Aldrich Química S.A., Spain).

In all the experiments using L-DOPA, the dose of L-DOPA was preceded 1 min earlier by an injection of benserazide, a peripheral DOPA decarboxylase inhibitor, at a dose of 6.25 mg kg^{-1} i.p. in order to avoid the peripheral metabolism of L-DOPA.

All compounds except haloperidol were dissolved in 0.9% w/v NaCl (saline). Haloperidol was suspended in peanut oil. Control animals were injected with the appropriate vehicle. All drugs were injected i.p. in a volume of 1 ml kg^{-1} . Doses are always quoted in terms of the base.

Measurement of rectal temperature

Temperature was measured by use of a digital readout thermocouple (Type K thermometer, Portec, U.K.) with a resolution of $\pm 0.1^\circ\text{C}$ and accuracy of $\pm 0.2^\circ\text{C}$ attached to a CAC-005 Rodent Sensor which was inserted 2.5 cm into the rectum, the rat being lightly restrained by holding in the hand. A steady readout was obtained within 10 s of probe insertion.

Elevation of body temperature with a homeothermic blanket

In one experiment rats were injected with MDMA + haloperidol and had their rectal temperature kept elevated to near that seen in rats given MDMA alone. This was achieved by placing the rats in a cage with a Harvard Homeothermic Blanket (Model 50-7087) covering the base.

Measurement of monoamines and their metabolites in cerebral tissue

Rats were killed by cervical dislocation and decapitation, the brains rapidly removed and cortex, hippocampus and striatum dissected out on ice. Tissue was homogenized and 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) measured by high performance liquid chromatography (h.p.l.c.). Briefly, the mobile phase consisted of: KH_2PO_4 0.05 M, octanesulphonic acid 0.16 mM, EDTA 0.1 mM and methanol (16%), and was adjusted to pH 3 with phosphoric acid, filtered and degassed. The flow rate was 1 ml min^{-1} and the working electrode potential was set at 0.85 V.

The h.p.l.c. system consisted of a pump (Waters 510) linked to an automatic sample injector (Loop 200 μl , Waters 712 WISP), a stainless steel reversed-phase column (Spherisorb ODS2, 5 μm , $150 \times 4.6 \text{ mm}$) with a precolumn and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

[^{3} H]-Paroxetine binding in tissue homogenates

[^{3} H]-Paroxetine binding was measured by the method described in detail by Hewitt & Green (1994). The animals were killed, the brain rapidly removed and dissected on ice within 2 min. Tissue from individual animals was homogenized in ice-cold Tris-HCl (50 mM; pH 7.4) containing (in mM): NaCl 120 and KCl 5, using an Ultra-Turrax. The homogenate was centrifuged at $30,000 \times g$ for 10 min at 4°C . The supernatant was discarded and the wash procedure

repeated twice more. The pellet was finally resuspended in the Tris buffer at a concentration of 10 mg tissue ml⁻¹. In order to obtain an estimate of the maximal density of [³H]-paroxetine-labelled 5-HT uptake sites the assay solution (1 ml) contained a saturating concentration of [³H]-paroxetine (1 nM) and 800 µl tissue preparation with the addition of 5-HT (100 µM) for determination of non specific binding. Incubation was for 60 min at room temperature. Assays were terminated by rapid filtration and counting of the radioactivity by scintillation spectrometry. Protein concentrations were measured by the method of Lowry *et al.* (1951).

Implantation of microdialysis probe in the striatum

Rats were anaesthetized with sodium pentobarbitone (Euta-Lender, 40 mg kg⁻¹ i.p.) and secured in a Kopf stereotaxic frame with the tooth bar at -3.3 mm below the interaural zero. The dialysis probe (3.5 mm × 200 µm; Cuprophane) was implanted in the right striatum +7.9 mm from the interaural line, -2.5 mm lateral and -8 mm below the surface of the brain (König & Klippel, 1963). Probes were secured to the skull as described by Baldwin *et al.* (1994).

Measurement of dopamine and their metabolites in the striatal dialysate

Twenty-four hours after implantation, probes were perfused with artificial cerebrospinal fluid (in mM): KCl 2.5, NaCl 125, MgCl₂·6H₂O 1.18, CaCl₂·2H₂O 1.26, at a rate of 1 µl min⁻¹ and samples collected from the freely moving animals at 30 min intervals in tubes containing 5 µl of a solution composed of HClO₄ (0.01 M), cysteine (0.2%) and sodium metabisulphite (0.2%). The first 60 min sample was discarded and the next three 30 min baseline samples collected.

Dopamine, DOPAC and HVA were measured in the dialysate by h.p.l.c. and electrochemical detection. The mobile phase consisted of KH₂PO₄ (0.05 M), octanesulphonic acid (1 mM), EDTA (0.1 mM) and methanol (14%), and was adjusted to pH 3 with phosphoric acid, filtered and degassed. The flow rate was 1 ml min⁻¹. The h.p.l.c. system consisted of a pump (Waters 510) linked to manual sample injector (Loop 20 µl Rheodyne), a stainless steel reversed-phase column (Spherisorb ODS2, 5 µm, 150 × 4.6 mm) with a precolumn and a coulometric detector (Coulchem 5100A) with a 5011 analytical cell. The working electrode potential was set at 400 mV with 500 nA gain. The current produced was monitored by using a computer data handling system (AXXIOM 747).

The limit of the sensitivity of these assays was approximately 8 pg (dopamine), 30 pg (DOPAC) and 125 pg (HVA). The per cent recovery of a solution containing dopamine (1 pg µl⁻¹), DOPAC (100 pg µl⁻¹) and HVA (150 pg µl⁻¹) was 26.5 ± 5.5% (*n* = 4) for dopamine, 25.8 ± 1.7% (*n* = 4) for DOPAC and 28.0 ± 1.4% (*n* = 4) for HVA. The percentage of recovery is maintained constant for the following ranges of concentrations in the external medium: dopamine 1–27 pg µl⁻¹, DOPAC 100–2700 pg µl⁻¹, and HVA 150–4050 pg µl⁻¹. These ranges were comparable to the amounts of dopamine and metabolites recovered from the brain *in vivo*.

Implantation of microdialysis probe in the hippocampus

Rats were anaesthetized with sodium pentobarbitone ('Euta-Lender', 40 mg kg⁻¹ i.p.) and secured in a Kopf stereotaxic frame with the tooth bar at -3.3 mm below

the interaural zero. The dialysis probe (3.5 mm × 200 µm; Cuprophane) was implanted in the right hippocampus +2.2 mm from the interaural line, -4.3 mm lateral and -8 mm below the surface of the brain (König & Klippel, 1963). Probes were secured to the skull as described by Baldwin *et al.* (1994).

Measurement of free radical formation *in vivo* using microdialysis

Free radical formation in the brain *in vivo* was determined by the method recently described in detail by Colado *et al.*

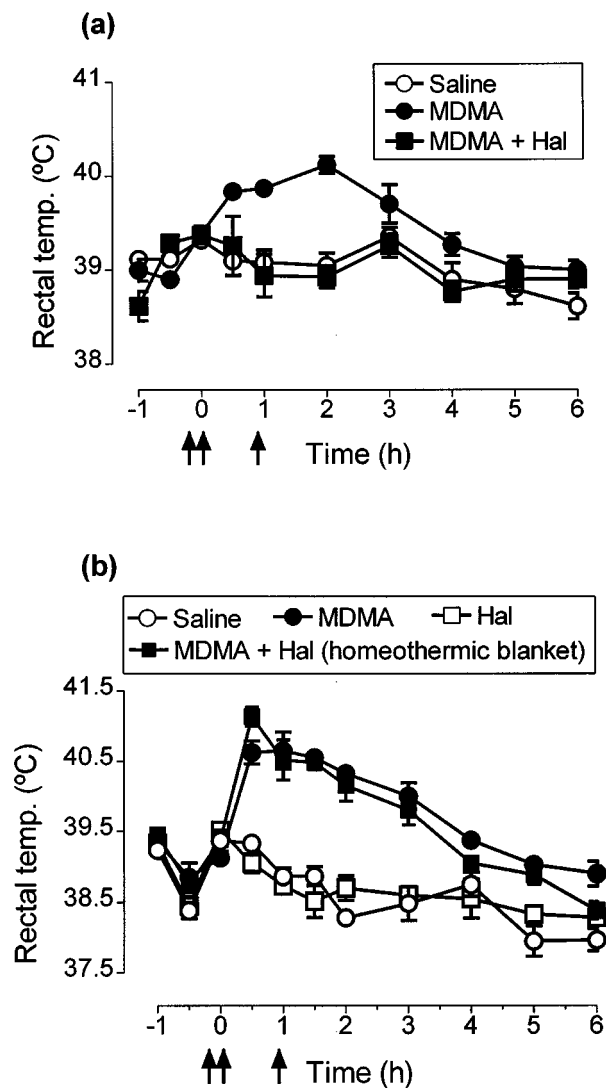


Figure 1 Effect of haloperidol (Hal) on MDMA-induced hyperthermia. (a) Rectal temperature of rats injected with saline or MDMA (15 mg kg⁻¹ i.p.) at time zero and rats injected with haloperidol (2 mg kg⁻¹ i.p.) at -5 min and +55 min and treated with MDMA at time zero. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature ($F(1,10)=19.89$, $P<0.01$) compared with the saline injected group. Administration of haloperidol to MDMA-treated rats prevented the MDMA-induced hyperthermia ($F(1,11)=27.16$, $P<0.001$). (b) Effect of haloperidol on MDMA-induced hyperthermia when rats injected with haloperidol+MDMA were kept at high ambient temperature. Temperature of rats given haloperidol and saline is also shown. There was no difference in basal temperature of the groups. Using the homeothermic blanket, the rectal temperature of the haloperidol+MDMA-treated rats was significantly higher than that of the saline group ($F(1,10)=141.69$, $P<0.001$). All results shown as means ± s.e. mean of *n* = 4–6 rats.

(1997b). The method relies on the fact that hydroxyl free radicals react with salicylic acid to generate 2,3- and 2,5-dihydroxybenzoic acids (2,3-DHBA and 2,5-DHBA) and this reaction is utilized by measuring the formation of 2,3- and 2,5-DHBA in the dialysate of a microdialysis probe implanted in the hippocampus (see above) and which is being perfused with salicylic acid (see Chieuh *et al.*, 1992; Giovanni *et al.*, 1995). Twenty-four hours after implantation, probes were perfused with artificial cerebrospinal fluid (in mM): KCl 2.5, NaCl 125, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.18, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.26, containing salicylic acid (0.5 mM) at a rate of $1 \mu\text{l min}^{-1}$ and samples collected from the freely moving animals at 30 min intervals. The first 60 min sample was

discarded and the next three 30 min baseline samples collected.

The two compounds 2,3-DHBA and 2,5-DHBA were measured by h.p.l.c. and electrochemical detection. The mobile phase consisted of KH_2PO_4 (0.025 M), acetonitrile (20%) and methanol (10%) and was adjusted to pH 3.7 with phosphoric acid, filtered and degassed. The flow rate was 1 ml min^{-1} .

The h.p.l.c. system consisted of a pump (Waters 510) linked to manual sample injector (Loop $20 \mu\text{l}$ Rheodyne), a stainless steel reversed-phase column ($250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$ C8 Ultracarb, Phenomenex) with a precolumn ($30 \times 4.60 \text{ mm}$, $5 \mu\text{m}$ C8 Ultracarb, Phenomenex) and a coulometric detector (Coulo-

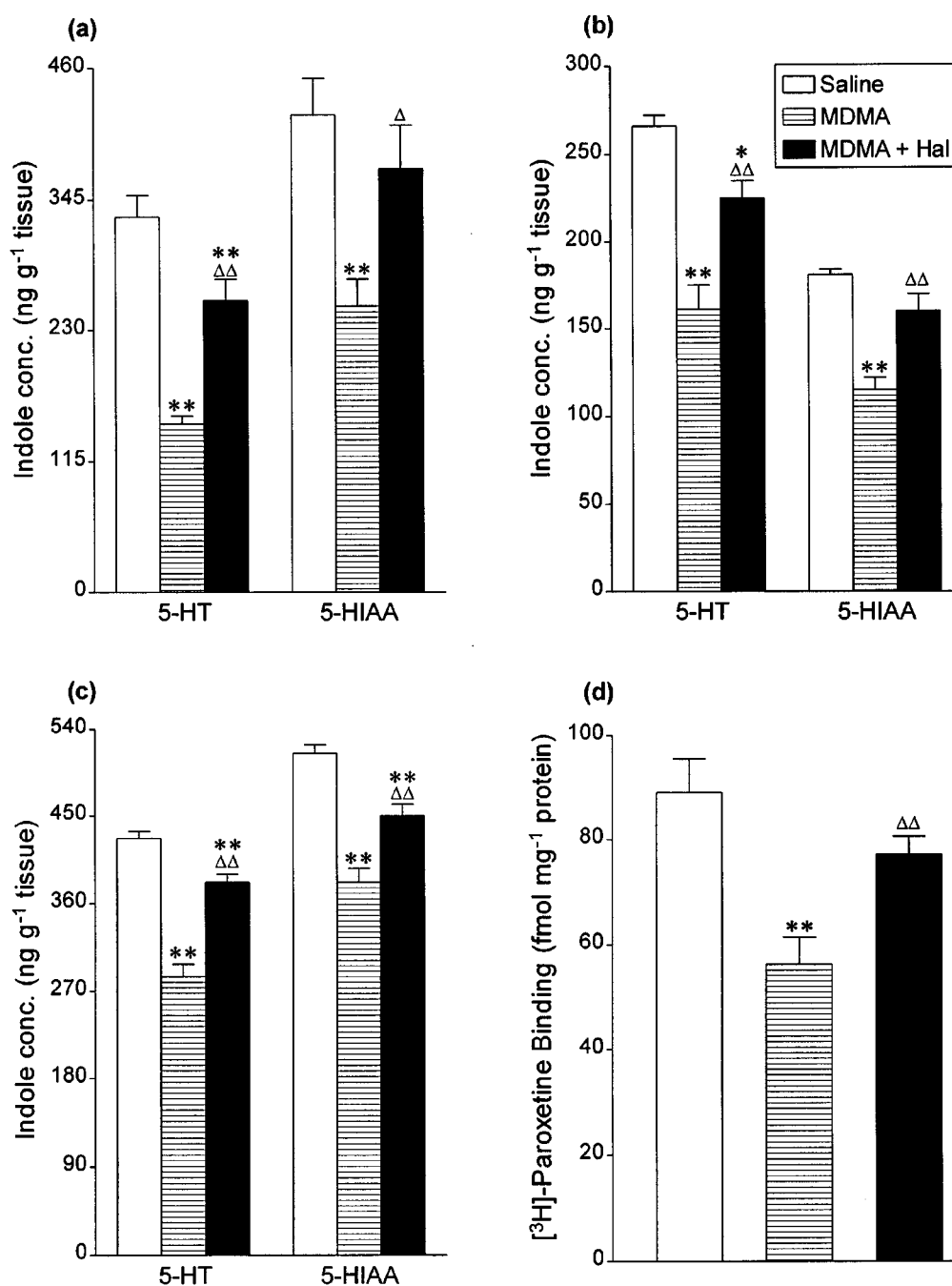


Figure 2 Indole concentrations in (a) hippocampus, (b) cortex and (c) striatum and (d) density of ^3H -paroxetine labelled 5-HT uptake sites in cortex 7 days following saline or haloperidol (Hal, 2 mg kg^{-1} i.p.) 5 min before and 55 min after MDMA (15 mg kg^{-1} i.p.). Results shown as means $\pm$ s.e.mean, $n = 4-7$. Different from saline-treated: * $P < 0.05$, ** $P < 0.01$. Different from MDMA-treated: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$.

chem 5100A) with a 5011 analytical cell. The working electrode potential was set at 400 mV with 1 μ A gain. The current produced was monitored by using a computer data handling system (AXXIOM 747).

Statistics

Statistical analyses of the microdialysis experiments were performed using the statistical computer package BMDP/386 Dynamic (BMDP Statistical Solutions, Cork, Eire). Data were analysed by analysis of variance (ANOVA) with repeated measures (program 2 V) or, where missing values occurred, an unbalanced repeated measure model (program 5 V) was used. Both used treatment as the between subjects factor and time as the repeated measure. ANOVA was performed on both pre-treatment and post-treatment data. Temperature data were also analysed by ANOVA with repeated measures. Data from

monoamine and [^{3} H]-paroxetine binding experiments were analysed by the Newman-Keuls test.

Results

The effect of haloperidol on rectal temperature of MDMA-treated rats kept at normal ambient temperature

Administration of MDMA (15 mg kg⁻¹ i.p.) produced a clear and sustained hyperthermia (Figure 1a). In contrast, when haloperidol (2 mg kg⁻¹ i.p.) was injected 5 min before and 55 min after the MDMA injection the hyperthermic response was totally abolished (Figure 1a). Administration of haloperidol (2 mg kg⁻¹ i.p.) alone had no effect on rectal temperature (Figure 1b).

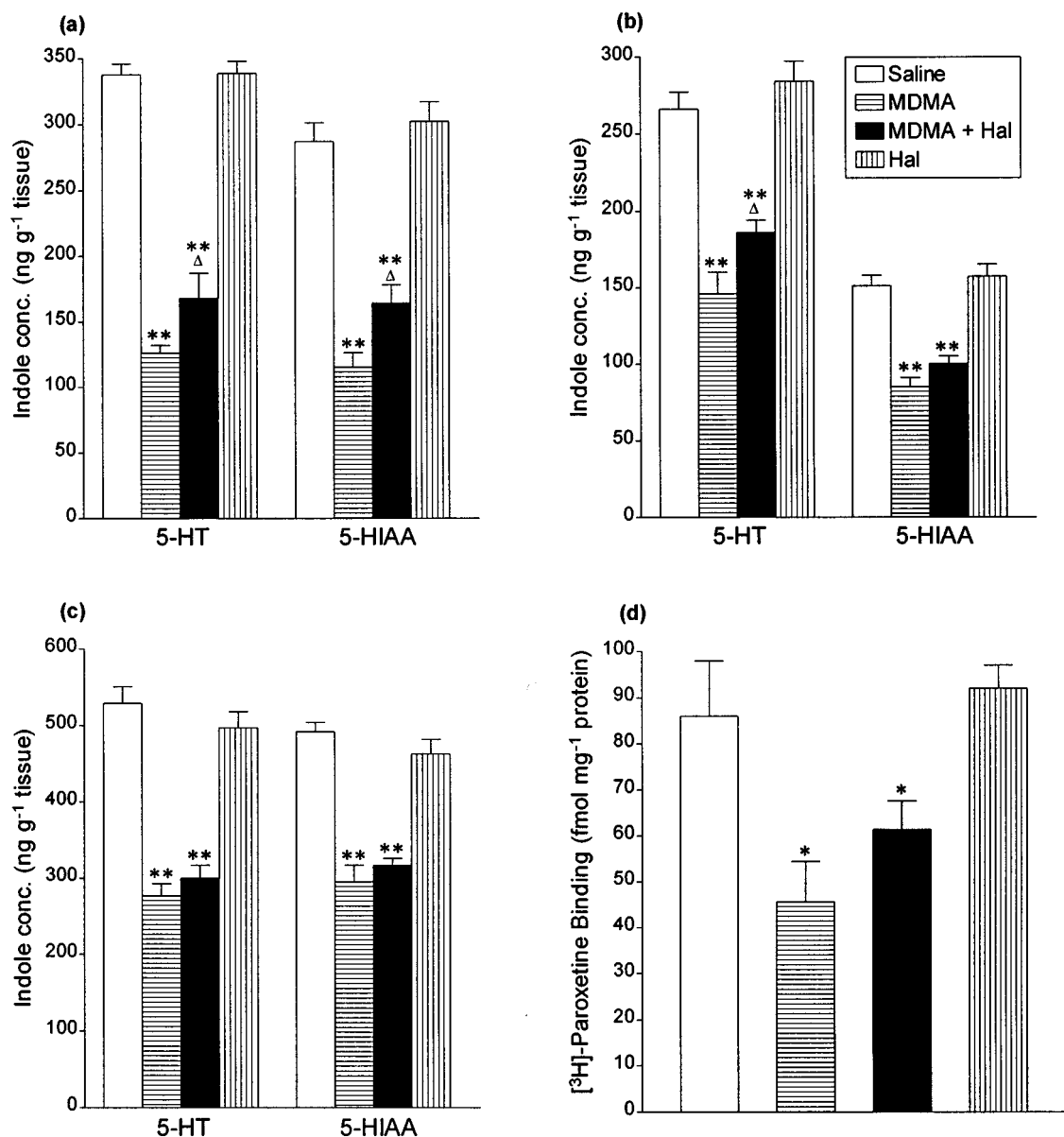


Figure 3 Indole concentrations in (a) hippocampus, (b) cortex and (c) striatum and (d) density of ^{3} H-paroxetine labelled 5-HT uptake sites in cortex 7 days following saline or haloperidol (Hal, 2 mg kg⁻¹ i.p.) 5 min before and 55 min after saline or MDMA (15 mg kg⁻¹ i.p.). The MDMA + haloperidol group was kept at high ambient temperature during the 6 h after the first haloperidol injection. Results shown as means $\pm$ s.e. mean, $n = 4-6$. Different from saline-treated: * $P < 0.05$, ** $P < 0.01$. Different from MDMA-treated: $\Delta P < 0.05$.

The effect of haloperidol on the cerebral monoamine content and [^3H]-paroxetine binding in MDMA-treated rats kept at normal ambient temperature

MDMA (15 mg kg^{-1} i.p.) administration produced a substantial loss in 5-HT and 5-HIAA concentration in the three brain areas examined and also in [^3H]-paroxetine binding values in the cortex (Figure 2) 7 days later. Haloperidol administration provided significant protection against this neurotoxic degeneration (Figure 2).

The effect of haloperidol on rectal temperature of MDMA-treated rats kept at high ambient temperature

The experiment above was repeated except that the rats which were injected with both MDMA and haloperidol were kept at high ambient temperature (see Methods) in order to prevent the decrease in rectal temperature that occurred when this group was kept at normal ambient temperature. This aim was achieved (Figure 1b).

The effect of haloperidol on the cerebral monoamine content and [^3H]-paroxetine binding in MDMA-treated rats kept at high ambient temperature

Administration of MDMA (15 mg kg^{-1} i.p.) again produced a substantial loss in 5-HT and 5-HIAA concentration and [^3H]-paroxetine binding in the brain 7 days later (Figure 3). However little or no protection was seen in the group given haloperidol plus MDMA when the temperature of the rats had been kept elevated to match the temperature seen in the rats given only MDMA (Figure 3).

Effect of L-DOPA on rectal temperature of MDMA treated rats

Administration of L-DOPA (25 mg kg^{-1} + benserazide 6.25 mg kg^{-1}) had no effect on rectal temperature (Figure 4). However, when this dose was administered 2 h after MDMA (15 mg kg^{-1}) it extended the duration of the major MDMA-induced hyperthermia (Figure 4). Higher doses of L-DOPA or administering this dose of the compound at the same time as MDMA resulted in death of the animals, apparently as the result of severe hyperthermia so such protocols could not be investigated further.

Effect of MDMA and L-DOPA on dopamine metabolism

Injection of MDMA (15 mg kg^{-1}) produced a rapid and substantial increase in extracellular dopamine in the striatum, as measured by microdialysis (Figure 5a). This peak was followed by a sustained decrease in both HVA and DOPAC, the decrease in the latter starting earlier than that of HVA (1 h after MDMA, the DOPAC and HVA levels compared to basal values were $23 \pm 2\%$ and $60 \pm 3\%$, respectively, $P < 0.01$) (Figure 5b and c). Injection of L-DOPA (25 mg kg^{-1} + benserazide 6.25 mg kg^{-1}) alone produced no change in the concentration of dopamine in the dialysate, but did produce a sustained and large increase in both HVA and DOPAC (Figure 5b and c). When L-DOPA was injected 2 h after MDMA it did produce a clear increase in dopamine, thereby enhancing the size of the total dopamine response (Figure 5a). A prolonged increase in both HVA and DOPAC was also seen, thereby reversing the MDMA-induced decrease in the concentration of these two metabolites (Figure 5b and c).

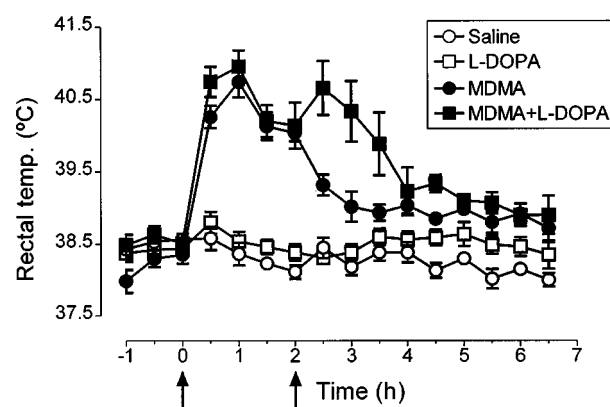


Figure 4 Rectal temperature of rats receiving L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) 2 h after MDMA (15 mg kg^{-1} i.p.) or saline. Also shown are the rectal temperature of animals injected with saline instead of L-DOPA and administered 2 h before saline or MDMA. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature ($F(1,9) = 129.36$, $P < 0.001$) compared with the saline injected group. Administration of L-DOPA to MDMA-treated rats produced a prolongation of the hyperthermic response induced by MDMA ($F(1,11) = 4.12$, $P < 0.05$).

Effect of MDMA and L-DOPA on free radical formation in the brain

In confirmation of previous findings (see Discussion) MDMA injection increased the concentration of 2,3- and 2,5-DHBA in the dialysate from a microdialysis probe implanted in the hippocampus and perfused with salicylic acid (Figure 6). L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) alone did not produced significant changes in the concentration of 2,3 DHBA or 2,5 DHBA (Figure 6). The increase in formation of 2,3- and 2,5-DHBA resulting from MDMA injection was unaltered by an injection of L-DOPA (25 mg kg^{-1}) 2 h later (Figure 6).

The effect of L-DOPA on MDMA-induced degeneration of 5-HT neurones

A single injection of MDMA (15 mg kg^{-1}) resulted in a major loss of 5-HT and 5-HIAA in the hippocampus, cortex and striatum 7 days later and a similar loss of [^3H]-paroxetine binding in the cortex (Figure 7). The degenerative loss of 5-HT was similar in rats injected with MDMA and those given MDMA plus L-DOPA, while L-DOPA alone was without effect on the concentration of either the indole concentration or [^3H]-paroxetine binding in the brain (Figure 7). MDMA injection either alone or with L-DOPA also had no effect on the concentration of dopamine or DOPAC in the striatum 7 days later (Table 1).

Effect of low dose MDMA and L-DOPA on rectal temperature, dopamine metabolism and subsequent neurotoxicity of cerebral 5-HT neurones

Rats were injected with L-DOPA (25 mg kg^{-1} + benserazide 6.25 mg kg^{-1}) followed 30 min later with a sub-neurotoxic dose of MDMA (5 mg kg^{-1}) to examine whether pretreatment with the amino acid would result in MDMA now producing toxicity. Pretreatment with L-DOPA did not enhance the hyperthermic effect of MDMA (Figure 8). Although L-DOPA did not enhance the MDMA-induced increase in dopamine in the striatal dialysate, it induced marked increases in both HVA

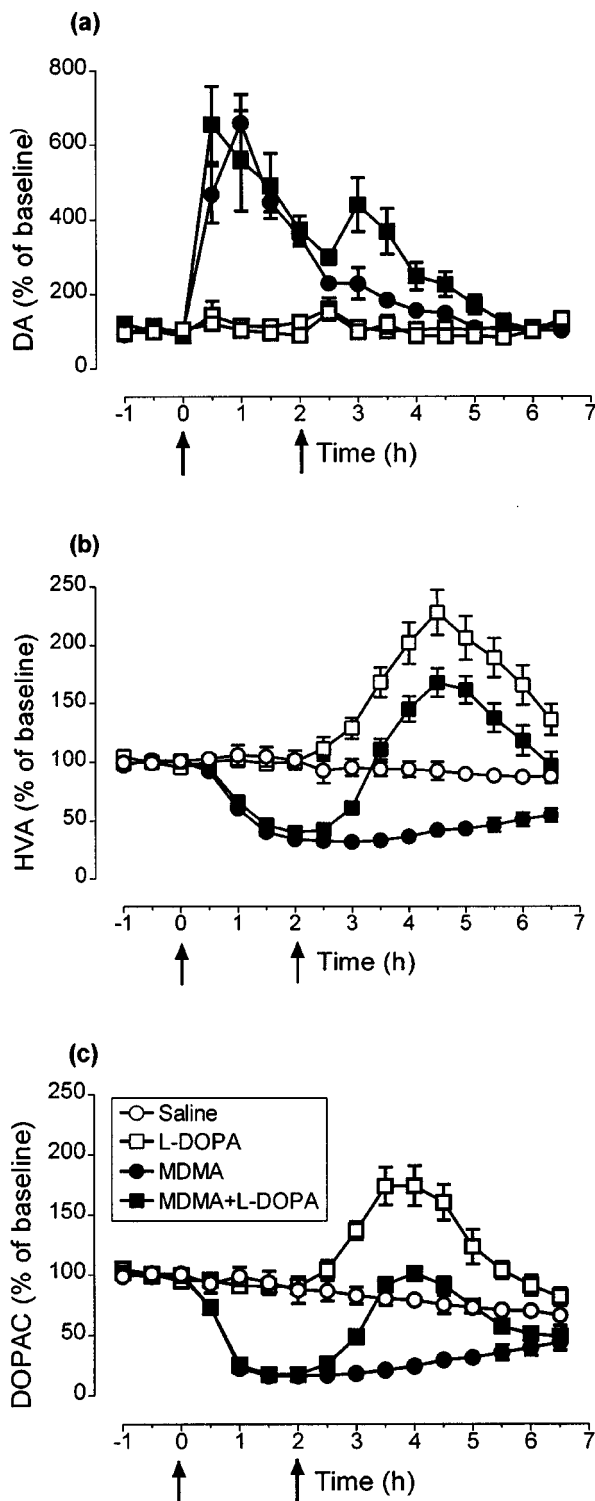


Figure 5 Changes in the levels of (a) dopamine (DA), (b) homovanillic acid (HVA) and (c) 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatal dialysate of rats treated with L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) or saline 2 h after MDMA (15 mg kg^{-1} i.p.). MDMA produced an increase in the content of DA ($F(1,9)=74.34$, $P<0.001$) and a decrease in the levels of HVA ($F(1,9)=81.07$, $P<0.001$) and DOPAC ($F(1,9)=52.61$, $P<0.001$). Administration of L-DOPA enhanced the effect of MDMA on extracellular dopamine ($F(1,10)=9.57$, $P<0.001$) and reversed the MDMA-induced decrease in HVA ($F(1,10)=66.72$, $P<0.001$) and DOPAC ($F(1,10)=43.10$, $P<0.001$). L-DOPA, when given alone, increased the extracellular levels of HVA ($F(1,10)=27.30$, $P<0.001$) and DOPAC ($F(1,10)=24.54$, $P<0.001$). Values are expressed as a percentage of the mean of three measurements before drug administration. Each value is the means $\pm$ s.e. mean of 5–7 experiments. The basal concentrations in saline-

and DOPAC in place of the decrease in the concentration of these metabolites produced by MDMA alone (Figure 9). The low dose of MDMA did not result in neurodegeneration occurring 7 days later, nor did the pretreatment with L-DOPA result in this dose of MDMA producing neurodegeneration (Figure 10).

Effect of clomethiazole on the MDMA-induced change in dopamine metabolism in vivo

The increase in dopamine and decrease in HVA and DOPAC induced by MDMA (15 mg kg^{-1}) was totally unaltered by administration of clomethiazole (50 mg kg^{-1}) 5 min prior and 55 min post MDMA injection (Figure 11).

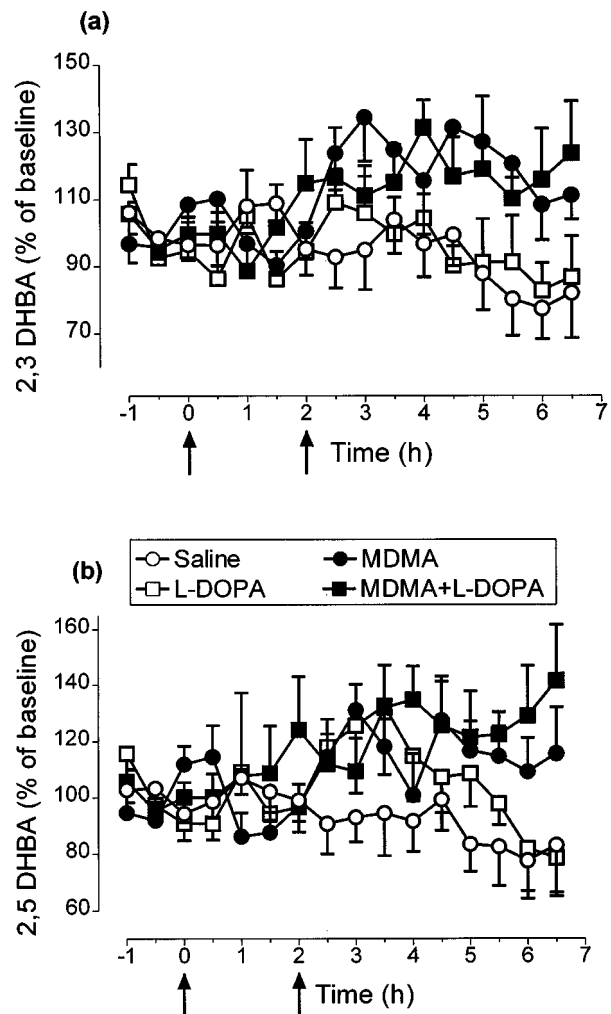


Figure 6 Changes in the levels of (a) 2,3 dihydroxybenzoic acid (DHBA) and (b) 2,5 DHBA in the hippocampal dialysate of rats treated with L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) or saline 2 h after MDMA (15 mg kg^{-1} i.p.). MDMA increased the extracellular levels of 2,3 DHBA ($F(1,11)=10.34$, $P<0.001$) and 2,5 DHBA ($F(1,11)=4.66$, $P<0.05$). This effect was not modified by L-DOPA injection 2 h after MDMA. L-DOPA, when given to saline-treated rats did not significantly modify the extracellular levels of 2,3 DHBA or 2,5 DHBA. Values are expressed as a percentage of the mean of three measurements before drug administration. Each value is the means $\pm$ s.e. mean of 6–10 experiments. The basal concentrations in saline-treated rats ($n=6$) were: 2,3 DHBA ($10.1 \pm 1.1 \text{ pg } \mu\text{L}^{-1}$) and 2,5 DHBA ($11.6 \pm 1.22 \text{ pg } \mu\text{L}^{-1}$).

treated rats were: DA ($0.83 \pm 15 \text{ pg } \mu\text{L}^{-1}$), HVA ($246 \pm 16 \text{ pg } \mu\text{L}^{-1}$) and DOPAC ($252 \pm 23 \text{ pg } \mu\text{L}^{-1}$).

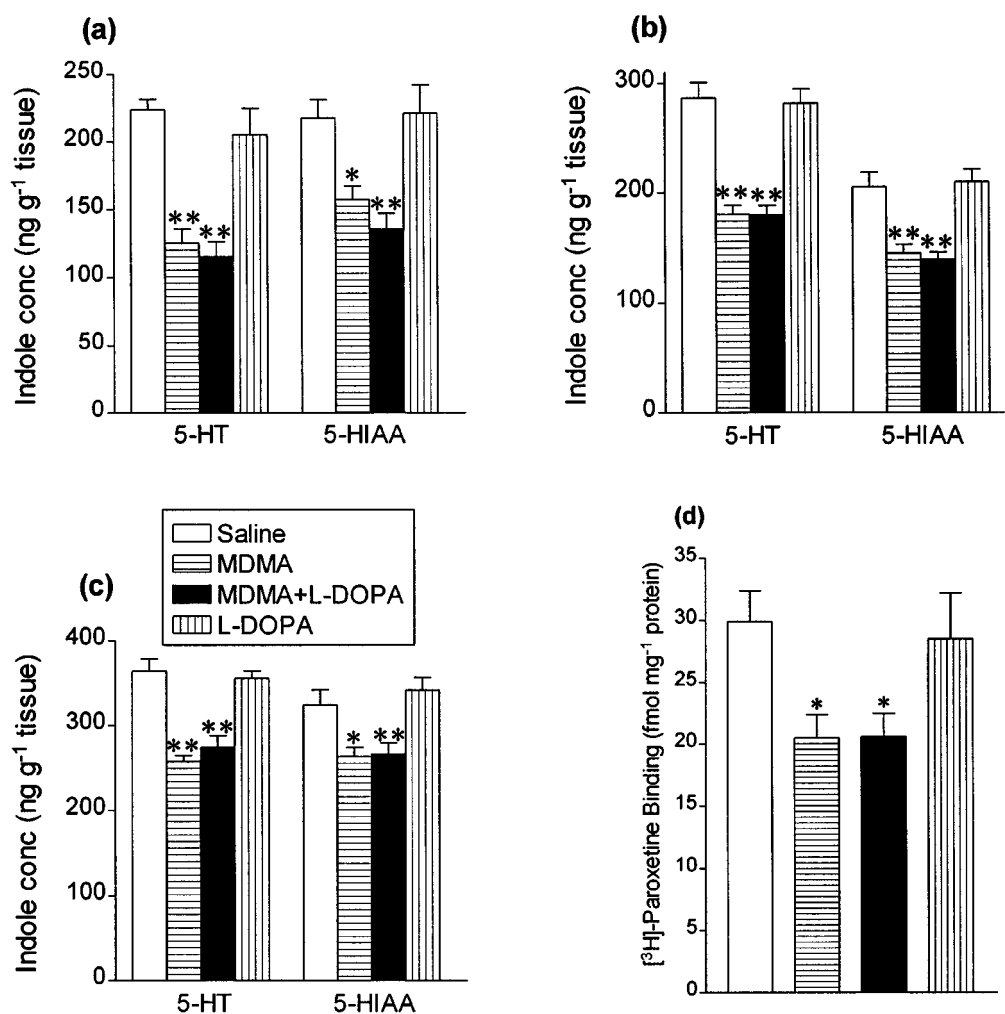


Figure 7 Indole concentrations in (a) hippocampus, (b) cortex and (c) striatum and (d) density of ^3H -paroxetine labelled 5-HT uptake sites in cortex 7 days following saline or L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) 2 h after saline or MDMA (15 mg kg^{-1} i.p.). Results shown as means $\pm$ s.e. mean, $n = 5-8$. Different from saline-treated: * $P < 0.05$, ** $P < 0.01$.

Table 1 The concentration of dopamine and DOPAC in striatum 7 days after injection of MDMA (15 mg kg^{-1} i.p.) followed 2 h later by L-DOPA (25 mg kg^{-1} i.p.)

1st injection	2nd injection	n	Catechol concentration (ng g^{-1} tissue)	
			Dopamine	DOPAC
Saline	Saline	5	6110 ± 423	1588 ± 212
MDMA	Saline	5	6200 ± 204	1600 ± 78
MDMA	L-DOPA	8	6771 ± 310	1403 ± 137
Saline	L-DOPA	5	6495 ± 170	1662 ± 142

Results shown as means $\pm$ s.e. mean. No significant differences in tissue concentrations of the catechols were found between experimental groups.

Discussion

Both Schmidt *et al.* (1990b) and Hewitt & Green (1994) have reported that administration of haloperidol concurrently with MDMA attenuates the neurodegeneration of 5-HT terminals in the brain produced by this neurotoxic amphetamine and this observation was confirmed by the current study. Schmidt *et al.* (1990b) did not measure body temperature but Hewitt & Green (1994) observed that the combination of haloperidol and MDMA, whilst not producing hypothermia, did markedly attenuate the hyperthermia normally produced by the drug, an

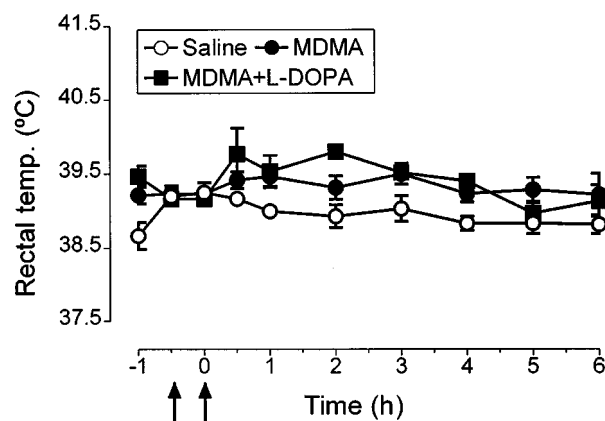


Figure 8 Rectal temperature of rats receiving L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) 30 min before MDMA (5 mg kg^{-1} i.p.). Also shown are the rectal temperature of animals injected with saline instead of L-DOPA and administered saline or MDMA 30 min later. There was no difference in the basal temperature of the groups. MDMA produced a rise in body temperature ($F(1,10) = 7.15$, $P < 0.05$). Pretreatment with L-DOPA did not alter the hyperthermic response induced by MDMA.

observation also confirmed by the present study. We therefore repeated the experiment on the neuroprotective effect of

haloperidol but used a homeothermic blanket, thereby keeping the rectal temperature of the animals elevated to a similar level to that seen in the group given only MDMA (Figure 1). The

protective effect of haloperidol in this hyperthermic group was marginal. These data support the findings in our other recent investigations (Colado *et al.*, 1998a, b) which have indicated strongly that drugs can provide substantial neuroprotection not only by producing hypothermia as has previously been demonstrated by others (Farfel & Seiden 1995a, b; Malberg *et al.*, 1996; Colado *et al.*, 1998a) but also by merely preventing the MDMA-induced hyperthermia (Colado *et al.*, 1998b). Haloperidol therefore can be concluded to be protective because of its effect on temperature and not because of a selective neuroprotective mechanism.

This finding in turn raised the question as to the validity of earlier proposals that dopamine plays a key role in the expression of MDMA-induced neurotoxicity. Various observations have been used to support this tenet, including the fact that MDMA alters dopamine release (Johnson *et al.*, 1986; Schmidt *et al.*, 1987; Hiramatsu & Cho, 1990; Nash, 1990; Nash & Brodtkin, 1991) and the observation that L-DOPA administration potentiates MDMA-induced damage (Schmidt *et al.*, 1991). We therefore re-investigated both these claims and also investigated whether L-DOPA administration alters the increase in free radical formation which has been shown to follow MDMA injection (Colado *et al.*, 1997b; 1998c).

With regard to the alteration of dopamine metabolism which follows MDMA injection, both Johnson *et al.* (1986) and Schmidt *et al.* (1987) noted that MDMA released [³H]-dopamine from striatal slices and Steele *et al.* (1987) reported that the drug inhibited [³H]-dopamine uptake into striatal synaptosomes. Subsequent studies using either *in vivo* voltammetry (Yamamoto & Spanos, 1988) or microdialysis (Nash, 1990; Hiramatsu & Cho, 1990; Gough *et al.*, 1991) found that MDMA administration resulted in a rapid rise in extracellular dopamine and decrease in DOPAC and HVA. Both the size and the time course of these changes as reported by Nash (1990) and Gough *et al.* (1991) were replicated in the current study. The size of the dopamine increase and DOPAC decrease are considerably greater than those observed in postmortem tissue 4 h after a single injection of MDMA (Colado & Green, 1994).

The current investigation found that following MDMA the concentration of HVA in the dialysate was decreased in a similar fashion to DOPAC, but that the change in the extracellular level of HVA lagged behind the DOPAC change. The difference in the time courses of changes in the extracellular DOPAC and HVA levels following MDMA may reflect the fact that HVA is a secondary metabolite of dopamine, being predominantly derived from intraneuronally formed DOPAC but also from the released dopamine *via* 3-methoxytyramine (Westerink & Spaan, 1982). Therefore, the delayed decrease in HVA compared to DOPAC might be due to an initial rise in 3-methoxytyramine and its subsequent conversion to HVA by catechol O-methyl transferase. These data suggest that MDMA exerts two separate actions on dopaminergic activity *in vivo*, one causing an increase in dopamine release and another producing a decrease in the intraneuronal dopamine metabolism (reflected by the extracellular levels of DOPAC and HVA). These changes on dopamine metabolites are probably the results of MDMA blocking dopamine re-uptake (causing less dopamine to be metabolized to DOPAC) and also by inhibiting monoamine oxidase (MAO). Dopamine is preferentially metabolized by MAO-A in the rat striatum *in vivo* (Kato *et al.*, 1986) and MDMA acts as a competitive inhibitor of MAO-A activity (Leonardi & Azmitia, 1994). An additional explanation of the decrease in DOPAC and HVA would be a reduction in dopamine synthesis following MDMA. However this hypoth-

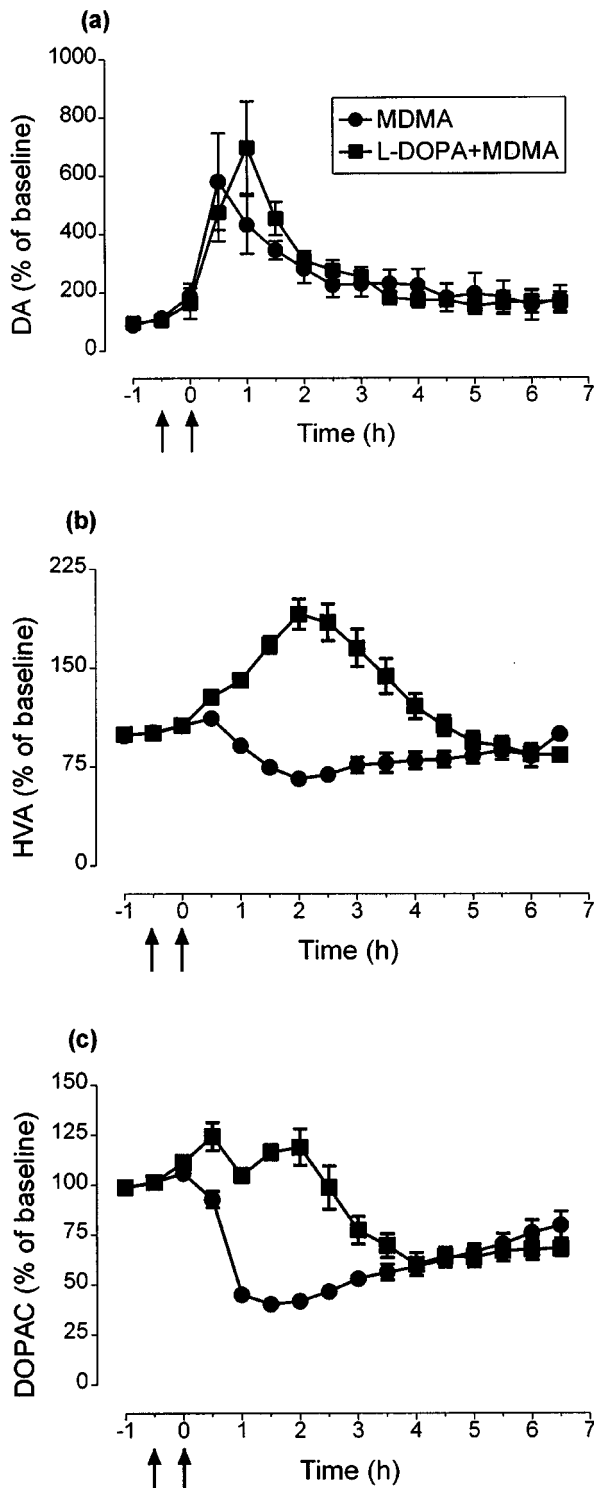


Figure 9 Changes in the levels of (a) dopamine (DA), (b) homovanillic acid (HVA) and (c) 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatal dialysate of rats treated with L-DOPA (25 mg kg^{-1} i.p. + benserazide 6.25 mg kg^{-1} i.p.) 30 min before MDMA (5 mg kg^{-1} i.p.). L-DOPA did not modify the effect of MDMA on extracellular dopamine ($F(1,8)=0.0908$, n.s.) but reversed the MDMA-induced decrease in HVA ($F(1,9)=47.82$, $P<0.001$) and DOPAC ($F(1,10)=20.55$, $P<0.001$). Values are expressed as a percentage of the mean of two measurements before drug administration. Each value is the means $\pm$ s.e. mean of 4–6 experiments.

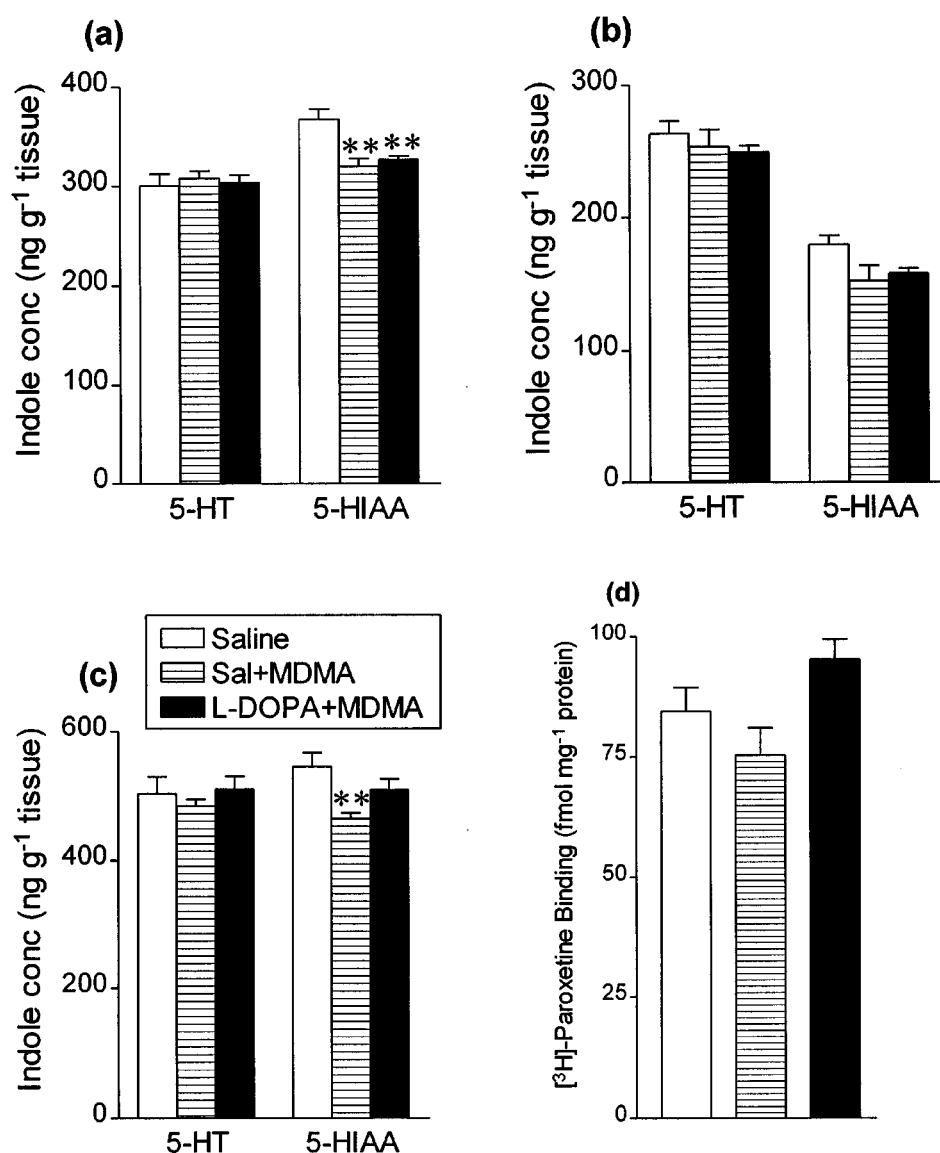


Figure 10 Indole concentrations in (a) hippocampus, (b) cortex and (c) striatum and (d) density of ³H paroxetine labelled 5-HT uptake sites in cortex 7 days following saline or L-DOPA (25 mg kg⁻¹ i.p. + benserazide 6.25 mg kg⁻¹ i.p.) 30 min before MDMA (5 mg kg⁻¹ i.p.). Results shown as means $\pm$ s.e. mean, $n = 5-8$. Different from saline-treated: ** $P < 0.01$.

esis is unlikely since MDMA does not alter tyrosine hydroxylase activity in the striatum (Stone *et al.*, 1986).

The data raise a further point; that is, the nature of the mechanism of MDMA-induced dopamine release. It has often been assumed that the dopamine release is by a carrier-mediated exchange mechanism as occurs with other amphetamine-like drugs (Raiteri *et al.*, 1979; Fischer & Cho, 1979; also see McMillen, 1983). However it is worth noting that several investigators have provided convincing evidence that 5-HT₂ agonists enhance MDMA-induced dopamine release while 5-HT₂ antagonists inhibit release (Nash, 1990; Schmidt *et al.*, 1990a, 1994; Yamamoto *et al.*, 1995; Gudelsky *et al.*, 1994). MDMA-induced 5-HT release therefore appears to play a major role in the release of dopamine. When one adds the known effects of MDMA on dopamine uptake inhibition (Steele *et al.*, 1987) and MAO inhibition (Leonardi & Azmitia, 1994) then it becomes plausible to suggest that carrier-mediated exchange need not be a major determinant in the increase of extracellular dopamine which follows MDMA administration, a suggestion supported by recent *in vitro* studies (Crespi *et al.*, 1997). Further support for this proposal

comes from our study on the effect of clomethiazole. This drug, which enhances GABA_A receptor activity (see Green, 1998), inhibited the increase in extracellular dopamine which follows administration of methamphetamine, a known carrier-mediated mechanism (McMillen, 1983). Therefore, the failure of this drug to alter the MDMA-induced increase in extracellular dopamine may reflect a difference in the release mechanism between these two amphetamine derivatives. The implications of this to the selective neurotoxic action of MDMA are discussed later.

Interestingly the size of the increase in extracellular dopamine in the striatum following MDMA seen *in vivo* was similar in magnitude to that seen after a high dose of methamphetamine (Baldwin *et al.*, 1993) which raises questions as to why the locomotor and behavioural activity seen after MDMA administration appears to be 5-HT rather than dopamine receptor mediated, having a different profile to that seen after amphetamine (Callaway *et al.*, 1990; Callaway & Geyer, 1992). It could be that the serotonin-mediated behaviours predominate over any dopamine-mediated effects. While MDMA releases dopamine in both the accumbens

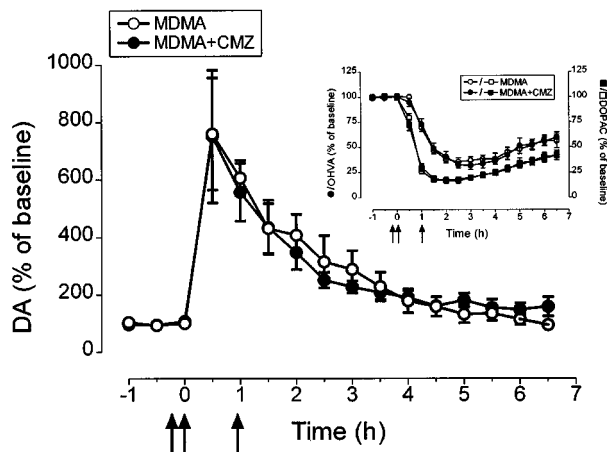


Figure 11 Changes in the levels of dopamine (DA) and the metabolites (shown in the insert) in the striatal dialysate of rats treated with clomethiazole (CMZ, 50 mg kg⁻¹ i.p.) or saline 5 min before and 55 min after MDMA (15 mg kg⁻¹ i.p.). Clomethiazole did not modify the effect of MDMA on DA, DOPAC and HVA extracellular levels. Values are expressed as a percentage of the mean of three measurements before drug administration. Each value is the means $\pm$ s.e. mean of 6–7 experiments.

(Callaway & Geyer, 1992) and striatal regions (Nash, 1990; this paper) the accumbens is the locus for locomotor activity following amphetamine administration (Kelly *et al.*, 1975). However, recent data suggest that the predominant action of MDMA in this region may be that of inhibiting dopamine neuronal firing (Gifford *et al.*, 1996). Since the drug can induce Ca²⁺ dependent dopamine release (Crespi *et al.*, 1997) dopamine mediated function would be suppressed, thereby allowing the expression of 5-HT mediated behaviours including the serotonin syndrome (Spanos & Yamamoto, 1989; Colado *et al.*, 1993; Green *et al.*, 1995).

When L-DOPA was given 2 h after MDMA we observed that extracellular dopamine was increased above that seen in the MDMA alone treated group for approximately 3 h and that HVA and DOPAC concentrations also increased markedly over a similar period. These changes presumably reflect increased dopamine synthesis and release. Despite this clear *in vivo* indication of increased dopamine release and possibly also function, no enhancement of MDMA neurotoxicity was observed 7 days later. These data are in contradiction of the report of Schmidt *et al.* (1991) that MDMA-induced neurotoxicity is enhanced by L-DOPA. While Schmidt and colleagues used higher doses of L-DOPA these proved impossible to administer in the current study as they proved fatal. This is probably because of the use of the Dark Agouti strain of rat in our study since we have previously found that neurotoxicity occurs in these animals after much lower doses of MDMA and p-chloroamphetamine (PCA) than are required with other strains (see Colado *et al.*, 1995; 1997b; Murray *et al.*, 1996). Nevertheless using the highest practical dose of L-DOPA, no potentiation of MDMA-induced toxicity was detected. We were also unable to induce neurotoxicity in rats given a sub-toxic dose of MDMA by also administering L-DOPA, as was also reported by Schmidt *et al.* (1991).

In order to investigate further this apparent discrepancy between our data and that of Schmidt *et al.* (1991) we also examined whether L-DOPA altered MDMA-induced free radical formation. The MDMA-induced increase in the dialysate concentration of 2,3- and 2,5-DHBA that has previously been reported to occur after MDMA administra-

tion (Colado *et al.*, 1997b; 1998c) was again seen in the current study. While the concentration of 2,5-DHBA appeared to be increased by L-DOPA, this change was not statistically significant. In any event, as Halliwell *et al.* (1991) have pointed out, a 2,3-DHBA increase is the only reliable indicator of increased free radical formation because of enzyme-induced conversion of salicylate to 2,5-DHBA. Nevertheless we found no evidence that the MDMA-induced increase in either 2,3- or 2,5-DHBA was even modestly enhanced by L-DOPA administration. Thus one plausible explanation for the results obtained by Schmidt *et al.* (1991), namely that tissue damage resulting from free radicals produced by MDMA metabolism (see Colado *et al.*, 1997b) would be further potentiated by administration of L-DOPA, a compound that through its metabolism to dopamine would also increase free radical production through auto-oxidation (see Chiueh *et al.*, 1992; 1993), could not be supported.

What does remain a possible explanation for the discrepant findings is the degree of hyperthermia produced by addition of L-DOPA to MDMA-treated rats. As we and others have shown, sustained hyperthermia markedly enhances the neurotoxic damage produced by MDMA (Colado *et al.*, 1998b; Broening *et al.*, 1995) presumably because hyperthermia is conducive to enhancing free radical production (Kil *et al.*, 1996). If L-DOPA produced a sustained hyperthermia in the strain of rats used by Schmidt *et al.* (1990) rather than the more transient effect we observed, then neurotoxic damage would probably be increased. However, such a mechanism does not indicate that dopamine has a core role in MDMA-induced neurodegeneration as has been proposed by others (Stone *et al.*, 1988; Schmidt *et al.*, 1991). In this regard it is also worth pointing out that in our current study the increase in extracellular dopamine was almost the same whether a neurotoxic dose of MDMA of 15 mg kg⁻¹ or a non-toxic dose of 5 mg kg⁻¹ was given.

Finally we examined the effect of injecting clomethiazole on MDMA-induced changes in dopamine metabolism. Previously in an *in vivo* study we found that clomethiazole inhibited both the increase in dopamine and reduction in HVA and DOPAC which followed an injection of methamphetamine (Baldwin *et al.*, 1993). In contrast, clomethiazole appeared to have no effect on MDMA-induced changes in dopamine metabolism. This finding is perhaps surprising in the light of a report suggesting that MDMA-induced dopamine release is regulated by the activity of a GABAergic input into the substantia nigra (Yamamoto *et al.*, 1995). Clomethiazole is one of the few compounds to have been shown unequivocally to protect against MDMA-induced damage by a mechanism which does not involve a reduction in body temperature (Colado *et al.*, 1998b). It is also a compound that has been shown to have a powerful neuroprotective action in a wide variety of animal models of acute ischaemic stroke (Green, 1998). These data suggest that dopamine is not involved in the neuroprotective effect of clomethiazole against MDMA-induced neurotoxicity.

Finally it is worth pointing out that a major conundrum is raised by the current study. In rats, but not mice (see Laverty & Logan 1990), MDMA produces a neurodegenerative loss of 5-HT nerve terminals, whilst sparing dopamine terminals (Schmidt & Kehne, 1990; Colado *et al.*, 1997a, b), a fact again supported by the findings in this current study (see Table 1). The question arises as to the mechanisms involved in this selectivity. It is noteworthy that methamphetamine which also increases extracellular 5-HT and dopamine (see for example Baldwin *et al.*, 1993) and also increases free radical formation (Giovanni *et al.*, 1995) produces neurotoxic degeneration of both 5-HT and dopamine neurones (Gibb *et al.*, 1990; Green *et*

al., 1992; Baldwin *et al.*, 1993). If we are correct and the extracellular dopamine increase does not reflect carrier-mediated exchange then relatively little MDMA may be entering the dopamine nerve ending and being metabolised to the neurotoxic metabolites which are then auto-oxidised thereby producing free radicals (see Colado *et al.*, 1997b). This may, in part, explain its lack of neurotoxic action on dopamine nerve endings since it would be reasonable to assume that MDMA would be metabolized in the same way in dopamine and 5-HT neurones. Against this hypothesis however there is good evidence that dopamine uptake inhibitors markedly inhibit MDMA-induced dopamine release (Nash & Brodtkin, 1991). This observation would strongly support the idea that MDMA acts to release dopamine by a facilitated exchange mechanism which utilises the dopamine uptake carrier mechanism (see Raiteri *et al.*, 1979; Fischer & Cho, 1979; McMillen, 1983).

Another possible explanation for the selective damage which follows MDMA is that the neurotoxic metabolites of

methamphetamine and MDMA differ in their pattern of damage. A major postulated neurotoxic metabolite of MDMA, namely dihydroxymethylamphetamine (Hiramatsu *et al.*, 1990; Colado *et al.*, 1995, 1997b), has been reported not to be toxic to dopamine neurones (Johnson *et al.*, 1992).

In conclusion, the current results demonstrate that MDMA has a major acute effect on dopamine metabolism in the brain but we suggest that there is little evidence to indicate that the changes reported are crucially involved in the long term neurotoxic degeneration of 5-HT neurones produced by this commonly used recreational drug.

M.I. Colado thanks CICYT (SAF 98-0074) and Astra Arcus for financial support. The authors are grateful to Servicio de Restricción de Estupefacientes, Ministry of Health, Spain for supplying MDMA.

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(Received April 24, 1998

Revised November 16, 1998

Accepted November 19, 1998)



Enhanced role for the opening of potassium channels in relaxant responses to acetylcholine after myocardial ischaemia and reperfusion in dog coronary arteries

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1 Anaesthetized dogs were subjected to 1 h occlusion of the left circumflex coronary artery followed by 2 h of reperfusion. Relaxant responses were examined in coronary artery rings removed proximal (nonischaemic) or distal (ischaemic) to the site of occlusion.

2 Relaxant responses to acetylcholine (ACh) were similar in nonischaemic and ischaemic artery rings. In addition ACh-induced relaxation of nonischaemic and ischaemic artery rings was equally susceptible to inhibition of nitric oxide (NO) synthase using L-N^G-nitroarginine (L-NOARG, 10⁻⁴ M), or to inhibition of soluble guanylate cyclase using 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10⁻⁵ M).

3 In nonischaemic arteries, the relaxation to ACh was unaffected by high K⁺ (67 mM) but in ischaemic arteries, the maximum relaxation to ACh was significantly reduced from 113 ± 6 to 60 ± 2% (ANOVA, *P* < 0.05). Tetraethylammonium (TEA, 10⁻³ M), an inhibitor of large conductance calcium activated potassium (BK_{Ca}) channels did not inhibit the response to ACh in nonischaemic arteries but in ischaemic arteries TEA significantly shifted the concentration response curve to ACh to the right (pEC₅₀; nonischaemic, 7.07 ± 0.25; ischaemic, 6.54 ± 0.21, *P* < 0.01, ANOVA) without decreasing the maximum relaxation. TEA did not affect the responses to sodium nitroprusside in either nonischaemic or ischaemic arteries.

4 In conclusion, ischaemia/reperfusion did not change the sensitivity of endothelium-dependent relaxation to L-NOARG or ODQ indicating that ischaemia did not affect the contribution of NO or cyclic GMP to ACh-induced relaxation. However, in ischaemic arteries the opening of the BK_{Ca} channels contributed to relaxation caused by ACh whereas TEA had no effect in nonischaemic arteries. The factor responsible for the opening of this potassium channel was a factor other than NO and may be endothelium derived hyperpolarizing factor (EDHF).

Keywords: Acetylcholine; dog coronary arteries; ischaemia and reperfusion; nitric oxide; potassium channels; sodium nitroprusside

Abbreviations: BK_{Ca}, large conductance calcium activated potassium channel; DMSO, dimethyl sulphoxide; EDHF, endothelium-derived hyperpolarizing factor; K_{ATP}, ATP-sensitive potassium channel; L-NOARG, L-N^G-nitroarginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; SNP, sodium nitroprusside; TEA, tetraethylammonium; U46619, 9, 11-dideoxy-9 α , 11 α -epoxymethano-prostaglandin F_{2 α}

Introduction

The endothelium regulates vascular tone by the release of a number of factors including endothelium-derived relaxing factor (EDRF), characterized as nitric oxide (NO, Palmer *et al.*, 1987), endothelium-derived hyperpolarizing factor (EDHF, Taylor & Weston, 1988; Furchgott & Vanhoutte, 1989; Komori & Vanhoutte, 1990; Garland *et al.*, 1995; Mombouli & Vanhoutte, 1997) which remains to be identified and prostaglandins such as PGI₂ (Moncada *et al.*, 1976). EDHF opens potassium channels to hyperpolarize cells leading to closure of voltage-operated calcium channels to cause relaxation (Garland *et al.*, 1995). The type of potassium channels involved remains unclear but the calcium-sensitive potassium channels and ATP-sensitive potassium (K_{ATP}) channels are most commonly proposed to be involved (Garland *et al.*, 1995).

Even though EDHF can cause relaxation, its effect is transient (Komori & Vanhoutte, 1990) and in some studies it contributes to relaxation only when the NO system is blocked (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). This

suggests that EDHF may operate as a reserve system when the NO system is impaired which, for example, can occur in artery diseases (Garland *et al.*, 1995). Hence the contribution of EDHF to vasodilatation may be increased where there is endothelial dysfunction such as in the presence of hypercholesterolaemia and perhaps after myocardial ischaemia and reperfusion.

Myocardial ischaemia/reperfusion has been reported to cause selective attenuation of the endothelium-dependent relaxation of isolated coronary arteries (Ku, 1982; Sobey *et al.*, 1990; Dauber *et al.*, 1992; Sobey *et al.*, 1992) without impairing the function of the smooth muscle cells. However, Quillen *et al.*, (1990) and Winn & Ku (1992) both reported that ACh-induced relaxation of dog coronary arteries was unaffected by ischaemia and reperfusion. This then raises the question as to whether EDHF might contribute to endothelium-dependent relaxation after ischaemia and reperfusion to help to maintain responses to endothelium-dependent dilators, however the mechanism of endothelium-dependent relaxation has not previously been investigated in ischaemic arteries. The aim of this study was to compare the sensitivity of endothelium-dependent relaxation in nonischaemic and pre-

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viously ischaemic arteries to inhibition of NO synthase, guanylate cyclase and K⁺ channel opening to determine whether ischaemia and reperfusion altered the contribution of NO and EDHF to relaxation of the coronary arteries.

Methods

All experimental procedures were performed within the guidelines of the National Health & Medical Research Council of Australia and were approved by the Pharmacology and Physiology subcommittee of the University of Melbourne Animal Experimentation Ethics Committee.

Experimental preparation

Mongrel dogs ($n=23$) of either sex (14–47 kg) were sedated with an intramuscular injection of xylazine (0.2–0.6 mg kg⁻¹, Bayer, Australia) and anaesthetized by intravenous injection of sodium thiopentone (25–30 mg kg⁻¹, Abbott Australasia, Australia) followed by α -chloralose (70 mg kg⁻¹, i.v.) which was supplemented as necessary. The dogs were ventilated artificially with room air plus additional oxygen as necessary to maintain arterial blood gas and pH levels within the normal physiological range. Arterial pressure was measured by inserting a catheter into the left femoral artery and connecting it to a pressure transducer (Spectromed, U.S.A.) and mean and phasic arterial pressure were recorded using a Grass chart recorder (model # 7E, U.S.A.).

A left thoracotomy was performed and the heart was suspended in a pericardial cradle. Left ventricular pressure (LVP) was measured with a pressure tip transducer (Millar, U.S.A.) passed through the left atrium into the left ventricle. Peak positive LV dp/dt, an index of myocardial contractility, was obtained by a differential coupler. The heart rate was measured using a cardiometer coupler. A 1–2 cm segment of the left circumflex coronary artery was dissected clear of connective tissue for placement of an electromagnetic flow probe (internal diameter 2.0 or 2.5 mm) which was connected to a flow meter (AE Delft, Netherlands) for measurement of mean and phasic coronary blood flow. Commencing at the start of the surgery, normal saline (10 ml kg⁻¹ h⁻¹, i.v.) was infused to compensate for fluid loss throughout the experiments. A stabilization period of 15 min was allowed following the surgery.

Lignocaine hydrochloride (50 mg, i.v., Astra, Australia) was given 20 min prior to coronary artery occlusion. Total occlusion of the left circumflex artery, distal to both the probe and the first main branch, was performed with artery forceps. After 60 min of artery occlusion, the forceps were removed to start reperfusion for 2 h. The blood was allowed into the myocardium gradually to limit the ischaemia-induced hyperaemia which reduces the incidence of reperfusion arrhythmias. In sham ischaemic preparations, five dogs were instrumented and remained anaesthetized for the same period of time as dogs subjected to ischaemia/reperfusion.

Experimental protocol

At the end of the ischaemia/reperfusion (or sham) protocol, the dogs were killed with an anaesthetic (pentobarbitone, Boehringer, Australia) overdose. The heart was excised and the circumflex artery was isolated. Sections of the coronary artery proximal (nonischaemic) and distal (ischaemic/reperfused) to the point of occlusion were isolated and cleared of superficial connective tissue. Rings were stored in Krebs-bicarbonate

solution (composition (mM): NaCl 118.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, (+)-glucose 5.0, NaHCO₃ 25.0, CaCl₂·2H₂O 2.5) overnight at 4°C.

Coronary artery rings, 5 mm in width, were mounted between two stainless steel wires with one end linked to an isometric force transducer (model # FT03, Grass Medical Instruments, U.S.A.) connected to a chart recorder (model # R-02, Rikadenki Kogyo Co., Japan). The other end was anchored to a glass rod submerged in a siliconized, jacketed, 10 ml organ bath filled with Krebs-bicarbonate solution containing indomethacin (3×10^{-5} M) maintained at 37°C with a pH of 7.4 and continuously aerated with 95% O₂, 5% CO₂. Rings were equilibrated to a resting tension of 4 g for 30 min. All artery rings were submaximally precontracted using 9, 11-dideoxy-9 α , 11 α -epoxymethano-prostaglandin F_{2 α} (U46619, $3-30 \times 10^{-9}$ M) and the endothelial integrity was confirmed with a single addition of acetylcholine (ACh, 10^{-5} M) and all rings showed relaxation of at least 65% of active tension. After washout and re-equilibration for 30 min, U46619 ($3-30 \times 10^{-9}$ M) was added to induce active tension which was matched between various treatment groups. Relaxant responses were examined in proximal and distal coronary artery rings removed from 18 dogs subjected to ischaemia/reperfusion and in five dogs subjected to sham ischaemia. Control relaxant responses to ACh were determined in artery rings from all dogs and control responses to sodium nitroprusside (SNP) in artery rings from four ischaemic/reperfused dogs. In artery segments isolated from ischaemic/reperfused dogs relaxant responses were also examined in the presence of the following treatments: (i) L-N^G-nitroarginine (L-NOARG, 10^{-4} M, ACh $n=7$, SNP $n=4$) to inhibit NO synthase; (ii) L-NOARG (10^{-4} M) plus haemoglobin (Hb, 2×10^{-5} M, ACh $n=4$) to bind NO; (iii) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M, ACh $n=7$, SNP $n=4$) to inhibit soluble guanylate cyclase; (iv) High K⁺ (67 mM) solution plus nifedipine (3×10^{-7} M, ACh $n=4$). Concentrations of K⁺ higher than 25 mM inhibit the activity of K⁺ channels (Chen & Suzuki, 1989) and concentrations of 60–80 mM K⁺ have previously been used to investigate the role of EDHF in coronary artery relaxation (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). The L-type Ca²⁺ channel blocker, nifedipine, was included to prevent contraction in response to the K⁺-induced hyperpolarization (Kilpatrick & Cocks, 1994); (v) Tetraethylammonium (TEA, 10^{-3} M, ACh $n=5$, SNP $n=4$) to inhibit large conductance calcium activated potassium (BK_{Ca}) channels; (vi) Glibenclamide (10^{-6} M, ACh $n=4$) to inhibit ATP-sensitive potassium (K_{ATP}) channels; or (vii) TEA (10^{-3} M) plus L-NOARG (10^{-4} M, ACh $n=5$).

In artery rings from sham ischaemic dogs relaxant responses to ACh ($n=5$) were examined in the presence of the following treatments: (i) L-NOARG (10^{-4} M); (ii) ODQ (10^{-5} M); or (iii) TEA (10^{-3} M).

The response to ACh was also examined in the presence of dimethyl sulphoxide (DMSO), the vehicle for ODQ and glibenclamide. DMSO had no effect on the ACh-induced relaxation and therefore this data was included in the control group.

Drugs and chemical used

Bovine haemoglobin, glibenclamide, indomethacin, nifedipine, L-N^G-nitroarginine, sodium nitroprusside, tetraethylammonium bromide and U46619 were obtained from Sigma Co. (U.S.A.). Acetylcholine perchlorate was obtained from BDH Chemicals (England) and ODQ was purchased from Sapphire

(Australia). All drugs were dissolved in normal saline (0.9% w v⁻¹ NaCl), except for acetylcholine perchlorate which was dissolved in distilled water, nifedipine and U46619 which were prepared in ethanol (100%) and U46619 further diluted in sodium bicarbonate (0.25 M). ODQ and glibenclamide were dissolved in 100% DMSO and glibenclamide was further diluted in 50% DMSO.

Data presentation and statistical analysis

The results are expressed as the mean $\pm$ s.e.mean and *n* indicates the number of experiments (dogs). Where possible, concentration-response curves from isolated coronary artery rings were computer fitted to a sigmoidal curve (REAP, Gamma Research Systems, Australia) to enable the calculation of the sensitivity (pEC₅₀) to agonists. However, where there is a substantial inhibition of the maximal relaxation that prevents the data being fitted to a sigmoid curve, pEC₅₀ cannot be determined. The calculated values for the pEC₅₀ and maximum relaxation were compared using one way analysis of variance (ANOVA) with *post hoc* multiple comparisons using the Bonferroni test (Instat, GraphPad Software, U.S.A.). The concentration-response curves generated before and after antagonist pretreatments were also compared with two-way ANOVA (CSS, StatSoft Inc., U.S.A.). In experiments where data was paired, that is where responses in nonischaemic (proximal) and ischaemic (distal) arteries from the same dog were compared, statistical analysis was carried out using a two tailed Student's *t*-test (Instat). In all cases, statistical differences were accepted when *P* $\leq$ 0.05.

Results

Effect of ischaemia on relaxant responses to ACh and SNP

The responses to ACh and SNP were similar in non-ischaemic (proximal) and ischaemic (distal) segments of coronary artery taken from dogs subjected to coronary artery occlusion and reperfusion (Figure 1). ACh-induced relaxation was similar in

proximal and distal segments of coronary artery from sham ischaemic dogs and responses were also similar to those observed in corresponding artery segments from ischaemic dogs (Table 1).

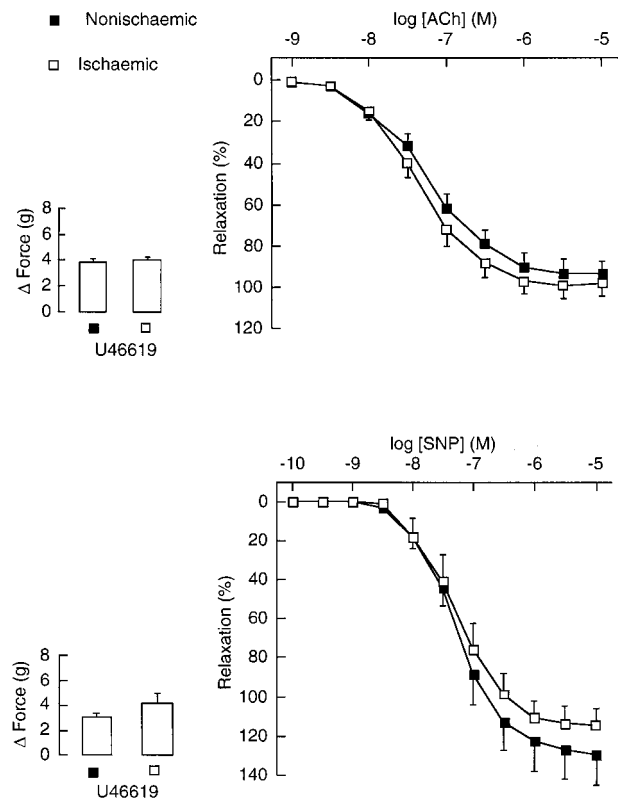


Figure 1 Concentration response curves to ACh (*n*=18) or SNP (*n*=4) in nonischaemic or ischaemic coronary artery rings. The bar graphs show that the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: ACh 12 $\pm$ 2; SNP 11 $\pm$ 2, ischaemic: ACh 14 $\pm$ 2; SNP 11 $\pm$ 2]. The responses to ACh or SNP were not affected by myocardial ischaemia/reperfusion. All values are means $\pm$ s.e.mean shown by vertical lines.

Table 1 A comparison of responses to ACh in proximal (to the occlusion site) and distal (to the occlusion site) artery segments obtained from dogs subjected to ischaemia/reperfusion (I/R) and sham ischaemia/reperfusion (Sham)

Treatment		n	pEC ₅₀	R _{max} (%)	pEC ₅₀	R _{max} (%)
			Nonischaemic (proximal)		Ischaemic (distal)	
I/R	Control	18	7.20 $\pm$ 0.10	94 $\pm$ 6	7.30 $\pm$ 0.10	99 $\pm$ 6
	L-NOARG (10 ⁻⁴ M)	8	6.70 $\pm$ 0.12	39 $\pm$ 6*	6.67 $\pm$ 0.26	30 $\pm$ 8*
	L-NOARG (10 ⁻⁴ M) + Hb (2 $\times$ 10 ⁻⁵ M)	4	6.61 $\pm$ 0.09	23 $\pm$ 10*	n.d.	15 $\pm$ 9*
	ODQ (10 ⁻⁵ M)	7	6.76 $\pm$ 0.21	50 $\pm$ 11*	7.13 $\pm$ 0.15	46 $\pm$ 8*
	High K ⁺ (67 mM)	4	7.36 $\pm$ 0.18	85 $\pm$ 6	7.50 $\pm$ 0.14	60 $\pm$ 2*#
	TEA (10 ⁻³ M)	5	7.07 $\pm$ 0.25	107 $\pm$ 9	6.54 $\pm$ 0.21*	94 $\pm$ 8
	Glibenclamide (10 ⁻⁶ M)	4	7.23 $\pm$ 0.06	90 $\pm$ 6	7.54 $\pm$ 0.08	112 $\pm$ 4
	TEA (10 ⁻³ M) + L-NOARG (10 ⁻⁴ M)	5	6.14 $\pm$ 0.22*	51 $\pm$ 5*	n.d.	20 $\pm$ 7*#
			Sham (proximal)		Sham (distal)	
	Control	5	7.36 $\pm$ 0.16	98 $\pm$ 4	7.18 $\pm$ 0.20	93 $\pm$ 6
Sham	L-NOARG (10 ⁻⁴ M)	5	7.00 $\pm$ 0.11	26 $\pm$ 16†	n.d.	15 $\pm$ 8†
	ODQ (10 ⁻⁵ M)	5	6.84 $\pm$ 0.21	39 $\pm$ 11†	7.00 $\pm$ 0.25	39 $\pm$ 19†
	TEA (10 ⁻³ M)	5	6.92 $\pm$ 0.15	101 $\pm$ 5	6.81 $\pm$ 0.15	97 $\pm$ 6

n = the total number of experiments, the number in parentheses indicates the number of experiments where a pEC₅₀ was calculated if not equal to *n*. n.d. = not determined due to inability to fit data to a sigmoid curve. * = significantly different from I/R control (*P* < 0.01, ANOVA, Bonferroni multiple comparisons tests). † = significantly different from sham control (*P* < 0.05, ANOVA, Bonferroni multiple comparison tests). # = significantly different from I/R nonischaemic (*P* < 0.05, Student's paired *t*-tests).

Effect of inhibition of NO on the responses to ACh and SNP in nonischaemic and ischaemic arteries

In the nonischaemic and ischaemic arteries, L-NOARG, an inhibitor of NO synthase, significantly reduced the maximum relaxation to a similar extent (Table 1), indicating non-competitive inhibition of the ACh-induced relaxation (Figure 2). In the presence of L-NOARG plus Hb, the maximum relaxation to ACh was reduced to $23 \pm 10\%$ in nonischaemic

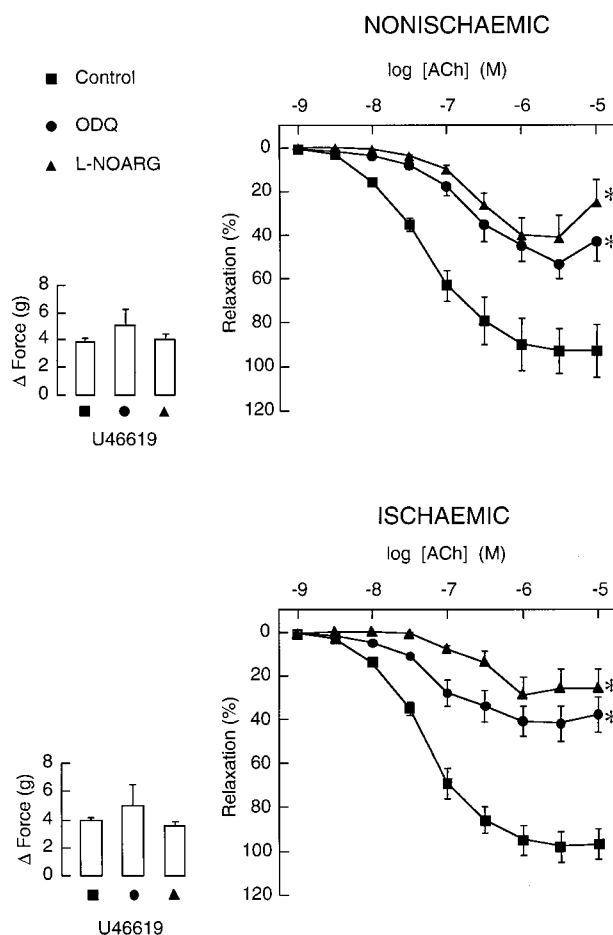


Figure 2 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of ODQ ($n=7$, 10^{-5} M) or L-NOARG ($n=8$, 10^{-4} M). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to a similar level with U46619 [(nm) nonischaemic: control 12 ± 3 ; ODQ 6 ± 1 ; L-NOARG 4 ± 1 , ischaemic: control 12 ± 3 ; ODQ 5 ± 1 ; L-NOARG 3 ± 1]. The maximum relaxation to ACh was reduced by all treatments. Nonischaemic and ischaemic coronary arteries were equally sensitive to L-NOARG and ODQ. All values are means $\pm$ s.e.mean shown by vertical lines. *Significantly different from control (ANOVA, $P < 0.05$).

arteries and $15 \pm 9\%$ in ischaemic arteries although in neither case did this achieve statistical significance in comparison to responses in the presence of L-NOARG alone. The effect of L-NOARG on ACh-induced relaxation of proximal and distal arteries from sham ischaemic dogs was similar (Table 1). L-NOARG did not affect the sensitivity or maximum relaxation to SNP in nonischaemic or ischaemic arteries (Table 2).

Effect of inhibition of guanylate cyclase on the responses to ACh and SNP in nonischaemic and ischaemic arteries

Concentration-response curves were generated for ACh in the presence of ODQ, a selective inhibitor of soluble guanylate cyclase in nonischaemic and ischaemic arteries precontracted by U46619 (Figure 2). In the presence of ODQ, the maximum relaxation was significantly depressed in nonischaemic and ischaemic arteries (Table 1). The sensitivity to ACh was not affected by ODQ in either nonischaemic or ischaemic arteries (Table 1). In addition ACh-induced relaxation was equally susceptible to inhibition by ODQ in proximal and distal artery segments from sham ischaemic dogs (Table 1). ODQ significantly reduced the pEC_{50} of SNP in both nonischaemic and ischaemic arteries (Table 2).

Effect of non-selective inhibition of potassium channels on the responses to ACh in nonischaemic and ischaemic arteries

Figure 3 shows the effect of high K⁺ plus nifedipine on concentration-response curves to ACh in nonischaemic and ischaemic arteries. L-type Ca²⁺ channels were blocked with nifedipine to prevent contraction in response to the K⁺-induced depolarization. Thus the level of precontraction of the artery rings using U46619 was similar in the absence or presence of K⁺. High K⁺ did not affect the sensitivity or maximum relaxation to ACh in the nonischaemic coronary artery (Table 1). In contrast, there was a significant reduction in the maximum relaxation in the ischaemic arteries (Table 1). In addition the maximum relaxation to ACh in the presence of high K⁺ in ischaemic arteries was significantly less than that observed in nonischaemic arteries under the same conditions (Table 1).

Effect of selective inhibition of potassium channels on the responses to ACh and SNP in nonischaemic and ischaemic arteries

Glibenclamide was used to inactivate the K_{ATP} channel and TEA to inhibit the BK_{Ca} channel. In the nonischaemic artery, consistent with the high K⁺ data, neither TEA nor glibenclamide affected the sensitivity or maximum relaxation to ACh (Figure 4). By contrast, in the ischaemic arteries, TEA shifted the ACh concentration response curves to the right (Figure 4) with a significant reduction of the pEC_{50} without

Table 2 A comparison of responses to SNP in nonischaemic and ischaemic artery segments

Treatment	n	Nonischaemic		Ischaemic	
		pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)
Control	4	7.28 ± 0.13	130 ± 15	7.27 ± 0.21	115 ± 9
ODQ (10^{-5} M)	4	$5.66 \pm 0.51^*$	$94 \pm 8^*$	$5.26 \pm 0.40^*$	117 ± 8
L-NOARG (10^{-4} M)	4	7.24 ± 0.26	112 ± 3	7.43 ± 0.22	130 ± 7
TEA (10^{-3} M)	4	7.19 ± 0.14	113 ± 3	7.30 ± 0.08	109 ± 3

n = total number of experiments. * = significantly different from control ($P < 0.001$, ANOVA, Bonferroni multiple comparisons test).

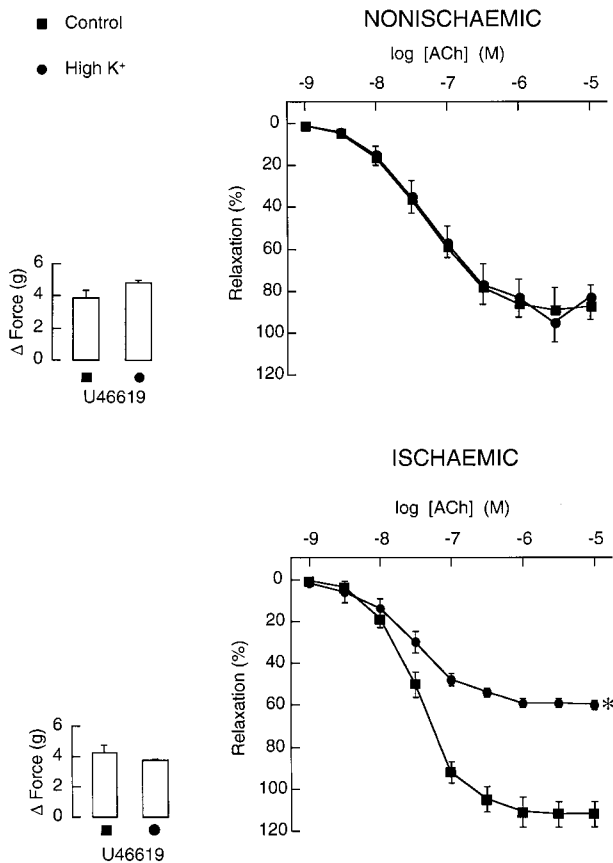


Figure 3 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of high K⁺ (67 mM) plus nifedipine (3×10^{-7} M, $n=4$). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: control 10 ± 1 ; high K⁺ 14 ± 2 ; ischaemic: control 12 ± 3 ; high K⁺ 13 ± 2]. Responses to ACh were unaffected by high K⁺ in the nonischaemic arteries but significantly impaired in the ischaemic vessels. All the values are means $\pm$ s.e. mean shown by vertical line. *Significantly different from control (ANOVA, $P < 0.05$).

depressing the maximum response (Table 1). TEA had no effect on ACh-induced relaxation of proximal or distal artery segments from sham ischaemic dogs (Table 1). Unlike TEA, glibenclamide did not inhibit the ACh-induced response (Table 1, Figure 4).

To determine whether it was NO or another factor that opened the potassium channels, responses to SNP were examined in the absence or presence of TEA. In both nonischaemic and ischaemic coronary arteries, TEA did not affect the response to SNP (Table 2) suggesting that NO did not open the BK_{Ca} channel to cause relaxation.

Effect of combined inhibition of NO and potassium channels on the responses to ACh in nonischaemic and ischaemic arteries

The response to ACh was examined after blocking the contribution of NO and opening of BK_{Ca} channels to cause relaxation simultaneously using L-NOARG plus TEA. In nonischaemic coronary arteries, L-NOARG plus TEA inhibited the response to ACh with a significant depression in the maximum relaxation but the level of inhibition was similar to that achieved by L-NOARG alone (Table 1). In

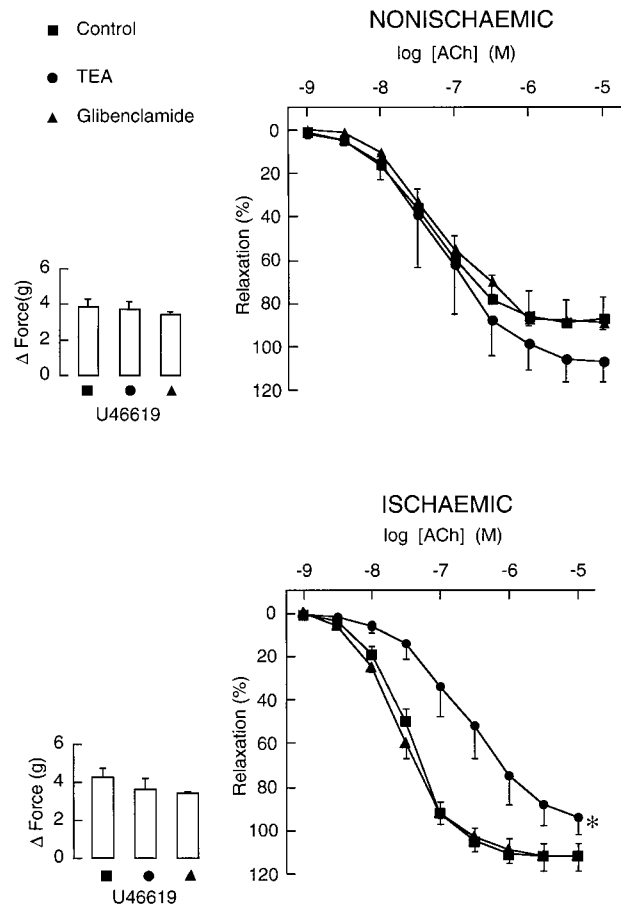


Figure 4 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of TEA ($n=5$, 10^{-3} M) or glibenclamide ($n=4$, 10^{-6} M). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: control 10 ± 1 ; TEA 7 ± 2 ; glibenclamide 9 ± 1 ; ischaemic: control 10 ± 2 ; TEA 6 ± 1 ; glibenclamide 7 ± 1]. In the nonischaemic coronary arteries ACh-induced relaxation was unaffected by TEA or glibenclamide. By contrast in the ischaemic artery rings TEA inhibited the response to ACh. All values are means $\pm$ s.e. mean shown by vertical lines. *Significantly different from control (ANOVA, $P < 0.05$).

contrast, in the ischaemic arteries, TEA plus L-NOARG almost abolished the response to ACh such that the maximum relaxation in the ischaemic artery was significantly less in comparison to the nonischaemic and control ischaemic arteries (Table 1).

Discussion

We investigated the effect of 60 min of coronary artery occlusion and 120 min reperfusion *in vivo* on subsequent endothelium-dependent relaxation of dog coronary arteries *in vitro*. Ischaemia/reperfusion did not affect the responses to the endothelium-dependent vasodilator ACh. In addition the responses to ACh in nonischaemic and ischaemic coronary arteries were equally impaired by inhibition of NO or soluble guanylate cyclase. However, whereas responses to ACh in nonischaemic arteries were unaffected by depolarization with high K⁺, in ischaemic arteries the same treatment caused a significant reduction in the endothelium-dependent relaxation. TEA, but not glibenclamide, significantly reduced endothe-

lium-dependent relaxation in ischaemic arteries indicating that opening of BK_{Ca} channels was involved in the relaxation.

In the present study, the responses were compared between nonischaemic artery segments which were isolated proximal to the occlusion site and ischaemic/reperfused artery rings which were dissected distal to the occlusion site. Studies in which dogs were submitted to sham ischaemia confirmed that there were no differences in the responses to agonists generated in proximal and distal portions of the circumflex coronary arteries as reported earlier (Coughlan *et al.*, 1994). In addition, in the sham ischaemic tissue the sensitivity of proximal and distal segments to inhibition of ACh-induced relaxation by L-NOARG, ODQ and TEA was similar. Therefore if there is a difference in the responses generated from nonischaemic and ischaemic arteries, this is not caused by tissue variation across artery segments, but arises due to prior exposure to ischaemia and reperfusion.

There are several studies that have demonstrated that myocardial ischaemia and reperfusion selectively impairs endothelium-dependent relaxation of isolated coronary arteries (for reviews Hearse *et al.*, 1993; Sobey & Woodman 1993) although there are also reports that the coronary conductance arteries are unaffected by ischaemia (Quillen *et al.*, 1990; Winn & Ku, 1992). In the present study ischaemia/reperfusion did not affect the absolute response to ACh even though we employed a protocol similar to previous studies from this laboratory where endothelium-dependent relaxation was impaired (Sobey *et al.*, 1990; 1992). Ischaemia was confirmed by an absence of blood flow and an impairment of cardiac contractility measured *in vivo* (data not shown). Bauer *et al.*, (1993) have suggested that administration of vasodilators before ischaemia may prevent vascular dysfunction but in this study no vasodilators were administered *in vivo*. Thus the divergent effects of ischaemia on vascular function remain unexplained. Even though ischaemia/reperfusion did not alter the total response to ACh we hypothesized that an enhanced contribution of K⁺ channel opening could compensate for impaired NO release. As no previous studies have investigated the effect of ischaemia on the mechanism of endothelium-dependent dilatation we compared the contribution of NO and BK_{Ca} channel opening in non-ischaemic and ischaemic coronary arteries.

L-NOARG caused a non-competitive inhibition of the relaxant response to ACh with a 70% reduction in the maximum response in both nonischaemic and ischaemic arteries. The incomplete blocking effect observed with L-NOARG is not due to an inadequate concentration of L-NOARG (10^{-4} M) since a higher concentration (3×10^{-4} M) did not produce any greater inhibitory effect (data not shown) and additional treatment with Hb did not cause a further significant inhibition. Thus, these results suggest that the ACh-induced relaxation of the dog coronary artery is predominantly mediated by NO and that ischaemia/reperfusion does not affect the sensitivity of the relaxation to inhibition of NO synthesis or action. Whether ischaemia/reperfusion may affect the contribution of cyclic GMP, the second messenger for NO, to endothelium-dependent dilatation was investigated by determining the sensitivity of ACh-induced relaxation to ODQ, a selective inhibitor of soluble guanylate cyclase (Garthwaite *et al.*, 1995; Schrammel *et al.*, 1996). ACh-induced relaxation of nonischaemic and ischaemic/reperfused arteries were equally sensitive to inhibition by ODQ indicating that ischaemia/reperfusion does not affect the activity of guanylate cyclase.

There is now considerable evidence that NO may not be the only factor released by ACh to cause relaxation. A second

factor that could contribute to endothelium-dependent relaxation is EDHF (Garland *et al.*, 1995; Mombouli & Vanhoutte, 1997), an unidentified factor which causes relaxation by opening K⁺ channels to cause hyperpolarization. To determine whether the opening of K⁺ channels might be contributing to the endothelium-dependent relaxation we examined the responses to ACh in the presence of a depolarizing concentration of K⁺ (67 mM). To prevent K⁺-induced contraction we also added the L-type Ca²⁺ channel blocker nifedipine (Kilpatrick & Cocks, 1994). The rings were then precontracted with the thromboxane mimetic U46619 to the same level as in previous treatment groups so that the relaxant responses were not affected by differences in active tension. In the nonischaemic coronary arteries ACh-induced relaxation was unaffected by high K⁺ whereas in the ischaemic/reperfused arteries there was a significant inhibition of the endothelium-dependent relaxation. This suggests that the opening of K⁺ channels only contributes to ACh-induced relaxation after the coronary arteries have been exposed to a period of ischaemia/reperfusion.

The type of K⁺ channel that mediates vascular smooth muscle relaxation remains a matter of debate. The types most commonly proposed to mediate endothelium-dependent relaxation are the K_{ATP} channel (Garland *et al.*, 1995) and BK_{Ca} channel (Najibi *et al.*, 1994; Najibi & Cohen, 1995). Therefore we examined the effects of two K⁺ channel blockers TEA, which at the concentration used (10^{-3} M) is selective for the BK_{Ca} channel (Nelson, 1993) and glibenclamide (Kuramaru & Sakai, 1996), which is selective for the K_{ATP} channel. Neither inhibitor affected ACh-induced relaxation of the nonischaemic coronary arteries, as expected given that responses in nonischaemic tissue were insensitive to high K⁺. However TEA caused a significant decrease in the sensitivity to ACh in the ischaemic/reperfused artery rings whereas glibenclamide had no effect. Thus it appears that the BK_{Ca} and not the K_{ATP} channel, was involved in the ACh-induced relaxation of the ischaemic/reperfused artery.

Several previous studies have reported that endothelium-dependent relaxation of coronary arteries in normal circumstances does not involve K⁺ channel opening but if NO synthesis is inhibited, a K⁺ sensitive component of the relaxation is revealed (Kilpatrick & Cocks 1994; Drummond & Cocks, 1996). Thus it was proposed that an endothelium-derived K⁺ channel opener, possibly EDHF, acted as a reserve to NO to maintain vasodilatation. In this study however we did not observe any effect of K⁺ on ACh-induced relaxation in the absence or presence of NO synthase inhibition in nonischaemic arteries. The reason for the difference between the present observation and those of Cocks and colleagues (Kilpatrick & Cocks 1994; Drummond & Cocks 1996) is not known but may involve the use of different agonists (ACh versus bradykinin) or species (dog versus pig and cow).

There are no other reports of the effect of ischaemia on the mechanism of endothelium-dependent dilatation. However, the results of this study are similar to the observations of Cohen and his colleagues (Najibi *et al.*, 1994; Najibi & Cohen, 1995) who investigated the effect of hypercholesterolaemia on the mechanism of endothelium-dependent relaxation of rabbit carotid arteries. They reported that hypercholesterolaemia did not affect the relaxant responses to ACh nor was the sensitivity of that relaxation to inhibition of NOS different between normal and hypercholesterolaemic arteries. However elevated K⁺ impaired ACh-induced relaxation in hypercholesterolaemic, but not normal arteries (Najibi *et al.*, 1994). Further it was demonstrated that in hypercholesterolaemic arteries charybotoxin also inhibited the endothelium-dependent relaxation

indicating that it was the BK_{Ca} channel that was involved in the relaxation (Najibi & Cohen, 1995). Thus it appears that pathological states such as ischaemia/reperfusion and hypercholesterolaemia may alter the mechanism of endothelium-dependent relaxation even in the absence of any change in the absolute magnitude of the relaxation.

The question then arises as to what factor is responsible for the opening of the potassium channels. In the study of Tare *et al.*, (1990), hyperpolarization and relaxation caused by NO infusion in guinea-pig uterine artery were reduced by a NO synthase inhibitor, L-N^G-monomethylarginine or Hb. Bolotina *et al.*, (1994) also demonstrated that relaxation to NO was blocked by charybdotoxin, a specific inhibitor of calcium-dependent potassium channels, in rabbit aortic smooth muscle cells. By contrast other studies reported that endothelium-dependent relaxation but not endothelium-dependent hyperpolarization was inhibited by methylene blue or Hb in rat (Chen *et al.*, 1988) or rabbit arteries (Huang *et al.*, 1988) indicating a factor other than NO caused hyperpolarization. Thus, both NO and EDHF have been suggested to open the potassium channels leading to hyperpolarization in response to ACh. In the present study, the NO released by SNP did not cause the opening of BK_{Ca} channels as the responses were not affected by the presence of TEA in either nonischaemic or ischaemic arteries. It might be argued that the form of NO

released by SNP is not the same as that released endogenously by ACh, since studies showing that the endogenous form of EDRF may be a precursor of NO (Greenberg *et al.*, 1990). This would imply that the exogenous NO might not be able to interact with the potassium channels to cause hyperpolarization. However, there are other studies showing the capability of SNP to cause hyperpolarization (Huang *et al.*, 1988; Tare *et al.*, 1990). Therefore the present findings indicate that most likely a factor other than NO is released upon ACh stimulation in ischaemic arteries. This factor might be EDHF since the factor of interest here and EDHF share some common features in that both cause opening of the K_{Ca} channels and are released from endothelium upon agonist stimulation (Mombouli & Vanhoutte, 1997).

In conclusion, NO is the predominant factor mediating endothelium-dependent relaxation upon agonist-stimulation in nonischaemic dog isolated coronary arteries. By contrast, after ischaemia and reperfusion, in addition to the release of NO upon agonist stimulation, there is another factor released from the endothelium. This factor is possibly EDHF which then acts on the BK_{Ca} channels to cause hyperpolarization leading to relaxation. Thus this study provides evidence that ischaemia and reperfusion activates a reserve system, hyperpolarization to contribute to endothelium-dependent relaxation through the opening of BK_{Ca} channels.

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(Received August 28, 1998

Revised November 6, 1998

Accepted November 20, 1998)



Decreased vascular permeability response to substance P in airways of genetically hypertensive rats

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1 The inbred genetically hypertensive strain (GH) of the Otago Wistar rat possesses more sensory neurons containing the neuropeptide substance P (SP) than does its genetically related control normotensive strain.

2 As SP contributes to airway inflammation by increasing microvascular permeability, we assessed the extravasation of Evans Blue dye in trachea and main bronchus of anaesthetized GH and control rats, in the presence of endogenous (capsaicin-liberated) or exogenous SP.

3 Following intravenous administration of either capsaicin (75 µg kg⁻¹) or SP (3.3 nmol kg⁻¹), extravasation of Evans Blue in airways from GH rats was only about 60% of that in airways of control rats. This difference was not gender-specific and responses to capsaicin were abolished by pretreatment with a selective NK₁ receptor antagonist SR 140333 (360 nmol kg⁻¹).

4 By contrast, the extravasation of dye caused by intravenous 5-hydroxytryptamine (0.5 µmol kg⁻¹) was similar in magnitude in both GH and control strains.

5 Falls in systemic arterial blood pressure in response to exogenous SP (0.1–3 nmol kg⁻¹) or acetylcholine (0.2–2 nmol kg⁻¹) were also very similar between strains, but those in response to capsaicin (75 µg kg⁻¹) in the GH rats were about double those in control rats. The hypotensive response to SP was abolished by SR 140333, but that to capsaicin was unaffected.

6 Our results indicate that the increased peripheral innervation density by SP nerves in GH rats is accompanied by reduced inflammatory responses to SP. This does not involve decreased vasodilator potency of SP and is therefore probably related to altered endothelial responsiveness.

Keywords: Neuropeptides; trachea; sensory nerve; capsaicin; Evans Blue; hypotension; NK₁ receptor antagonist; substance P; hypertensive rat model

Abbreviations: ACh, Acetylcholine; CGRP, calcitonin gene-related peptide; GH, genetically hypertensive; 5-HT, 5-hydroxytryptamine; NGF, nerve growth factor; NO, nitric oxide; NOS, NO synthase; PACAP, pituitary adenylate cyclase activating peptide; SP, substance P

Introduction

Neuropeptides such as substance P (SP) are thought to be important inflammatory mediators in asthma (Barnes & Belvisi, 1997). Asthmatics have been reported as having increased numbers of intrapulmonary SP-containing axons (Ollerenshaw *et al.*, 1991) and as exhibiting increased levels of SP in bronchoalveolar lavage (Nieber *et al.*, 1992; Khwaja & Rogers, 1996). SP probably exerts its major effect in asthma by inducing airway oedema consequent to an increase in microvascular permeability (Joos *et al.*, 1994; Barnes & Belvisi, 1997). In rat trachea, microvascular permeability increase due to SP release can be induced by electrical stimulation of the vagal trunk (Lundberg & Saria, 1982) or by capsaicin (Germonpre *et al.*, 1995), making these manoeuvres an appropriate model for studies of inflammatory responses to SP.

The Otago strain of genetically hypertensive rat (GH) possesses selectively increased numbers of the polymodal C-fibre class of spinal sensory neurons. As a consequence, some peripheral tissues, including the trachea, are hyperinnervated by SP-containing sensory nerves and contain abnormally high levels of SP (Bakhle & Bell, 1994). We were therefore interested to determine whether the chronic presence of excessive amounts of neurogenic SP had an influence on the inflammatory responses of the airways in the GH strain. Some

preliminary results of this work have been communicated to the British Pharmacological Society (Bakhle *et al.*, 1998).

Methods

Animals

The rats used were bred in the Trinity College BioResources Unit from stock obtained in 1995 from the Wellcome Medical Research Institute, University of Otago (New Zealand) and consisting of GH animals and normal (N) Wistar animals from the colony that had produced the original GH strain. The integrity of each line was confirmed by establishing that the arterial blood pressures of each generation, as measured by tail-cuff plethysmography or by direct arterial cannulation, were consistently normotensive in N strain animals (<145 mmHg) and hypertensive in the GH animals (>190 mmHg).

Age-matched rats of each strain were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (Sagatal; Servier Merieux, 60 mg kg⁻¹), placed on a stainless steel operating plate maintained at 35°C and allowed to breathe spontaneously. In all experiments, a polyethylene catheter (PE50) was passed down one external jugular vein to the region of the right atrium for injection of drugs. In experiments concerned with vascular responses, a second catheter was

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placed in the left common carotid artery for monitoring arterial blood pressure.

Chemicals

Acetylcholine (ACh), capsaicin, Evans Blue, formamide (SigmaUltra grade), heparin, 5-hydroxytryptamine creatinine phosphate (5-HT) and substance P (SP) were obtained from Sigma. The selective NK₁ antagonist, SR 140333, ((S)-1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]octane chloride; Emonds *et al.*, 1993) was generously provided by Sanofi. Evans Blue was dissolved (30 mg ml⁻¹) in isotonic saline containing heparin (300 IU ml⁻¹) and the solution was filtered before use. Capsaicin was dissolved in 96% ethanol-Tween 80 (1:1, v/v) to give a stock solution of 10 mg ml⁻¹; SR 140333 was dissolved in ethanol to give a stock solution of 3.7 mM. All other solutions were made in saline.

Microvascular permeability measurements

Evans Blue (30 mg kg⁻¹) was given intravenously and was followed, 90 s later, with an injection of capsaicin (75 µg kg⁻¹), 5-HT (0.5 µmol kg⁻¹) or SP (3.3 nmol kg⁻¹), or an equal volume (0.2 ml) of saline. After a further 5 min, the chest was opened and the rat was perfused transcardially with 200 ml isotonic saline at a pressure of 100 cm water. The trachea and main bronchus were dissected free, blotted dry, weighed and extracted in 1.5 ml formamide at 37°C overnight. The optical density of the extract was measured at 620 nm and the Evans Blue concentration calculated from a standard curve of Evans Blue in formamide determined on the same day. Extravasated dye was expressed as ng dye (mg wet weight tissue)⁻¹.

In some animals given capsaicin or SP, the NK₁ antagonist SR 140333 (360 nmol kg⁻¹; Siney & Brain, 1996) was given as a single intravenous injection, 30 min before Evans Blue administration.

Blood pressure measurements

Arterial blood pressure was measured *via* a Statham pressure transducer and recorded using a MacLab data acquisition system. After allowing at least 10 min after arterial cannulation for stabilization of the preparation, blood pressure responses to intravenous injections of capsaicin (75 µg kg⁻¹), SP (0.1–3 nmol kg⁻¹) and ACh (0.2–2 nmol kg⁻¹) were obtained, before and after administration of SR 140333 (360 nmol kg⁻¹) intravenously.

Statistical analysis

Results are given as mean (±s.e.mean) values from the number of assays shown. Differences between means were assessed by Welch's test (no assumption of equal variances) and values of *P* < 0.05 were taken as significant.

Results

Changes in microvascular permeability

A small amount of extravasation of Evans Blue was detectable in tissues from animals that had received only saline challenge. This extravasation was similar in both trachea and bronchus and for both control (N) and GH strains (trachea; N, 33 ± 6 vs

GH, 33 ± 3; main bronchus; N, 38 ± 8 vs GH, 28 ± 3, all expressed as ng dye (mg wet weight)⁻¹; *n* = 8) and these results have been combined to give the baseline value shown as a dotted line in Figures 1 and 2.

Preliminary studies showed that 75 µg kg⁻¹ i.v. capsaicin produced a substantial and reproducible degree of extravasation without excessive mortality and this dose was used in all subsequent experiments. In both the trachea and the main bronchus, capsaicin caused a marked increase in extravasation above the baseline value, with a greater response in the bronchus. There was however a substantial strain difference in the magnitude of the effect of capsaicin, with the responses in both tissues of the GH animals being only about 60% of those seen in the comparable control (N) tissues (Figure 1). Capsaicin produced no visible blueing of the lung parenchyma, although blueing was clearly visible in the skin around the nostrils and in the ears.

Both SP (3.3 nmol kg⁻¹ i.v.) and 5-HT (0.5 µmol kg⁻¹) produced permeability increases similar in magnitude to those induced by capsaicin. As with capsaicin-induced extravasation, the responses to SP in both trachea and bronchus were substantially less in GH than in N animals (Figure 2a). By contrast, responses to 5-HT were of similar magnitude for both strains (Figure 2b).

The results described above were obtained using male rats. A limited series of experiments using female rats confirmed that the reduced extravasation in the trachea of GH animals to capsaicin (N, 72 ± 13; GH, 28 ± 5) or exogenous SP (N, 134 ± 6; GH, 105 ± 8; expressed as ng dye (mg wet weight)⁻¹; *P* < 0.05, *n* = 5 for each set) was independent of gender.

In another series of animals, the role of the NK₁ receptor in extravasation mediated by capsaicin was tested by pre-treatment with SR 140333 for 30 min before challenge. In these experiments, the responses to capsaicin were abolished in both N and GH strains (Figure 3).

Systemic blood pressure responses to capsaicin or to exogenous SP

Extravasation of plasma protein in areas of increased microvascular permeability may be modified by blood flow changes secondary to changes in systemic arterial pressure. We

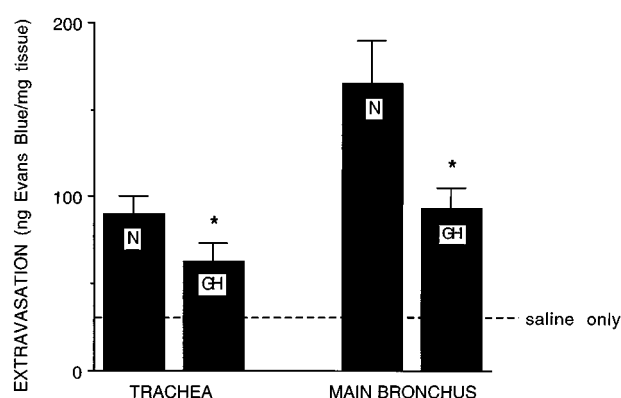


Figure 1 Increased microvascular permeability in the upper airways of GH and control rats following capsaicin. Dye extravasation in trachea and main bronchus was markedly increased by i.v. injection of capsaicin (75 µg kg⁻¹) over that induced by injection of saline (dotted line). However the effect of capsaicin was consistently less in the GH rats than in the control (N) animals. The height of the bars represents the mean values (±s.e.mean; *n* = 8) of tissue content of extravasated dye. *Significantly different from value in control rats; *P* < 0.05.

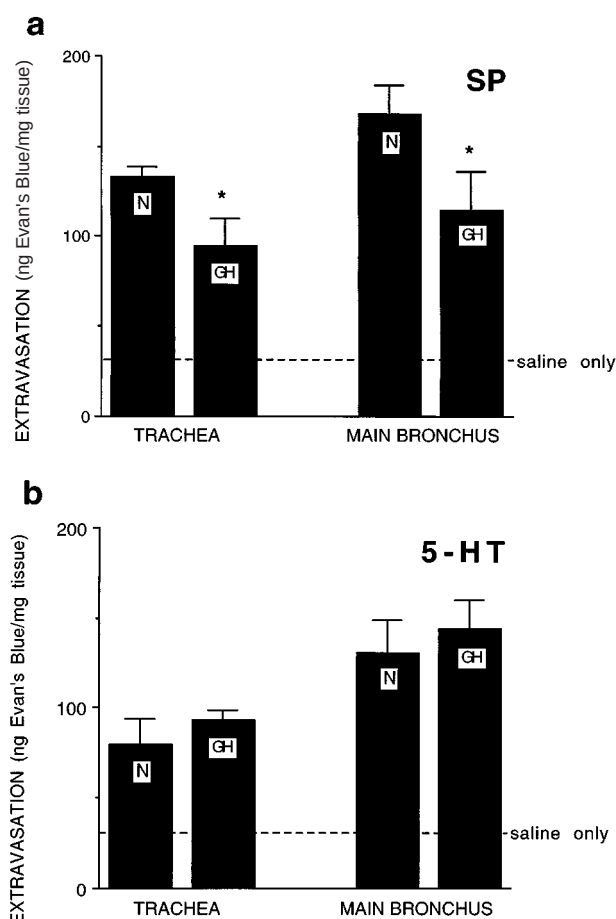


Figure 2 Increased microvascular permeability following exogenous SP (3.3 nmol kg^{-1} i.v.) (a) or 5-HT ($0.5 \mu\text{mol kg}^{-1}$) (b). Both these exogenous pro-inflammatory agents increased dye extravasation, but only with SP was there a strain-related difference in response. Saline control values shown by the dotted line. The height of the bars represents the mean values ($\pm$ s.e. mean; $n=5$ for SP, $n=6$ for 5-HT) of tissue content of extravasated dye. *Significantly different from value in control rats; $P<0.05$.

therefore compared blood pressure responses to capsaicin and to SP between GH and control (N) strains and used ACh as an independent index of vascular reactivity. Comparison of vasodepressor responses to submaximal intravenous doses of either SP or ACh (Figure 4) showed no consistent difference between strains but, overall, there was a trend towards a greater response in the GH rats.

Intravenous injection of capsaicin ($75 \mu\text{g kg}^{-1}$) produced a depressor response which was more prolonged than that to SP (Figure 5). The magnitude of response to a second injection was frequently less than that to the first dose but responses to subsequent doses of capsaicin remained similar in size (Figure 5). Comparison of maximal hypotensive responses to the first dose of capsaicin showed a larger fall in GH animals ($111 \pm 11 \text{ mmHg}$, $n=5$) than in the N strain ($57 \pm 9 \text{ mmHg}$; $n=4$; $P<0.05$). In addition to the immediate depressor response, the first dose of capsaicin given invariably caused a maintained reduction in resting blood pressure. In control (N) animals, the resting arterial pressure fell after the first dose of capsaicin from a value of $129 \pm 5 \text{ mmHg}$ to a sustained value of $102 \pm 3 \text{ mmHg}$ ($n=5$). A closely similar magnitude of response was seen in GH rats, with the pre-capsaicin value of $186 \pm 3 \text{ mmHg}$ falling to a new baseline of $152 \pm 6 \text{ mmHg}$ ($n=6$).

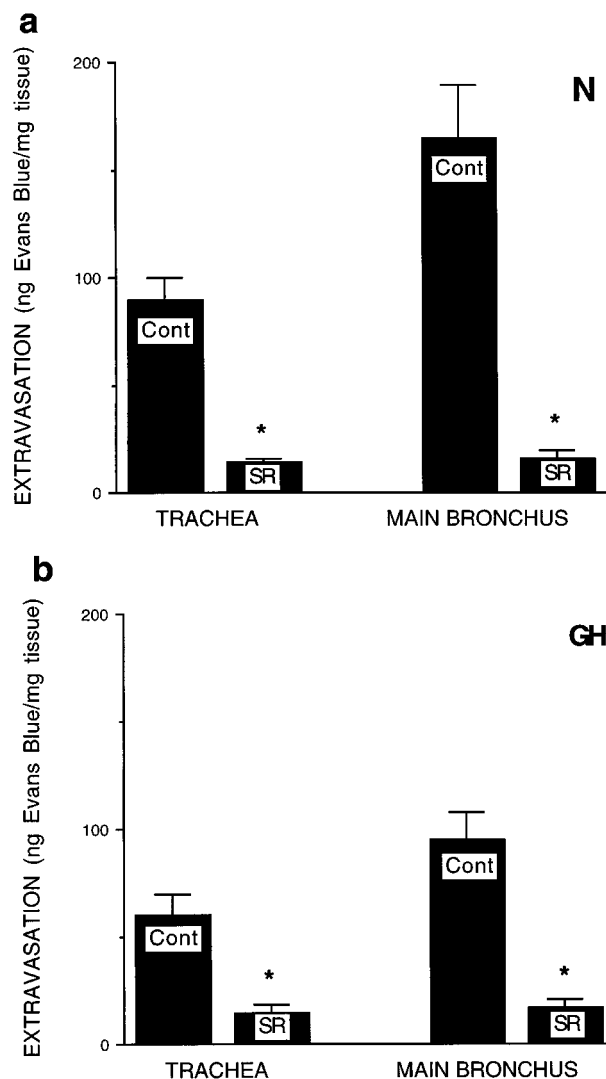


Figure 3 Prevention of capsaicin-induced dye extravasation in rat upper airways by SR 140333. Pre-treatment with the selective NK₁ receptor antagonist SR 140333 (360 nmol kg^{-1}) completely prevented the effects of capsaicin ($75 \mu\text{g kg}^{-1}$) in either strain. The left hand bar of each pair represents dye extravasation induced by capsaicin alone (Cont) and the right hand bar extravasation induced by capsaicin after SR 140333 (SR). The height of the bars represents the mean values ($\pm$ s.e. mean; $n=8$) of tissue content of extravasated dye. *Significantly different from value in rats treated with capsaicin alone; $P<0.05$.

Involvement of the NK₁ receptor in the hypotensive responses to SP and capsaicin was tested in eight animals (4 N and 4 GH) by treatment with SR 140333 (360 nmol kg^{-1}). In all animals, SR 140333 abolished the depressor response to SP whereas the response to capsaicin was unaffected. The antagonist effect of SR 140333 on SP responses persisted for at least 30 min. Results from a typical experiment are shown in Figure 5. In an additional series of five animals (2 N and 3 GH), intravenous administration of the same dose of SR 140333, before capsaicin, was seen not to have any effect on resting blood pressure in either strain.

Discussion

Our experiments demonstrate that the upper airways of GH rats, which are hyperinnervated by sensory axons containing

SP (Bakhle & Bell, 1994), exhibit a markedly reduced capacity to increase microvascular permeability in response to either endogenous or exogenous SP. By contrast, the GH airways exhibited a normal capacity for microvascular permeability

increase in the presence of another pro-inflammatory mediator, 5-HT. Furthermore, the decreased inflammatory response was not likely to be due to altered circulatory dynamics in the GH, since both strains exhibited similar vasodepressor sensitivities to exogenous SP or ACh.

The inflammatory response to capsaicin may involve release not only of SP but also of other neuropeptides including calcitonin gene-related peptide (CGRP) and pituitary adenylylate cyclase-activating peptide (PACAP), which are powerful vasodilators (Brain *et al.*, 1985; Mulder *et al.*, 1994) and therefore potentiate extravasation of plasma proteins when microvascular permeability has been increased (Brain & Williams, 1989; Delay Goyet *et al.*, 1992). It does not seem likely, however, that the lesser inflammatory response to capsaicin in GH animals was due to an absence of contribution from these other peptides, since a similar strain difference was seen with the inflammatory responses to SP itself. Furthermore, involvement of receptors for peptides other than SP in the response to capsaicin was ruled out by the fact that this response was abolished by SR 140333, a selective NK₁ receptor antagonist (Emonds Alt *et al.*, 1993).

Another possible explanation for reduced inflammatory capacity in GH airways would be a reduced ability of the venular endothelium to change its structure and hence its permeability (Kenins *et al.*, 1984) in response to inflammatory mediators. However, the normal inflammatory response to 5-HT that we observed argues against a non-specific loss of the microvascular response in the GH strain.

A third possibility would be that the endothelium of the GH microvasculature is selectively insensitive to SP. The reduced permeability responses of GH animals to exogenous SP supported this explanation and it seems likely, therefore, that a subsensitivity to SP is responsible for the low level of permeability responses to capsaicin in GH airways.

Further possibilities include differences in receptor coupling to intracellular signalling pathways or in co-mediator biosynthesis, particularly nitric oxide (NO). Oedema, induced by exogenous SP or by stimulation of sensory nerves, has been shown to be decreased by inhibition of NO synthase (NOS) (Hughes *et al.*, 1990; Kajekar *et al.*, 1995). Indirect evidence of decreased NO synthesis has been described for blood vessels of the GH rat, *ex vivo* (Winquist *et al.*, 1984) and *in vivo* (Ledingham & Laverty, 1997) and similar changes in NOS in the vasculature of the airways could modulate permeability responses.

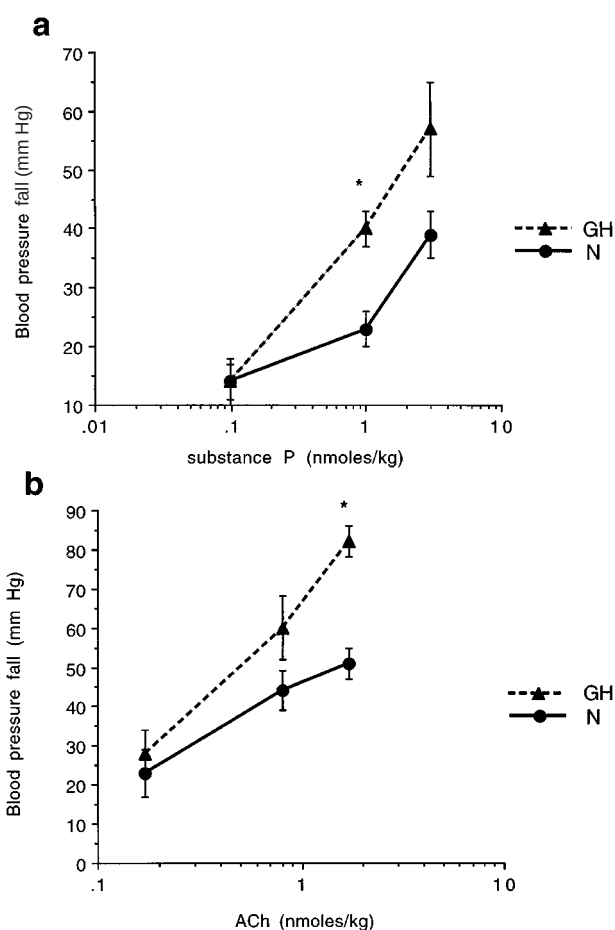


Figure 4 Vasodepressor responses to i.v. SP (a) or to ACh (b) in control (N) and GH rats. Over the range of doses shown, no consistent strain-related difference in hypotensive effect was evident. Where there was a difference, the responses of the GH rats were the greater. The values shown are the mean ($\pm$ s.e. mean) results from 3–4 animals at each dose. *Significantly different from value in control rats; $P < 0.05$.

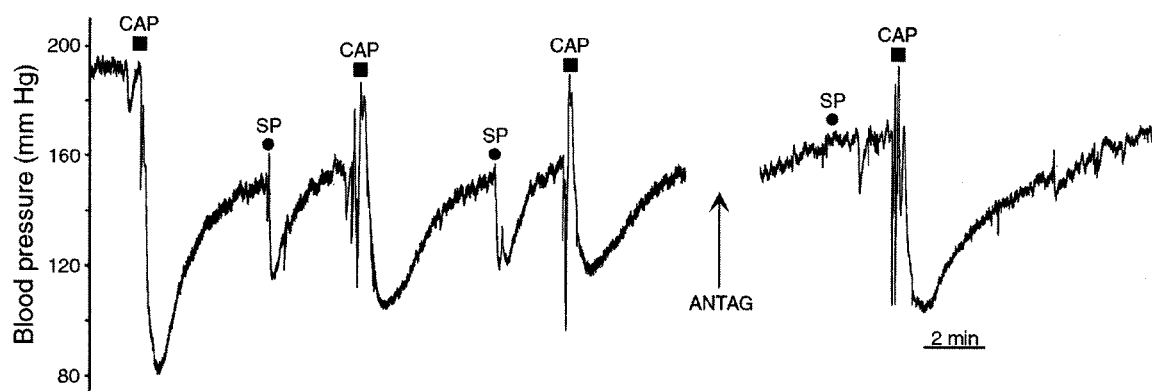


Figure 5 Depressor effects of substance P (SP, 0.2 nmol kg^{-1}) and capsaicin (CAP, $75 \mu\text{g kg}^{-1}$) on arterial blood pressure in a GH rat. Note that resting blood pressure was maintained at a lower level following the first injection of capsaicin. Subsequent doses of capsaicin or SP caused reproducible blood pressure responses, with responses to capsaicin being longer lasting. Five minutes after i.v. injection of the NK₁ antagonist SR 140333 (360 nmol kg^{-1}), the response to SP was abolished whereas that to capsaicin was unaffected.

Another cause of subsensitivity to SP might be a decreased number of SP receptors in the GH. An acute loss of NK₁ receptors due to internalization occurs within minutes of agonist binding *in vivo* or *in vitro* (Garland *et al.*, 1994) and functional tachyphylaxis, compatible with receptor loss from the cell membrane, is a well established feature of SP-NK₁ receptor interactions (Quartara & Maggi, 1997). Since the GH is known to have elevated numbers of SP-containing sensory neurons and these provide an elevated innervation density in a number of peripheral tissues, including the airways (Bakhle & Bell, 1994), increased basal release of SP could result in a lower resting number of NK₁ receptors at the cell surface. Thus the response to the acute release of high levels of SP by capsaicin or to addition of exogenous SP could be attenuated by a pre-existing state of tachyphylaxis.

Alternatively, a chronic loss of NK₁ receptors could involve nerve growth factor (NGF). The overgrowth of SP-containing sensory neurons in the GH is coupled with reduced numbers of sympathetic neurons (Gurusinghe *et al.*, 1990) and both defects are normalized by administration of exogenous NGF in the neonatal period (Messina & Bell, 1991). Exogenous NGF increases the sensitivity of sensory neurons to capsaicin, probably by up-regulating capsaicin receptors (Bevan & Winter 1995; Hu-Tsai *et al.*, 1996). In view of the specificity of capsaicin for neurokinin-containing neurons, it is possible that neurokinin receptors may also be sensitive to modulation by NGF and that, therefore, reduced NGF levels may down-regulate receptor numbers.

SP is known to cause long-lasting excitation of sympathetic motoneurons (Holzer, 1988) and these neurons receive inputs from SP-containing sensory axons (Matthews & Cuello, 1984; Gurusinghe & Bell, 1989). In view of the increased density of SP-containing axons in sympathetic ganglia in GH animals, it has been suggested that enhancement of sympathetic drive by intra-ganglionic release of SP may contribute to the elevated blood pressure in this strain (Gurusinghe & Bell, 1989). However, this is not supported by our present observations. Thus, administration of SR 140333 in a dose that abolished

depressor responses to SP had no effect on resting blood pressure in the GH strain. Furthermore, the sustained reduction of resting blood pressure after an initial dose of capsaicin was similar in N and GH animals, although neurochemical data indicate that there is tonically elevated sympathetic drive in the GH rat (see Bell, 1996).

While the hypotensive response to exogenous SP was abolished by the NK₁ antagonist SR 140333, that due to capsaicin was hardly affected. One explanation for this difference could be that the depressor effect of capsaicin was mediated *via* a central, rather than a peripheral, mechanism. Centrally mediated cardiovascular effects of capsaicin have been demonstrated in rats (Donnerer & Lembeck, 1983), as have been centrally localized capsaicin-sensitive stores of SP (Virus *et al.*, 1982) and NK receptors (Otsuka & Yoshioka, 1993). However, intravenous SR 140333 is known to block the responses of thalamic neurons to mechanical nociception (Emonds Alt *et al.*, 1993). An equally viable explanation would be to attribute most of the systemic hypotensive effect of capsaicin to the release of CGRP. This neuropeptide is a potent vasodilator in rats (Claing *et al.*, 1992; Hall *et al.*, 1995) but as its effects are not mediated by the NK₁ receptor, they would not be susceptible to blockade by SR 140333.

Finally, the well documented ability of SP to degranulate mast cells (Metcalfe *et al.*, 1997) makes it of some interest to note that mast cells in the Kyoto strain of genetically hypertensive rat exhibit abnormal responses to a range of degranulating stimuli, such as deficient NO synthesis (Masini *et al.*, 1991) and lack of mediator release (Kwasniewski *et al.*, 1998). In view of these observations and our present results, it is possible that subsensitivity to the inflammatory actions of SP is a general characteristic of genetic hypertension.

We are grateful to the Health Research Board (Ireland), the British Council, Trinity College Dublin and the Royal Irish Academy for their support of this work and the Wellcome Trust for a Summer Studentship to J.D. Brogan (Glasgow Caledonian University). We thank Dr J.E. Brelière (Sanofi) for a generous supply of SR 140333.

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(Received October 29, 1998

Accepted November 20, 1998)



G-protein activation by putative antagonists at mutant Thr³⁷³Lys α_{2A} adrenergic receptors

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1 Replacement of a threonine by a lysine at position 373 in the C-terminal portion of the third intracellular loop of the human α_{2A} -adrenergic receptor (α_{2A} AR) has been reported to generate a constitutively active mutant receptor in analogy with similar mutations in the α_{1B} and β_2 AR (Ren *et al.*, 1993). In the present study, the mutant Thr³⁷³Lys α_{2A} AR receptor was investigated by measuring the formation of inositol phosphates in either the absence or presence of mouse $G_{\alpha 15}$ protein in Cos-7 cells.

2 Increased affinity, potency and/or efficacy for the agonists [(–)-adrenaline, UK 14304, clonidine, guanabenz and oxymetazoline] was observed, consistent with a precoupled mutant α_{2A} AR: G-protein state. The basal inositol phosphates response was similar at the wild-type (wt) and mutant α_{2A} AR, but was enhanced at the mutant α_{2A} AR upon co-expression with the mouse $G_{\alpha 15}$ protein. This enhanced response could not be attenuated in the presence of any of the tested α_2 AR antagonists (10 μ M), suggesting that inverse agonist activity did not occur at the mutant α_{2A} AR.

3 Ligands that so far have been identified as antagonists at the wt α_{2A} AR demonstrated either no intrinsic activity (MK 912, WB 4101, RS 15385, RX 811059 and RX 821002) or positive efficacy [$E_{\max}$, % vs. 1 μ M UK 14304: dexefaroxan (27 ± 7), idazoxan (34 ± 9), atipamezole (27 ± 4), BRL 44408 (59 ± 5) and SKF 86466 (54 ± 9)] at the mutant α_{2A} AR, but only in the presence of the mouse $G_{\alpha 15}$ protein. The ligand potencies corresponded with their respective pK_i values at the mutant α_{2A} AR receptor.

4 The partial agonist effect of SKF 86466 was resistant to pertussis toxin treatment (100 ng ml^{–1}) and not affected by co-expression of the rat $G_{\alpha i1}$ protein. It was virtually absent in the presence of 10 μ M RS 15385. SKF 86466 was without intrinsic activity upon co-expression of the mouse $G_{\alpha q}$ protein.

5 Some putative α_2 AR antagonists exerted a partial agonist activity that was highly dependent on the presence of specific G-protein α -subunits, suggesting that these ligands cause selective G-protein activation at the mutant α_{2A} AR.

Keywords: α_{2A} -adrenergic receptor; G-protein coupling; intrinsic ligand activity; antagonist trafficking

Abbreviations: α_{2A} AR, α_{2A} -adrenergic receptor; BRL 44408, ($\pm$)-2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole; CSS, controlled salt solution; DMEM, Dulbecco's modified Eagle Medium; MK 912, (2S,12bS) 1',3'-dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine)-2,4'-pyrimidin-2'-one; RS 15385, (+)-(8aR,12aS,13aS)-3-methoxy-12-(methylsulphonyl)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-6H-isoquino[2',1-g][1,6]naphthyridine; RX 811059, 2-[2-(2-ethoxy-1,4-benzodioxan-2-yl)-1-imidazoline; [³H] RX 821002, [³H] (1,4-[6,7(n)-[³H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride; SKF 86466, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine; UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)aminoethyl-1,4-benzodioxane hydrochloride

Introduction

α_2 -Adrenergic receptors (α_2 AR) are implicated in the control of noradrenergic neurotransmission in the brain and modulate a number of physiological processes peripherally, including aggregation of platelets, release of insulin from the pancreas, and inhibition of lipolysis (Timmermans *et al.*, 1990; Szabadi & Bradshaw, 1996). Pharmacological results including ligand-binding data suggest that α_2 AR are not a homogeneous class of receptors. At least three distinct genes encoding α_2 AR subtypes ($\alpha_{2C10/A}$ AR, $\alpha_{2C2/B}$ AR, $\alpha_{2C4/C}$ AR) have been isolated in man and characterized in heterologous expression systems (Regan *et al.*, 1988; Fraser *et al.*, 1989; Lomasney *et al.*, 1990). The α_2 AR belong to the superfamily of G-protein-coupled receptors, mediating their effects through the activation of heterotrimeric G-proteins. The α_{2A} AR, *via* its interaction with

the pertussis toxin-sensitive G_i/G_o subclass of G-proteins, modulates multiple effector systems, including inhibition of adenylate cyclase, opening of Ca^{2+} channels and activation of K^+ channels (Lakhlani *et al.*, 1996). Eason *et al.* (1992) and Federman *et al.* (1992) have demonstrated paradoxical α_2 AR-mediated increases in cyclic AMP level *via* coupling to G_s . The degree of G_s coupling depends initially on the agonist's structural features, and ligands that act as full agonists for G_i coupling are not necessarily full agonists for G_s coupling (Eason *et al.*, 1994). The α_2 AR may also couple to other intracellular pathways involving Na^+/H^+ exchange, phospholipase A_2 and phospholipase C activation (Limbird, 1988; Cotecchia *et al.*, 1990).

Mutagenesis studies of several G-protein-coupled receptors indicate that the coupling to specific G-proteins is predominantly mediated by the intracellular loops (Dalman and Neubig, 1991). The second and third intracellular loops are clearly involved in α_{2A} AR:G-protein interactions (Dalman &

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Neubig, 1991; Näsman *et al.*, 1997). Replacement of a threonine by a lysine or a cysteine at position 373 in the BBXXB motif (where B represents a basic residue and X a non-basic residue) of the third intracellular loop of the human α_{2A} AR, results in a constitutively active mutant receptor in analogy with mutations affecting the same region in the α_{1B} AR and β_2 AR (Ren *et al.*, 1993). The mutant α_{2A} AR demonstrates an increased affinity for the agonist UK 14304 associated with an agonist-independent inhibition of adenylyl cyclase in HEK 293 cells. The functional consequences of this mutant receptor have also been studied in a stably transfected 3T3F442A preadipose cell line (Bétuing *et al.*, 1997). Similar (in the absence of agonist) and amplified (in the presence of agonist) cellular responses were measured at the preadipocyte stage indicating constitutive activation of the mutant receptor. However, the α_{2A} AR-dependent antilipolysis at the adipocyte stage was neither reproduced nor amplified by the mutant receptor. Conversely, down-regulation of $G_{\alpha i}$ -proteins and an increase in the maximal response to lipolytic agents have been observed in cell lines expressing the mutant α_{2A} AR (Bétuing *et al.*, 1997). Other than the agonist UK 14304, very few α_2 AR ligands have been evaluated at this mutant α_2 AR. Also, it has yet to be determined whether the inhibition of constitutive activity by α_2 AR inverse agonists can be reversed. Tian *et al.* (1994) demonstrated that some α_2 AR antagonists display inverse agonist activity at precoupled wild-type (wt) rat α_{2D} AR in recombinant PC12 cells; the rank order of maximal effectiveness differed from that of receptor binding affinities.

Our studies of constitutively active mutant α_{2A} AR provide apparently opposite results for ligands that so far have been characterized as α_2 AR antagonists. By measuring the positive coupling of the mutant receptor to the formation of inositol phosphates, partial agonist instead of either neutral antagonist or inverse agonist activity was observed for a series of α_2 AR ligands at the mutant Thr³⁷³Lys α_{2A} AR in the presence of mouse $G_{\alpha 15}$ protein. Experiments were performed in parallel with the wt α_{2A} AR, and were compared to receptor activity as measured by inhibition of stimulated cyclic AMP formation in stably transfected C6-glia cells. We found an increased affinity of the mutant α_{2A} AR receptor for diverse agonists, whereas no functional evidence could be observed for inverse agonist activity. In contrast, a number of putative α_2 AR antagonists displayed positive efficacy and behaved as partial agonists at the mutant α_{2A} AR in the presence of mouse $G_{\alpha 15}$ protein. The partial agonist activity is discussed with regard to the receptor binding properties, G-protein coupling and partial agonist activity of antagonists at other G-protein-coupled receptor mutants.

Methods

Cloning of human wt and mutant Thr³⁷³Lys α_{2A} -adrenergic receptors, rat $G_{\alpha i}$ protein, and mouse $G_{\alpha 15}$ and G_q protein genes

Genes were cloned by PCR using primers designed at the start and stop codons of the respective nucleotide sequences (Fraser *et al.*, 1989; Jones & Reed, 1987; Strathmann & Simon, 1990; Wilkie *et al.*, 1991). The PCR mixture (50 μ l) consisted of 250 ng of reverse-transcribed poly(A⁺) RNA from either human, mouse or rat total brain, or 1 μ g of mouse genomic DNA, dNTP (350 μ M), primers (400 nM), DMSO (10% for the α_{2A} AR gene only) and 1 μ l of Expand long template DNA polymerase mix in PCR buffer [mM: (NH₄)₂SO₄, 16; MgCl₂, 1.75; Tris-HCl, 50 (pH 9.2)]. The PCR program consisted of 30

repetitive cycles with a strand separation step at 96°C for 30 s, an annealing step at 60°C for 1 min and an elongation step at 68°C for 1.5 min. The amplification cycle was preceded by a hot start step at 96°C for 5 min in which the DNA polymerase mix was omitted. PCR fragments were purified by 1% agarose gel electrophoresis, purified using a Geneclean II kit and subsequently cloned into 50 ng of a pCR3.1 vector. Sequencing was performed automatically on an ABI Prism 310 Genetic analyser using a dichloro-rhodamine terminator cycle sequencing kit. Nucleotide sequences were identical to the published sequences. Site-directed mutagenesis of the α_{2A} AR was performed using a modified overlap extension technique based on PCR (Wurch *et al.*, 1998) using appropriate complementary primers carrying the Thr (ACG codon) to Lys (AAA codon) mutation. The obtained PCR fragment contained the presence of the mutated codon: Thr³⁷³Lys.

Transient co-transfection of α_{2A} -adrenergic receptors and various G-protein genes in Cos-7 cells

Ten micrograms of plasmid containing either wt α_{2A} AR or mutant Thr³⁷³Lys α_{2A} AR and 10 μ g of the indicated G-protein plasmid were co-transfected with 5×10^6 exponentially-growing Cos-7 cells using a Bio-Rad electroporator (250 mV, 250 μ F) in DMEM supplemented with 1% DMSO. Cultures were grown in 24-well tissue culture plates with DMEM supplemented with 10% foetal calf serum, washed and treated after 24 h with 2% dialysed foetal calf serum and [2-³H](N) myo-inositol (2 μ Ci per well) for a period of 24–48 h before functional receptor analysis. For radioligand experiments, cells were grown for 48 h in DMEM supplemented with 10% foetal calf serum.

Stable transfection of wt α_{2A} -adrenergic receptor gene in C6-glia cells

C6-glia cells were transfected as described above with 10 μ g pCR3.1/human α_{2A} AR and were subcultured after 48 h in DMEM in the presence of 1.25 mg geneticin ml⁻¹. After about 10 days, individual clones were isolated and subcultured as described (Pauwels *et al.*, 1996). Intact cells were assayed for [³H] (1,4-[6,7(n)-³H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002) binding and inhibition of forskolin-induced cyclic AMP formation. Results presented in this study were obtained with a clone that expresses 1.3 pmol mg⁻¹ protein of [³H] RX 821002 binding sites.

Receptor binding assay for α_{2A} -adrenergic receptors

Membrane preparations were prepared in Tris-HCl pH 7.6 (50 mM) as described (Pauwels *et al.*, 1996). Binding assays were performed with [³H] RX 821002 (2 nM). Incubation mixtures consisted of 0.4 ml of cell membranes (6 μ g protein), 0.05 ml of radioligand and 0.05 ml of displacing compound or phentolamine (10 μ M) to determine nonspecific binding. K_i values were calculated according to the equation $K_i = IC_{50}/(1 + C/K_d)$ with C representing the concentration and K_d the equilibrium dissociation constant of the [³H] ligand. pK_d values were obtained from saturation binding studies performed as previously described (Pauwels *et al.*, 1996).

α_{2A} -adrenergic receptor-mediated inhibition of forskolin-induced cyclic AMP formation

Inhibition of forskolin (100 μ M)-stimulated cyclic AMP formation in the C6-glia cell line stably expressing the wt α_{2A} AR was measured as previously described (Pauwels *et al.*,

1996). The cellular cyclic AMP content was assayed using a radioimmunoassay kit. Inhibition of forskolin-induced cyclic AMP formation was expressed as the percentage of that obtained with UK 14304 (1 μ M). EC₅₀ values (concentrations of the compounds producing 50% inhibition of their own maximal inhibition) were derived. Antagonists were added 15 min prior to agonists. pK_B values were calculated as $K_B = (B)/(A'/A) - 1$ where B is the concentration of the antagonist, and A and A' are the EC₅₀ values of agonist concentration measured in the absence and presence of antagonist, respectively.

α_2 A-adrenergic receptor-mediated phosphatidyl inositol phosphates hydrolysis

Cos-7 cells were washed with 1.0 ml CSS, and were incubated for 1 h at 37°C with 1.0 ml CSS containing LiCl (10 mM) either in the presence or absence of agonist. The reaction was stopped by the addition of 0.1 ml 1 N HClO₄ and neutralized prior to the addition of 0.25 ml of sample buffer (mM: Na₂B₄O₇, 30; EDTA, 3). The fraction of total [³H]-inositol phosphates produced after agonist stimulation was separated from free [³H]-myoinositol and [³H]-glycerophosphoinositol by chromatography on a strong anion exchange AG1-X8 resin equilibrated in water. A 1.3 ml aliquot of the cell lysate was loaded on a column as described (Pauwels *et al.*, 1990). After two washes with 10.0 ml water, the glycerophosphoinositol was washed out in 15.0 ml of [mM: Na₂B₄O₇, 5; CH₃NO₂, 60]. The total [³H]-inositol phosphates were eluted with 2.0 ml of [mM: CH₃NO₂, 2; HCOOH, 0.1]. The radioactivity was extracted in 16 ml Ultima Gold XR and counted with a Tri-Carb 2500 liquid scintillation counter. Maximal stimulation of the total production of inositol phosphates was defined in the presence of UK 14304 (1 μ M). EC₅₀ values were defined as the concentrations of compounds producing 50% of their own maximal response. Antagonist studies were performed by pre-incubating the cells with the antagonist for 10 min before the addition of the agonist. pK_B values were calculated as described above. In the case of pertussis toxin treatment (100 ng ml⁻¹), cells were pretreated for 16 h before inositol phosphates were measured.

Statistical analysis

Statistical comparison of ligand binding affinities, agonist and antagonist potencies, and basal and UK 14304-mediated responses between wt α_2 A and mutant Thr³⁷³Lys α_2 A AR were made using the two-tailed Student's *t*-test.

Materials

Cos-7 and C6-glia cells were obtained from ATCC (Rockville, U.S.A.). The ABI Prism 310 Genetic analyzer and the dichloro-rhodamine terminator cycle sequencing kit were from Perkin Elmer (Forster City, U.S.A.). The pCR3.1 vector was from Invitrogen (San Diego, U.S.A.). The GeneClean II kit was purchased from Bio 101 Inc. (La Jolla, U.S.A.). The Expand long template PCR kit was from Boehringer Mannheim (Mannheim, Germany). The AG1-X8 resin was from Bio-Rad Laboratories (Hercules, U.S.A.). [³H] RX 821002 (50.6 Ci mmol⁻¹) and [2-³H](N)-myoinositol (20 Ci mmol⁻¹) were obtained respectively from Amersham (Les Ulis, France) and New England Nuclear (Les Ulis, France). 2-(2,6-dimethoxyphenoxyethyl)aminoethyl-1,4-benzodioxane hydrochloride (WB 4101) was from RBI (Natick, U.S.A.). Clonidine, (–)-adrenaline, oxymetazoline and gua-

nabenz were from Sigma. (±)-2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole (BRL 44408) and 6-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine (SKF 86466) were from Smith Kline Beecham (Herts, U.K.). (2S,12bS) 1',3'-dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine)-2,4'-pyrimidin-2'-one (MK 912) was from Merck (Essex, U.K.). 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate (UK 14304), dexefaroxan, (–)-efaroxan, atipamezole, (+)-(8aR,12aS,13aS)-3-methoxy-12-(methylsulphonyl)-5,8,8a,9,10,11,12,12a, 13,13a-decahydro-6H-isoquino [2',1-g][1,6]naphthyridine (RS 15385) and 2-[2-(2-ethoxy-1,4-benzodioxan-2-yl)-1-imidazoline (RX 811059) were prepared intra-muros. Idazoxan and (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002) were from Reckitt and Colman (Kingston-upon-Hull, U.K.).

Results

Binding properties of α_2 -adrenergic ligands at wt α_2 A and mutant Thr³⁷³Lys α_2 A-adrenergic receptors

Saturation binding experiments with [³H] RX 821002 in membrane preparations of Cos-7 cells transfected with either a wt α_2 A or mutant Thr³⁷³Lys α_2 A AR gene indicated the presence of a single high affinity binding site with similar affinity for [³H] RX 821002 (pK_d: 8.98 ± 0.05 and 8.84 ± 0.02 for wt α_2 A and mutant α_2 A AR receptors, respectively). The maximal binding capacity for [³H] RX 821002 in these transfected cells was similar (4.0 ± 0.4 and 4.5 ± 1.0 pmol mg⁻¹ protein, for the wt and mutant α_2 A AR, respectively). A series of eleven α_2 -adrenergic antagonists and five agonists were characterized for inhibition of [³H] RX 821002 binding to wt α_2 A and mutant α_2 A AR (Table 1). The mutation significantly decreased (1.8–2.9 fold) the affinity of RS 15385, RX 811059 and WB 4101, and left unchanged that of the other antagonists. In contrast, each of the agonists appeared to be significantly more potent at the mutant α_2 A AR; the following rank order of increased affinity was obtained (fold-increase): (–)-adrenaline (20) > UK 14304 (8.7) > clonidine (3.5) = guanabenz (3.2) > oxymetazoline (1.9) (Table 1). Co-expression of either the wt α_2 A or mutant Thr³⁷³Lys α_2 A AR with the mouse G_{z15} protein enhanced the maximal binding capacity for [³H] RX 821002 without affecting the affinity constant for [³H] RX 821002. Binding affinities of the ligands tested were not affected upon co-expression with mouse G_{z15} protein or co-incubation with GTP (100 μ M) (not shown).

Intrinsic activity of α_2 -adrenergic ligands at wt α_2 A and mutant Thr³⁷³Lys α_2 A-adrenergic receptors

In contrast to nontransfected Cos-7 cells, UK 14304 produced a concentration-dependent stimulation of inositol phosphates formation in cells transfected with either wt α_2 A or mutant Thr³⁷³Lys α_2 A AR. The basal activity as well as the maximal response to UK 14304 were the highest with the mutant Thr³⁷³Lys α_2 A AR in the presence of mouse G_{z15} protein (Table 2). Otherwise, for the wt α_2 A AR, the basal and maximal responses to UK 14304 were similar in the presence and absence of mouse G_{z15} protein. The UK 14304-mediated responses are likely to occur *via* different G-protein subclasses as they could be differentially blocked by pretreatment of the cells with pertussis toxin. The responses at wt α_2 A and mutant Thr³⁷³Lys α_2 A AR were respectively blocked by 84 and 67% in the presence of pertussis toxin but in the absence of mouse G_{z15} protein. In contrast, the UK 14304 responses were less affected

upon pertussis toxin treatment in the presence of mouse $G_{\alpha 15}$ protein, although this effect was variable between experiments (Table 2). Further studies were conducted unless otherwise specified in the presence of mouse $G_{\alpha 15}$ protein. UK 14304 and clonidine stimulated the production of inositol phosphates to the same magnitude as (–)-adrenaline at the wt α_{2A} AR, whereas guanabenz and oxymetazoline were less active (73 and 51%, respectively; Figure 1). The corresponding potencies and $E_{\max}$ values are summarized in Table 3, and are compared with the respective agonist potencies as measured by inhibition of 100 μ M forskolin-stimulated cyclic AMP formation in C6-gial cells stably transfected with the wt α_{2A} AR, and stimulation of the production of inositol phosphates at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells. Agonist potencies were systematically lower in the cyclic AMP assay, in particular for (–)-adrenaline (21 fold) in the presence of propranolol (1 μ M) (to block β AR). This may be due to the lower levels of receptor expression in C6-gial cells (1.3 pmol mg^{–1} protein) versus Cos-7 cells (4.0–

4.5 pmol mg^{–1} protein). The cyclic AMP assay with the C6-gial cell line appeared to be also less sensitive in differentiating between the maximal responses of the agonists. Nonetheless, the $E_{\max}$ values of guanabenz and oxymetazoline were 24 and 20% inferior to that of UK 14304. Potencies for each of the agonists in the inositol phosphates assay for the mutant Thr³⁷³Lys α_{2A} were significantly greater than at wt α_{2A} AR, and their maximal responses were similar to that of UK 14304 (Figure 1 and Table 3).

Table 4 summarizes the intrinsic activities of the α_2 -adrenergic antagonists as measured at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR. Two distinct subgroups were revealed within the antagonists by measuring their stimulation of inositol phosphates at the mutant Thr³⁷³Lys α_{2A} AR. One group consisted of RS 15385, RX 811059, RX 821002 and WB 4101 which displayed neither positive nor negative efficacy at mutant and wt α_{2A} AR. The other group consisted of atipamezole, dexefaroxan, SKF 86466, idazoxan and BRL 44408 which demonstrated between +27 to +59% stimulation of inositol phosphates (versus 1 μ M of UK 14304) at the mutant α_{2A} AR. These ligands were apparently free of intrinsic activity (–1.3 to +13.0% versus 1 μ M of UK 14304) at the wt α_{2A} AR receptor. The observed potencies for the partial agonist activities of these ligands corresponded with their respective pK_i values at the mutant α_{2A} AR (Tables 1 and 4). The partial intrinsic activity of these ligands was attenuated or absent in the presence of RS 15385 (10 μ M, Figure 2). The partial agonist effect was also resistant to pertussis toxin treatment and virtually absent without expression of the mouse $G_{\alpha 15}$ protein. This is shown for SKF 86466 when compared to RS 15385 in Table 5.

In order to demonstrate that the partial agonist effect of SKF 86466 is selective for the mouse $G_{\alpha 15}$ protein, further experiments were performed by co-expression of the mutant α_{2A} AR with either mouse $G_{\alpha q}$ protein or mouse $G_{\alpha 15}$ plus rat $G_{\alpha i1}$ protein. The data summarized in Table 6 illustrate that SKF 86466 (1 μ M) was without intrinsic activity upon co-expression of the mouse $G_{\alpha q}$ protein. The partial agonist effect of SKF 86466 was not affected by co-expression of the rat $G_{\alpha i1}$ and the mouse $G_{\alpha 15}$ proteins. SKF 86466 and RS 15385 did not display intrinsic activity at the wt α_{2A} AR by co-expression of mouse $G_{\alpha q}$ or mouse $G_{\alpha 15}$ + rat $G_{\alpha i1}$ proteins (not shown).

Blockade of UK 14304-mediated responses by α_2 -adrenergic antagonists at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} -adrenergic receptors

UK 14304 was selected as a highly efficacious agonist to characterize the potencies of the antagonists at either wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR as measured both by stimulation

Table 1 pK_i values of α_2 -adrenergic ligands at human wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in transfected Cos-7 cells

Ligand	Mean pK_i values $\pm$ s.e.mean	
	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR
<i>Putative antagonist:</i>		
RS 15385	9.19 $\pm$ 0.04	8.94 $\pm$ 0.04*
RX 811059	8.95 $\pm$ 0.07	8.49 $\pm$ 0.06**
MK 912	8.89 $\pm$ 0.08	8.76 $\pm$ 0.07
RX 821002	8.87 $\pm$ 0.07	8.67 $\pm$ 0.05
Atipamezole	8.60 $\pm$ 0.06	8.47 $\pm$ 0.07
Dexefaroxan	8.28 $\pm$ 0.02	8.29 $\pm$ 0.07
SKF 86466	8.00 $\pm$ 0.23	7.88 $\pm$ 0.08
Idazoxan	7.86 $\pm$ 0.06	7.79 $\pm$ 0.09
WB 4101	7.86 $\pm$ 0.08	7.42 $\pm$ 0.07*
BRL 44408	7.80 $\pm$ 0.03	7.64 $\pm$ 0.06
(–)-efaroxan	<5	<5
<i>Agonist:</i>		
Oxymetazoline	7.73 $\pm$ 0.05	8.00 $\pm$ 0.09*
Guanabenz	7.53 $\pm$ 0.03	8.04 $\pm$ 0.03***
Clonidine	7.21 $\pm$ 0.07	7.76 $\pm$ 0.16*
UK 14304	7.15 $\pm$ 0.11	8.09 $\pm$ 0.09***
(–)-Adrenaline	5.91 $\pm$ 0.05	7.21 $\pm$ 0.09***

[³H]RX 821002 binding was performed with membrane preparations of Cos-7 cells as described in Methods. Total and nonspecific binding represented 1639 $\pm$ 223 and 204 $\pm$ 57 d.p.m. for wt α_{2A} AR, and 2409 $\pm$ 136 and 167 $\pm$ 41 d.p.m. for mutant Thr³⁷³Lys α_{2A} AR, respectively. Values were obtained from 3–4 independent experiments, performed in duplicate. Statistical analysis was performed using Student's *t*-test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 2 Comparison between inositol phosphates formation in Cos-7 cells transfected with either wt α_{2A} or mutant Thr³⁷³Lys α_2 AR in the absence or presence of mouse $G_{\alpha 15}$ protein and blockade by pertussis toxin

Mouse $G_{\alpha 15}$	Inositol phosphates formation			
	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR
	–	+	–	+
Basal (d.p.m.)	455 $\pm$ 104	378 $\pm$ 135	401 $\pm$ 28	1310 $\pm$ 244*
1 μ M UK 14304 (d.p.m.)	2021 $\pm$ 471	2248 $\pm$ 664	1932 $\pm$ 598	5124 $\pm$ 1083**
1 μ M UK 14304 + pertussis toxin treatment (%)	15.6 $\pm$ 2.8	81.6 $\pm$ 13.0	32.8 $\pm$ 9.9	81.0 $\pm$ 18.0

Transfection of Cos-7 cells was performed as described in Methods. Pertussis toxin treatment (100 ng ml^{–1}) was performed 16 h before stimulation of inositol phosphates in Cos-7 cells and was measured as described in Methods. Basal and UK 14304 (1 μ M)-mediated inositol phosphates formation are expressed as d.p.m. $\pm$ s.e.mean obtained from 3–4 independent experiments, each one performed in quadruplicate. UK 14304 (1 μ M) + pertussis toxin treatment data, expressed versus the corresponding maximal response obtained with UK 14304 (1 μ M) in nontreated cells, are shown as mean $\pm$ s.e.mean obtained from 5–8 independent experiments, performed in quadruplicate. **P* < 0.05 versus the other basal responses; ***P* < 0.05 versus UK 14304 response at mutant α_{2A} AR without mouse $G_{\alpha 15}$ protein expression (Student's *t*-test).

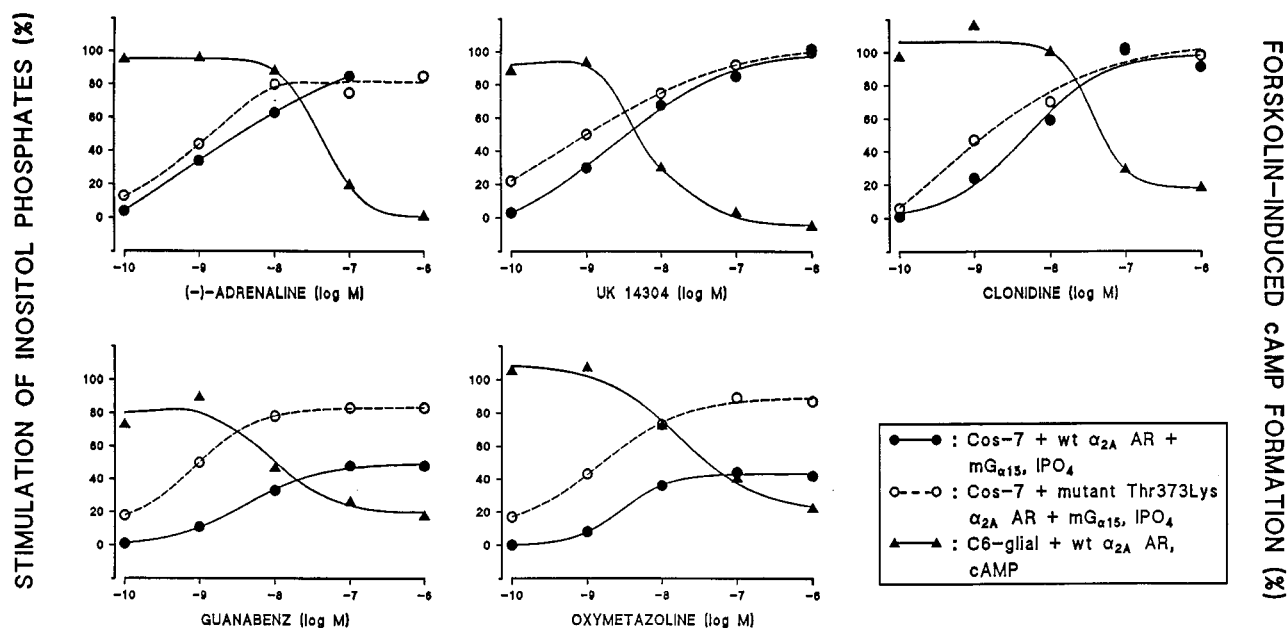


Figure 1 Stimulation of inositol phosphates formation and inhibition of forskolin-induced cyclic AMP formation by α_2 AR agonists in transfected cells expressing either wt α_2 A AR or mutant Thr³⁷³Lys α_2 A AR. Stimulation of agonist-stimulated inositol phosphates formation in Cos-7 cells expressing either wt α_2 A AR or mutant Thr³⁷³Lys α_2 A AR upon co-expression of mouse $G_{\alpha 15}$, and agonist-mediated inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells expressing wt α_2 A AR were measured as described in Methods. Representative dose-response curves are shown from 3–4 independent experiments. pEC₅₀ and E_{max} values are summarized in Table 3.

Table 3 pEC₅₀ and E_{max} values of α -adrenergic agonists for stimulating inositol phosphates formation in the presence of mouse $G_{\alpha 15}$ protein, or for inhibiting forskolin-induced cyclic AMP formation in transfected cells expressing either wt α_2 A or mutant Thr³⁷³Lys α_2 A AR

Ligand	wt α_2 A AR Stimulation of inositol phosphates formation in Cos-7 cells		wt α_2 A AR Inhibition of forskolin-induced cyclic AMP formation in C6-glia cells		Mutant Thr ³⁷³ Lys α_2 A AR Stimulation of inositol phosphates formation in Cos-7 cells	
	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)
UK 14304	8.69 ± 0.10	100	8.23 ± 0.13	100	8.99 ± 0.07*	100
Clonidine	8.11 ± 0.10	91.0 ± 14.0	7.36 ± 0.18	89.0 ± 7.0	8.95 ± 0.06***	96.4 ± 2.2
(-)-Adrenaline	8.51 ± 0.04	90.0 ± 5.0	7.18 ± 0.18	94.0 ± 12.0	8.85 ± 0.05**	89.2 ± 6.3
Guanabenz	8.13 ± 0.11	73.0 ± 11.5	7.34 ± 0.21	76.0 ± 10.0	8.91 ± 0.22*	89.0 ± 3.6
Oxymetazoline	8.26 ± 0.07	51.0 ± 3.5	7.34 ± 0.21	80.0 ± 13.0	8.89 ± 0.06***	96.8 ± 5.5

Stimulation of inositol phosphates in Cos-7 cells in the presence of mouse $G_{\alpha 15}$ protein, and inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells were measured as described in Methods. E_{max} values are expressed *versus* the response obtained with UK 14304 (1 μ M). pEC₅₀ values are defined as the concentration at which 50% of the maximal response was obtained for each individual compound. Basal and forskolin-stimulated cyclic AMP levels in C6-glia cells were 5.07 ± 0.79 and 85.5 ± 6.26 pmol ml⁻¹ respectively, for wt α_2 A AR. Maximal inhibition with UK 14304 represented 81 ± 2% of forskolin-stimulated cyclic AMP formation. Results are expressed as means ± s.e.mean obtained from 3–4 independent experiments, performed in triplicate. Statistical analysis was performed using Student's *t*-test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *versus* pEC₅₀ values for stimulation of inositol phosphates at wt α_2 A AR in Cos-7 cells.

of inositol phosphates in the presence of mouse $G_{\alpha 15}$ protein (Figure 3), and by inhibition of stimulated cyclic AMP formation at wt α_2 A AR (Table 7). In both assay systems, antagonists (1 μ M) inhibited the UK 14304-mediated response at the wt α_2 A AR. (-)-Efarafoxan (1 μ M) did not affect the UK 14304-mediated response. The calculated pK_B values (Table 7) for antagonism of the UK 14304-mediated stimulation of inositol phosphates were similar to their corresponding pK_i values. The pK_B values obtained in the cyclic AMP assay were inferior compared to their pK_i values and the pK_B values in the inositol phosphates assay. Partial blockade of the UK 14304-mediated response at the mutant Thr³⁷³Lys α_2 A AR was obtained with atipamezole, dexefarafoxan, SKF 86466, idazoxan and BRL 44408 (Figure 3). In contrast, RS 15385, RX 811059,

RX 821002 and WB 4101 antagonized the UK 14304-mediated inositol phosphates response at the mutant α_2 A AR in a manner that appeared competitive (Figure 3) and consistent with their lack of intrinsic activity. The antagonist potencies were inferior (2–7 fold) to their respective pK_i values, and the pK_B values (5–13 fold) at the wt α_2 A AR (Table 7).

Discussion

The present study compares the intrinsic activities of α_2 AR ligands at the wt α_2 A and mutant Thr³⁷³Lys α_2 A AR. This receptor mutant has previously been reported to be constitutively active in HEK 293 cells as well as in a 3T3F442A

preadipose cell line (Ren *et al.*, 1993; Bétuing *et al.*, 1997). We also found evidence for constitutive activity at the mutated α_{2A} AR. Enhanced affinity, potency and/or efficacy for the agonists was observed in line with a precoupled mutant α_{2A} AR:G-protein state. The basal inositol phosphates response was also enhanced but only upon co-expression of the mutant α_{2A} AR

Table 4 Intrinsic activity of antagonists at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in the presence of mouse G_{z15} protein in Cos-7 cells

Ligand	Inositol phosphates formation		
	wt α_{2A} AR <i>E</i> _{max} (%)	Mutant Thr ³⁷³ Lys α_{2A} AR <i>E</i> _{max} (%)	<i>pEC</i> ₅₀
RS 15385	-5.1 ± 6.5	3.2 ± 2.6	
RX 811059	1.2 ± 1.0	-0.4 ± 2.5	
MK 912	0.8 ± 2.1	13.0 ± 1.1	
RX 821002	-0.3 ± 0.7	-1.3 ± 2.2	
Atipamezole	13.0 ± 4.2	27.2 ± 3.5	8.59
Dexefaroxan	3.5 ± 1.3	27.4 ± 6.9	7.52
SKF 86466	3.1 ± 1.4	54.4 ± 9.0	8.00
Idazoxan	3.5 ± 0.5	33.6 ± 9.1	7.52
WB 4101	2.7 ± 2.9	7.2 ± 1.4	
BRL 44408	-1.3 ± 2.0	59.2 ± 4.5	7.62
(-)-Efaroxan		2	

Stimulation of inositol phosphates in Cos-7 cells in the presence of mouse G_{z15} protein was measured as described in Methods. Ligands were tested at 10 μ M, whereas dose-response curves were performed for atipamezole, dexefaroxan, SKF 86466, idazoxan and BRL 44408 at the mutant Thr³⁷³Lys α_{2A} AR. Mean *E*_{max} values $\pm$ s.e.mean are expressed *versus* the maximal response obtained with UK 14304 (1 μ M). *pEC*₅₀ values were derived from the mean dose-response curves and were defined as the concentrations at which 50% of the maximal response was obtained for each individual ligand. Results were obtained from 2–5 independent experiments for wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR, performed in triplicate.

with the mouse G_{z15} protein. However, this response could not be attenuated in the presence of any of the tested antagonists. Indeed, ligands previously characterized as antagonists at the

Table 5 Effects of pertussis toxin treatment and mouse G_{z15} protein on the intrinsic activity of SKF 86466 and RS 15385 at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells

	Inositol phosphates formation Mutant Thr ³⁷³ Lys α_{2A} AR (<i>E</i> _{max} , %)		
	Mouse G _{z15}	+	+
Pertussis toxin	-	-	+
SKF 86466	6.8 ± 3.1	43.8 ± 8.2	54.0 ± 8.8
RS 15385	6.8 ± 7.2	6.8 ± 6.9	-1.3 ± 4.8

Pertussis toxin treatment (100 ng ml⁻¹) was performed 16 h before stimulation of inositol phosphates in Cos-7 cells. Ligands were tested at 1 μ M. Mean *E*_{max} values $\pm$ s.e.mean, expressed *versus* the maximal response of UK 14304 (1 μ M), were obtained from 4 independent experiments, performed in triplicate.

Table 6 Effects of various G-protein subunits on the intrinsic activity of SKF 86466 and RS 15385 at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells

G protein subtype	Inositol phosphates formation Mutant Thr ³⁷³ Lys α_{2A} AR (<i>E</i> _{max} , %)	
	SKF 86466	RS 15385
Mouse G _{z15}	48.2 ± 6.1	6.2 ± 2.5
Mouse G _{zq}	4.3 ± 4.1	-0.7 ± 2.2
Mouse G _{z15} + rat G _{z11}	41.3 ± 4.7	-0.7 ± 2.1

Stimulation of inositol phosphates in Cos-7 cells was measured as described in Methods. Ligands were tested at 1 μ M. Mean *E*_{max} values $\pm$ s.e.mean, expressed *versus* the maximal response of UK 14304 (1 μ M), were obtained from 2–6 independent experiments, performed in triplicate.

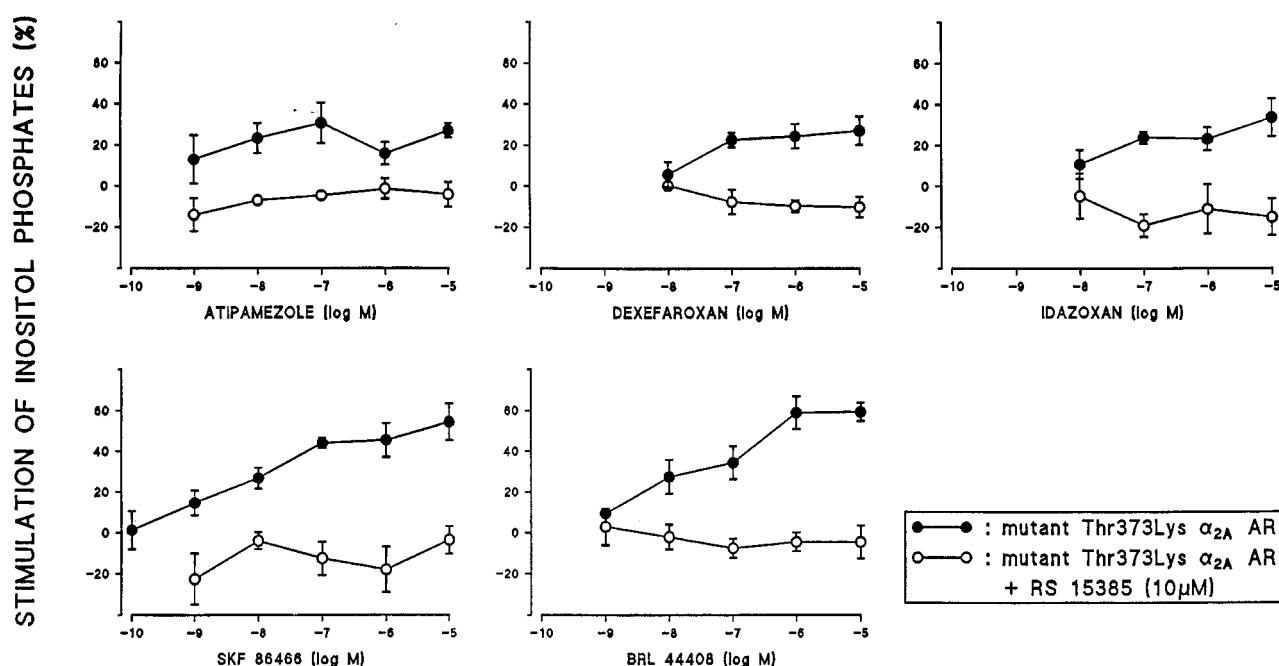


Figure 2 Intrinsic activity of putative α_{2A} AR antagonists at mutant Thr³⁷³Lys α_{2A} AR upon co-expression of mouse G_{z15} protein. Stimulation of inositol phosphates formation by putative α_{2A} AR antagonists in Cos-7 cells expressing mutant Thr³⁷³Lys α_{2A} AR and mouse G_{z15} protein, in either the absence or presence of RS 15385 (10 μ M), was measured as described in Methods. Dose-response curves were constructed using mean $\pm$ s.e.mean of five independent experiments. *pEC*₅₀ values are summarized in Table 4.

wt α_{2A} AR demonstrated either no intrinsic activity or positive efficacy at the mutant α_{2A} AR upon co-expression with the mouse $G_{\alpha_{15}}$ protein. This observation suggests that some of these ligands may actually possess a partial agonist activity at the wt α_{2A} AR receptor, which is augmented by the facilitating Thr³⁷³Lys mutation. Importantly, this partial agonist effect is dependent on the presence of specific G-protein α -subunits. In particular, SKF 86466 and BRL 84408 displayed in the presence of mouse $G_{\alpha_{15}}$ protein, an intrinsic activity with maximal responses similar to that of oxymetazoline at the wt α_{2A} AR. The partial agonist effect was not attenuated by the

co-expression of the rat $G_{\alpha_{11}}$ protein, and was not observed upon co-expression of the mouse G_{α_q} protein.

The partial agonists did not display modifications in their binding affinities for the mutant α_{2A} AR. The ligands characterized as antagonists at both the wt α_{2A} and mutant α_{2A} AR showed less affinity at the mutant α_{2A} AR, in line with what would be expected for inverse agonists (Westphal & Sanders-Bush, 1994). However, no direct functional evidence was obtained for the occurrence of such inverse agonist activity. The α_2 AR antagonists appear under the present experimental conditions to behave as either neutral antagonists

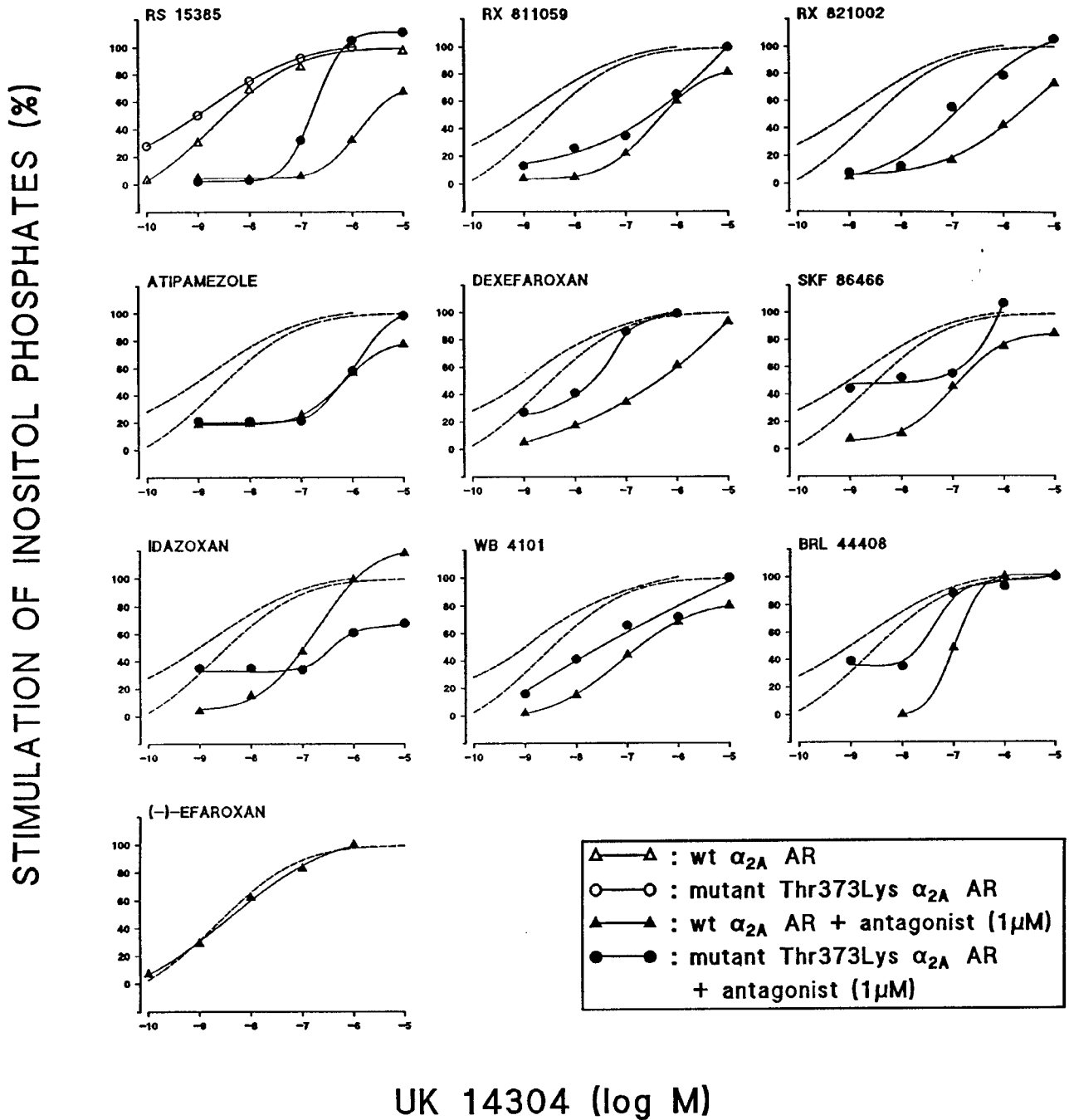


Figure 3 Antagonism of UK 14304-mediated stimulation of inositol phosphates formation by α_{2A} AR antagonists in transfected Cos-7 cells expressing either wt α_{2A} AR or mutant Thr³⁷³Lys α_{2A} AR and mouse $G_{\alpha_{15}}$ protein. Stimulation of UK 14304-mediated inositol phosphates formation in Cos-7 cells upon co-expression of mouse $G_{\alpha_{15}}$ was measured in either the absence and presence of 1 μ M antagonist as described in Methods. Representative curves are shown from 3–5 independent experiments. pK_B values are summarized in Table 6. In each panel, the dotted lines represent the UK 14304-mediated dose-responses for stimulation of inositol phosphates at wt α_{2A} AR and mutant Thr³⁷³Lys α_{2A} AR, as shown in the upper left-hand panel for RS 15385.

Table 7 Antagonist potencies of α_2 -adrenergic ligands against UK 14304-mediated stimulation of inositol phosphates formation in the presence of mouse $G_{\alpha 15}$ protein or inhibition of forskolin-induced cyclic AMP formation in transfected cells expressing either wt α_{2A} or mutant Thr³⁷³Lys α_{2A} AR

Ligand	wt α_{2A} AR Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells pK_B	wt α_{2A} AR Antagonism of UK 14304-mediated inhibition of forskolin-induced cyclic AMP formation in C6-glia cells pK_B	Mutant Thr ³⁷³ Lys α_{2A} AR Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells pK_B
RS 15385	9.32 ± 0.14	8.29 ± 0.18	8.47 ± 0.13*
RX 811059	8.74 ± 0.08	8.07 ± 0.15	8.05 ± 0.04**
MK 912		8.10 ± 0.26	
RX 821002	8.75 ± 0.07	8.07 ± 0.22	7.83 ± 0.09**
Atipamezole	8.76 ± 0.06	8.10 ± 0.26	
Dexefaroxan	8.27 ± 0.10	7.66 ± 0.32	
SKF 86466	7.69 ± 0.00	7.28 ± 0.20	
Idazoxan	7.70 ± 0.08	7.18 ± 0.24	
WB 4101	8.14 ± 0.05	7.08 ± 0.20	7.04 ± 0.11**
BRL 44408	8.23 ± 0.16	6.96 ± 0.22	

Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells in the presence of mouse $G_{\alpha 15}$ protein and inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells were measured as described in Methods. Mean pK_B values $\pm$ s.e.mean were obtained from 3–5 independent experiments, performed in triplicate. Statistical analysis was performed using Student's *t*-test: * $P < 0.01$, ** $P < 0.001$ versus pK_B values for antagonism of UK 14304-mediated stimulation of inositol phosphates at wt α_{2A} AR in Cos-7 cells.

or partial agonists at the mutant α_{2A} AR, depending on the co-expressed G-protein α -subunit.

Specificity of the α_{2A} AR-mediated inositol phosphates responses in Cos-7 cells

The following findings suggest that the observed inositol phosphates responses in transfected Cos-7 cells are mediated by stimulation of a human α_{2A} AR: (i) the absence of a UK 14304-mediated inositol phosphates response in the non-transfected cell type together with a lack of specific [³H] RX 821002 binding; (ii) the observed agonist stimulation of inositol phosphates at wt α_{2A} AR which is consistent with the agonist data on inhibition of forskolin-stimulated cyclic AMP formation in C6-glia cells; (iii) the competitive blockade of UK 14304-mediated responses by the various antagonists at the wt α_{2A} AR, and in particular the rank order of potency of dexefaroxan and its inactive enantiomer (–)-efaroxan, at concentrations similar to their binding affinities; and (iv) the correlation of the binding affinities for UK 14304 at the wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in membrane preparations of Cos-7 cells with those measured in transfected HEK 293 cells (Ren *et al.*, 1993). This further extends the observation that α_{2A} AR may mediate multiple second messenger pathways, including the stimulation of polyphosphoinositide hydrolysis (Cotecchia *et al.*, 1990). The ligand binding characteristics at the wt α_{2A} AR in either the absence or the presence of mouse $G_{\alpha 15}$ protein were similar, thus excluding a spontaneous coupling of the wt α_{2A} receptor to this particular G-protein. Inositol phosphates responses at wt α_{2A} AR could be more readily measured in the presence of mouse $G_{\alpha 15}$; the intrinsic activity of the various ligands was apparently not affected by this G-protein. However, the partial agonist effect for some of the antagonists at the mutant α_{2A} AR was only observed upon co-expression of mouse $G_{\alpha 15}$. Therefore, inositol phosphates responses mediated by the coupling of mutant α_{2A} AR to pertussis-toxin insensitive mouse $G_{\alpha 15}$ protein (Milligan *et al.*, 1996; Table 2) are distinct from the responses evoked by mutant α_{2A} AR- pertussis-toxin sensitive G protein coupling in Cos-7 cells. Specific coupling to multiple second messenger pathways by synthetic agonists has previously been observed for α_{2A} , α_{2B} and α_{2C} AR (Eason *et al.*, 1994). Differential G-

protein activation by agonists has been suggested for a splice variant of the pituitary adenylate cyclase activating polypeptide (PACAP) receptor (Spengler *et al.*, 1993) and the *Drosophila* octopamine-tyramine receptor (Robb *et al.*, 1994). In the present study, $G_{\alpha 15}$ protein activation by putative α_2 AR antagonists was observed. The concept that G-protein-coupled receptors can assume different conformations that can selectively and differentially permit their coupling to specific second messenger pathways also receives support from mutagenesis studies. Conversion of Asp79 to asparagine in the second transmembrane domain of the α_{2A} AR results in a selective uncoupling of the receptor from K^+ currents while retaining the receptor's ability to inhibit cyclic AMP production and open voltage-sensitive Ca^{2+} channels (Lakhilani *et al.*, 1996). Ligands may differentially affect multiple active receptor states. As a consequence, multiple receptor:G-protein interactions, each of them specific for a particular G-protein/effector pathway, can be induced and lead to different, maybe distinct, receptor-mediated responses. Therefore, the possibility exists for the development of new ligands for these receptors which could selectively activate a desired second messenger pathway and reduce collateral side effects mediated via other undesired pathways.

Partial agonist activity of antagonists at mutant G-protein-coupled receptors

Partial agonist activity of antagonists has been reported (Cho *et al.*, 1996; Groblewski *et al.*, 1997) at constitutively active mutant human dopamine D₁ and rat AT_{1A} angiotensin II receptors. R(+)SCH 23390, a classical antagonist at the wt D₁ receptor, behaves as a partial agonist at the mutant Leu²⁸⁶Ala D₁ receptor, with an efficacy reaching 40% of the maximal stimulation attained with the full D₁ agonist ($\pm$)SKF 82958. However, like ($\pm$)SKF 82958, R(+) SCH 23390 failed to demonstrate increased affinity at the mutated D₁ receptor, similar to our observations for the partial agonists at the mutant α_{2A} AR. In a similar way, the (Sar¹, Ile⁸)AII and (Sar¹, Ala⁸)AII derivatives, which are peptidic antagonists of the wt rat AT_{1A} angiotensin II receptor, display maximal activities of 52 and 27%, respectively, with unchanged affinities for the mutant Asn¹¹¹Ala AT_{1A} angiotensin II receptor. Otherwise,

induction of partial agonist properties of the AT₂-specific peptide ligand CGP 42112A [27% of the response to the agonist (Sar¹)AII] is associated with an increased affinity for the mutant AT_{1A} angiotensin II receptor (Grobowski *et al.*, 1997). Thus, with the exception of CGP 42112A, partial agonist activity was not accompanied by an increased binding affinity at this latter receptor. However, Noda *et al.* (1996) described a large increase in affinity with the antagonist (Sar¹, Ile⁸) A II for the mutant AT₁ angiotensin receptor. The apparent absence of increased binding affinity may be related to a lack of sensitivity in the models being investigated.

According to the extended ternary complex model of Lefkowitz and colleagues (Lefkowitz *et al.*, 1993; Samama *et al.*, 1993) and refined by Kenakin (1995), the receptor is capable of either spontaneous or ligand-facilitated isomerization to an activated state, which is then capable of coupling to G-proteins. It is hypothesized that the adoption of this activated conformation is favoured by certain mutations that display not only increases in basal activity but also increases in agonist activity, proportional to the intrinsic activity of the agonist. The model described by Lefkowitz and colleagues may hold for constitutively activating mutations that structurally mimic the ligand-activated state of the receptor throughout its entire tertiary structure. Different mutational substrates may very well result in different local effects on binding and G-protein-coupling geometries. It is conceivable that structural alterations near the ligand-binding pocket may modify the nature of ligand/receptor interactions altogether, such that some antagonists are capable of stabilizing the active conformation at the new binding interface generated by the mutation. The Leu²⁸⁶Ala mutation in transmembrane domain VI is within the ligand-binding transmembrane core of the D₁ receptor. The mutation Thr³⁷³Lys, located in the carboxyl-terminal region of the third intracellular loop of the α_2 A AR, seems to favour the coupling of the mutated receptor to the mouse G_{z15} protein. This receptor:G-protein interaction may modify the tertiary structure of the mutated receptor in such a way that some antagonists are capable of stabilizing the active receptor conformation, thereby generating positive efficacy. The Asn¹¹¹Ala mutation in transmembrane domain III of the AT_{1A} angiotensin II receptor is more difficult to interpret since peptidic ligands bind the receptor *via* both transmembrane residues and extracellular loops (Hjorth *et al.*, 1994; Noda *et al.*, 1995).

Partial agonist activity for certain antagonists has also been measured at mutated G-protein-coupled receptors which do not display constitutive activity. The inverse agonist 11-cis-retinal behaves as a partial agonist at the Gly¹²¹Leu mutation in transmembrane domain III of rhodopsin (Han *et al.*, 1997). The single amino acid substitution Asp¹¹³Glu in the third transmembrane domain of the hamster β_2 AR promotes partial agonist activity for pindolol, oxyprenolol and alprenolol, ligands that act as antagonists at the wt β_2 AR (Strader *et al.*, 1989). The other β AR antagonists being investigated did not demonstrate intrinsic activity at the mutant β_2 AR. However, the affinity of the agonist isoproterenol was also reduced 100 fold, suggesting that this mutant receptor has partially lost both affinity and specificity for activation by ligands. This contrasts with our binding data at the α_2 A AR where both ligand affinity and specificity are conserved. Strader *et al.* (1989) explained their findings by suggesting the existence of overlapping binding sites for agonists and antagonists. Claude *et al.* (1996) hypothesize that antagonists with high structural homology to agonists are capable of agonist activity if the receptor is permissive. This was based on their observation that the mutation of conserved serine residues (Ser¹⁷⁷Leu or Ser¹⁹⁶Leu) in transmembrane domain IV of μ -opioid receptors confers full agonistic properties to peptide (TIPP ϕ) and alkaloid antagonists (naloxone, naltrexone and naltriben). A similar explanation may also account for atipemazole and BRL 44408 in our study since their structures resemble the α_2 AR agonist medetomidine.

In conclusion, the results provided in this study imply that the pharmacology of constitutively active receptors is more complex than is commonly assumed. The mutant Thr³⁷³Lys α_2 A AR facilitates detection of partial agonist activity for a subclass of putative antagonists. This further supports the hypothesis that pure neutral antagonists may be relatively uncommon. Targeting of these mutant receptors may open new possibilities to manipulate the α_2 A AR receptor.

We acknowledge the cooperation of Mrs C. Palmier and Dr I. Rauly. We sincerely thank the excellent technical assistance of M.-C. Ailhaud, J.-C. Blanchet, C. Cathala, F. Finana and F. Lestienne, and fruitful secretarial assistance of S. Cecco.

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(Received July 23, 1998

Revised October 30, 1998

Accepted November 20, 1998)



Inhibition of the production of endothelium-derived hyperpolarizing factor by cannabinoid receptor agonists

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1 The endogenous cannabinoid, anandamide, has been reported to induce an 'endothelium-derived hyperpolarizing factor (EDHF)-like' relaxation *in vitro*. We therefore investigated the effects of cannabinoid CB1 receptor agonists; HU 210, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and anandamide, and a CB1 antagonist/inverse agonist, SR 141716A, on nitric oxide (NO) and EDHF-mediated relaxation in precontracted rings of porcine coronary, rabbit carotid and mesenteric arteries.

2 In rings of mesenteric artery HU 210 and Δ^9 -THC induced endothelium- and cyclo-oxygenase-independent relaxations which were sensitive to SR 141716A. Anandamide (0.03–30 μ M) induced a slowly developing, endothelium-independent relaxation which was abolished by diclofenac and was therefore mediated by cyclo-oxygenase product(s). None of the CB1 agonists tested affected the tone of precontracted rings of rabbit carotid or porcine coronary artery.

3 In endothelium-intact segments, HU 210, Δ^9 -THC and anandamide did not affect NO-mediated responses but under conditions of continuous NO synthase/cyclo-oxygenase blockade, significantly inhibited acetylcholine and bradykinin-induced relaxations which are attributed to the production of EDHF. The effects of HU 210 and Δ^9 -THC were not observed when experiments were performed in the presence of SR 141716A suggesting the involvement of the CB1 receptor.

4 In a patch clamp bioassay of EDHF production, HU 210 decreased the EDHF-mediated hyperpolarization of detector smooth muscle cells when applied to the donor segment but was without effect on the membrane potential of detector cells. The inhibition of EDHF production was unrelated to alterations in Ca^{2+} -signalling or cytochrome P450 activity.

5 These results suggest that the activation of endothelial CB1 receptors appears to be negatively coupled to the production of EDHF.

Keywords: Endothelium-derived hyperpolarizing factor (EDHF); nitric oxide (NO); cannabinoid CB1 receptor; electrophysiology

Abbreviations: ACh, acetylcholine; CB1, cannabinoid CB1 receptor; CYP, cytochrome P450; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; EDHF, endothelium-derived hyperpolarizing factor; HUVEC, human umbilical vein endothelial cells; L-NA, N^G -nitro-L-arginine; NO, nitric oxide

Introduction

Δ^9 -Tetrahydrocannabinol (Δ^9 -THC) has long been known to exert distinct effects on the cardiovascular system inducing a maintained decrease in blood pressure (Graham & Li, 1973; Estrada *et al.*, 1987). Δ^9 -THC- and the endogenously generated cannabinoid, anandamide, induce hypotension and bradycardia which are generally attributed to a centrally mediated reduction in sympathetic tone (Berdyshev *et al.*, 1996; Varga *et al.*, 1995). However, in anaesthetized rats the cannabinoid CB1 receptor agonist, HU 210, induces a long-lasting hypotension and bradycardia which cannot be attributed to sympathetic withdrawal (Vidrio *et al.*, 1996). Indeed, cannabinoid receptors have recently been reported in the peripheral organs (Galiegue *et al.*, 1995; Munro *et al.*, 1993) as well as in endothelial cells (Deutsch *et al.*, 1997). Thus, the activation of the peripheral CB1 receptor on endothelial cells may well influence vascular tone and the generation of endothelium-derived autacoids. While some of the effects of anandamide may be attributed to its metabolism to arachidonic acid and subsequently to cyclo-oxygenase or cytochrome P450 (CYP) products *via* a receptor-independent mechanism (Ellis *et al.*, 1995; Pratt *et al.*, 1998), others are clearly mediated by CB1 receptor activation (Hunter & Burstein, 1997). The recent discovery of cannabinoid

receptors and some of their endogenous ligands has in turn focused attention on the possible signalling pathways that may be activated by these seven transmembrane, G protein-coupled receptors. Evidence for at least three, apparently different pathways has been proposed and depending on the cell type under investigation, cannabinoids are able to stimulate eicosanoid synthesis to inhibit the activation of adenylyl cyclase and to affect Ca^{2+} signalling (Pertwee, 1997). CB1 receptors are also involved in G protein receptor cross talk and anandamide is, for example, able to inhibit the activation of 5-HT₃ receptors in rat nodose ganglion neurones (Fan, 1995).

Endothelial cells generate and release anandamide (Deutsch *et al.*, 1997) as well as a second endogenous cannabimimetic molecule, 2-arachidonoylglycerol (Sugiura *et al.*, 1998), and as such may also be involved in the local regulation of vascular responsiveness. Indeed, just such a role for anandamide was recently proposed when it was suggested that a CB1 agonist produced by endothelial cells could account for the, as yet still elusive, endothelium-derived hyperpolarizing factor (EDHF) (Randall *et al.*, 1996). The aim of the present study was to determine whether a crosstalk occurs between CB1 receptor activation or inactivation and endothelial autacoid production. To this end we studied the effects of CB1 receptor agonists (HU 210, Δ^9 -THC and anandamide) and the selective antagonist/inverse agonist SR 141716A (Bouaboula *et al.*,

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1997) on the vascular tone of three different arteries all of which synthesize both nitric oxide (NO) and an EDHF which is characterized as a CYP-dependent metabolite of arachidonic acid (Bauersachs *et al.*, 1994; Popp *et al.*, 1996; 1998).

Methods

Materials

Bradykinin was from Bachem Biochemica GmbH (Heidelberg, Germany), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and N^G-nitro-L-arginine (L-NA) from Serva (Heidelberg, Germany). Diclofenac (Voltaren injection solution) was from Ciba-Geigy, (Wehr, Germany), M-199 medium from GIBCO (Berlin, Germany), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α}) was provided by Upjohn (Ann Arbor, MI, U.S.A.) and SR 141716A was provided by Sanofi Research (Montpellier, France). Rp-cyclic AMPS was from Alexis Biochemicals (Grünberg, Germany), 3-(1,1-dimethylheptyl)-(-)-11-hydroxy- Δ^8 -tetrahydrocannabinol (HU 210) was purchased from Tokris Cookson (Langford, England). Arachidoylethanolamide (anandamide), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), acetylcholine (ACh), isobutylmethyl-xanthine (IBMX), sodium nitroprusside and all other chemicals were purchased from Sigma (Deisenhofen, Germany).

Vessel preparation

Porcine coronary arteries: Porcine hearts were obtained from a local slaughterhouse, placed immediately into ice-cold Krebs-Henseleit solution and transported to the laboratory. The coronary arteries were dissected, cleaned of adventitial adipose and connective tissue and cut into rings (4 mm) for organ bath studies. Coronary segments (40 mm length) were used for the bioassay of EDHF.

Rabbit carotid and mesenteric arteries: New Zealand White rabbits of either sex (1.5–2.5 kg body weight) were anaesthetized with pentobarbitone sodium (90 mg kg⁻¹ i.v.) and exsanguinated by cutting through both the abdominal aorta and vena cava. The carotid arteries and the main mesenteric artery were dissected, cleaned of adventitial adipose and connective tissue, and cut into rings (4 mm). In a number of experiments the endothelium was removed mechanically using blunt forceps.

Vascular reactivity studies Porcine coronary artery, rabbit carotid or mesenteric artery rings were mounted between force transducers (Scaime, France) and a rigid support for measurement of isometric force and incubated in organ baths containing warmed (37°C), oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit solution (pH 7.4) of the following composition (mM): NaCl, 119; NaHCO₃, 25.0; KCl, 4.7; CaCl₂, 1.6; KH₂PO₄, 1.2; MgSO₄, 1.2; glucose 12.

Porcine coronary arteries Passive tension was adjusted over a 60 min equilibration period to 5 g; thereafter the segments were exposed to a Krebs-Henseleit solution rich in KCl (80 mM) until stable contractions were obtained. After washing, arterial rings were exposed to U46619 (0.1–0.3 μ M) to achieve a stable contraction which was approximately 80% of the maximal KCl-induced contraction. Thereafter, the integrity of the endothelium was assessed by the application of bradykinin (1 μ M) and vessels exhibiting less than 90% relaxation were discarded.

Rabbit carotid and mesenteric arteries Passive tension was adjusted over a 60 min equilibration period (2 g for carotid arteries and 2.5 g for mesenteric arteries), thereafter the segments were exposed twice to K⁺-rich Krebs solution. After washing and a further equilibration period of 40–60 min the arterial rings were contracted to 80% of the maximal KCl response using phenylephrine (0.1–1 μ M). The integrity of the endothelium was assessed using ACh (1 μ M) and vessels exhibiting less than 90% relaxation were discarded. As some of the relaxant responses observed were transient, the concentration-response curves presented represent the peak response observed to cumulative applications of the agonists.

Cell culture Human umbilical vein endothelial cells were seeded on fibronectin-coated culture dishes containing medium M-199 and 20% heat-inactivated foetal calf serum (Greiner) supplemented with penicillin (50 u ml⁻¹), streptomycin (50 μ g ml⁻¹) and L-glutamine (1 mM). CYP activity, cyclic AMP levels and [Ca²⁺]_i were estimated in confluent cells.

Rat aortic smooth muscle cells were isolated and cultured as described (Popp *et al.*, 1996). Experiments were performed using confluent smooth muscle cells grown on glass coverslips between passages 6 and 16.

Electrophysiological measurements The membrane potential of cultured rat aortic smooth muscle cells was recorded using the slow whole-cell configuration of the patch-clamp technique. The patch pipettes used had an input resistance of 8–10 M Ω when filled with standard KCl solution (mM: KCl, 140; MgCl₂, 1; CaCl₂, 1.3; HEPES, 10; D-glucose, 5; pH 7.4). G Ω seals (approximately 10 G Ω) were established by gentle suction. The slow whole-cell configuration was obtained using nystatin (100 μ g ml⁻¹) in the pipette. This nystatin concentration provides a low resistance access to the cytosol to measure intracellular potentials under current clamp conditions. The electrical contact with the cytosol was established within 1–2 min of seal formation, during this time the input resistance decreased to approximately 1 G Ω . The cell membrane potential, measured in the current clamp mode, was recorded continuously and only detector smooth muscle cells which had a stable resting membrane potential for more than 2 min and exhibited no further change in the input resistance were used.

Detection of EDHF release The release of EDHF was detected by recording changes in membrane potential of cultured rat aortic smooth muscle cells exposed to the effluve of a perfused porcine coronary artery. The transit time between the coronary artery and the detector smooth muscle cells was approximately 2 s. Briefly, a segment of porcine coronary artery was cannulated at both ends, mounted in an organ chamber and perfused intraluminally (1 ml min⁻¹) with HEPES-modified Tyrode's solution of the following composition (in mM): NaCl, 132; KCl, 4; CaCl₂, 1; MgCl₂, 0.5; HEPES, 9.5 and glucose, 5 (pH 7.4, 37°C) containing the NO synthase inhibitor L-NA (100 μ M) and diclofenac (10 μ M). The perfusate from a bradykinin-stimulated, L-NA/diclofenac-treated porcine coronary artery was directed over cultured rat aortic smooth muscle cells, the membrane potential of which was recorded using the slow whole-cell configuration of the patch clamp technique as described above. The resting membrane potential as determined in the whole cell configuration was approximately -48.2 mV (n = 30) in cultured rat aortic smooth muscle cells. Continuous superfusion with HEPES-Tyrode (1 ml min⁻¹) did not alter the resting

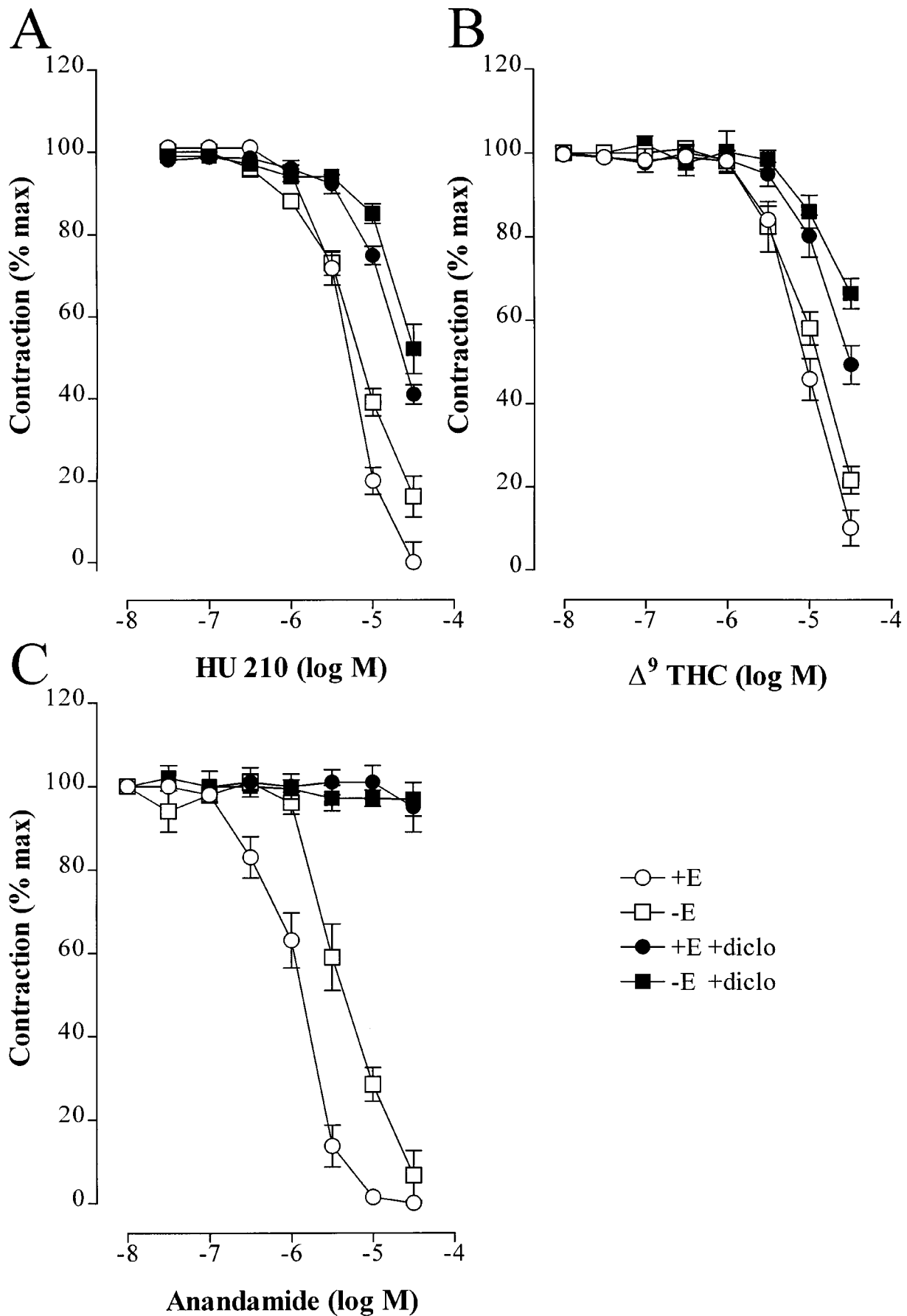


Figure 1 Effect of CB1 agonists on the tone of phenylephrine-precontracted mesenteric artery rings. Concentration-response curves showing the relaxant effect of (A) HU 210, (B) Δ^9 -THC and (C) anandamide on rings of rabbit mesenteric artery precontracted to 80% of the maximal KCl-induced response with phenylephrine. Experiments were performed using endothelium-intact (+E) and -denuded (–E) arteries in the absence and presence of diclofenac (diclo, 10 μ M). Results are presented as the mean $\pm$ s.e. mean of 12 separate experiments.

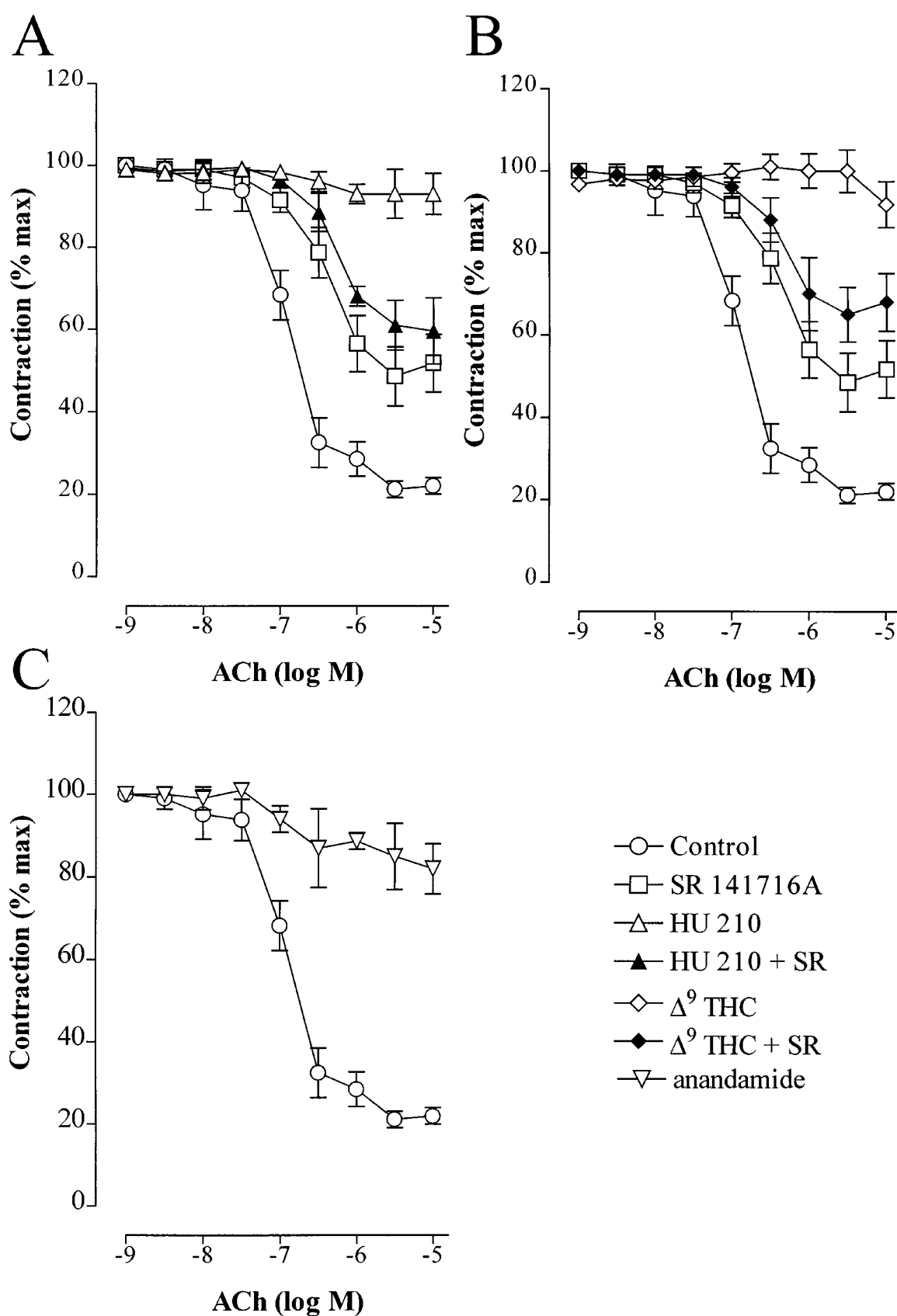


Figure 2 Effect of CB1 agonists on the EDHF-mediated relaxation of the rabbit mesenteric artery. Concentration-response curves to acetylcholine (ACh) were obtained using phenylephrine precontracted mesenteric artery rings in the presence of either solvent (0.06% ethanol), SR 141716A (30 μ M), HU 210 (30 μ M; A), Δ^9 -tetrahydrocannabinol (Δ^9 -THC, 30 μ M; B), anandamide (30 μ M; C) or combinations of CB1 agonist and SR 141716A. All experiments were performed in the continuous presence of N^G -nitro-L-arginine (100 μ M) and diclofenac (10 μ M) and the results are presented as the mean $\pm$ s.e. mean of nine separate experiments.

membrane potential. We have previously reported that freshly isolated porcine coronary smooth muscle cells and cultured rat aortic smooth muscle cells respond in a similar manner to the luminal incubate from endothelium-intact porcine coronary arteries (Popp *et al.*, 1998).

Cell culture Human umbilical vein endothelial cells (HUVEC), isolated from umbilical cords as described previously (Hopkins & Gorman, 1981) were seeded on coverslips in culture dishes containing medium M-199 and 20% heat-inactivated foetal calf serum supplemented with penicillin (50 u ml⁻¹), streptomycin (50 µg ml⁻¹), L-glutamine (1 mM), glutathione (5 mg ml⁻¹) and L(+)ascorbic acid (5 mg ml⁻¹) (GIBCO) and cultured until confluent.

Measurement of [Ca²⁺]_i For the measurement of [Ca²⁺]_i, HUVEC were loaded with the fluorescent Ca²⁺-sensitive dye fura 2 by incubation with 3 µM fura 2-AM and 0.025% (w/v) Pluronic F-127 at 37°C for 60 min. Thereafter the coverslips were washed in HEPES-Tyrode solution and [Ca²⁺]_i was determined fluorometrically in thermostatically controlled cuvette as described previously (Fleming *et al.*, 1996).

Measurement of cytochrome P450 (CYP) activity CYP-dependent metabolic activity was assayed as the dealkylation of 7-ethoxyresorufin (Yatomi *et al.*, 1992) in confluent HUVEC in which P450 activity had been induced by incubation with β-naphthoflavone (3 µM, 48 h). Briefly, cells were washed twice with HEPES-Tyrode and thereafter incubated with 7-ethoxyresorufin (3.4 µM) for 10 min. The supernatant was then removed and the resorufin formed was measured at an excitation of 522 nm and emission at 586 nm using a fluorescence spectrophotometer (Deltascan, Photon-Technology, Wedel, Germany). Cell-mediated conversion of ethoxyresorufin to resorufin was linear over at least 30 min and was abolished by the P450-inhibitor clotrimazole (3 µM).

Determination of cyclic AMP concentration In order to assess the effects of CB1 agonists on the isoprenaline-induced increase in HUVEC cyclic AMP, cells were pretreated with the phosphodiesterase inhibitor IBMX (0.1 mM) in the absence and presence of either HU 210 (30 µM) or Δ⁹-THC (50 µM). Cells were then incubated with isoprenaline (0.3 µM, 37°C), and after 5 min the incubation was stopped by the aspiration of the buffer and addition of 600 µl of ice cold 6% (v/v) trichloroacetic acid. Samples were left on ice for 30 min, cells and supernatants were then harvested by scraping and the precipitates centrifuged at 10,000 × g (15 min, 4°C). There-

after, the supernatants were extracted four times with 5 volumes of water-saturated diethyl ether and the concentration of cyclic AMP was determined by a specific radioimmunoassay. Cyclic AMP content is expressed as pmol per mg protein.

Statistical analysis Unless otherwise indicated data are expressed as mean ± s.e.mean. Statistical evaluation was performed using Student's *t*-test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test, or ANOVA for repeated measures where appropriate. Values of *P* < 0.05 were considered statistically significant.

Results

Effects of HU 210, Δ⁹-THC and anandamide on vascular tone

In phenylephrine-precontracted rabbit mesenteric artery rings, the selective non-hydrolysable CB1 agonist, HU 210, induced an endothelium-independent decrease in mesenteric artery tone which was slightly, but not significantly, attenuated by diclofenac (Figure 1A) and was abolished by the CB1 antagonist SR 141716A (data not shown). Δ⁹-THC also induced an endothelium-independent relaxation which was mostly insensitive to diclofenac (Figure 1B). The endocannabinoid, anandamide, elicited a slowly developing relaxation, with a threshold of 0.1 µM (Figure 1C). Although the removal of the endothelium induced a distinct rightward shift in the concentration-response curve to anandamide (EC₅₀ -6.0 ± 0.05 and -5.39 ± 0.02 M, in endothelium-intact and endothelium denuded rings respectively, *P* < 0.05), the maximal relaxation (R_{max}) of vessels precontracted to 80% of the maximal KCl-induced contraction was comparable in both the presence and absence of the endothelium. Inclusion of diclofenac (10 µM) in the organ bath abolished the anandamide-induced relaxation of both endothelium-intact and -denuded mesenteric artery rings (Figure 1C).

Neither HU 210, Δ⁹-THC nor anandamide relaxed rabbit carotid or porcine coronary artery rings (data not shown).

Effect of SR 141716A, HU 210, Δ⁹-THC and anandamide on EDHF-mediated relaxations

In endothelium-intact phenylephrine-contracted rabbit mesenteric artery rings under conditions of combined NO synthase/cyclo-oxygenase blockade (L-NA 100 µM; diclofenac 10 µM), ACh induced a concentration-dependent relaxation which was

Table 1 Effect of CB1 agonists and a CB1 antagonist on the ACh-induced, EDHF-mediated relaxation of the rabbit mesenteric and carotid arteries

	Mesenteric artery		Carotid artery	
	EC ₅₀ (M)	R _{max} (%)	EC ₅₀ (M)	R _{max} (%)
Control	-7.10 ± 0.25	78.00 ± 2.00	-7.18 ± 0.40	78.02 ± 4.06
SR 141716A	-6.41 ± 0.10	48.21 ± 6.20***	-6.49 ± 0.28*	65.23 ± 7.63
HU 210	-	3.30 ± 5.03***, †	-6.59 ± 0.25	28.00 ± 8.02***, †
HU 210 + SR 141716A	-6.31 ± 0.12	40.4 ± 7.6***	-6.53 ± 0.20	64.04 ± 8.25
Δ ⁹ -THC	-	8.2 ± 5.0***, †	-	4.44 ± 2.50***, †††
Δ ⁹ -THC + SR 141716A	-6.47 ± 0.31	33.8 ± 7.03***	-6.66 ± 0.33	45.25 ± 2.54
Anandamide	-	18.6 ± 6.33***	-6.58 ± 0.36	40.00 ± 8.31*

The results are presented as the mean ± s.e.mean of nine (mesenteric artery) and ten (carotid artery) experiments. *Indicates a significant difference from control (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) and † a significant difference between experiments performed in the absence and presence of SR 141716A (†*P* < 0.05, †††*P* < 0.001).

attributed to the synthesis and release of EDHF (Figure 2). The CB1 antagonist, SR 141716A (30 μ M) did not alter the basal tension of mesenteric arteries or significantly affect tone

in precontracted arteries. However, SR 141716 (30 μ M) attenuated the ACh-induced EDHF-mediated relaxation (Figure 2, Table 1).

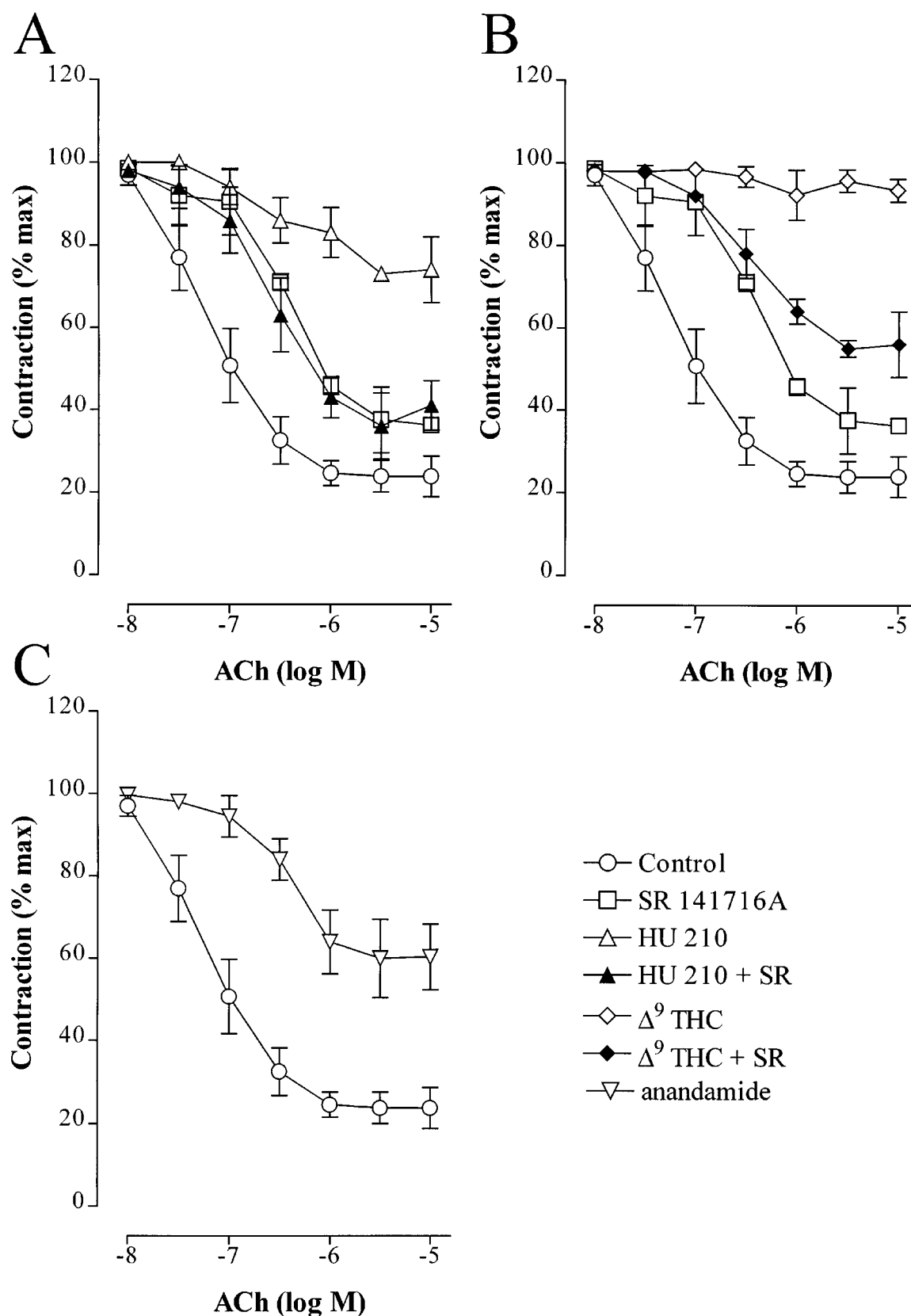


Figure 3 Effect of CB1 agonists on the EDHF-mediated relaxation of the rabbit carotid artery. Concentration-response curves to acetylcholine (ACh) were obtained using phenylephrine precontracted carotid artery rings in the presence of either solvent (0.06% ethanol), SR 141716A (30 μ M), HU 210 (30 μ M; A), Δ^9 -tetrahydrocannabinol (Δ^9 -THC, 30 μ M; B), anandamide (30 μ M; C) or combinations of CB1 agonist and SR 141716A. All experiments were performed in the continuous presence of N^G-nitro-L-arginine (100 μ M) and diclofenac (10 μ M) and the results are presented as the mean $\pm$ s.e. mean of ten separate experiments.

The ACh-induced, EDHF-mediated relaxations of mesenteric arteries was concentration-dependently inhibited by HU 210 (1–30 μM) and was abolished at the highest concentration studied. This effect was not observed in experiments performed in the presence of SR 141716A (Figure 2A, Table 1). The sensitivity of arterial rings to an exogenous NO donor (sodium nitroprusside, 1 μM) was unaffected by HU 210 (R_{max} was $103 \pm 6\%$ and $98 \pm 10\%$ in vessels with solvent and HU 210 respectively, $n=9$). Similarly, Δ^9 -THC (30 μM ; Figure 2B) also resulted in the SR 141716A-sensitive inhibition of EDHF-mediated relaxation, while the anandamide-induced inhibition (30 μM ; Figure 2C) was not sensitive to SR 141716A (not shown).

In the rabbit carotid artery, which did not relax in response to either HU 210, Δ^9 -THC or anandamide, all of these agents inhibited EDHF-mediated relaxation to a similar extent as in the rabbit mesenteric artery (Figure 3, Table 1). Similar results were obtained using the porcine coronary artery.

To determine whether the effect of the CB1 agonists on EDHF-mediated relaxations was specific, the NO-mediated relaxation to ACh was determined in the absence and presence of HU 210 (30 μM , Figure 4). The CB1 agonist failed to affect the ACh-induced, NO-mediated relaxation of vessels precontracted to 80% of the maximal KCl-induced contraction, demonstrating that these compounds selectively affect EDHF production. The subsequent addition of L-NA completely abolished ACh-induced relaxation (not shown). Although the second concentration response curve to ACh was shifted slightly to the right with respect to the first (Figure 4), this was the case in vessels treated with either solvent or HU 210. Similar results were obtained using the rabbit carotid artery (data not shown).

Effect of HU 210 on the production and activity of EDHF

A patch clamp bioassay for EDHF production was used to assess the effects of the CB1 agonists on the production and actions of EDHF. In this model the perfusate from a bradykinin-stimulated, L-NA/diclofenac-treated porcine coronary artery is directed over cultured rat aortic smooth muscle

cells, the membrane potential of which is continuously monitored using the patch clamp technique (Popp *et al.*, 1996; Bauersachs *et al.*, 1996).

The effects of 'authentic EDHF', anandamide and HU 210 on smooth muscle membrane potential were determined. The EDHF present in the perfusate of bradykinin-stimulated arteries induced an immediate biphasic change in smooth muscle membrane potential which consisted of an initial hyperpolarization of -11.4 ± 1.0 mV, followed by a depolarization of 4.7 ± 1.0 mV, as previously described (Popp *et al.*, 1996). Anandamide used at a concentration (30 μM) which induced complete relaxation of rabbit mesenteric artery rings induced a small and slowly developing smooth muscle cell hyperpolarization which reached a maximum of -3.04 ± 0.5 mV approximately 3 min after its application (Figure 5A). Neither the resting membrane potential nor the magnitude of the EDHF-induced hyperpolarization were markedly altered when detector cells were pre-treated with HU 210 (30 μM) or the CYP inhibitor miconazole (3 μM , Figure 5B). However, incubation of donor segments with either HU 210 or miconazole attenuated the production of EDHF, as demonstrated by a decrease in the hyperpolarization of detector smooth muscle cells induced by the perfusate from HU 210 or miconazole-treated, bradykinin-stimulated porcine coronary artery segments (Figure 5B).

Effects of CB1 agonists on endothelial $[\text{Ca}^{2+}]_i$, CYP activity and cyclic AMP levels

Since HU 210 decreased the synthesis and/or release of EDHF we determined its effect on endothelial $[\text{Ca}^{2+}]_i$ and CYP activity. These responses were assessed using human umbilical vein endothelial cells which produce an EDHF which we have previously characterized as a CYP-dependent metabolite of arachidonic acid (Popp *et al.*, 1996). In fura 2-loaded human umbilical vein endothelial cells, bradykinin (10 nM) induced a biphasic increase in $[\text{Ca}^{2+}]_i$ as previously described (Fleming *et al.*, 1995). Neither basal $[\text{Ca}^{2+}]_i$ nor the bradykinin-induced Ca^{2+} -response were altered by HU 210 (50 μM , Figure 6A) irrespective of whether experiments were performed in the absence or presence of L-NA and diclofenac (data not shown).

As the production of EDHF by all of the arteries tested as well as that produced by cultured endothelial cells stimulated with bradykinin (Bauersachs *et al.*, 1994; 1996; Popp *et al.*, 1996), could be attenuated by inhibitors of CYP we determined whether the CB1 agonists affected P450 activity in cultured human umbilical vein endothelial cells. CYP activity, assayed as the dealkylation of 7-ethoxyresorufin, was slightly attenuated by anandamide and Δ^9 -THC. HU 210 (30 μM) did not affect ethoxyresorufin dealkylase activity in intact endothelial cells, whereas miconazole inhibited the formation of resorufin by approximately 70% (Figure 6B). None of the CB1 agonists tested inhibited CYP activity in purified endothelial cell microsome preparations (data not shown).

As CB1 receptors are negatively coupled to adenylyl cyclase, we determined the effect of the CB1 agonists on the isoprenaline-induced increase in endothelial cyclic AMP. In confluent human umbilical vein endothelial cells HU 210 markedly reduced cyclic AMP levels under basal conditions and following stimulation with isoprenaline. Δ^9 -THC induced a similar, though less pronounced effect on cyclic AMP levels (Figure 6C).

In order to determine whether an increase in cellular cyclic AMP is a prerequisite for the production of EDHF we performed experiments using a specific protein kinase A inhibitor. Rp-cyclic AMPs (25 μM) was without effect on the

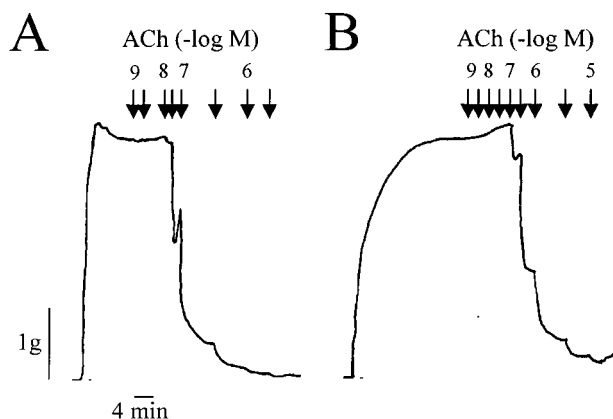


Figure 4 Effect of HU 210 on the acetylcholine-induced, NO-mediated relaxation of precontracted rabbit mesenteric arteries. Concentration-response curves to acetylcholine (1 nM to 3 μM) were obtained in rings of rabbit mesenteric artery precontracted to 80% of the maximal KCl-induced tone with phenylephrine. Experiments were performed in (A) the absence (0.06% ethanol) and (B) the presence of HU 210 (30 μM). Diclofenac (10 μM) was present throughout. The tracings shown are representative of results obtained in four separate experiments.

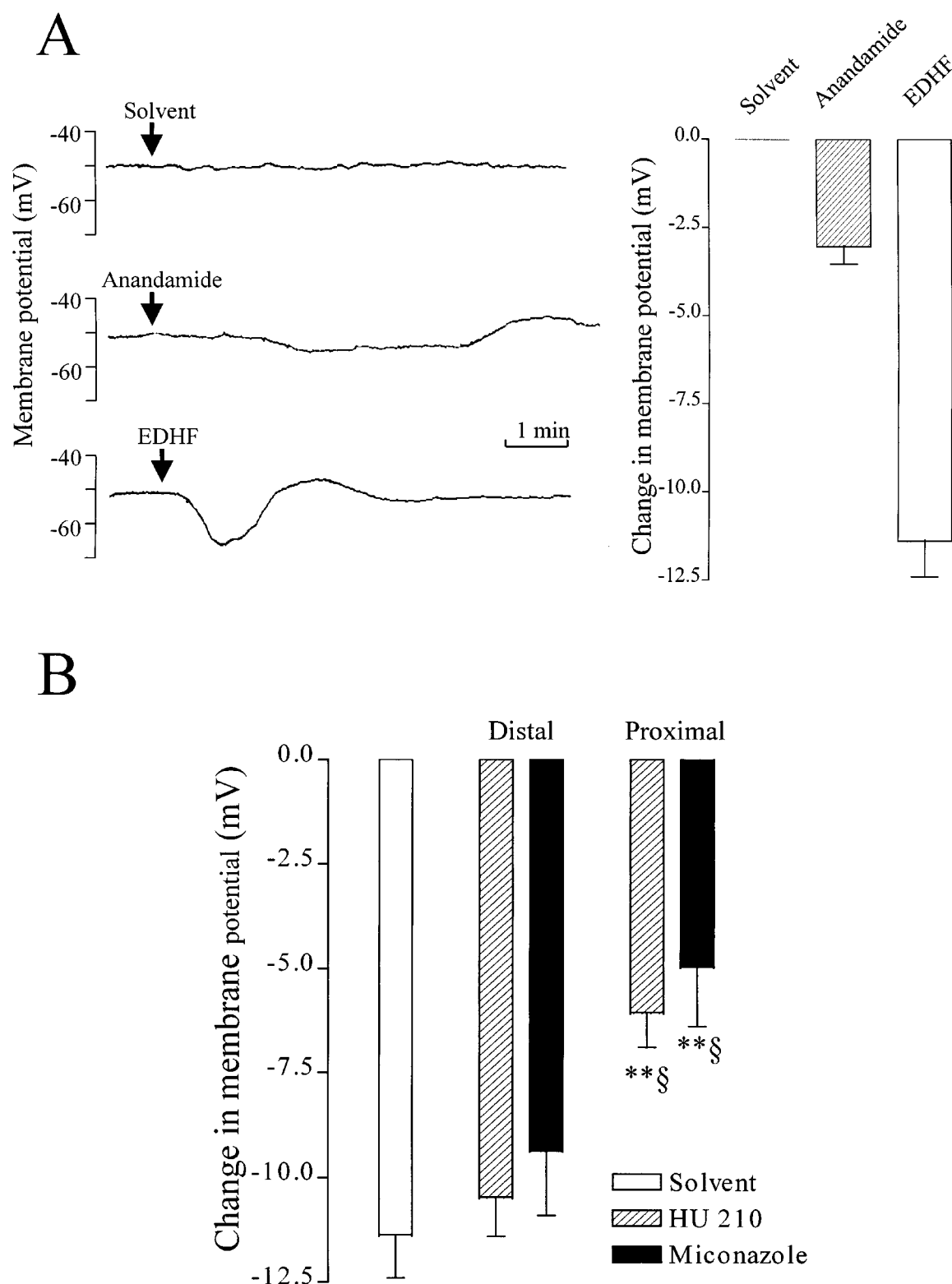


Figure 5 Effect of anandamide and HU 210 on smooth muscle membrane potential and the generation of EDHF from porcine coronary arteries. (A) Comparison of the effects on anandamide and 'authentic EDHF' on the membrane potential of cultured rat aortic smooth muscle cells. Original tracings (left panel) and statistical summary (right panel) illustrating the hyperpolarization of rat aortic smooth muscle cells induced by either the direct application of solvent (0.06% ethanol), anandamide (30 μ M) or the luminal perfusate removed from bradykinin-stimulated porcine coronary artery segments in the continuous presence of N^G-nitro-L-arginine (100 μ M) and diclofenac (10 μ M). Results are expressed as the mean $\pm$ s.e. mean of four separate experiments. (B) Effect of HU 210 and miconazole on the generation of EDHF by bradykinin-stimulated porcine coronary arteries. The membrane potential of cultured vascular smooth muscle cells superfused with the effluent from bradykinin-stimulated porcine coronary arteries was monitored (open columns). Experiments were performed in the absence (0.06% ethanol) and presence of HU 210 (30 μ M) and miconazole (3 μ M) added to the perfusate either proximal or distal to the donor segment (incubation time 15 min). Results are presented as the mean $\pm$ s.e. mean of six separate experiments, ** P < 0.01 vs control and § P < 0.05 vs response when the substance was applied distal to the donor artery.

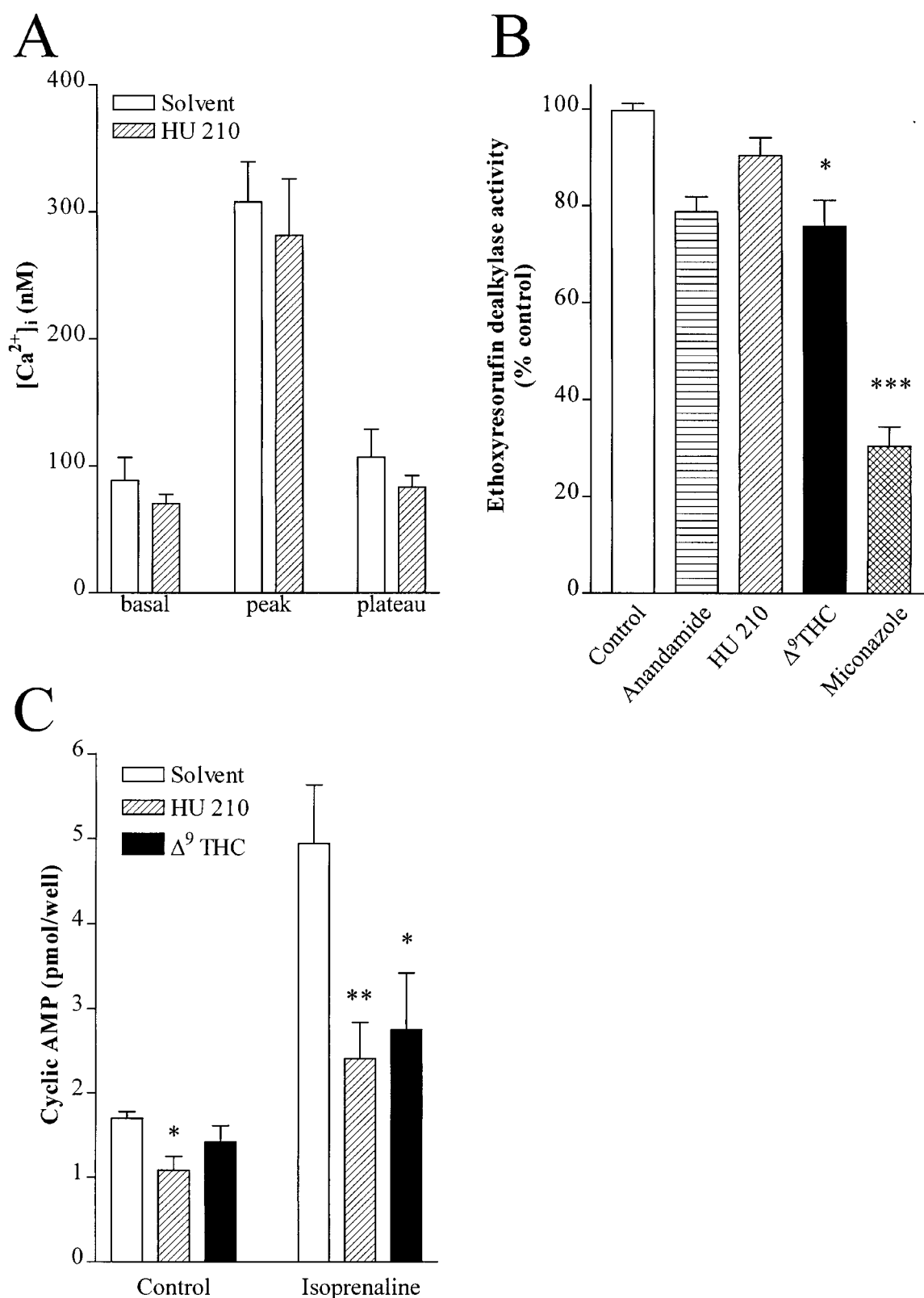


Figure 6 Effect on CB1 agonists on endothelial $[Ca^{2+}]_i$, P450 activity and cyclic AMP levels. (A) Basal $[Ca^{2+}]_i$ and the bradykinin (10 nM)-induced Ca^{2+} response in fura 2-labelled human endothelial cells. Experiments were performed in the absence (0.06% ethanol) and presence of HU 210 (30 μ M). Results are presented as the mean $\pm$ s.e. mean of eight separate experiments. (B) Ethoxyresorufin dealkylase activity was assessed in endothelial cells pre-incubated with either solvent, anandamide (30 μ M), HU 210 (30 μ M), Δ^9 -THC (30 μ M) or miconazole (3 μ M). Results are presented as the mean $\pm$ s.e. mean of five separate experiments. (C) Intracellular cyclic AMP levels in endothelial cells under basal and isoprenaline (0.3 μ M)-stimulated conditions. Experiments were performed in the absence and presence of HU 210 (30 μ M) or Δ^9 -THC (30 μ M). Results are presented as the mean $\pm$ s.e. mean of six separate experiments * P < 0.05, ** P < 0.01, *** P < 0.001 vs the appropriate solvent.

EDHF-mediated relaxation of the phenylephrine-precontracted rabbit mesenteric artery (data not shown).

Discussion

The results of the present study demonstrate that in rabbit mesenteric and carotid arteries as well as porcine coronary arteries, the CB1 agonists HU 210, Δ^9 -THC and anandamide inhibit the production of EDHF without affecting the synthesis of NO. The inhibitory effect of these agonists on EDHF production was not related to changes in endothelial $[Ca^{2+}]_i$ or CYP activity and could be reversed by the inclusion of the CB1 receptor antagonist, SR 141716A. Thus, the activation of endothelial CB1 receptors appears to be negatively coupled to the production of EDHF.

Anandamide has been reported to dilate rat mesenteric and coronary vascular beds *via* an EDHF-like (i.e. NO/ PGI_2 -independent) mechanism (Randall *et al.*, 1996; Randall & Kendall, 1997). The observation that the selective CB1 antagonist SR 141716A inhibited EDHF-mediated relaxations in these preparations led to the suggestion that EDHF may be an endocannabinoid. This hypothesis is however a matter of debate, as other investigators have since reported that anandamide-induced relaxations are not sensitive to SR 141617A and appear not to be mediated by the CB1 receptor or Ca^{2+} -dependent K^+ channel activation (Plane *et al.*, 1997; Chataigneau *et al.*, 1998). Although anandamide does hyperpolarize vascular smooth muscle cells, the response obtained in the present study bore characteristics markedly different to that induced by EDHF, being delayed in onset and exhibiting a different kinetic. Anandamide also relaxed rabbit mesenteric arteries but as this effect was sensitive to diclofenac the reduction in vascular tone was more likely to be mediated by a cyclo-oxygenase product. Indeed, the cyclo-oxygenase-dependent metabolism of anandamide underlies its vasodilator action in cerebral arterioles (Facci *et al.*, 1995) and bovine coronary arteries (Pratt *et al.*, 1998; Ellis *et al.*, 1995).

While the discussion relating to the role played by anandamide in EDHF-mediated relaxations seems to be concluded, endocannabinoids do appear to play a significant role in the regulation of vascular tone in certain vascular beds. In the rat kidney, which has previously been reported to express both anandamide amidase and the CB1 receptor (Deutsch & Chin, 1993; Shire *et al.*, 1995), anandamide vasodilates juxtamedullary afferent arterioles by a process involving the generation of endothelium-derived NO (Deutsch *et al.*, 1997). This anandamide-induced production of NO inhibited the release of norepinephrine from sympathetic nerve endings in the renal artery (Deutsch *et al.*, 1997) demonstrating that anandamide possesses both vasorelaxant and neuromodulatory properties. Similarly, anandamide is also reported to stimulate the production of NO from the vascular endothelium of the rat portal plexus (Prevot *et al.*, 1998). The effects of CB1 agonists appear to vary with the vascular bed under investigation since none of the CB1 agonists used affected tone in rabbit carotid or porcine coronary arteries. Only rings from the rabbit mesenteric artery relaxed in response to CB1 agonists. HU 210 and Δ^9 -THC relaxed both endothelium-intact and denuded mesenteric arterial rings to a similar extent, thus excluding a role for endothelium-derived NO. The inclusion of diclofenac shifted the concentration-response curve of both agonists to the right suggesting that both cyclo-oxygenase-dependent and -independent processes were involved in mediating the response observed. However, the fact that the relaxation induced by HU 210 and Δ^9 -THC was

resistant to L-NA and diclofenac does not suggest that these agents represent an EDHF-like substance as neither compound hyperpolarized vascular smooth muscle cells.

The data in Figures 2 and 3 clearly show that SR 141716A alleviated (between 70 and 100%) the inhibition of EDHF-mediated responses by HU 210 and Δ^9 -THC suggesting that this effect may be mediated by the CB1 receptor. However, since high concentrations of the inverse agonist (i.e., a compound which exhibits biological activity by blocking the signal transduction mediated by constitutively activated receptors) were required to observe any effect, we cannot rule out the possibility that the effects observed may be indirect. Indeed, high concentrations of SR 141716A have recently been reported to block the agonist-induced release of arachidonic acid (Pratt *et al.*, 1998) and to hyperpolarize guinea-pig carotid arteries and thus attenuate the hyperpolarization observed in response to ACh (Chataigneau *et al.*, 1998).

The patch clamp bioassay, in which HU 210 attenuated EDHF-mediated hyperpolarizations only when applied to the donor artery, provided the most convincing evidence that CB1 agonists interfere with the generation of EDHF by endothelial cells rather than its effect on smooth muscle membrane potential. Exactly how the CB1 agonists interfere with the production of EDHF is at the moment unclear but cannabinoids are able to intercalate into cell membranes and have been reported to alter the properties of cellular proteins including G protein-coupled receptors. Such effects are however unlikely to account for the results observed in the present study as neither anandamide nor HU 210 markedly altered basal $[Ca^{2+}]_i$ or the bradykinin-induced Ca^{2+} -response in cultured human umbilical vein endothelial cells, which express mRNA for the CB1 receptor (data not shown), and neither Δ^9 -THC nor HU 210 affected ACh or bradykinin-induced, NO-mediated relaxations.

Given that the exact chemical identity of EDHF remains to be elucidated, it is difficult to investigate the signal transduction cascade which results in the activation of the 'EDHF synthase'. Current opinion is that the synthesis of EDHF is secondary to the activation of phospholipase A_2 (PLA_2), and the liberation of arachidonic acid (Quilley *et al.*, 1997). Indeed, the synthesis of EDHF is thought to be Ca^{2+} -dependent by virtue of the reported Ca^{2+} -dependency of PLA_2 . Thereafter, the signalling pathway is obscure and although EDHF may be generated from arachidonic acid by CYP enzymes in some vascular beds this is almost certainly not the case in others (Mombouli & Vanhoutte, 1997; Quilley *et al.*, 1997). Since the EDHF produced in all of the arteries under investigation has been pharmacologically characterized as a CYP-dependent metabolite of arachidonic acid, it seemed plausible to suggest that the CB1 agonists attenuated EDHF production by decreasing the activity of the 'EDHF synthase'. However, although cannabidiol has been reported to inactivate several CYP enzymes (Bornheim *et al.*, 1993), and anandamide and Δ^9 -THC did attenuate ethoxyresorufin dealkylase activity in intact endothelial cells, HU 210 was without effect. Thus CYP inhibition seems unlikely to be the major mechanism by which the CB1 agonists attenuate the production of EDHF. It is nonetheless impossible to rule out the possibility that HU 210, Δ^9 -THC and anandamide affect the activity of CYP enzymes which do not metabolise 7-ethoxyresorufin.

The binding of anandamide to cannabinoid receptors leads to the inhibition of adenylyl cyclase and anandamide has been shown to inhibit forskolin-stimulated cyclic AMP accumulation in a number of cell systems expressing the CB1 receptor (Felder *et al.*, 1993; Vogel *et al.*, 1993; Childers *et al.*, 1994). One previous study showed that isoprenaline induced an

endothelium-dependent relaxation in rat aortic rings which appeared to be mediated by the release of a CYP-dependent metabolite of arachidonic acid (Satake *et al.*, 1997). Although HU 210 and Δ^9 -THC attenuated both basal cyclic AMP levels as well as the isoprenaline-stimulated increase in cyclic AMP in cultured endothelial cells, a selective inhibitor of protein kinase A did not affect EDHF-mediated dilation.

Novel effects of CB1 receptor activation are continuously being described, for example it was recently reported that coupling exists between CB1 receptors and receptor tyrosine kinase-activated transduction pathways (Bouaboula *et al.*, 1997). Since SR 141716A has been shown to interfere with the activation of mitogen-activated protein (MAP) kinases following receptor tyrosine kinase activation by insulin and IGF1 (Bouaboula *et al.*, 1997), it is feasible that CB1 agonists affect signalling pathways which may be involved in the generation of EDHF. Although it is tempting to suggest that CB1 agonists interfere with the signal transduction pathway initiated by ACh and bradykinin, such a general effect cannot account for the selective inhibition of EDHF production. The only way that signal crosstalk could influence the production of EDHF selectively is if the pathways for EDHF production are regulated independently of that resulting in an increase in NO production. Such an idea would contradict the general concept that EDHF synthesis is activated simply in response to an increase in $[Ca^{2+}]_i$.

Assuming that an increase in $[Ca^{2+}]_i$ is the essential signal to initiate the generation of EDHF, one might speculate that the CB1 agonists interfere with EDHF-mediated responses by uncoupling cell-cell communication. The possibility that

EDHF is a substance which is capable of passing through gap junctions is not necessarily at odds with the observations made using bioassay models, as it is conceivable that this factor may spill over into the extracellular fluid. Anandamide has been reported to inhibit gap junction conductance and dye permeability in astrocytes in a pertussis toxin-sensitive manner (Venance *et al.*, 1995; Schmilinsky-Fluri *et al.*, 1997). Moreover, cell-cell communication in connexin 43-expressing cells is rapidly and transiently disturbed by G protein-coupled receptors (Postma *et al.*, 1998) via a process involving the activation of $p60^{src}$. Other indications that CB1 agonists interfere with gap junction conductance can be indirectly inferred from observations that MAP kinases are involved in the regulation of connexin 43-based cell-cell communication (Kanemitsu & Lau, 1993) and that anandamide (Wartmann *et al.*, 1995) and HU 210 (authors unpublished observations) can activate MAP kinase in cells expressing the CB1 receptor. Given that peptides homologous to extracellular loop motifs of connexin 43 attenuate EDHF-mediated relaxations (Chaytor *et al.*, 1997), an inhibitory effect of CB1 agonists on myo-endothelial and/or inter-endothelial gap junctions is an attractive hypothesis, currently under investigation, which may explain the abolition of EDHF-mediated relaxations by HU 210 and Δ^9 -THC.

The authors are indebted to Isabel Winter for expert technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (SFB 553, B1) and the Commission of the European Communities (BMH4-CT96-0979).

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(Received October 15, 1998

Revised November 17, 1998

Accepted November 20, 1998)



Effect of cerivastatin sodium, a new inhibitor of HMG-CoA reductase, on plasma lipid levels, progression of atherosclerosis, and the lesional composition in the plaques of WHHL rabbits

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1 The aim of this study was to examine whether cerivastatin sodium, a new inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, affects the lesional composition of spontaneously developed atherosclerosis due to hypercholesterolaemia and delays progression of the lesions.

2 We administered cerivastatin to 2-month-old WHHL rabbits, a low-density lipoprotein receptor-deficient animal model, at a dose of 0.6 mg kg⁻¹ day⁻¹ for 32 weeks. We examined the plasma lipid levels, the severity of atherosclerosis, and composition of atherosclerotic lesions. Lesional composition was determined using immunohistostaining for macrophages and smooth muscle cells, and Azan-Mallory staining for collagen fibres and extracellular lipid deposits.

3 Compared to the control group, the plasma cholesterol levels were decreased in the treated group by 39% (12.7 ± 0.6 mmol L⁻¹ versus 20.9 ± 1.0 mmol L⁻¹, *P* < 0.001). Atherosclerosis was suppressed by about 37% as measured by the thickness of the aortic lesions (158 ± 13 µm versus 250 ± 15 µm, *P* < 0.001), and by 28% as measured by coronary stenosis (62.7 ± 11.4 versus 86.9 ± 12.2, *P* < 0.05). In the cerivastatin group, regarding the per cent areas of lesional components in the lesion area, the macrophages (21.0 ± 1.5% versus 27.9 ± 1.9%, *P* < 0.01) and extracellular lipid deposits (3.2 ± 0.4% versus 5.1 ± 0.4%, *P* < 0.001) were decreased in the aortic lesions, and the per cent area of macrophages in the coronary lesions was also decreased (4.9 ± 1.4% versus 11.6 ± 2.4%, *P* < 0.05). The per cent area of smooth muscle cells and collagen fibres did not significantly decrease.

4 These results indicate that cerivastatin contributed to the plaque stabilization and delayed progression of early atherosclerosis in young WHHL rabbits, in addition to the potent hypolipidemic effects.

Keywords: Cerivastatin; HMG-CoA reductase inhibitor; plasma cholesterol; atherosclerosis; coronary stenosis; macrophage; smooth muscle cell; WHHL rabbit

Abbreviations: AUC, area under concentration curve; EDTA, ethylenediaminetetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high-pressure liquid chromatography; LCX, left circumflex artery; LMT, main trunk of left coronary artery; RCA, right coronary artery; RCO, origin portion of right coronary artery

Introduction

Clinical trials have demonstrated that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase prevent development of coronary events, in addition to having a hypolipidemic effect (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995; Downs *et al.*, 1998; Rosenson & Tangney, 1998). Vaughan *et al.* (1996) reported that HMG-CoA reductase inhibitors do more than just lower cholesterol regarding prevention of coronary events.

We have previously shown that an inhibitor of HMG-CoA reductase, pravastatin sodium, reduces plasma cholesterol levels, and suppresses atherosclerosis in WHHL rabbits, a low-density lipoprotein receptor-deficient animal model (Watanabe *et al.*, 1988; Shiomi *et al.*, 1990; 1995; 1998). In our recent study using mature WHHL rabbits that suffered from established atherosclerosis, pravastatin stabilized atherosclerotic plaques (Shiomi *et al.*, 1995). WHHL rabbits are a useful animal model for examination of antiatherosclerotic effects of drugs, since atherosclerosis is developed due to spontaneous hypercholesterolemia (Watanabe, 1980; Shiomi *et al.*, 1992).

Recently, it was reported that cerivastatin sodium (cerivastatin, BAY w 6228), a newly synthesized inhibitor of

HMG-CoA reductase, potently inhibits the activity of HMG-CoA reductase in the microgram range (Angerbauer *et al.*, 1994; Bischoff *et al.*, 1997). In a study of inhibition of cholesterol synthesis in rat hepatocytes, the dose of cerivastatin causing 50% inhibition was less than one-hundredth of the required dose of lovastatin (Bischoff *et al.*, 1997). In addition, cerivastatin did not affect the blood pressure and heart rate in dogs or the hematological parameters of rats (Dr H. Bischoff, Bayer AG, Germany, personal communication). On the other hand, *in vitro* studies demonstrated that although pravastatin has a weak effect on inhibition of smooth muscle cell growth, cerivastatin caused a potent inhibition of proliferation of human smooth muscle cells (Negre-Aminou *et al.*, 1997; Corsini *et al.*, 1996). Igarashi *et al.* (1997a,b) also reported that cerivastatin suppressed neointimal thickening by attenuating intimal smooth muscle cell proliferation and infiltration of macrophages in balloon-injured rabbit models. In addition, Wada *et al.* (1996) reported that the plasma tissue factor levels and tissue factor pathway inhibitor levels were decreased in 21 Japanese patients treated with cerivastatin, although the other coagulation parameters did not change.

The inhibitory effect on smooth muscle cell proliferation varies among various HMG-CoA reductase inhibitors. In our previous observations, pravastatin sodium, an HMG-CoA

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reductase inhibitor which shows a low inhibitory effect on smooth muscle cell proliferation *in vitro* (Corsini *et al.*, 1996) caused no decrease in the smooth muscle cell content in the atheromatous plaques of WHHL rabbits. On the other hand, fluvastatin sodium, an inhibitor of HMG-CoA reductase which potently inhibits smooth muscle cell proliferation *in vitro* (Corsini *et al.*, 1996) reduced the smooth muscle cell content in the atheromatous plaque of WHHL rabbits (Shiomi *et al.*, 1998). Several researchers have discussed the conclusion that inhibition of smooth muscle cell proliferation is unfavourable for the prevention of coronary events (Van der Wal *et al.*, 1994; Davies, 1995; Libby, 1995; Weissberg *et al.*, 1996). Therefore, we considered it important to examine whether cerivastatin affects the lesional composition of atherosclerosis, especially smooth muscle cells.

In this study, we examined whether cerivastatin affects lesional composition, especially smooth muscle cells, in the spontaneously developed atheromatous plaques due to hypercholesterolaemia, and confirmed the preventive effect on atherosclerosis. In addition, we compared the effects of cerivastatin on the lesional composition of atherosclerosis with those of other HMG-CoA reductase inhibitors previously studied.

Methods

Reagents

Cerivastatin was supplied by Bayer Yakuhin, Ltd. (Osaka, Japan). HHF35, a monoclonal antibody specific for muscle actin, was purchased from Enzo Diagnostics, Inc. (NY, U.S.A.), RAM-11, a monoclonal antibody specific for rabbit macrophages, was purchased from Dako A/S (Glostrup, Denmark), and avidin-biotinylated enzyme complex (ABC) kits was purchased from Vector Laboratories Inc. (CA, U.S.A.).

Animals

In order to measure plasma cerivastatin concentration, we used 12 normal male Japanese white rabbits aged 3 months (Kitayama Labes Co., Ltd., Ina, Nagano Prefecture, Japan). To examine the antiatherogenic effects of cerivastatin, we used 20 WHHL rabbits aged 2 months, which were bred at Kobe University and had a high incidence of severe coronary atherosclerosis (Shiomi *et al.*, 1992). These rabbits were divided into control group (five females and five males) and cerivastatin group (five females and five males) so as to make the plasma lipid level, body weight, and litter distribution uniform. The rabbits in the cerivastatin group were subcutaneously injected with cerivastatin solution (1 mg ml^{-1} in saline) at a dose of $0.6 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 32 weeks. Since plasma drug concentration does not increase in rabbits when cerivastatin is given orally, we administered the drug to rabbits by subcutaneous injection. The control rabbits were injected subcutaneously with saline. Rabbits were housed individually in metal cages in a room maintained at a constant temperature and humidity, and were fed 100 g of laboratory diet (RC4, Oriental Yeast Co., Tokyo, Japan) per day. Water was given *ad libitum*. All aspects of animal experimentation and care were conducted according to the Guidelines for Animal Experimentation of the Kobe University School of Medicine, the Law Concerning the Protection and Control of Animals (Law No. 105, 1973), and Standards Relating to the Care and

Management, etc. of Experimental Animals (Notification No. 6, 1980).

Plasma lipid measurement

Blood samples were taken from the marginal ear vein after overnight fasting and plasma was obtained by addition of sodium-EDTA and centrifugation. Plasma total cholesterol, triglyceride and phospholipid levels were measured by enzymatic methods.

Preparation of histological sections

At the end of 32 weeks of drug administration, rabbits were anaesthetized by intravenous injection of sodium pentobarbital (25 mg kg^{-1}) and perfused with lactated Ringer's solution. After the perfusion, the aortas were excised, and then the coronary arteries were perfused with Bouin's fixative at a constant pressure of 100 mmHg. The aortas were divided into aortic arch, thoracic aorta, and abdominal aorta. The thoracic aortas and hearts were immersed in Bouin's fixative for at least 24 h. The remainders of the aortas were frozen and used for other purposes. After immersion-fixation, aortas were longitudinally cut open and the surface area of the intima and the lesion were determined with a colour image analyzer, SP-500 (Olympus, Ltd., Osaka, Japan). Aortic atherosclerosis was histologically examined at five locations: the regions beneath the first, third, fifth, seventh and ninth orifices of the intercostal artery. The segments in each portion were embedded in paraffin, and ten sections, $4 \mu\text{m}$ thickness, were cut serially from each segment.

Coronary atherosclerosis was examined in the main trunk of the left coronary artery (LMT, four segments), the origin portion of the right coronary artery (RCO, four segments), the left circumflex artery (LCX, 30 segments), and the right coronary artery (RCA, 20 segments). The hearts were divided into six blocks, and the blocks were embedded in paraffin. The blocks containing LMT or RCO were sectioned at $200 \mu\text{m}$ intervals and the other blocks were sectioned at $500 \mu\text{m}$ intervals. A total of 58 segments from each heart were examined and ten sections, $4 \mu\text{m}$ in thickness, were cut serially from each segment.

One section of each segment was stained with elastic van Gieson's stain. Sections from each aortic segment and from the coronary segments showing the most severe stenosis in the LMT, RCO and LCX were stained with monoclonal antibodies, HHF35 and RAM-11, and with Azan-Mallory's stain. Immunostaining was carried out with a Vectastain ABC kit according to the manufacturer's instructions.

Quantitative analysis of atherosclerosis and lesional composition

All parameters for atherosclerotic lesions were measured with a colour image analyzer. The degree of atherosclerosis was evaluated by the per cent of lesion area in the surface of intima (the surface area of lesions/the surface area of the whole intima) and the thickness of atherosclerotic lesions (area of intimal lesions/length of external elastic lamina) in the thoracic aortas (Shiomi *et al.*, 1990), and by the score of stenosis in the coronary arteries (Shiomi *et al.*, 1995). Intimal lesion area was estimated using sections stained with elastic van Gieson's stain. We measured coronary stenosis in 58 segments per heart and calculated the coronary stenosis score of each segment. The coronary score was graded as follows: no lesion, 0 points; stenosis of 10% or less narrowing, 1

point; stenosis of 10–20% narrowing, 2 points; stenosis of 20–30% narrowing, 3 points; stenosis of 30–40% narrowing, 4 points; stenosis of 40–50% narrowing, 5 points; stenosis of 50–60% narrowing, 6 points; stenosis of 60–70% narrowing 7 points; stenosis of 70–80% narrowing, 8 points; stenosis of 80–90% narrowing, 9 points; stenosis of over 90% narrowing, 10 points. These points were summed for each portion, and the total points for each rabbit were also counted.

Lesional components were quantitatively evaluated with the colour image analyzer at a magnification of $\times 56$ to $\times 280$, and the lesion area and the area of each lesional component was measured according to the method reported previously (Shiomi *et al.*, 1994). We defined cells stained with HHF35 as smooth muscle cells and cells stained with RAM-11 as macrophages. In sections treated with Azan-Mallory's stain, fibres stained with cobalt blue were defined as collagen, and extracellular vacuoles and lacunae as extracellular lipid deposits. The per cent area of each lesional component was calculated by dividing the area of each lesional component by the lesion area and multiplying by 100%.

Evaluation of xanthoma

Xanthomas were observed macroscopically on the digital joints in each leg after the skin had been removed. The severity of xanthoma was graded as follows: no lesions (–), slight lesion (+) showing diffuse granular lesions, moderate lesion (++) showing one massive lesion with or without diffuse granular ones, and severe lesion (+++) showing more than one massive lesion with or without diffuse granular ones (Shiomi *et al.*, 1990).

Plasma concentration of cerivastatin

The plasma concentration of cerivastatin was measured after a single administration of the drug. Normal Japanese white rabbits were divided into four groups. Three rabbits were given cerivastatin orally at a dose of 0.15 mg kg^{-1} , and three rabbits each in the other three groups were subcutaneously injected with the drug at a dose of 0.15 mg kg^{-1} , 0.3 mg kg^{-1} or 0.6 mg kg^{-1} . Blood samples were obtained from the marginal ear vein of each rabbit at 0.5, 1.0, 2.0, 4.0 and 6.0 h after drug injection. The plasma cerivastatin concentration was determined using HPLC equipped with a Lichrospher 100 RP-18 column according to the method of Krol *et al.* (1993) with slight modification. We analysed T max, C max, and the area under the concentration curve (AUC) from 0–6 h.

Statistical analysis

Data are presented as means $\pm$ s.e.mean. Statistical analysis was carried out by the Student's *t*-test or Aspin-Welch's *t*-test for the means of plasma lipid levels, Mann-Whitney U-test for

the means of atherosclerosis, Fischer's exact probability test for incidences of xanthoma, and Wilcoxon's rank sum test for xanthoma severity.

Results

There were no findings of myopathy or hepatic disorders in any rabbits. In the atherosclerosis prevention study, one rabbit in the control group died suddenly at the 30th week of treatment. Autopsy of this rabbit showed no sign of infection or any other abnormality.

Plasma cerivastatin concentration

In preliminary experiments, plasma cholesterol levels did not decrease in WHHL rabbits administered cerivastatin orally at a dose of 0.15 mg kg^{-1} . Accordingly, we examined plasma cerivastatin concentrations in normal rabbits. Table 1 shows the plasma concentrations of cerivastatin. C max and AUC were remarkably higher in the subcutaneous injection group than in the oral administration group at the same dose. These data show that oral administration of cerivastatin is not suitable for rabbits. With subcutaneous injection, C max and AUC increased dose-dependently.

Plasma lipid levels

Table 2 shows plasma lipid levels of WHHL rabbits treated with cerivastatin. At the start of this experiment, plasma lipid levels were similar in the cerivastatin and control groups. At the end of the experiment, the total cholesterol levels of the cerivastatin group were significantly decreased, by 39%, compared to the control group. Although the phospholipid levels were also significantly decreased, by 33%, the decrease in triglyceride levels of the cerivastatin group was not significant. In the control group, the plasma lipid levels were decreased with progression of the study. In general, the plasma lipid levels of WHHL rabbits are decreased with ageing due to an age-associated decrease in VLDL secretion from the liver (unpublished results).

Table 1 Plasma cerivastatin concentration in normal rabbits after a single administration

	Dose (mg kg^{-1})	T max (h)	C max (ng ml^{-1})	AUC _{0–6} (ng ml^{-1})
Oral administration	0.15	0.5	1.9 ± 0.4	3.1 ± 1.3
Subcutaneous administration	0.15	0.5	60 ± 11	159 ± 36
	0.30	0.5	117 ± 5	175 ± 8
	0.60	0.5	276 ± 26	405 ± 60

Data are presented as means $\pm$ s.e.mean ($n=3$). AUC_{0–6} means area under concentration curve from 0–6 h after drug administration.

Table 2 Plasma lipid levels of WHHL rabbits

	Total cholesterol (mmol L^{-1})		Triglyceride (mmol L^{-1})		Phospholipid (mmol L^{-1})	
	Start	End	Start	End	Start	End
Control ($n=9$)	30.2 ± 0.8	20.9 ± 1.0	3.4 ± 0.5	2.6 ± 0.3	7.2 ± 0.2	5.8 ± 0.3
Cerivastatin ($n=10$)	30.5 ± 0.5	12.7 ± 0.6	3.5 ± 0.3	2.0 ± 0.1	7.3 ± 0.1	3.9 ± 0.1
		$P < 0.001$				$P < 0.001$

Data are represented as means $\pm$ s.e.mean. Statistical analysis was carried out with Student's *t*-test or Aspin-Welch's *t*-test.

Table 3 Atherosclerosis of the thoracic aorta of WHHL rabbits

	Orifice 1	Lesion thickness at each orifice of the intercostal artery (μm)					Per cent lesion area in the intima surface (%)
		Orifice 3	Orifice 5	Orifice 7	Orifice 9	Mean	
Control ($n=9$)	241 $\pm$ 40	283 $\pm$ 23	275 $\pm$ 36	248 $\pm$ 32	205 $\pm$ 34	250 $\pm$ 15	67.7 $\pm$ 6.8
Cerivastatin ($n=10$)	162 $\pm$ 25	186 $\pm$ 28	173 $\pm$ 38	156 $\pm$ 30	115 $\pm$ 22	158 $\pm$ 13	43.4 $\pm$ 8.2
		$P<0.05$			$P<0.05$	$P<0.001$	

Data are represented as means $\pm$ s.e.mean. Statistical analysis was carried out with the Mann-Whitney U-test. Lesional thickening was evaluated using cross sections prepared beneath orifice No. 1, No. 3, No. 5, No. 7 and No. 9. The mean value was calculated using all the data ($n=45$ in the control group and $n=50$ in the cerivastatin group). [Per cent lesion area in the intimal surface] = [the surface area of lesions]/[the surface area of intima of longitudinally cut-opened aorta] $\times$ 100.

Severity of aortic and coronary atherosclerosis

Table 3 shows measurements of the extent of atherosclerotic lesions of the thoracic aorta. The lesion thickness of the cerivastatin group was significantly lower at the third and ninth orifices of the intercostal artery, and the mean value of all portions was also significantly decreased, by 37%, compared to the control group ($158 \pm 13 \mu\text{m}$ versus $250 \pm 15 \mu\text{m}$, $P<0.001$). The per cent lesion area in the surface of the intima that was calculated by dividing the surface area of lesions by the surface area of the whole intima was decreased by 37%; these were not significantly different. Figure 1 shows the coronary stenosis in control and treated rabbits. We examined 58 sections per heart. The coronary stenosis score of the cerivastatin group was significantly decreased, by 28% in the total heart (62.7 ± 11.4 versus 86.9 ± 12.2 , $P<0.05$), compared to the control group. The average of the most severe stenosis in each portion was $44.9 \pm 4.5\%$ ($n=40$) in the cerivastatin group and $50.9 \pm 4.4\%$ ($n=36$) in the control group; these were not significantly different.

Lesional composition

Figure 2 shows the lesional composition of atherosclerotic plaques of the thoracic aorta. Although the per cent areas of collagen and smooth muscle cell were not altered, those of macrophages and extracellular lipid deposits were significantly decreased, by 25% (21.0 ± 1.5 versus 27.9 ± 1.9 , $P<0.01$) and 37% (3.2 ± 0.4 versus 5.1 ± 0.4 , $P<0.01$), respectively, in the cerivastatin group compared to the control group. Typical findings are shown in Figure 3. The control rabbits showed accumulation of large foam cells and extracellular lipid deposits in the atheromatous plaques, while the area occupied by macrophages and extracellular lipid deposits were smaller in the cerivastatin-treated rabbits. In addition, the atherosclerotic lesions were not as thick in the cerivastatin group as in the control group. Although the frequency of disappearance of internal elastic lamina, accumulation of cholesteryl crystals, and calcification did not decrease, the frequency of fragmentation of the internal elastic lamina was significantly decreased in the cerivastatin group (76% versus 96%, $P<0.01$).

Figure 4 shows the lesional composition of the coronary atherosclerosis. The per cent area of macrophages in the cerivastatin group was significantly decreased, by 58%, compared to the control group (4.9 ± 1.4 versus 11.6 ± 2.4 , $P<0.05$). Other lesional components tended to be decreased by the cerivastatin treatment. Typical findings are shown in Figure 5. The frequency of fragmentation and disappearance of the internal elastic lamina were significantly decreased in the cerivastatin group (19% versus 48%, $P<0.05$ and 7% versus 33%, $P<0.05$, respectively).

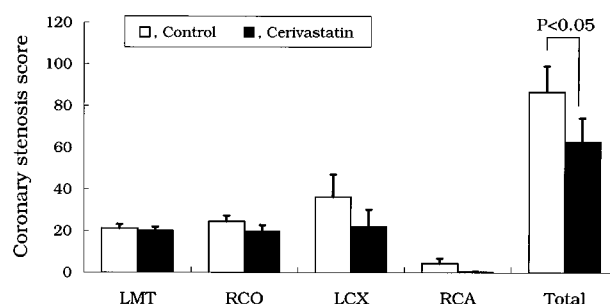


Figure 1 Coronary atherosclerosis of WHHL rabbits treated with cerivastatin sodium. Statistical analysis was carried out with the Mann-Whitney U-test. Bars indicate means $\pm$ s.e.mean. LMT, the main stem of left coronary artery; RCO, the origin portion of right coronary artery; LCX, left circumflex artery; RCA, right coronary artery. For calculation of coronary stenosis score see Methods.

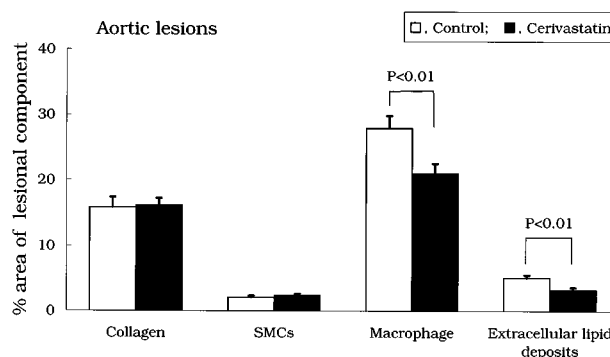


Figure 2 Lesional composition of aortic atherosclerosis of WHHL rabbits treated with cerivastatin sodium. Statistical analysis was carried out with the Mann-Whitney U-test. Bars indicate means $\pm$ s.e.mean. [per cent area of lesional composition] = [area of each lesional component]/[lesion area] $\times$ 100.

Xanthoma

Table 4 summarizes findings about the xanthoma of WHHL rabbits. The incidence of xanthoma in the cerivastatin group was significantly lower in the hind legs and total legs than in the control group. The distribution of severity of xanthoma shows that the degree of xanthoma in the cerivastatin group was significantly less than in the control group ($P<0.001$). Although the frequency of degree of severity over (+) was 61% in the control group, it was only 3% in the cerivastatin group.

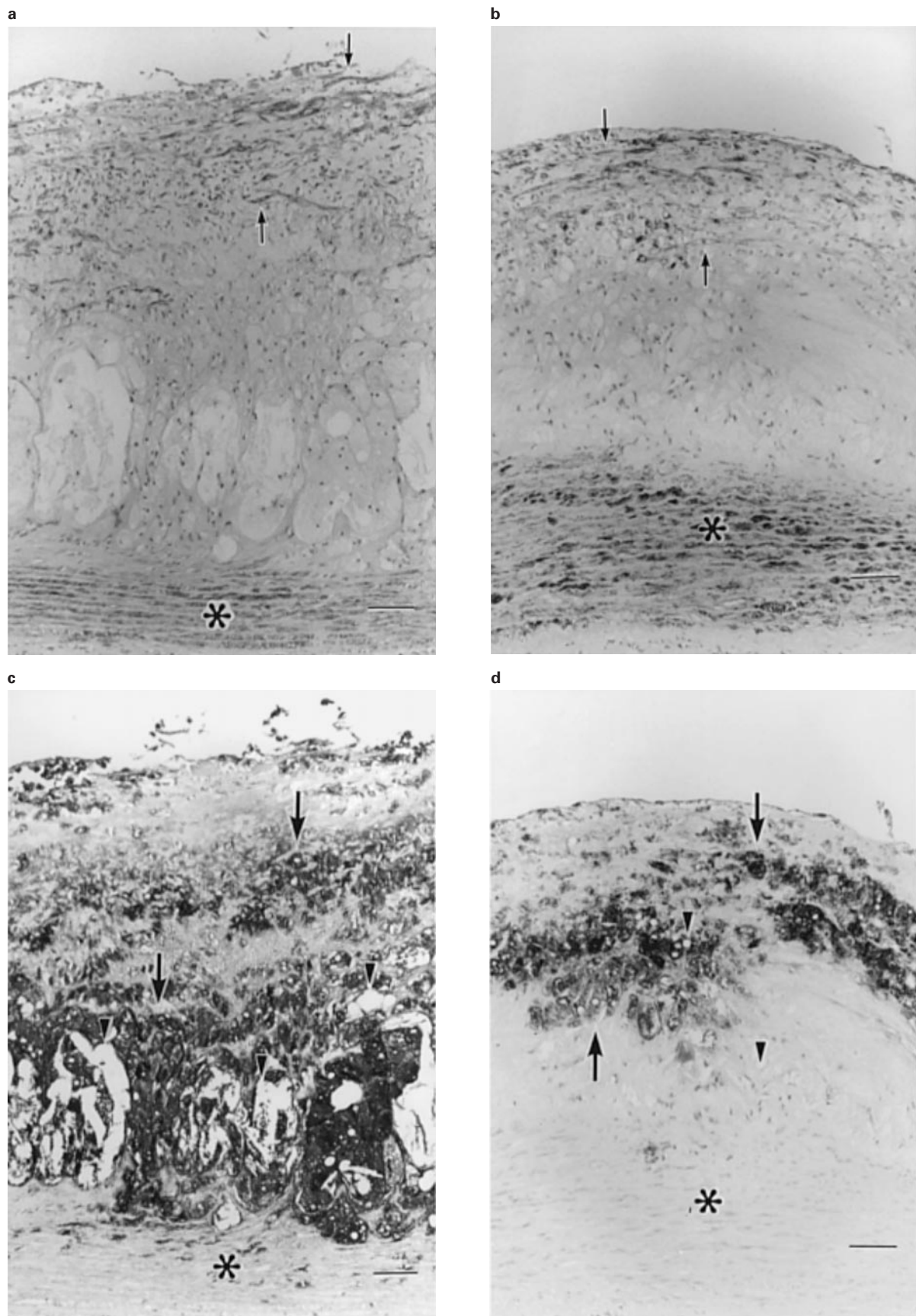


Figure 3 Cross-sections of the thoracic aortas of WHHL rabbits treated with cerivastatin sodium. Sections were immunohistochemically stained with HHF35, a monoclonal antibody specific for muscle actin, or RAM-11, a monoclonal antibody specific for rabbit macrophages. The positive staining shows as black, and vacuoles and lacunae show extracellular lipid deposits. (a) Control; HHF35 staining. (b) Cerivastatin; HHF35 staining. (c) Control; RAM-11 staining. (d) Cerivastatin; RAM-11 staining. Small arrows indicate smooth muscle cells, large arrows indicate macrophages, arrowheads indicate extracellular lipid deposits, and asterisks indicate arterial media. Bars indicate 100 μ m.

Discussion

In the present study, cerivastatin decreased the plasma total cholesterol levels and suppressed progression of the early atherosclerosis and xanthoma in young WHHL rabbits. In

addition, we showed a decrease in the per cent areas of macrophages and extracellular lipid deposits and no decrease in the per cent areas of smooth muscle cells and collagen fibres in the early atherosclerotic lesions of young WHHL rabbits treated with cerivastatin.

In a similar study using fluvastatin sodium, another HMG-CoA reductase, while the per cent areas of smooth muscle cells, collagen fibres, and extracellular lipid deposits were decreased, the per cent area of macrophages was increased in the early atherosclerotic lesions of young WHHL rabbits (Shiomi *et al.*, 1998). Both cerivastatin and fluvastatin show inhibitory effects on proliferation of smooth muscle cells in *in vitro* studies (Corsini *et al.*, 1996; Negre-Aminou *et al.*, 1997; Bellosta *et al.*, 1998) and *in vivo* studies using normocholesterolaemic rabbits which had neointimal thickening of arteries caused by injury (Soma *et al.*, 1993; Bandoh *et al.*, 1996; Igarashi *et al.*, 1997b). However, in our studies using young WHHL rabbits, fluvastatin caused a decrease in the per cent area of smooth muscle cells in the atherosclerotic lesions whereas cerivastatin did not, despite the similar hypolipidemic effects of the two drugs. On the other hand, pravastatin, which shows little inhibitory effect on smooth muscle cell proliferation *in vitro* (Soma *et al.*, 1993; Corsini *et al.*, 1996; Bellosta *et al.*, 1998) decreased the per cent area of macrophages and extracellular lipid deposits, but did not affect smooth muscle cells in the established lesions of mature WHHL rabbits (Shiomi *et al.*,

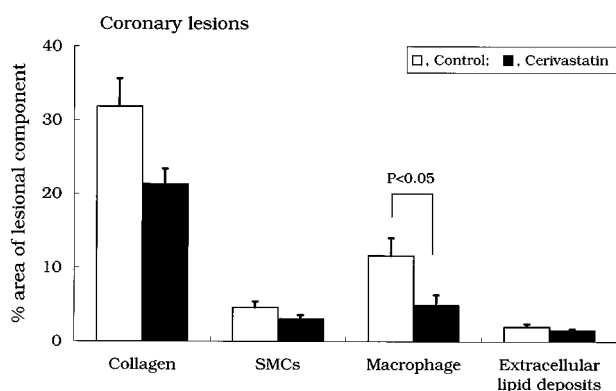


Figure 4 Lesional composition of coronary atherosclerosis of WHHL rabbits treated with cerivastatin sodium. Statistical analysis was carried out with the Mann-Whitney U-test. Bars indicate means $\pm$ s.e.mean. [per cent area of lesional composition] = [area of each lesional component]/[lesion area] $\times$ 100.

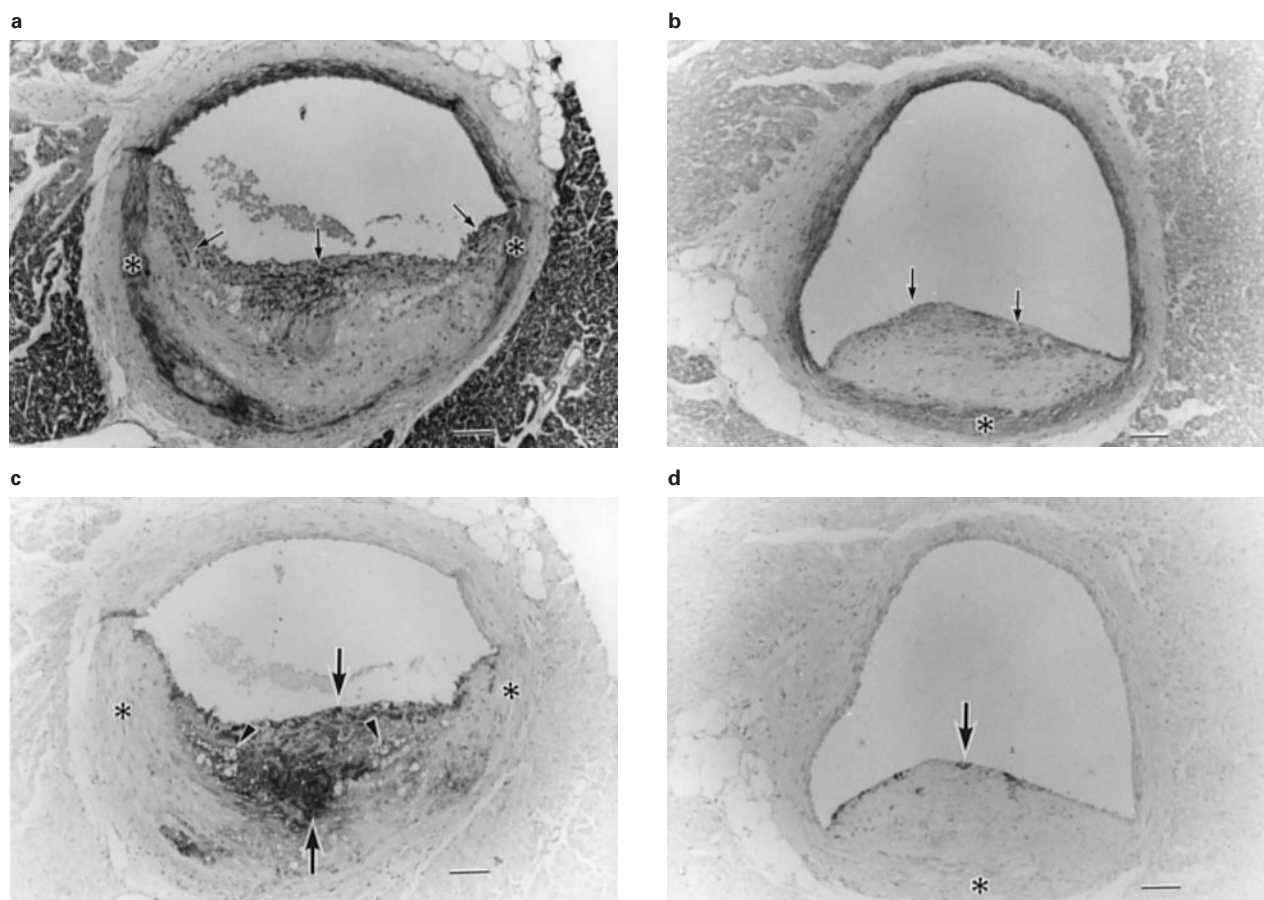


Figure 5 Cross-sections of the left circumflex arteries of WHHL rabbits treated with cerivastatin sodium. Sections were immunohistochemically stained with HHF35, a monoclonal antibody specific for muscle actin, or RAM-11, a monoclonal antibody specific for rabbit macrophages. The positive staining shows as black, and vacuoles and lacunae show extracellular lipid deposits. (a) Control; staining with HHF35. (b) Cerivastatin; staining with HHF35. (c) Control; staining with RAM-11. (d) Cerivastatin; staining with RAM-11. Small arrows indicate smooth muscle cells, large arrows indicate macrophages, arrowheads indicate extracellular lipid deposits, and asterisks indicate arterial media. Bars indicate 100 μ m.

Table 4 Xanthoma of WHHL rabbits

	Forelegs	Incidence (%)		Total	—	Distribution of severity (%)		
		Hind legs				+	++	+++
Control	100 (18/18)	100 (18/18)	100 (36/36)	0 (0/36)	39 (14/36)	44 (16/36)	17 (6/36)	
Cerivastatin	100 (20/20)	70 (14/20)	85 (34/40)	15 (6/40)	83 (33/40)	3 (1/40)	0 (0/40)	
		$P < 0.05$	$P < 0.05$					

Values in parentheses are represented as (xanthoma-positive legs)/(examined legs). The xanthoma of the treated group was significantly milder than that of the control group by Wilcoxon's rank sum test ($P < 0.001$). Statistical analysis of the incidence was carried out with Fisher's exact probability test. For distribution of severity see Methods.

1995). These results suggest that the effects of various HMG-CoA reductase inhibitors on the composition of atherosclerotic lesions differ, despite the similar hypolipidemic effects. Although we have no data to explain these inconsistencies, each HMG-CoA reductase inhibitor may have yet unknown properties. Rosenson and Tangney (1998) also reported that the properties of various HMG-CoA reductase inhibitors differ. They reviewed the antiatherosclerotic properties of various HMG-CoA reductase inhibitors, especially lovastatin, pravastatin and simvastatin. In their review, the effects of different HMG-CoA reductase inhibitors varied regarding endothelial function, inflammatory responses, antioxidant capacity, fibrinogen levels, blood and plasma viscosity, levels of plasminogen activator inhibitor, and Lp(a) levels.

Since smooth muscle cells regulate synthesis of intercostal collagens, no change or an increase in smooth muscle cell content in the atheromatous plaques should render a plaque less liable to disruption than would a decrease in smooth muscle cells. Libby (1995) and Weissberg *et al.* (1996) have reviewed evidence that intimal smooth muscle cells protect plaques from rupturing and render unstable, potentially lethal lesions more stable. Moreover, Davies (1995) and Van der Wal *et al.* (1994) showed that regions of the fibrous cap of plaques that have ruptured contain relatively few smooth muscle cells. Therefore, the lack of decrease of smooth muscle cells and collagen fibres in the atheromatous plaques suggests that cerivastatin did not make the plaques prone to disruption in young WHHL rabbits.

On the other hand, *in vitro* studies (Negre-Aminou *et al.*, 1997; Corsini *et al.*, 1996; Igarashi *et al.*, 1997b) and an *in vivo* study using balloon-injured arteries of normocholesterolaemic rabbits (Igarashi *et al.*, 1997a,b) showed that cerivastatin inhibited proliferation of smooth muscle cells. Regarding the disagreement on inhibition of smooth muscle cell proliferation between our present results and previous reports, we think that the mechanism regulating smooth muscle cell proliferation in spontaneously developed atherosclerosis may be different from those in injured arteries or culture conditions, and cerivastatin may effectively inhibit smooth muscle cell proliferation in culture conditions or injured arteries. Alternatively, the stimulation of smooth muscle cell proliferation may be more potent in culture conditions and injured arteries than in spontaneously developed atherosclerosis due to hypercholesterolaemia. Cerivastatin may inhibit smooth muscle cell proliferation more effectively in response to strong proliferative stimuli than in response to weak proliferative stimuli. In a previous study, we observed that the content of smooth muscle cells in the atherosclerotic lesions was low in WHHL rabbits aged about 10 months (Shiomi *et al.*, 1994). Therefore, the inhibitory effect of cerivastatin on smooth muscle cell proliferation may not have been observed in this study.

In our previous study using mature WHHL rabbits given pravastatin from 10 to 22 months of age, pravastatin prevented

decreases in collagen fibres and smooth muscle cells in the established atherosclerotic lesions, despite decreases in collagen and smooth muscle cells in the lesions of the placebo group (Shiomi *et al.*, 1995). In the present study, however, the per cent areas of collagen fibres and smooth muscle cells in the early atheromatous plaques did not decrease significantly in the cerivastatin group, compared to the control groups. This disagreement regarding the effects of HMG-CoA reductase inhibitors on collagen and smooth muscle cells may be due to the difference in the stage of atherosclerosis in the two studies. The present study used young rabbits which had lesions of early to transitional stages (Shiomi *et al.*, 1994), while mature WHHL rabbits which had lesions of transitional to advanced stages (Shiomi *et al.*, 1994) were used in the previous study.

Our results showed that cerivastatin reduced the atheromatous core, macrophages and extracellular lipid deposits in the centre of the lesion. These results are similar to those of previous studies using mature WHHL rabbits which suffered from established atherosclerosis treated with pravastatin alone (Shiomi *et al.*, 1995) and in combination with cholestyramine (Shiomi *et al.*, 1990). Davies *et al.* (1993) reported that unstable plaques had large lipid cores, and Galis *et al.* (1995) reported that macrophages secreted matrixdegradation proteinases, and collagen fibres might be degraded. Therefore, reduction of atheromatous cores in the lesions is considered favourable with respect to stabilization of the plaque.

Although it was reported that the plasma concentration of cerivastatin was increased by an oral administration in humans (Krol *et al.*, 1993) and various animals (Steinke *et al.*, 1996), an extremely low plasma drug concentration and no decrease in the plasma lipid levels were observed when cerivastatin was given to WHHL rabbits orally. Therefore, we administered cerivastatin to rabbits by subcutaneous injection in this study. By subcutaneous injection of cerivastatin, we achieved reduction of plasma cholesterol levels and suppression of atherosclerosis and xanthoma. We consider that an adequate plasma drug level led to the large effect on reduction of plasma cholesterol level and preventing atherosclerosis.

In conclusion, this study showed that cerivastatin suppressed progression of early atherosclerosis and qualitatively ameliorated and stabilized the atheromatous plaques, in addition to exerting potent hypolipidemic effects. Furthermore, our results suggest that the effects of various HMG-CoA reductase inhibitors on atherosclerotic lesions are somewhat different.

We are grateful to Professor Peter Libby, Department of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, for his helpful discussions. Cerivastatin sodium was kindly supplied by Bayer Yakuhin, Ltd., Osaka, Japan.

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(Received June 1, 1998
Revised November 2, 1998
Accepted November 20, 1998)



A_{2B} adenosine receptors mediate relaxation of the pig intravesical ureter: adenosine modulation of non adrenergic non cholinergic excitatory neurotransmission

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1 The present study was designed to characterize the adenosine receptors involved in the relaxation of the pig intravesical ureter, and to investigate the action of adenosine on the non adrenergic non cholinergic (NANC) excitatory ureteral neurotransmission.

2 In U46619 (10^{-7} M)-contracted strips treated with the adenosine uptake inhibitor, nitrobenzylthioinosine (NBTI, 10^{-6} M), adenosine and related analogues induced relaxations with the following potency order: 5'-N-ethylcarboxamidoadenosine (NECA) = 5'-(N-cyclopropyl)-carboxamidoadenosine (CPCA) = 2-chloroadenosine (2-CA) > adenosine > cyclopentyladenosine (CPA) = N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA) = 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680).

3 Epithelium removal or incubation with indomethacin (3×10^{-6} M) and L-N^G-nitroarginine (L-NOARG, 3×10^{-5} M), inhibitors of prostanoids and nitric oxide (NO) synthase, respectively, failed to modify the relaxations to adenosine.

4 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10^{-8} M) and 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385, 3×10^{-8} M and 10^{-7} M), A₁ and A_{2A} receptor selective antagonists, respectively, did not modify the relaxations to adenosine or NECA. 8-phenyltheophylline (8-PT, 10^{-5} M) and DPCPX (10^{-6} M), which block A₁/A₂-receptors, reduced such relaxations.

5 In strips treated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-NOARG (3×10^{-5} M) and indomethacin (3×10^{-6} M), both electrical field stimulation (EFS, 5 Hz) and exogenous ATP (10^{-4} M) induced contractions of preparations. 8-PT (10^{-5} M) increased both contractions. DPCPX (10^{-8} M), NECA (10^{-4} M), CPCA, (10^{-4} M) and 2-CA (10^{-4} M) did not alter the contractions to EFS.

6 The present results suggest that adenosine relaxes the pig intravesical ureter, independently of prostanoids or NO, through activation of A_{2B}-receptors located in the smooth muscle. This relaxation may modulate the ureteral NANC excitatory neurotransmission through a postsynaptic mechanism.

Keywords: Pig intravesical ureter; adenosine receptors; electrical field stimulation; 8-PT; DPCPX; ZM 241385; NANC neurotransmission

Abbreviations: 2-CA, 2-chloroadenosine; 8-PT, 8-phenyltheophylline; ACh, acetylcholine; CGS21680, 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine; CPA, cyclopentyladenosine; CPCA, 5'-(N-cyclopropyl)-carboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; IB-MECA, N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide; NBTI, nitrobenzylthioinosine; NECA, 5'-N-ethylcarboxamidoadenosine; ZM 241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

Introduction

ATP and related nucleotides and nucleosides have been described to be involved in non adrenergic non cholinergic (NANC) neurotransmission (Burnstock, 1972; Burnstock *et al.*, 1978). ATP is converted to adenosine by the action of ecto-ATPases and nucleotidases, which are present in the surroundings of the nerve terminal (McDonald & White, 1984; Cusack & Hourani, 1984; Cusack *et al.*, 1988). Once formed, adenosine acts on presynaptic receptors to depress further ATP release (Khakh & Kennedy, 1998).

Physiological and pharmacological effects of purines are mediated *via* two main classes of receptors, designated as P₁ and P₂, which primarily respond to adenosine and ATP,

respectively (Burnstock, 1978). Smooth muscle P₁-purinoceptors generally cause relaxation (Burnstock, 1978) although in a number of smooth muscle preparations adenosine cause contraction *via* these receptors (Bailey *et al.*, 1992). P₂-purinoceptors have recently been termed P2 receptors since they are receptors for a purine or pyrimidine nucleotide or dinucleotide. These receptors have been classified into the ionotropic P2X and the metabotropic P2Y. P2X operate *via* intrinsic ion channels mediating fast and transient responses while P2Y are G protein-coupled receptors which mediate slower responses (Abbracchio & Burnstock, 1994; Fredholm *et al.*, 1994; 1997).

P₁-receptors have been divided in four subtypes, designated as A₁, A_{2A}, A_{2B} and A₃ (Fredholm *et al.*, 1994; Olah & Stiles, 1995). All four are G protein-coupled receptors; the A₁ and A₃

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subtypes are coupled to inhibitory G proteins, while activation of the A_{2A}- and A_{2B}-receptors leads to adenylate cyclase stimulation (Collis & Hourani, 1993; Alexander *et al.*, 1994; Fredholm *et al.*, 1994). A₁- and A₂-receptor subtypes have been defined according to the relative potency on them of adenosine analogues, as well as considering the potency shown by A₁-selective antagonists such as 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX). Thus, on A₁-receptors, N⁶ substituted analogues such as N⁶-cyclopentyladenosine (CPA) are more potent than 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA). Xanthines such as 8-phenyltheophylline (8-PT) and 8-sulphophenyltheophylline (8-SPT) are antagonists at both A₁- and A₂-receptors, whereas DPCPX has nanomolar affinity at A₁-receptors and micromolar affinity at A₂ receptors, and thus effectively discriminates between the two types of receptors (Lohse *et al.*, 1987; Bruns, 1990; Collis *et al.*, 1989).

A₂-receptors have been divided further into A_{2A} and A_{2B} subtypes, based on the potency shown by some two-substituted adenosine analogues, such as 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680). Thus, CGS21680 is a potent agonist at A_{2A} receptors but is virtually inactive at A_{2B} sites (Jacobson, 1990). Recently, a non-xanthine antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol, (ZM 241385) had shown high selectivity for A_{2A} receptors (Poucher *et al.*, 1995).

A₃-receptors, which are considered to be xanthine-insensitive, have been cloned from rat striatum. These receptors are coupled to an inhibitory G protein (Meyerhof *et al.*, 1991; Zhou *et al.*, 1992). N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA) has been reported as a selective A₃-receptor agonist (Jacobson *et al.*, 1993; 1995; Gallo-Rodríguez *et al.*, 1994). A₃-receptors are resistant to blockade with methylxanthines, although it has been reported that compounds such as 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BWA1433) block them (Jacobson *et al.*, 1995).

The autonomic nervous system plays an essential role in the maintenance of ureteral peristalsis. Thus, a rich catecholaminergic innervation forming dense neuromuscular, perivascular and subepithelial plexuses has been found in the intravesical ureter (Schulman, 1985; Prieto *et al.*, 1993; 1994). Both noradrenaline (Hernández *et al.*, 1992) and acetylcholine (Hernández *et al.*, 1993; 1995a; Prieto *et al.*, 1994) stimulate the two contractile components of intravesical ureteral smooth muscle (phasic activity and tone). In addition to the adrenergic and cholinergic systems, several neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal peptide and somatostatin have been identified in ureteral nerves, coexisting with different neurotransmitters (Edyvane *et al.*, 1994; Smet *et al.*, 1994; Prieto *et al.*, 1997). These peptides act by modulating the neurotransmitter-evoked activity. Thus, NPY has been reported to enhance the noradrenaline-induced contraction in the equine intravesical ureter through Y₂-receptors (Prieto *et al.*, 1997). Together with the above mentioned peptides, nitric oxide (NO) (Hernández *et al.*, 1995b) and calcitonin gene related peptide (CGRP) (Maggi & Giuliani, 1991) have been involved in the ureteral non adrenergic non cholinergic (NANC) inhibitory neurotransmission, in processes operated through guanylate cyclase (Hernández *et al.*, 1997) and adenylate cyclase (Maggi *et al.*, 1994; 1995), respectively, involving in both cases the activation of glibenclamide-sensitive K⁺ channels.

Adenosine has been reported to modulate the NANC mouse urinary bladder neurotransmission (Acevedo *et al.*, 1992). The possible role exerted by adenosine in the

intravesical ureter has not been described. Therefore, the present study was designed to characterize the functionally active adenosine receptor subtypes involved in the relaxation of porcine ureter, and to investigate the possible modulatory action of adenosine in the NANC motor transmission of the pig intravesical ureter.

Methods

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders with attached ureters were removed immediately after the animals were killed, and kept in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection, as previously described (Hernández *et al.*, 1992). The ureteral strips were suspended horizontally and placed parallel between two platinum electrodes, with one end connected to an isometric transducer (Grass FT 03C) and the other one to a micrometer screw which regulates the tension applied to the preparations, in 5 ml organ baths. The signal was continuously recorded on a polygraph (Graphtec Multicorder MC 6621, Hugo Sachs Elektronik, Germany). Passive tension of 2 g was applied to the ureteral preparations and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile ability of the preparations was determined by exposing the ureteral strips to 124 mM potassium-rich physiological saline solution (KPSS). Relaxations to adenosine and structurally related analogues were performed on strips incubated for 30 min with the adenosine uptake inhibitor nitrobenzylthioinosine (NBTI, 10⁻⁶ M) and further contracted with the thromboxane analogue U46619 (10⁻⁷ M). Afterwards, the tissues were washed every 20 min during 1.5 h. To study the effect of antagonists on adenosine or NECA relaxations, the preparations were incubated with NBTI alone (control curve) or NBTI plus the antagonist (30 min, except for 8-PT, which was incubated for 45 min). At the end of each experiment with adenosine and its structural analogues, papaverine (10⁻⁴ M) was added to the organ bath with the aim of obtaining the maximal relaxation of preparations.

In electrical field stimulation (EFS) experiments, adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostaglandins were blocked by incubation with guanethidine (10⁻⁵ M), atropine (10⁻⁷ M), L-N^G-nitroarginine (L-NOARG, 3 × 10⁻⁵ M) and indomethacin (3 × 10⁻⁶ M), respectively, during a period of 1 h, washing every 20 min, and these drugs were present throughout the experiment. EFS was performed with rectangular pulses (1 ms duration, 5 Hz, 20 s trains), at 3 min intervals, from a Cibertec CS20 stimulator (Barcelona, Spain) with constant current output adjusted to 75 mA. The strips were repeatedly washed and allowed to equilibrate for at least 1 h before they were incubated with blocking agents.

Drugs and solutions

The following drugs were used: acetylcholine (ACh), adenosine, adenosine 5'-triphosphate (ATP), atropine sulphate, guanethidine, 5-hydroxytryptamine (5-HT), indomethacin, L-NOARG, papaverine, 9, 11-dideoxy-11 α , 9 α -epoxymethano-

prostaglandin F_{2α} (U46619) and tetrodotoxin (Sigma, U.S.A.). N⁶-cyclopentyladenosine (CPA), 5'-(N-cyclopropyl)-carboxamidoadenosine (CPCA), 2-chloroadenosine (2-CA), 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680), 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA), nitrobenzylthioinosine (NBTI), 5'-N-ethylcarboxamidoadenosine (NECA), 8-phenyl-theophylline (8-PT) (RBI, U.S.A), 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) (Tocris Cookson, U.S.A.). ACh, adenosine, ATP, guanethidine, 5-HT, L-NOARG and tetrodotoxin were dissolved in double distilled water. CGS21680, DPCPX, NBTI, NECA, IB-MECA and ZM 241385 were dissolved in dimethyl sulphoxide (DMSO). CPCA was dissolved in 0.1N HCl. 8-PT was dissolved in 25% ethanol plus 30 mM NaOH. U46619, indomethacin, 2-CA, CPA and papaverine were dissolved in 96% ethanol. These solvents, in the amount applied, had no effect on pig intravesical ureteral activity. The composition of PSS was (mM): NaCl 119, KCl 4.6, MgCl₂ 1.2, NaHCO₃ 24.9, glucose 11, CaCl₂ 1.5, KH₂PO₄ 1.2, EDTA (ethylenediamine tetraacetic acid) 0.027. The solution was continuously gassed at 37°C with 95% O₂ and 5% CO₂, to maintain pH at 7.4. KPSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in double-distilled water.

Calculations and statistics

For each concentration-response relaxation curve to adenosine and related analogues, the drug concentration required to give 40% relaxation (EC₄₀) of U46619 induced contraction was estimated by regression of the linear portion of the curve, by computerized linear regression analysis (Inplot, GraphPad, U.S.A.). The sensitivity of the drugs is expressed in terms of pEC₄₀, where pEC₄₀ is defined as the negative logarithm of EC₄₀ (pEC₄₀ = -logEC₄₀ (M)). Each parameter was determined from ureters of at least 4–6 different animals. Statistical significance of differences was calculated by Student's *t*-test, for paired observations for individual concentrations or frequencies and variance analysis (ANOVA) for multiple comparisons, followed by an *a posteriori* Bonferroni test (Wallestein *et al.*, 1980). Differences were considered significant with a probability level of *P* < 0.05.

Results

The pig intravesical ureteral preparations were equilibrated to a passive tension of 1.8 ± 0.3 g (*n* = 87). In NBTI (10⁻⁶ M)-pretreated tissues, the thromboxane analogue, U46619 (10⁻⁷ M) induced sustained contractions of 1.9 ± 0.3 g (*n* = 75). Adenosine, NECA, 2-CA, CPCA, CGS 21680, CPA and IB-MECA all concentration-dependently relaxed the U46619-contracted intravesical ureteral strips, the order of potency being: NECA = CPCA = 2-CA > adenosine > CPA = IB-MECA = CGS21680 (Figure 1, Table 1). This order of potency suggests that the adenosine receptors that relax the pig intravesical ureteral smooth muscle belong to the A_{2B} subtype.

The relaxations to adenosine (10⁻⁷–10⁻³ M) and NECA (10⁻⁷–10⁻⁴ M) were reproducible. Thus, pEC₄₀ values and the relaxations to 10⁻³ M adenosine were 4.1 ± 0.2 and 73.2 ± 7.0%, and 4.0 ± 0.2 and 70.2 ± 6.8% (*P* > 0.05, paired *t*-test, *n* = 9), in a first and second concentration-response curve performed in the same preparation, respectively. pEC₄₀ values and the relaxations to 10⁻⁴ M NECA were 5.2 ± 0.2 and

92.1 ± 5.2% and 5.1 ± 0.3 and 88.3 ± 4.8% (*P* > 0.05, paired *t*-test, *n* = 7), in two consecutive curves.

Removal of ureteral epithelium did not modify the adenosine concentration-relaxation curve, the pEC₄₀ values being 4.0 ± 0.2 and 4.1 ± 0.2, (*P* > 0.05, paired *t*-test, *n* = 6) in the absence and in the presence of epithelium, respectively. Epithelium removal had also no effect on the relaxations to 10⁻³ M adenosine. These relaxations were 70.1 ± 4.7% and 63.2 ± 5.2% (*P* > 0.05, paired *t*-test, *n* = 6), in the absence and in the presence of epithelium, respectively (Figure 2a).

Indomethacin (3 × 10⁻⁶ M) and L-NOARG (3 × 10⁻⁵ M), inhibitors of prostanoid production and NO synthase activity, respectively, did not modify the relaxations to adenosine. The pEC₄₀ values and the relaxations to 10⁻³ M adenosine were 4.1 ± 0.1 and 75.7 ± 4.8% and 4.1 ± 0.2 and 76.4 ± 4.3%

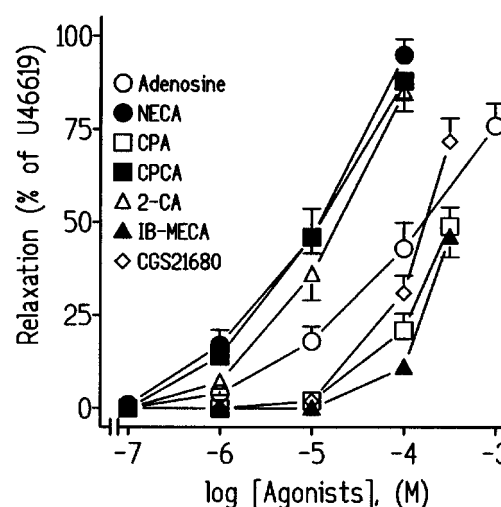


Figure 1 Log concentration-response relaxation curves to adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), N⁶-cyclopentyladenosine (CPA), 5'-(N-cyclopropyl)-carboxamidoadenosine (CPCA), 2-chloroadenosine (2-CA), N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA) and 2-[p-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680), in pig intravesical ureteral strips contracted to 10⁻⁷ M U46619. Relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e. of the mean of 5–17 preparations.

Table 1 Relaxations induced by adenosine and related structural analogues in pig intravesical ureteral strips contracted with 10⁻⁷ M U46619

Agonist	n	pEC ₄₀	R(%)
Adenosine	17	4.5 ± 0.1	75.9 ± 6.1
NECA	11	5.4 ± 0.2*	95.1 ± 4.2
CPCA	7	5.4 ± 0.1*	88.1 ± 5.4
2-CA	6	5.2 ± 0.3*	85.0 ± 5.1
CPA	6	4.2 ± 0.1*†,‡,‡,‡	48.9 ± 6.1
CGS21680	7	4.1 ± 0.1*†,‡,‡,‡	71.8 ± 6.3
IB-MECA	5	4.0 ± 0.1*†,‡,‡,‡	46.1 ± 5.2

Results are expressed as mean ± s.e. mean of *n* experiments. Differences were calculated by one-way analysis of variance (ANOVA) followed by an *a posteriori* Bonferroni *t*-test in case of significance; **P* < 0.05 compared to adenosine, †*P* < 0.05 compared to NECA, ‡*P* < 0.05 compared to CPCA, §*P* < 0.05 compared to 2-CA. pEC₄₀ = -log EC₄₀, where EC₄₀ is the concentration of agonist producing 40% relaxation of U46619 induced contraction. R: relaxation obtained at the highest concentration applied of each drug: Adenosine, 10⁻³ M; NECA 10⁻⁴ M; CPCA 10⁻⁴ M; 2-CA, 10⁻⁴ M; CPA 3 × 10⁻⁴ M; CGS21680, 3 × 10⁻⁴ M and IB-MECA, 3 × 10⁻⁴ M.

($P > 0.05$, paired t -test, $n = 6$), in control conditions and in the presence of indomethacin, respectively (Figure 2b). pEC_{40} values and the relaxations to 10^{-3} M adenosine in the experiments with L-NOARG (3×10^{-5} M) were 4.1 ± 0.2 and $73.4 \pm 5.5\%$ and 4.0 ± 0.2 and $68.8 \pm 4.7\%$ ($P > 0.05$, paired t -test, $n = 7$), in the absence and in the presence of L-NOARG, respectively (Figure 2c).

Effects of 8-PT, DPCPX and ZM 241385 on relaxations to adenosine and NECA

We examined whether 8-PT (10^{-5} M), an inhibitor of both A₁- and A₂-adenosine receptors, and DPCPX (10^{-8} M and 10^{-6} M), inhibited the relaxations to adenosine or NECA in U46619-precontracted strips obtained from pig intravesical ureter. Pretreatment of strips with 8-PT (10^{-5} M), did not modify the ureteral basal tone (1.6 ± 0.4 g, in control conditions and in the presence of 8-PT), but reduced the relaxations to adenosine (Figure 4a, Table 2) and NECA (Figure 3 and 4b, Table 1). Incubation with 10^{-8} M DPCPX, a concentration selective for A₁-receptors, did not modify the relaxations to adenosine or to NECA. However, the use of 10^{-6} M DPCPX, a concentration which blocks the A₂-receptors, significantly reduced the relaxations to adenosine or NECA on ureteral strips (Figure 5a and b, Table 2). ZM 241385 (3×10^{-8} M and 10^{-7} M), a non-xanthine antagonist which shows high selectivity for A_{2A} did not modify the relaxations to adenosine or to NECA (Figure 5c and d, Table 2).

Effects of 8-PT and DPCPX on contractions evoked by EFS and exogenous ATP

Pig intravesical ureteral strips were treated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-NOARG (3×10^{-5} M) and indomethacin (3×10^{-6} M), with the aim to block adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostanoids, respectively. In such conditions, both EFS (5 Hz) and exogenous ATP (10^{-4} M) evoked reproducible contractions of 1.3 ± 0.2 g and 1.2 ± 0.2 g ($P > 0.05$, paired t -test, $n = 7$) and 0.4 ± 0.1 g and 0.4 ± 0.2 g ($P > 0.05$, paired t -test, $n = 5$), in a first and second addition, respectively, on the basal tone of preparations. Treatment of ureteral strips with 8-PT (10^{-5} M) significantly potentiated the contractions induced by both EFS (1.1 ± 0.3 g and 1.7 ± 0.4 g, in control conditions and in the presence of 10^{-5} M 8-PT, respectively, $n = 9$, $P < 0.05$, paired t -test) (Figure 6a and b) or exogenous ATP (0.5 ± 0.2 g and 1.1 ± 0.2 g in the absence and in the presence of 10^{-5} M 8-PT, respectively, $n = 11$, $P < 0.05$, paired t -test) (Figure 6a and c). The incubation with a selective A₁-receptor antagonist, DPCPX (10^{-8} M), did not modify the contractions induced by EFS (0.9 ± 0.2 g and 1.0 ± 0.3 g, $P > 0.05$, paired t -test, $n = 7$, in control conditions and in the presence of 10^{-8} M DPCPX, respectively) or exogenous ATP (0.6 ± 0.2 g and 0.5 ± 0.2 g, $P > 0.05$ paired t -test, $n = 8$, in the absence and in the presence of 10^{-8} M DPCPX, respectively). The contractions elicited by EFS (5 Hz) were abolished by including

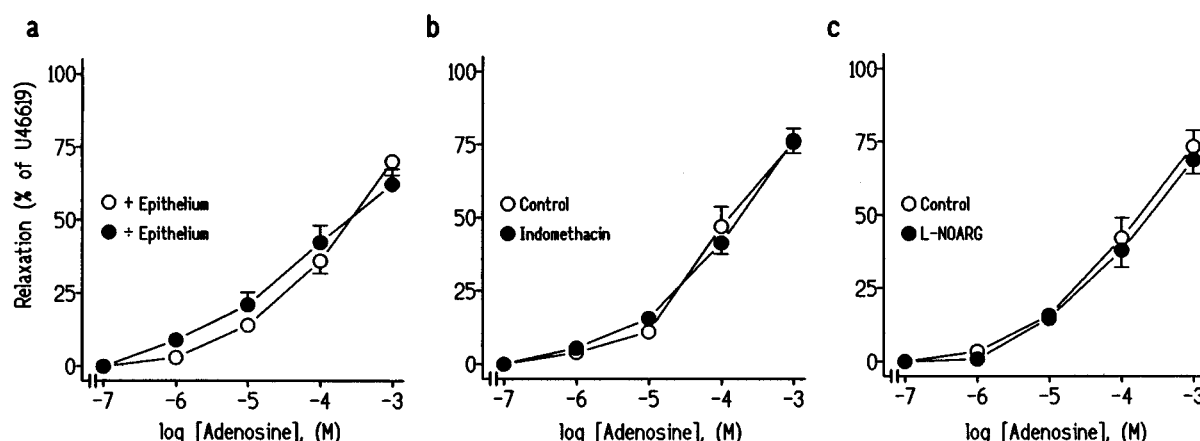


Figure 2 (a) Log concentration-response relaxation curves to adenosine in pig intravesical ureteral strips contracted to 10^{-7} M U46619 in the presence and in the absence of ureteral epithelium. (b and c) Log concentration-response relaxation curves to adenosine in control conditions or in the presence of (b) 3×10^{-6} M indomethacin or (c) 3×10^{-5} M L-N^G-nitroarginine (L-NOARG). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–7 preparations.

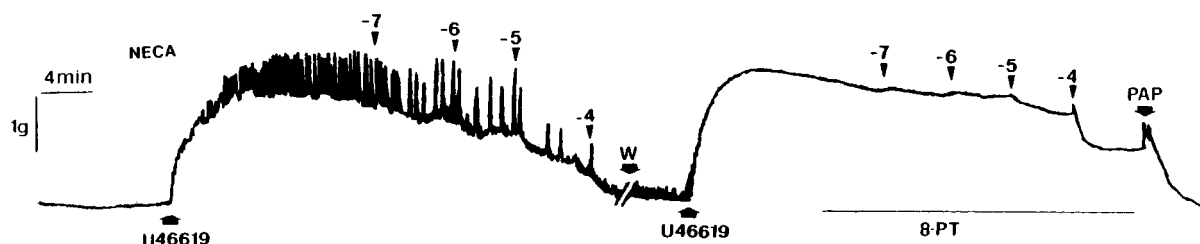


Figure 3 Isometric force recordings showing the effect of 8-phenyltheophylline (8-PT, 10^{-5} M) on the relaxant response to 5'-N-ethylcarboxamidoadenosine (NECA, 10^{-7} – 10^{-4} M), in U46619 (10^{-7} M)-contracted pig intravesical ureteral strips. At the end of the experiment, papaverine (PAP, 10^{-4} M) was added to obtain the maximal relaxation of the ureteral strips. Vertical bar shows tension in g and horizontal bar time in min. Numbers indicate molar concentration in the bath. W: wash out.

10^{-6} M tetrodotoxin in the bath, thus providing evidence for the neurogenic origin of these contractions.

Effects of NECA, CPCA and 2-CA on contractions evoked by EFS

In the conditions above mentioned, the incubation during 5 min of the preparations with a maximal (10^{-4} M) concentration of NECA, CPCA and 2-CA did not modify the contractions to EFS (5 Hz). Thus, EFS evoked contractions of 1.0 ± 0.3 g and 1.1 ± 0.3 g ($P > 0.05$, paired *t*-test, $n = 7$); 0.9 ± 0.2 g and 0.8 ± 0.2 g ($P > 0.05$, paired *t*-test, $n = 5$); 1.1 ± 0.3 g and 1.2 ± 0.4 g, ($P > 0.05$, paired *t*-test, $n = 5$) in control conditions and in the presence of NECA, CPCA and 2-CA, respectively.

Effects of NECA on contractions elicited by exogenous ATP

Pretreatment of ureteral strips for 5 min with NECA (10^{-4} M), reduced the contractions to exogenous ATP (10^{-4} M). Thus, ATP (10^{-4} M) induced contractions of 0.5 ± 0.2 g and

0.2 ± 0.2 g ($P < 0.05$, paired *t*-test, $n = 5$) in the absence or presence of NECA (10^{-4} M), respectively.

Effects of 8-PT on contractions induced by exogenous ACh and 5-HT

Incubation of preparations with 8-PT (10^{-5} M) increased the contractions to ACh (10^{-5} M) or 5-HT (10^{-5} M) in the pig intravesical ureter. ACh (10^{-5} M) induced contractions of 0.9 ± 0.2 g and 1.3 ± 0.3 g ($P < 0.05$, paired *t*-test, $n = 7$) in the absence or presence of 8-PT (10^{-5} M), respectively. 5-HT (10^{-5} M) evoked contractions of 1.3 ± 0.2 g and 1.8 ± 0.2 g ($P < 0.05$, paired *t*-test, $n = 5$) in control conditions and in the presence of 8-PT (10^{-5} M), respectively.

Discussion

In the present study adenosine and six of its related structural analogues, NECA, 2-CA, CPCA, CGS21680, CPA and IB-MECA concentration-dependently and epithelium-independently relaxed the pig intravesical ureteral strips precontracted

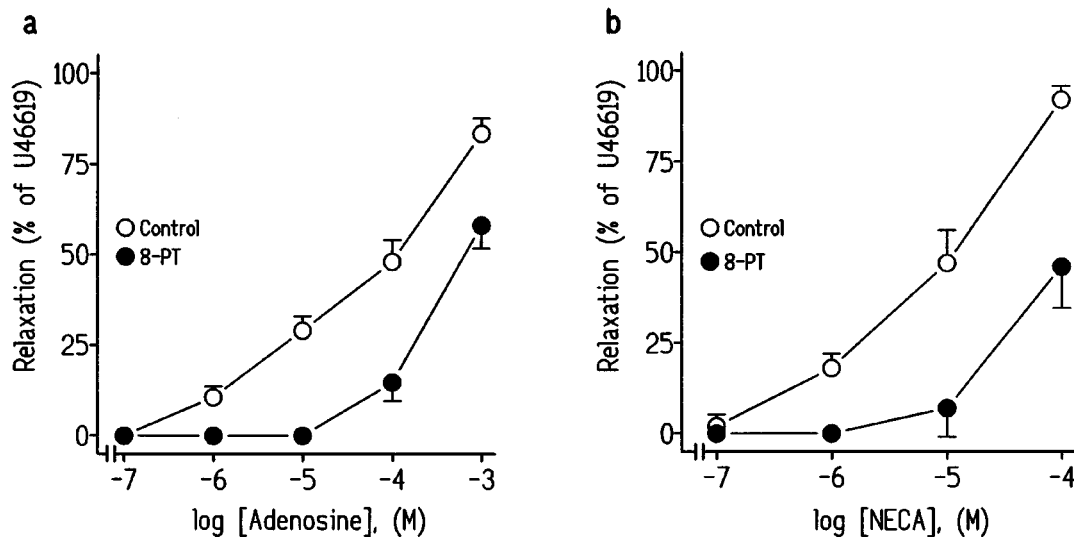


Figure 4 Log concentration-response relaxation curves to (a) adenosine and (b) 5'-N-ethylcarboxamido-adenosine (NECA) in pig intravesical ureteral strips contracted to 10^{-7} M U46619, in control conditions and in the presence of the A₁ and A₂ adenosine receptors inhibitor, 8-phenyltheophylline (8-PT, 10^{-5} M). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–7 preparations.

Table 2 Effect of 8-PT (10^{-5} M), DPCPX (10^{-8} M), DPCPX (10^{-6} M), ZM 241385 (3×10^{-8} M) and ZM 241385 (10^{-7} M) on relaxations to adenosine and NECA of pig intravesical ureter contracted to U46619 (10^{-7} M)

	n	Adenosine <i>pEC</i> ₄₀	R (%)	n	NECA <i>pEC</i> ₄₀	R (%)
Control	7	4.4 ± 0.2	83.3 ± 4.3	6	5.3 ± 0.2	92.1 ± 0.2
8-PT	7	—	$58.1 \pm 6.2^*$	6	—	$45.9 \pm 11.3^*$
Control	9	4.3 ± 0.1	77.2 ± 6.1	7	5.2 ± 0.2	94.7 ± 4.6
DPCPX (10^{-8} M)	9	4.3 ± 0.2	78.8 ± 5.3	7	5.2 ± 0.1	86.1 ± 5.2
DPCPX (10^{-6} M)	9	—	$52.2 \pm 4.1^\#$	7	—	$57.9 \pm 6.7^\#$
Control	6	4.2 ± 0.2	68.1 ± 4.7	7	5.2 ± 0.2	86.4 ± 4.8
ZM 241385 (3×10^{-8} M)	6	4.2 ± 0.2	70.1 ± 5.1	7	5.1 ± 0.3	85.1 ± 5.0
ZM 241385 (10^{-7} M)	6	4.0 ± 0.3	66.2 ± 6.3	7	4.9 ± 0.3	78.2 ± 5.5

Results are expressed as mean $\pm$ s.e.mean of *n* experiments. *Parameter significantly ($P < 0.05$) different compared to control value by paired *t*-test. #Parameter significantly ($P < 0.05$) different compared to control value by analysis of variance (ANOVA), followed by an *a posteriori* Bonferroni test. *pEC*₄₀ = $-\log EC_{40}$, where *EC*₄₀ is the concentration of agonist producing 40% relaxation of U46619 induced contraction. R: relaxation obtained at the highest concentration used: adenosine, 10^{-3} M and NECA, 10^{-4} M.

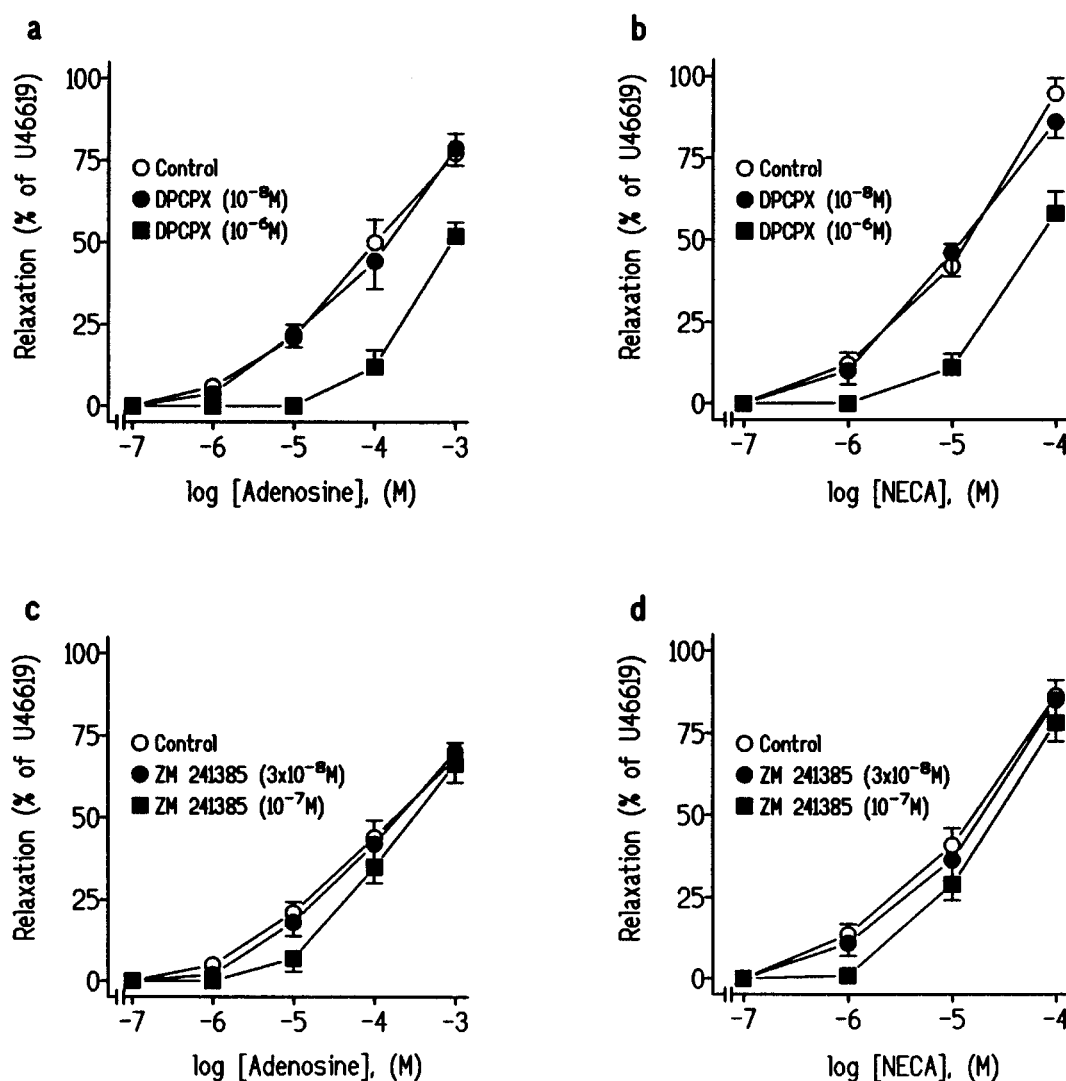


Figure 5 Log concentration-response relaxation curves to (a and c) adenosine and (b and d) 5'-N-ethylcarboxamidoadenosine (NECA) in pig intravesical ureteral strips contracted to 10^{-7} M U46619, in control conditions and in the presence of the A_1 -adenosine receptors inhibitor, 3-dipropyl-8-cyclopentylxanthine (DPCPX), (10^{-8} M) or (10^{-6} M) (a and b) and the A_{2A} selective receptors antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), (3×10^{-8} M) or (10^{-7} M) (c and d). The relaxations are expressed as a percentage of the U46619-induced contraction. Results represent means and vertical line s.e.mean of 6–9 preparations.

with U46619. The relaxing potency order obtained (NECA = CPCA = 2-CA > adenosine > CPA = IB-MECA = CGS21680) together with the lack of effect of indomethacin and L-NOARG is consistent with a prostanoid- and NO-independent activation of A_{2B} receptors by these agonists. The potentiation induced by 8-PT on the contraction evoked by EFS and exogenous ATP suggests that endogenous adenosine could be modulating the non adrenergic non cholinergic neurotransmission of the pig intravesical ureter.

The relaxation curves to adenosine and NECA were monophasic, suggesting that both agonists activate one receptor site to cause relaxation in the pig intravesical ureter. Removal of the ureteral epithelium did not change the relaxations to adenosine, indicating that these responses are mediated *via* a receptor located on the ureteral smooth muscle. Both prostanoids and NO have been reported to mediate the relaxation induced by adenosine in different tissues. Thus, part of the relaxing effect produced by adenosine in the rabbit isolated heart is mediated through release of prostaglandins (Ciabattini & Wennmalm, 1985; Karwatowska-Prokopczuk *et al.*, 1988). Adenosine, on the other hand, promotes the release

of NO from vascular endothelium (Vials & Burnstock, 1993; Abebe *et al.*, 1995; Li *et al.*, 1995; Danialou *et al.*, 1997) and smooth muscle (Ikeda *et al.*, 1997). However, in the pig intravesical ureter, indomethacin and L-NOARG, which inhibit prostanoid production and NO-synthase activity, respectively, did not modify the relaxations to adenosine, suggesting that adenosine relaxes the ureteral smooth muscle *via* a prostaglandins- and NO-independent mechanism. These results are consistent with those found in the frog aorta, where adenosine induces vasodilatation without involving prostanoid or NO production (Knight & Burnstock, 1996).

The blocker of A_1 - and A_2 -receptors, 8-PT (10^{-5} M) potently reduced the relaxations induced by adenosine and NECA, suggesting that these relaxations are mediated through P_1 -purinoceptors. The fact that 10^{-6} M DPCPX, a concentration high enough to block A_2 -receptors, inhibits the relaxations, indicates that they are A_2 -receptor-mediated. This hypothesis is in accordance with the general notion that smooth muscle A_2 -receptors mediate relaxation, whereas A_1 -receptors, which are presynaptically located, operate as a negative feedback mechanism, regulating transmitter release

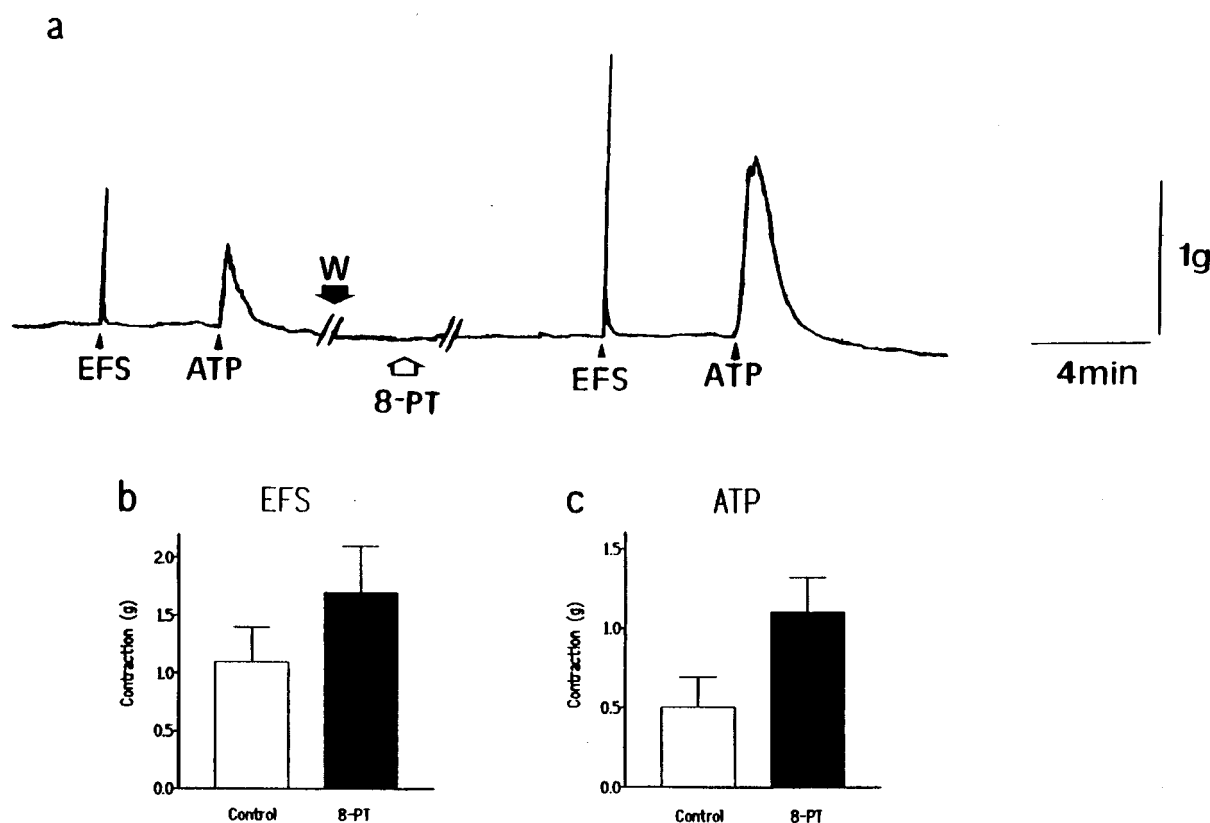


Figure 6 (a) Isometric force recordings showing the effect of 8-phenyltheophylline (8-PT, 10^{-5} M) on contractions induced by both EFS (5 Hz, 1 ms duration, 20 s trains, at 3 min intervals) and exogenous ATP (10^{-4} M), in pig intravesical ureteral strips pretreated with guanethidine (10^{-5} M), atropine (10^{-7} M), L-N^G-nitroarginine (L-NOARG, 3×10^{-5} M) and indomethacin (3×10^{-6} M), to block adrenergic neurotransmission, muscarinic receptors, NO synthase activity and prostaglandins, respectively. Vertical bar shows tension in g and horizontal bar time in min. W: wash out. Contraction (g) induced by (b) EFS (5 Hz) or (c) ATP (10^{-4} M), in the absence or presence of 10^{-5} M 8-phenyltheophylline in the pig intravesical ureter. Results represent means and vertical line s.e. mean of 9–11 preparations.

and/or postsynaptic contractile responses (White, 1988; Huidobro-Toro & Parada, 1989; Acevedo *et al.*, 1992). This notion, however, does not unequivocally mean that any adenosine mediated relaxation is exerted through an action at the A₂-receptor, as there are references describing A₁-receptor mediated relaxations. Thus, in the adenosine-mediated relaxation of the rat duodenum (Nicholls *et al.*, 1992), in the pig coronary arteries (Merkel *et al.*, 1992; Dart & Standen, 1993) and in rat diaphragmatic arterioles (Danialou *et al.*, 1997), the involvement of both the A₁- and A₂-receptor subtypes has been suggested. In the present study, the contribution of the A₁-receptor to pig intravesical ureter relaxation has been discarded because of the low potency shown by adenosine and CPA, as well as by the lack of effect of 10^{-8} M DPCPX, a concentration selective for blocking the A₁-receptor, on relaxations to adenosine and NECA.

A₂-receptors are divided into A_{2A}- and A_{2B}-subtypes on the basis of the potency of CGS21680 (15 nM on A_{2A} and practically inactive on A_{2B}) (Jacobson, 1990; Jacobson *et al.*, 1995). Thus, the rabbit aorta, mesenteric and coeliac arteries show A₂-receptor mediated vasodilatation, but in those preparations CGS21680 has a relaxing potency which is similar to that exhibited by NECA, so the A_{2A} subtype has been the one implicated in the vasodilatation (Balwierczak *et al.*, 1991). However, in vascular tissues such as the dog coronary artery and saphenous vein, guinea-pig aorta (Balwierczak *et al.*, 1991), rat mesenteric bed (Rubino *et al.*, 1995; Prentice *et al.*, 1997) as well as in the rat duodenum and

urinary bladder (Nicholls *et al.*, 1992) CGS21680 is very weak whereas NECA is a potent agonist, thus suggesting the presence of the A_{2B} subtype in those tissues. In the pig intravesical ureter, the low potency showed by CGS21680, as well as the lack of blocking effect produced by ZM 241385, a selective antagonist of A_{2A} adenosine receptors, on relaxations to adenosine or NECA (Poucher *et al.*, 1995), suggest that the adenosine-induced relaxation is mediated through activation of the A_{2B}-subtype. The low potency exhibited by CGS21680 is in accordance with those found in the rat duodenum and urinary bladder (Nicholls *et al.*, 1992).

A₃-receptors are clearly different from the A₁- and A₂-receptors, as the A₃-receptors show resistance to methylxanthine blockade. IB-MECA has been reported as a selective A₃-receptors agonist (Jacobson *et al.*, 1993; 1995; Gallo-Rodríguez *et al.*, 1994). In the pig intravesical ureter, the A₃-receptor subtype does not seem to be involved in the adenosine-mediated relaxation, as this relaxation was potently blocked by 8-PT, whereas the selective A₃-receptor agonist, IB-MECA (Gallo-Rodríguez *et al.*, 1994) produced relaxation of the ureteral smooth muscle only at high concentrations.

Adenosine is a regulator at a variety of synapses or neuroeffector junctions where ATP participates in neurotransmission, either as the transmitter itself or as a cotransmitter. Adenosine has also been reported as a paracrine neuromodulator (Acevedo *et al.*, 1992). Adenosine exerts its actions through a multiplicity of mechanisms. Thus, A₁-receptor activation may lead to modulation of adenylcyclase activity

and to a negative regulation of Ca²⁺ fluxes (Ribeiro *et al.*, 1979; Fredholm & Dunwiddie, 1988), protein kinase C stimulation, opening of K⁺ channels (Fredholm *et al.*, 1990), as well as to tonic inhibition of the release of ATP via presynaptic receptors (Sebastiao & Ribeiro, 1986; Huidobro-Toro & Parada, 1989). A₂-receptors may be concerned with blockade of Ca²⁺ currents or stimulation of adenylylase activity (Fredholm *et al.*, 1990). The multiplicity of mechanisms operated by adenosine, as well as the pre- and postsynaptic localization of the A₁- and A₂-receptors in the bladder, could explain the lack of selectivity of the adenosine-induced ATP antagonism. Thus, the adenosine analogue, NECA reduced the contractions induced not only by ATP but also those evoked by 5-HT or ACh (Acevedo *et al.*, 1992). These authors suggested that endogenous adenosine tone modulates the mouse urinary bladder neurotransmission. This conclusion has been reached on the basis of the potentiation evoked by 8-PT on the contraction induced by electrical stimulation and exogenous ATP (Acevedo *et al.*, 1992). In the present study, EFS and exogenous ATP evoked contractions of the intravesical ureteral strips, which were potentiated as a consequence of the blockade of adenosine receptors with 8-PT. These results suggest that adenosine could exert a physiological tonic influence on the ureteral NANC transmission and would thus be consistent with those obtained in the mouse (Acevedo *et al.*, 1992) and rat (Parija *et al.*, 1991) urinary bladder. The fact that 8-PT increased the contractions induced by two non purinergic agonists (ACh and 5-HT) could indicate that adenosine formed from ATP, either released from nerves or exogenously added, would be diminishing the contractions of the tissue nonspecifically. These results are in accordance with those found in the mouse urinary bladder smooth muscle

(Acevedo *et al.*, 1992) where NECA reduced the contractions induced by ATP, ACh, 5-HT and prostaglandin F_{2α}. The existence of a basal level of adenosine in the pig intravesical ureter does not seem likely as incubation of preparations with 8-PT failed to modify the ureteral basal tone. The lack of effect exhibited by 10⁻⁸ M DPCPX, on contractions evoked by EFS or exogenous ATP in the pig intravesical ureter, suggests the absence of an A₁-presynaptic receptor that could be inhibiting the release of excitatory transmitters from ureteral nerves. Moreover, the fact that adenosine analogues, NECA, CPCA and 2-CA did not modify the contractions to EFS suggests the absence of inhibitory A₂-receptors presynaptically located, which could be modulating the neurotransmitter release. NECA, however, reduced the contractions evoked by exogenous ATP. All these results suggest that adenosine could be acting *via* postsynaptic A_{2B}-receptors in the porcine ureter.

In summary, the results of the present investigation suggest that the adenosine receptors that cause relaxation of the intravesical ureteral smooth muscle are of the A_{2B}-subtype. Moreover, evidence is presented showing that adenosine could modulate the NANC excitatory neurotransmission of the pig intravesical ureter, possibly through a postsynaptic mechanism. The adenosine-induced modulation could be favouring the dilatation of ureteral lumen, thus contributing to urine boli transport through ureter and its discharge to urinary bladder.

The authors thank Mr Manuel Perales and Mr Francisco Puente for their technical assistance. They also wish to thank Municipal slaughterhouse (Villaviciosa de Odón, Madrid) for kindly donating the ureters.

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(Received July 7, 1998
Revised November 16, 1998
Accepted November 23, 1998)



The effects of phosphodiesterase type 4 inhibitors on tumour necrosis factor- α and leukotriene B₄ in a novel human whole blood assay

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1 The aim of this study was to assess the inhibitory activities of phosphodiesterase type 4 (PDE4) inhibitors on tumour necrosis factor- α (TNF- α) and leukotriene B₄ (LTB₄) production in a novel human whole blood assay.

2 Lipopolysaccharide (LPS) stimulation of human whole blood caused a time dependent increase in TNF- α and prostaglandin E₂ (PGE₂) plasma levels. Inhibition of LPS-induced TNF- α by the selective PDE4 inhibitor RP73401 was proportionally enhanced with endogenous PGE₂ (maximal after 24 h). In contrast, blocking endogenous PGE₂ production with indomethacin in blood stimulated with LPS for 24 h decreased the potency of RP73401 to that observed with a 4 h LPS incubation.

3 Non-selective and selective PDE4 inhibitors showed greater inhibition of LPS-induced TNF- α after 24 h compared to 4 h. Stereoselectivity was only achieved in the 24 h method.

4 LPS-stimulation of whole blood for either 30 min or 24 h followed by N-formyl-Met-Leu-Phe (fMLP) activation resulted in low plasma LTB₄ levels. Combination of both treatments resulted in a greater than 7 fold increase in plasma LTB₄ levels. Inhibition of the double LPS and fMLP-activated LTB₄ production was observed with non-selective and PDE4-selective inhibitors. Their LTB₄ inhibitory potencies were similar to that observed in the 24 h LPS-induced TNF- α assay. Thus, stimulation of human whole blood with two LPS stimulations followed by fMLP gives rise to both TNF- α and LTB₄ and their inhibition by various compounds can be assessed in the same blood sample.

5 Calcium ionophore (A23187) stimulation of whole blood resulted in plasma LTB₄ levels similar to the double LPS and fMLP method. Inhibition of A23187-induced LTB₄ biosynthesis was also achieved by PDE4-selective inhibitors as well as the direct 5-lipoxygenase (5-LO) inhibitor L-739,010.

6 These results confirm the anti-inflammatory properties of PDE4 inhibitors. Thus, this novel human whole blood can be used to assess the biochemical efficacy of PDE4 inhibitors in human subjects.

Keywords: Phosphodiesterase; tumour necrosis factor α ; leukotriene B₄; whole blood

Abbreviations: 5'-AMP, adenosine 5'-monophosphate; 5-LO, 5-lipoxygenase; A23187, calcium ionophore; BSA, bovine serum albumin; cyclic AMP, adenosine 3':5'-cyclic monophosphate; COX, cyclo-oxygenase; DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunosorbent assay; EP, PGE₂ receptor; FLAP, 5-lipoxygenase activating protein; fMLP, n-formyl-Met-Leu-Phe; IBD, inflammatory bowel disease; IBMX, 3-isobutyl-1-methylxanthine; IFN, interferon; IL-2, interleukin-2; LTB₄, leukotriene B₄; LPS, lipopolysaccharide; NSAIDs, non-steroidal anti-inflammatory drugs; PAF, platelet activating factor; PBS, phosphate buffered saline; PDE, phosphodiesterase; PGE₂, prostaglandin E₂; PKA, protein kinase A; PLA₂, phospholipase A₂; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear cell; TNF- α , tumour necrosis factor-alpha

Introduction

Phosphodiesterase 4 (PDE4), a member of the phosphodiesterase family of at least nine known distinct types (PDE1 to PDE9), is a cyclic adenosine monophosphate (cyclic AMP)-specific enzyme and is responsible for the hydrolysis of intracellular cyclic AMP to 5'-AMP. Inhibition of this enzyme leads to increases in intracellular cyclic AMP which can subsequently activate protein kinase A (PKA) (Torphy & Udem, 1991; Seldon *et al.*, 1995). It has been shown with various PDE4-specific inhibitors that an increase in intracel-

lular cyclic AMP can inhibit the expression and release of several inflammatory mediators, prevent superoxide anion formation in neutrophils, inhibit eosinophil chemotaxis and degranulation as well as lymphocyte proliferation, and causes relaxation of bronchial smooth muscles resulting in bronchodilation (Nielson *et al.*, 1990; Hartman *et al.*, 1993; Underwood *et al.*, 1994; Schudt *et al.*, 1995; Essayan *et al.*, 1997). PDE4 is predominantly expressed in monocytes, neutrophils and eosinophils (Schudt *et al.*, 1995) and its inhibition could be beneficial in treating the underlying inflammation associated with asthma.

Tumour Necrosis Factor- α (TNF- α) is a very powerful proinflammatory mediator produced by activated macrophages, blood monocytes and mast cells (Gordon & Galli,

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1991). In addition to its anti-tumour properties (Jia & Kleinerman, 1991), it plays a central role in many inflammatory processes (Warren *et al.*, 1988). It is a critical cytokine in the host defence of bacterial infections (Beutler & Cerami, 1989; Nakane *et al.*, 1992) but it is also associated with shock and tissue injury during sepsis leading to organ failure (Tracey *et al.*, 1986). It has many biological activities on a wide variety of cell types (endothelial cells, lymphocytes, osteoclasts) and can upregulate or induce expression of receptors (e.g. interferon (IFN)-gamma, TNF- α p55 receptors), cytokines, growth factors, inflammatory mediators and adhesion molecules as well as activate transcription factors (e.g. NF- κ B) (Dinarello *et al.*, 1986; Scheurich *et al.*, 1987; Doerfler *et al.*, 1989; Valyi-Nagy *et al.*, 1992; Krakauer & Oppenheim, 1993; Trefzer *et al.*, 1993; Mackay *et al.*, 1993; Arias-Negrete *et al.*, 1995; Schutze *et al.*, 1995; Batten *et al.*, 1996). This cytokine has been implicated in several inflammatory diseases (rheumatoid arthritis and Crohn's disease) and its inhibition has been the focus of many studies (Eigler *et al.*, 1997).

Leukotriene B₄ (LTB₄) is a product of arachidonic acid metabolism of the 5-lipoxygenase (5-LO) pathway (Borgeat & Samuelsson, 1979). Leukotrienes are also proinflammatory mediators and are synthesized by many cells such as macrophages, monocytes, eosinophils, mast cells and neutrophils. LTB₄ is a major product of activated neutrophils and a potent chemoattractant. It can exert its effects on cells and tissues by increasing aggregation and release of lysosomal enzymes, stimulating superoxide anion production in neutrophils, increasing interleukin-2 (IL-2) release and receptors in lymphocytes and enhancing vascular permeability in tissues (Ford-Hutchinson, 1990). Psoriasis, inflammatory bowel disease (IBD), rheumatoid arthritis and mostly asthma have all been associated with the potent inflammatory properties of LTB₄ (Davidson *et al.*, 1983; Brain *et al.*, 1984; Grabbe *et al.*, 1984; Sharon & Stenson, 1984). Inhibitors of 5-LO and 5-LO activating protein (FLAP) have been developed as therapeutic agents for the treatment of asthma and their effects on psoriasis and IBD have also been studied (Larsen & Jackson, 1996).

TNF- α has been shown to be inhibited at the transcriptional level by increases in cyclic AMP due to PDE4 inhibition or activation of adenylyl cyclase (Han *et al.*, 1990). A synergistic effect with both PDE4 inhibitors and activators of adenylyl cyclase (e.g. PGE₂) has been shown to greatly inhibit TNF- α (Schade & Schudt, 1993). The effects of PDE4 inhibitors on LTB₄ have not been studied as much as TNF- α but it has been shown that inhibition of LTB₄ in polymorphonuclear cells (PMNs) can be achieved by the non-selective PDE inhibitors IBMX (3-isobutyl-1-methylxanthine) and theophylline, the PDE4 selective inhibitor rolipram and the β -agonist isoproterenol. Studies have shown that arachidonic acid metabolites such as platelet activating factor (PAF) and LTB₄ are inhibited by increases in cyclic AMP *via* the regulation of phospholipase A₂ (PLA₂) activity and the modulation of intracellular calcium concentrations (Fonteh *et al.*, 1993; Villagrasa *et al.*, 1996). For the reason that TNF- α and LTB₄ are key mediators in inflammation and are both sensitive to intracellular cyclic AMP levels, they represent good surrogate markers of PDE4 activity.

Activation of isolated blood leukocytes is the most common *in vitro* method used to examine the intrinsic potency of PDE4 inhibitors, however these assays usually use low serum concentrations, hence a low protein environment, and cannot predict activity in whole blood. Furthermore, the effects of PDE4 inhibition have only been studied on one surrogate marker at a time per cell type using various stimulations such as LPS-induced TNF- α in monocytes or fMLP activated

PMNs. In this study, we have developed a human whole blood assay which can be used to examine the effects of non-selective and selective PDE inhibitors as well as activators of adenylyl cyclase on both TNF- α and LTB₄ simultaneously in a protein-rich environment.

Methods

LPS-induced TNF- α

Fresh blood was collected in heparinized tubes by venipuncture from both male and female volunteers with consent. The subjects had no apparent inflammatory conditions and had not taken any nonsteroidal anti-inflammatory drugs (NSAIDs) for at least 4 days prior to blood collection. Five hundred μ l aliquots of blood were pre-incubated with either 2 μ l vehicle or test compound at 37°C for 15 min. This was followed by incubation of the blood with 10 μ l lipopolysaccharide (LPS) (Sigma, St Louis, MO, U.S.A., from *E. coli* serotype 0111:B4, 1 μ g ml⁻¹ final concentration, diluted in 0.1% bovine serum albumin (BSA) (Sigma, St Louis, MO, U.S.A., fraction V, diluted in phosphate buffered saline (PBS))) for 0.5–24 h at 37°C. Appropriate PBS controls (no LPS) were used as blanks. After the desired incubation period, the samples were centrifuged at 1500 $\times$ g at 4°C for 10 min. Plasma TNF- α was quantified ELISA (Cistron Biotechnology, Pine Brook, NJ, U.S.A.). A 50 μ l aliquot of plasma was mixed with 200 μ l methanol for protein precipitation and centrifuged at 1500 $\times$ g at 4°C for 10 min. The supernatant was obtained and PGE₂ was determined by radioimmunoassay (Amersham, Oakville, Ont, Canada) after conversion of PGE₂ to its methyl oximate derivative according to manufacturer's procedure.

fMLP-stimulation of LTB₄

Heparinized human whole blood was pre-incubated with either vehicle or test compound as described above and then incubated with 10 μ l of LPS (1 μ g ml⁻¹ final concentration, diluted in 0.1% BSA) for either 30 min or 24 h at 37°C. This was followed by incubation of 10 μ l of the chemotactic peptide n-formyl-Met-Leu-Phe (fMLP) (Sigma, St Louis, MO, U.S.A., diluted in 1% BSA, 1 μ M final concentration), or PBS for blank controls, for 15 min at 37°C. For some experiments, the blood was incubated with fMLP immediately after drug pre-incubation without pre-stimulation with LPS. At the end of the fMLP incubation, the blood was immediately centrifuged in a pre-cooled centrifuge at 4°C for 10 min. The samples were left on ice during plasma transfer. The plasma was extracted in methanol as described above and the supernatant was assayed for LTB₄ by enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI, U.S.A.) according to manufacturer's instructions.

Double LPS-induced and fMLP-activated TNF- α and LTB₄ assay

Five hundred μ l aliquots of blood were pre-incubated with either vehicle or test compound at 37°C for 15 min. This was followed by incubation of the blood with 10 μ l LPS (1 μ g ml⁻¹ final concentration, diluted in 0.1% BSA) at 37°C in a humidified incubator (5% CO₂). Appropriate PBS control (no LPS) were used as blanks. After the 24 h incubation, another 10 μ l of LPS (1 μ g ml⁻¹ final concentration, diluted in 0.1% BSA) was added to blood (PBS for blanks) and incubated for 30 min at 37°C. The samples were then challenged for 15 min

at 37°C with either 10 μ l fMLP (diluted in 1% BSA, 1 μ M final concentration) or PBS for blanks. The blood was centrifuged as above and the plasma obtained was quantified for both TNF- α and LTB₄ content as described above.

A23187-stimulation of LTB₄

Heparinized human whole blood was pre-incubated with either vehicle or test compound as described above and then incubated with 2 μ l of the calcium ionophore A23187 (Sigma, St Louis, MO, U.S.A., diluted in DMSO, 25 μ M final concentration) or 2 μ l of dimethyl sulphoxide (DMSO) as blank controls for 30 min at 37°C. The following procedure was identical to the fMLP method for measurement of LTB₄.

Statistics

Results are expressed as mean and s.e.mean. Differences between controls and treatment groups were tested using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Materials

RS14203 (Wilhelm & Fatheree, 1994), RP73401 (Souness *et al.*, 1995), T-440 (Kaminuma *et al.*, 1996), R- and S-rolipram, SB207499 (Christensen *et al.*, 1998), CDP840 (Hughes *et al.*, 1996), CT1731 (Hughes *et al.*, 1996), Trequinsin, L-739,010 (Hamel *et al.*, 1997), MF-tricyclic (selective cyclo-oxygenase-2 (COX-2) inhibitor) (Oshima *et al.*, 1996) were synthesized by the Medicinal Chemistry departments at Merck Frosst Centre for Therapeutic Research and Celltech Therapeutics, U.K. Other compounds and their sources were: Indomethacin (Merck & Co., Rahway, NJ, U.S.A.), PGE₂ (Cayman Chemicals, Ann Arbor, MI, U.S.A.), amrinone, dibutylcyclic AMP, isoproterenol, 3-isobutyl-1-methylxanthine (IBMX), pentoxifylline, theophylline, zaprinast (Sigma, St Louis, MO, U.S.A.), dipyridamole, milrinone, quazinson, forskolin (Calbiochem, La Jolla, CA, U.S.A.) and cholera toxin (Biomol, Plymouth Meeting, PA, U.S.A.).

Results

Time course

Figure 1 shows the time course of TNF- α and PGE₂ production after LPS-stimulation of whole blood. During the incubation period, a sharp rise in TNF- α levels was observed from 2 h (2.70 ± 0.76 ng ml⁻¹) to 4 h (15.59 ± 3.61 ng ml⁻¹) and peaked at 8 h (22.92 ± 4.77 ng ml⁻¹). The levels obtained at 4, 6, 8 and 24 h were not significantly different from each other. Out of the three subjects used for this experiment, two had maximal TNF- α levels at 8 h whereas the other reached its peak at 4 h. In all cases the levels were lower after 24 h (17.33 ± 2.52 ng ml⁻¹) compared to levels at 8 h. PGE₂ levels did not increase significantly over baseline until 4 h (0.88 ± 0.15 nM) and rose steadily from 8 h (1.96 ± 0.16 nM) to 24 h (16.94 ± 2.79 nM) due to increased COX-2 expression as previously shown (Brideau *et al.*, 1996). Addition of 10 μ M RP73401 inhibited TNF- α production at 4, 6, 8 and 24 h by 48, 67, 75 and 78% respectively. Interestingly, this inhibition increased with rising levels of PGE₂ and was most significant at 24 h.

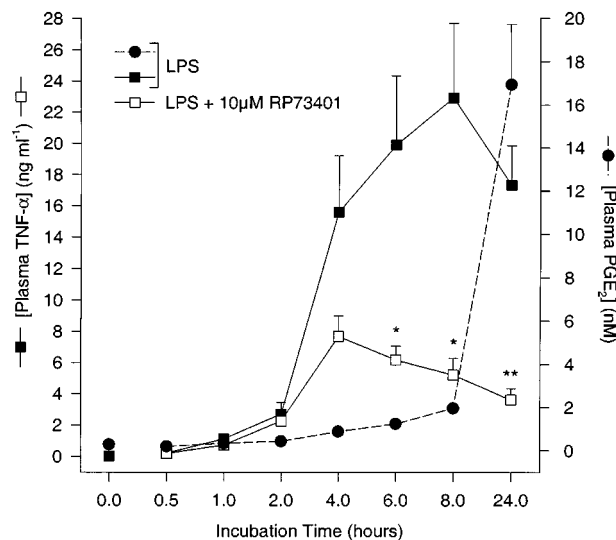


Figure 1 Time course of TNF- α and PGE₂ production in human whole blood following LPS (1μ g ml⁻¹) incubation in the presence or absence of a PDE4 inhibitor (10 μ M RP73401). *n*=3 donors, **P*<0.05 and ***P*<0.01 vs LPS-induced TNF- α alone.

TNF- α production: Effect of PGE₂ and LPS incubation time

Most of the published methods for TNF- α induction and inhibition by various agents in human whole blood use a 4 h LPS incubation period (Hartman *et al.*, 1993; 1995). To determine whether 4 h is optimal for characterizing PDE4 inhibitors, we examined the effects of LPS incubation time and PGE₂ on TNF- α inhibition. The concentration of PGE₂ required to inhibit half of the TNF- α produced (or IC₅₀) in the 4 h LPS-stimulation assay was 15 nM in whole blood (Figure 2a). In the same assay, the IC₅₀ for RP73401 was 9.5 μ M and the maximum inhibition observed was 68% at 10 μ M. The addition of 1 nM or 10 nM PGE₂ resulted in a dose dependent increase in TNF- α inhibition by RP73401 (Figure 2b). The maximal response observed with 10 μ M RP73401 rose to 97% in the presence of 10 nM PGE₂.

In many experiments performed in our laboratory using PDE4-selective inhibitors, maximal inhibitions of greater than 70% could not be achieved in the 4 h LPS-induced TNF- α assay (data not shown). Moreover, we showed greater inhibition of LPS-induced TNF- α with RP73401 after 8 h of incubation which coincided with increased PGE₂ levels (Figure 1). Therefore, we compared the potency of RP73401 after 4 and 24 h LPS incubations. As represented in Figure 3a, the mean plasma TNF- α levels increased at least 6 fold after 24 h LPS incubation compared to 4 h. In Figure 3b, the inhibitory effect of RP73401 on LPS-induced TNF- α was greater after 24 h. To establish the importance of PGE₂ in the 24 h assay, indomethacin was incubated simultaneously with LPS for 24 h to inhibit the synthesis of PGE₂. Indomethacin completely blocked the 24 h LPS-induced PGE₂ production and had no effect on TNF- α levels (Figure 3a). The potency of RP73401 on LPS-induced TNF- α was significantly reduced in the presence of indomethacin and was similar to that observed with the 4 h LPS incubation (Figure 3b).

Table 1 summarizes the IC₅₀ values of several PDE4 inhibitors in both the 4 and 24 h assays. All inhibitors, except CT1731 and CDP840, showed significantly higher potency in the 24 h assay. The maximum inhibitions observed in the 24 h assay were greater than 80% for all compounds. Significant (*P*<0.01) differences between enantiomeric pairs such as R-

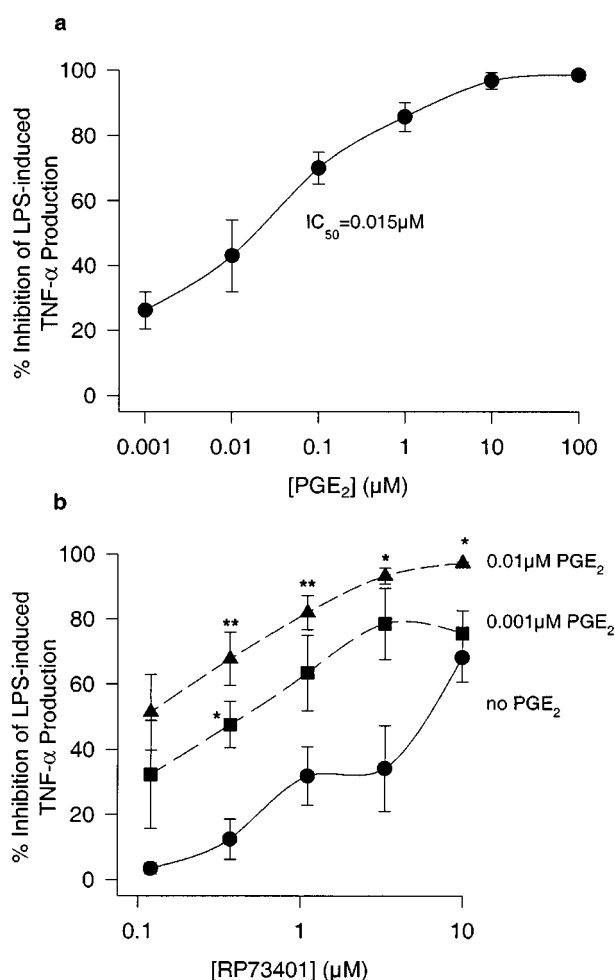


Figure 2 The potentiation of the inhibitory effect of a PDE4 inhibitor (RP73401) by exogenous PGE₂ on LPS-induced TNF-α production (4 h incubation). (a) Effect of exogenous PGE₂ alone. (b) Effect of RP73401 in the absence or presence of PGE₂. *n* = 3 donors. **P* < 0.05 and ***P* < 0.01 vs RP73401 alone.

rolipram and S-rolipram as well as CDP840 and CT1731 were only achieved in the 24 h LPS incubation method.

LTB₄ production: Effects of LPS and fMLP at different incubation times

We compared the effects of LPS stimulation, fMLP activation and incubation time on LTB₄ production in human whole blood. Figure 4 demonstrates the results of four different methods based on published procedures (Surette *et al.*, 1993) as well as procedures modified in our laboratory. Incubation of blood with 1 μM fMLP for 15 min failed to increase LTB₄ production compared to baseline and the negative PBS control (method A). Priming blood with LPS for 30 min prior to fMLP addition resulted in a significant increase in LTB₄ levels (3.44 ± 1.11 ng ml⁻¹) over baseline whereas a 30 min LPS incubation without fMLP had no effect (method B, Surette *et al.*, 1993). When LPS priming time was increased to 24 h as shown in method C, we obtained the similar results as in method B. More than a 6 fold increase (25.20 ± 7.92 ng ml⁻¹) in LTB₄ levels was observed when whole blood was pre-stimulated with LPS for 24 h and then again for 30 min followed by fMLP stimulation (method D). Thus, combining the LPS priming from methods B and C had a much greater effect on LTB₄ synthesis in human whole blood. We compared the effects of two PDE4 inhibitors on LTB₄ synthesis in whole

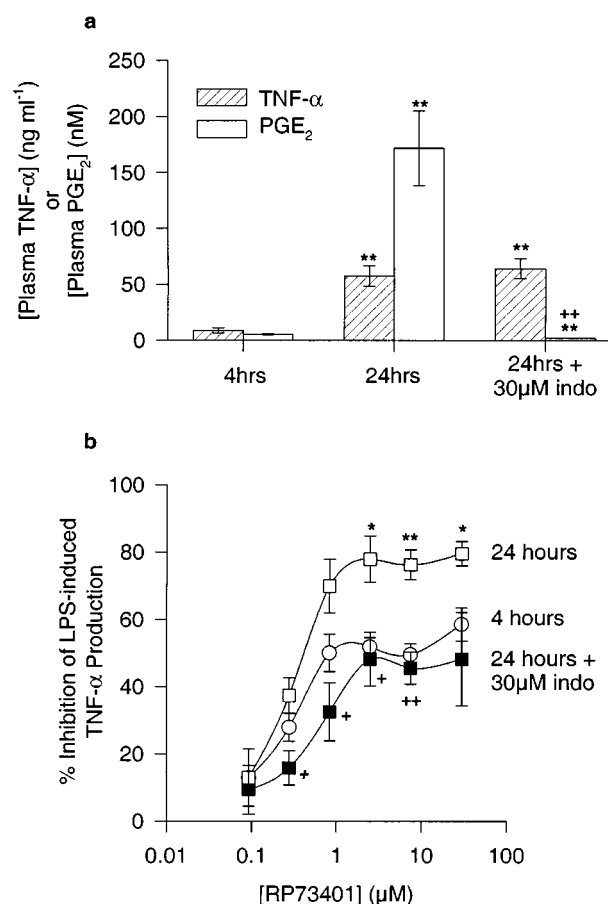


Figure 3 Comparison between 4 and 24 h incubation of human whole blood with 1 μg ml⁻¹ LPS in addition to a 24 h LPS incubation in the presence of 30 μM indomethacin. (a) plasma TNF-α and PGE₂ levels and (b) effect of RP73401 on LPS-induced TNF-α. Experiments performed in same blood sample. *n* = 3 donors. **P* < 0.05 and ***P* < 0.01 vs 4 h incubation and +*P* < 0.05 and ++*P* < 0.01 vs 24 h LPS only.

Table 1 Effect of incubation time on the potency of several PDE4 inhibitors in the LPS-induced TNF-α human whole blood assay. **P* < 0.05 and ***P* < 0.01

Inhibitor	4 h LPS IC ₅₀ (μM)	n	24 h LPS IC ₅₀ (μM)	n
RS14203	0.22 ± 0.08	11	0.048 ± 0.008*	15
RP73401	2.61 ± 0.62	22	0.34 ± 0.09*	12
R-Rolipram	15.22 ± 4.43	6	2.16 ± 0.20**	15
S-Rolipram	24.05 ± 2.78	5	8.55 ± 1.10**	15
IBMX	36.50 ± 6.65	6	9.11 ± 4.46*	3
CDP840	20.98 ± 1.65	59	15.44 ± 1.98	15
SB207499	81.32 ± 14.64	3	20.29 ± 3.39**	17
CT1731 (enantiomer of CDP840)	34.87 ± 7.31	3	35.12 ± 4.91	6
Pentoxifylline	256.0 ± 60.0	3	38.07 ± 6.94*	3

blood using methods B and D. Figure 5 demonstrates the inhibition curves for RS14203 (panel a) and RP73401 (panel b) in both methods. A greater maximal inhibition was observed for both compounds when the blood was pre-incubated with LPS for 24 h (method D). RS14203 exhibited a flat titration curve without the LPS pre-incubation and a plateau of approximately 66% inhibition was observed in the highest doses (method B) while 90% inhibition was achieved with the 24 h LPS pre-incubation (method D).

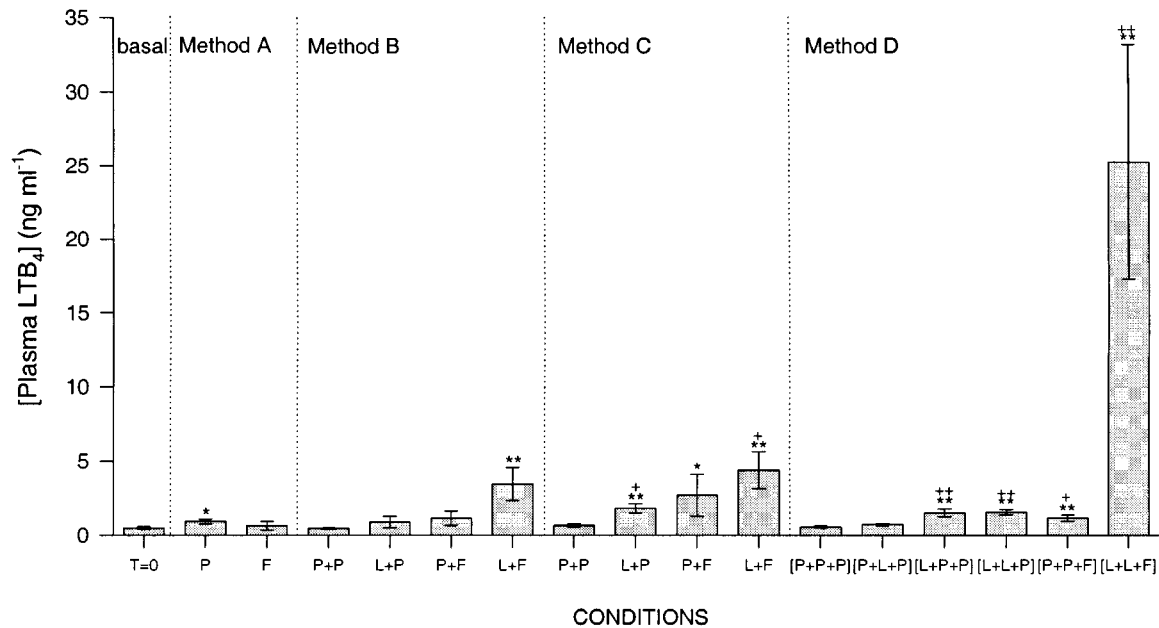


Figure 4 Plasma LTB₄ levels from different conditions. Basal represents unstimulated blood plasma at time 0. P=PBS (blanks), L=LPS (1 $\mu\text{g ml}^{-1}$) and F=fMLP (1 μM). Method A: human whole blood stimulated with fMLP for 15 min. Method B: pre-stimulation with either PBS or LPS (first letter on graph) for 30 min followed by incubation with either PBS or fMLP for 15 min (second letter on graph). Method C: pre-stimulation with either PBS or LPS for 24 h (first letter on graph) followed by incubation with either PBS or fMLP for 15 min (second letter on graph). Method D: pre-stimulation with either PBS or LPS (first letter on graph) for 24 h, then a 30 min stimulation with either PBS or LPS (second letter on graph) followed by incubation with either PBS or fMLP (third letter on graph) for 15 min. $n=3-6$ donor. * $P<0.05$ and ** $P<0.01$ vs time 0 and † $P<0.05$ and †† $P<0.01$ vs PBS blank for each condition.

Effect of double LPS pre-incubations and fMLP stimulation on TNF- α

As demonstrated above, TNF- α inhibition by PDE inhibitors was much more effective when whole blood was incubated with LPS for 24 h because of the presence of PGE₂. We examined whether an additional 30 min incubation with LPS and a 15 min fMLP-stimulation would alter the levels of TNF- α and the efficacy of PDE inhibitors. Figure 6 shows a very good correlation between the simple 24 h assay (TNF- α method) and the elaborated method used for LTB₄ (method D). TNF- α levels in the absence of PDE4 inhibitors were approximately 25% lower in method D but the inhibition responses of PDE inhibitors in the same blood sample were almost identical. Thus, method D can be used to study the effects of PDE4 inhibitors on both TNF- α and LTB₄.

Table 2 summarizes the IC₅₀ values of several PDE inhibitors, cyclic AMP elevating agents, inhibitors of cyclooxygenase (COX-1/COX-2) and a 5-LO inhibitor on inhibition of either TNF- α or LTB₄ from the same blood sample. All of the PDE4-selective inhibitors were able to inhibit both TNF- α and LTB₄ with similar potency except RS14203 and milrinone which were significantly more potent against TNF- α . Agents which elevate intracellular cyclic AMP by activating adenylyl cyclase are more potent inhibitors of TNF- α than LTB₄ except for cholera toxin which had similar potency. The non-selective PDE inhibitors and the PDE III-selective inhibitors were not potent against either mediator and the PDE V-selective inhibitors were not active. The activity observed with dipyrindamole was probably due to its inhibition on adenosine uptake by red blood cells rather than its inhibition of PDE V (Krump *et al.*, 1996; Eigler *et al.*, 1997). The 5-LO inhibitor L-739,010 had no effect on TNF- α but was very potent against LTB₄. The selective inhibition of COX-2 by MF-tricyclic had no effect on either TNF- α or LTB₄ whereas an increase in

TNF- α and LTB₄ production was observed with the non-selective COX inhibitor indomethacin.

Effects of PDE inhibitors on A23187-stimulated LTB₄

To confirm the LTB₄ inhibitory effects of PDE4 inhibitors, a non-receptor mediated whole blood assay was performed using the calcium ionophore, A23187, without LPS pre-stimulation. PDE4-selective inhibitors, cyclic AMP elevating agents and a 5-LO inhibitor were examined in whole blood challenged with 25 μM A23187 for 30 min. The plasma LTB₄ levels obtained by A23187 were very similar to the double LPS-fMLP-stimulated whole blood method (method D), $19.30 \pm 3.76 \text{ ng ml}^{-1}$ and $20.87 \pm 4.41 \text{ ng ml}^{-1}$ respectively (Figure 7a). Inhibition of LTB₄ by RS14203 was significantly greater in blood challenged with A23187 (Figure 7b). Several compounds were tested in both assays using the same blood sample and their results are summarized in Table 3. Although these compounds were more potent in A23187-challenged blood, with the exception of CDP840, none were found to be significantly different aside from S-rolipram and L-739,010.

Discussion

We have developed a novel method of assessing the effects of PDE4 inhibitors on both TNF- α and LTB₄ in the same human whole blood sample. Using this assay, it was possible to study PDE4 activity by measuring the levels of two surrogate markers in distinct cell types such as monocytes for TNF- α and neutrophils for LTB₄.

Inhibition of PDE4 using selective inhibitors was not sufficient to completely block the synthesis of TNF- α in LPS-stimulated blood in the absence of PGE₂. However, PGE₂ can completely block TNF- α synthesis albeit at concentrations

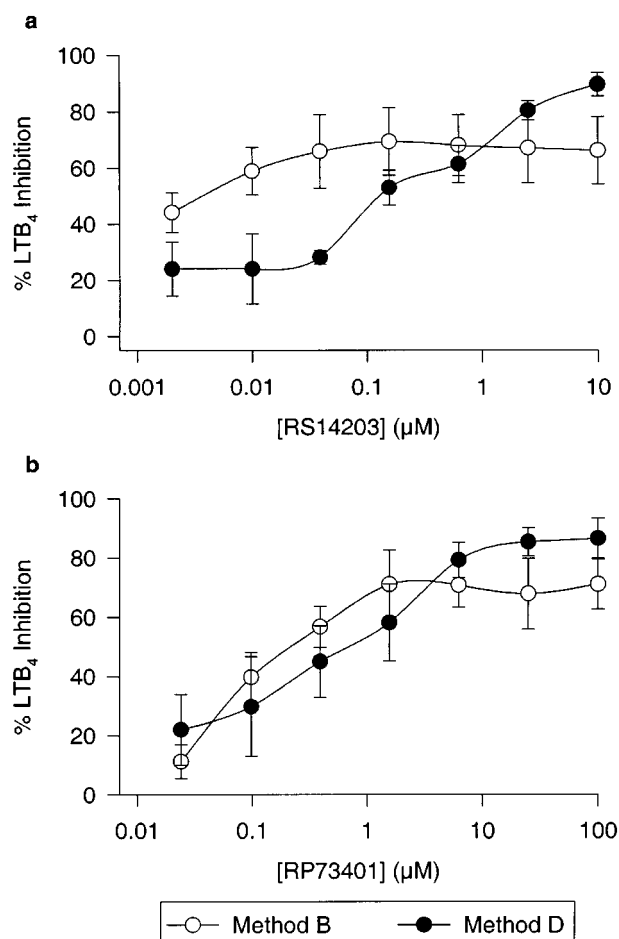


Figure 5 Comparison between two methods of fMLP-stimulated LTB₄ human whole blood assays using two different PDE4 inhibitors (RS14203 and RP73401). Method B: whole blood pre-stimulated with LPS ($1 \mu\text{g ml}^{-1}$) for 30 min followed by 15 min incubation with fMLP ($1 \mu\text{M}$) and Method D: whole blood pre-stimulated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h followed by 30 min stimulation with LPS ($1 \mu\text{g ml}^{-1}$) then a 15 min incubation with fMLP ($1 \mu\text{M}$). $n=3$ donors. Both methods were performed in the same donor sample.

greater than 500 fold the IC_{50} . As we did not use a direct method of establishing PDE4 inhibition in human blood cells such as measuring intracellular cyclic AMP or PKA activity, it is possible that complete inhibition of PDE4 may not raise intracellular cyclic AMP levels high enough for full inhibition of TNF- α synthesis. COX-2 enzyme expression and activity is upregulated in LPS-treated human monocytes (Patrignani *et al.*, 1994; Brideau *et al.*, 1996) and increases endogenous synthesis of PGE₂. PGE₂, an activator of adenylyl cyclase through the prostanoid receptor subtypes EP₂ or EP₄, leads to elevation of intracellular cyclic AMP (Coleman *et al.*, 1994), which, in combination with PDE4 inhibition, enhances inhibition of TNF- α synthesis. Complete TNF- α inhibition by PDE4 inhibitors was only observed in the 24 h LPS-stimulated whole blood assay. Using this assay, the best dose response curves for PDE4 inhibitors were achieved and thus more accurate IC_{50} values were calculated. This was particularly important when comparing the activity of enantiomeric pairs of PDE4 inhibitors. The activity of R-rolipram in whole blood was 4 fold more potent than its enantiomer, S-rolipram, which is consistent with previously published data on LPS-stimulated TNF- α in human monocytes (Souness *et al.*, 1996). In the absence of PGE₂ (4 h LPS incubation), the difference in activity between R-rolipram and S-rolipram and was not

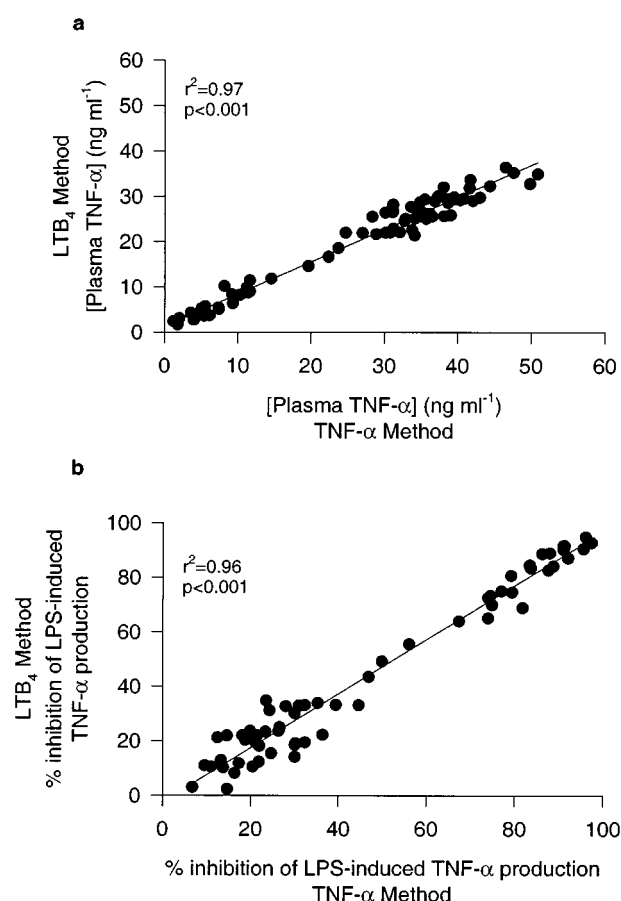


Figure 6 Linear correlation of results obtained from two methods of LPS-induced TNF- α in human whole blood. TNF- α Method: whole blood samples incubated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h. LTB₄ Method D: whole blood samples incubated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h followed by 30 min incubation of LPS ($1 \mu\text{g ml}^{-1}$) then a 15 min incubation with fMLP ($1 \mu\text{M}$). (a) represents plasma TNF- α levels from an experiment where both methods were done in the same blood sample in the presence and absence of varying concentrations of 10 different PDE4 inhibitors. $n=71$ data points. (b) represents the percentage inhibition of TNF- α of the same data points (except data points from positive no drug controls from which the percentage inhibition was calculated). $n=61$ data points. All data represented were blood samples from one donor.

significant. As demonstrated in the indomethacin experiment, PGE₂ is important for complete inhibition of TNF- α by PDE4 inhibitors since in its absence there was approximately 30% of residual TNF- α which could not be inhibited with higher doses of inhibitor. PGE₂ may be responsible for further activation of PKA through the direct activation of adenylyl cyclase. It has been shown that PKA is involved in the inhibition of TNF- α transcription by inhibiting NF- κ B-mediated transcription (Ollivier *et al.*, 1996), however, the mechanism is still unclear as there are other transcription factors involved in the regulation of TNF- α such as the p38 and p42 MAPK cascades (Foey *et al.*, 1998). It is possible that a 24 h incubation is longer than necessary and that 8 h may be sufficient for complete TNF- α inhibition by PDE4 inhibitors. The results from the time course experiment showed little difference in TNF- α inhibition by RP73401 at 8 and 24 h. However, the 24 h incubation method was considered more pragmatic taking into account the higher donor variability in expressing COX-2 and the subsequent release of PGE₂ in an 8 h method.

It has been shown that priming human whole blood with LPS for 30 min is necessary for the stimulation of LTB₄

Table 2 *In vitro* results of human whole blood pre-stimulated with LPS for 24 h then again for 30 min followed by fMLP (method D). * $P < 0.05$ and ** $P < 0.01$

Inhibitor	$TNF-\alpha$ IC_{50} (μM)	n	LTB_4 IC_{50} (μM)	n
PDE4-selective				
RS14203	0.048 ± 0.008	15	$0.30 \pm 0.19^*$	15
RP73401	0.34 ± 0.09	12	0.77 ± 0.38	11
T-440	1.13 ± 0.44	9	2.17 ± 0.62	9
R-Rolipram	2.16 ± 0.20	15	4.27 ± 1.58	15
S-Rolipram	8.55 ± 1.10	15	11.11 ± 2.50	15
CDP840	15.44 ± 1.98	15	14.46 ± 2.11	15
SB207499	20.29 ± 3.39	15	15.05 ± 4.51	18
CT1731 (CDP840 enantiomer)	35.12 ± 4.91	6	42.10 ± 10.57	6
Non-selective PDE				
IBMX	15.84 ± 5.19	6	44.94 ± 20.30	6
Pentoxifylline	38.07 ± 6.94	6	32.73 ± 17.88	3
Theophylline	56% @ 400 μM	3	50% @ 400 μM	3
PDE III-selective				
Trequinsin	22.04 ± 10.26	3	37.34 ± 23.94	3
Milrinone	28.97 ± 7.30	3	$122.5 \pm 18.12^{**}$	3
Amrinone	75.21 ± 18.41	3	51.77 ± 20.45	3
PDE V-selective				
Dipyridamole (adenosine uptake inh.)	37.44 ± 21.39	6	78.20 ± 15.68	6
Zaprinast	36% @ 400 μM	3	45% @ 400 μM	6
Quazinone	32% @ 400 μM	3	10% @ 400 μM	3
5-LO				
L-739,010	4% @ 10 μM	9	0.16 ± 0.02	9
COX				
Indomethacin (COX-1 & 2)	-54% @ 100 μM	9	-63% @ 100 μM	9
MF-Tricyclic (COX-2)	0% @ 100 μM	3	0% @ 100 μM	3
cyclic AMP elevating agents				
PGE ₂	0.008 ± 0.002	9	0% @ 10 μM	9
Isoproterenol	0.020 ± 0.013	3	6% @ 10 μM	3
Cholera toxin (μg ml ⁻¹)	2.67 ± 0.87	3	1.41 ± 0.34	3
Dibutyl-cyclic AMP	66% @ 10 μM	3	23% @ 10 μM	3
Forskolin	65% @ 80 μM	3	40% @ 80 μM	3

production by fMLP (method B) and that exogenous $TNF-\alpha$ has an additive effect with LPS (Surette *et al.*, 1993). We confirmed this by obtaining similar results when whole blood was incubated with LPS for 24 h (method C) to increase both $TNF-\alpha$ and PGE₂ levels before the subsequent fMLP-stimulated release of LTB_4 . The LTB_4 levels in either assay were approximately 7 fold higher compared to their blank controls. A greater than 50 fold window was obtained when whole blood was primed twice with LPS before fMLP-stimulation (method D). This substantial increase in LTB_4 may be due to further activation of cytosolic PLA₂ by the additional LPS incubation releasing a large pool of free arachidonate to be readily utilized by activated 5-LO. LPS-stimulated LTB_4 production was not $TNF-\alpha$ dependent since 10 μM PGE₂ totally abolished $TNF-\alpha$ but had no effect on LTB_4 . As observed in the $TNF-\alpha$ experiments, the presence of PGE₂ enhanced the inhibitory activity of PDE4 inhibitors on LTB_4 production. Compared to method B, LTB_4 inhibition was enhanced using method D (24 h LPS) even though LTB_4 levels were 7 fold higher. Furthermore, the potency of the PDE4-selective inhibitor, RP73401, was reduced in the presence of indomethacin using the method D assay (data not shown).

Of the non-selective PDE inhibitors, IBMX was most potent followed by pentoxifylline yet theophylline displayed marginal activity. For both $TNF-\alpha$ and LTB_4 , the PDE V-selective compounds and the COX-2 selective inhibitor, MF-tricyclic, showed no effect. The dual COX-1 and COX-2 inhibitor, indomethacin, potentiated $TNF-\alpha$ and LTB_4

production equally. In contrast, the 5-LO inhibitor, L-739,010, was most potent against LTB_4 and as expected was inactive against $TNF-\alpha$.

PDE4 is most pre-dominant in monocytes and neutrophils however PDE III is also present in monocytes (Souness *et al.*, 1996). PDE III-selective inhibitors have shown inhibitory activity on monocyte-derived $TNF-\alpha$ production and on neutrophil-derived leukotriene production (Banner *et al.*, 1996; Schudt *et al.*, 1991). In our assay, the PDE III-selective inhibitors were also active but to a lesser extent than the PDE4 inhibitors. The activity observed may also be due to the loss of selectivity of these inhibitors at high concentrations (IC_{50} s: $> 20 \mu M$).

While $TNF-\alpha$ levels were significantly decreased by 25% after an additional LPS priming and fMLP-stimulation following the initial 24 h LPS incubation, the effects of PDE4 inhibitors remained the same. Thus, this assay was used to determine the inhibitory effects of various agents on $TNF-\alpha$ and LTB_4 . Most of the PDE inhibitors were equipotent against $TNF-\alpha$ and LTB_4 . Among all of the agents tested in this assay, the PDE4-selective inhibitor, RS14203, was most potent against both $TNF-\alpha$ and LTB_4 despite the weaker activity seen on LTB_4 . Of the PDE4 selective compounds tested, CDP840 and SB207499, both being clinical development compounds, exhibited the least potency in whole blood. It is well recognized that synthetic small molecule inhibitors bind extensively to plasma protein. Experiments using isolated human mononuclear cells (PBMC) treated with LPS for 20 h in the presence of 1% human serum were performed to assess

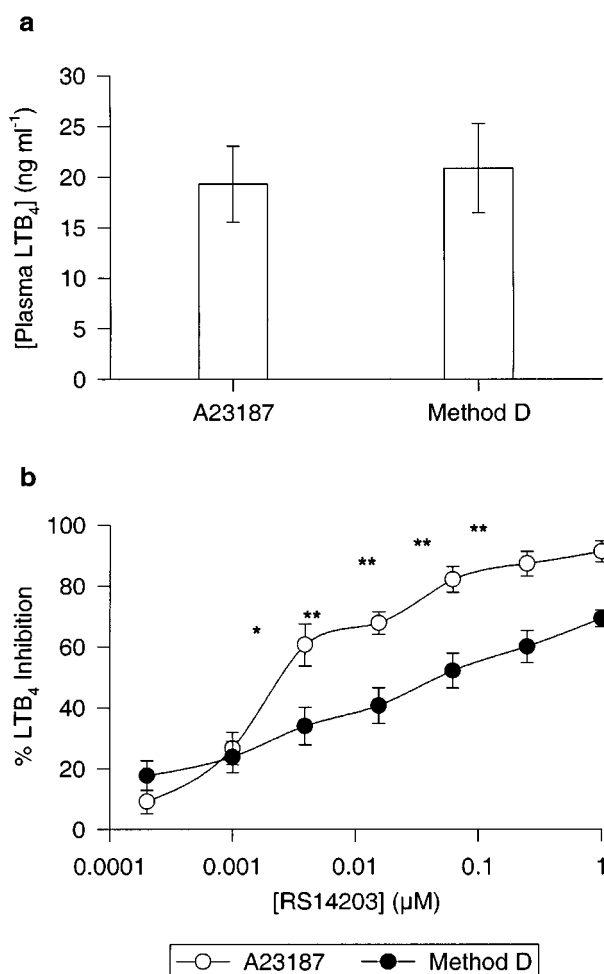


Figure 7 Comparison between two methods of LTB₄ human whole blood assays. (a) represents plasma LTB₄ levels for both assays and (b) represents the titration curve for RS14203 in both assays. A23187 method: whole blood challenged with 25 μM A23187 for 30 min. Method D: whole blood pre-stimulated with LPS (1 μg ml⁻¹) for 24 h followed by 30 min stimulation with LPS (1 μg ml⁻¹) then a 15 min incubation with fMLP (1 μM). *n* = 3 donors. Both methods were performed in the same donor sample. **P* < 0.05 and ***P* < 0.01.

protein shifts in terms of TNF-α inhibition. It was observed in our laboratory that CDP840 activity decreased by more than 40 fold in the whole blood assay compared to the PBMC assay (data not shown). However, SB207499 demonstrated lower intrinsic potency in the PBMC assay than CDP840 and was shifted approximately 26 fold in whole blood. Thus, high intrinsic potency does not always translate in whole blood assays.

CDP840 and SB207499 are the most interesting PDE4-selective inhibitors in that both were investigated in clinic. Of particular interest is CDP840 for it has been reported to be 20 fold more potent on the native PDE4 than (±)-rolipram (Hughes *et al.*, 1996) and our TNF-α assay results show a 7 fold difference when compared to R-rolipram. Using isolated human monocytes in the presence of 1% human serum such as described by Souness *et al.*, 1996, we found that CDP840 and R-rolipram had equivalent activity on the inhibition of LPS-stimulated TNF-α, 365 nm and 330 nm respectively (data not shown). Thus, it appears CDP840 is highly shifted (42 fold) in human whole blood compared to R-rolipram (7 fold).

SB207499 has been reported to be approximately 10 fold more selective on the D isoform of PDE4 than A, B or C isoforms (Torphy *et al.*, 1997). PDE4 inhibitors with greater

Table 3 LTB₄ *in vitro* results of human whole blood stimulated with LPS and fMLP (method D) compared to A23187 challenge. **P* < 0.05 and ***P* < 0.01

Inhibitor	LPS/LPS/fMLP IC ₅₀ (μM)	n	A23187 IC ₅₀ (μM)	n
PDE4-selective				
RS14203	0.12 ± 0.08	9	0.006 ± 0.002	9
RP73401	0.99 ± 0.51	8	0.16 ± 0.04	8
R-Rolipram	4.93 ± 2.31	9	0.18 ± 0.06	9
S-Rolipram	12.01 ± 3.86	9	1.82 ± 0.99*	9
CDP840	18.00 ± 6.03	3	23.25 ± 2.33	3
SB207499	22.96 ± 8.28	9	7.15 ± 3.19	9
5-LO				
L-739,010	0.20 ± 0.02	6	0.041 ± 0.005**	6
cyclic AMP elevating agents				
Isoproterenol	6% @ 10 μM	3	22% @ 1000 μM	3
Forskolin	40% @ 80 μM	3	46% @ 80 μM	3
Dibutyryl-cyclic AMP	23% @ 10 μM	3	56% @ 10 μM	3

selectivity for each of the four isoforms (at least > 100 fold) will be useful to investigate the expression and regulation of PDE4 isoenzymes and to establish which subtype is most involved in inflammation and the negative side effects of PDE4 inhibition such as emesis. Previously reported studies have already shown that PDE4 A, B and D are expressed in human monocytes (C was not detected) and that prolonged exposure of these cells to elevated cyclic AMP upregulated PDE4 A and B expression and activity (Souness *et al.*, 1996; Manning *et al.*, 1996).

Using the whole blood assay, it appears that monocytes are preferentially sensitive to PGE₂ and isoproterenol compared to neutrophils since both agents were very potent inhibitors of TNF-α but had no effect on LTB₄. Conversely, cholera toxin, a stimulator of G_s protein, had the same activity on both TNF-α and LTB₄ whereas forskolin and dibutyryl cyclic AMP showed weak activity. The different inhibitory activities of PGE₂ and isoproterenol in comparison to cholera toxin involve receptor binding and activation. The prostanoid receptor subtype (EP₁, 2, 3 or 4) distribution on human leukocytes is still unknown. Further studies are necessary to elucidate the mechanisms involved in neutrophil-derived LTB₄ modulation by cyclic AMP-elevating agents and PDE4 inhibition. Nonetheless, it is known that increases in cyclic AMP lead to a reduction in cytosolic free calcium (Torphy *et al.*, 1991) and it was shown that rolipram was an effective inhibitor of both LTB₄ synthesis and intracellular calcium elevation in human PMNs (Cortijo *et al.*, 1996; Villagrasa *et al.*, 1996). Other studies have shown that increases in cyclic AMP can regulate PLA₂ activity and inhibit the ability of activated cells to mobilize arachidonic acid (Fonteh *et al.*, 1993).

The non-receptor mediated A23187-stimulation of whole blood (without LPS priming) resulted in the same LTB₄ levels as the double LPS and fMLP-stimulation assay (method D) yet the potency of PDE4 inhibitors were enhanced in this assay. This increased inhibitory activity by PDE4 inhibitors may be due to lower levels of active PDE4 in comparison to a long exposure of blood cells to LPS. It has been shown that PDE4 expression and activity are upregulated during long exposures to β-adrenoceptor stimulation in the presence of rolipram (Manning *et al.*, 1996). Other studies have shown that PDE4 activity could be augmented by 8-bromo-AMP alone in U937 cells and that this increase was due to new protein synthesis (Torphy *et al.*, 1992).

L-739,010 was significantly more potent in the A23187-stimulated LTB₄ assay which may be due to different

sequestrations of intracellular calcium. Interestingly, isoproterenol, forskolin and dibutyryl cyclic AMP exhibited very little effect on LTB₄ in both the LPS-fMLP and A23187 assays.

In conclusion, we have established a method to assess the biochemical efficacy of PDE4-selective inhibitors in human whole blood. We have shown that PDE4-selective inhibitors are potent inhibitors of TNF- α and LTB₄ biosynthesis and thus should be effective anti-inflammatory agents. Since TNF-

α and LTB₄ are implicated in asthma, this assay shall be useful in clinical settings where *ex vivo* experiments can be performed on human subjects.

We wish to thank Cairine Brideau, R.N., Linda Godin, R.N. and Yvonne Baise, R.N. for their expert technical assistance.

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(Received July 10, 1998)

Revised September 23, 1998

Accepted November 24, 1998)



Influence of TASP-V, a novel neuropeptide Y (NPY) Y₂ agonist, on nasal and bronchial responses evoked by histamine in anaesthetized pigs and in humans

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1 In nine anaesthetized pigs we have studied the influence of intranasal or intrabronchial pretreatment with TASP-V, a neuropeptide Y (NPY) Y₂ agonist formed by the attachment of NPY 21–36 to a template-assembled synthetic peptide (TASP), on the functional responses to subsequent intranasal or intrabronchial histamine challenge.

2 In a parallel study, subjective and objective nasal airway resistance (NAR) increase following intranasal histamine challenge was evaluated in 11 healthy volunteers after TASP-V or placebo pretreatment.

3 In pigs, increase in sphenopalatine blood flow induced by histamine dihydrochloride nasal spray (0.25 mg kg⁻¹ in 3 ml of saline) was significantly reduced by 65% ($P < 0.05$) following intranasal pretreatment with 10 µg kg⁻¹ of TASP-V. Bronchoconstriction induced by histamine dihydrochloride nebulization (0.5 mg kg⁻¹ in 3 ml of saline) was significantly attenuated by 25 and 55% following aerosolized pretreatment with TASP-V analogue at 10 and 20 µg kg⁻¹, respectively.

4 In healthy volunteers, objective increase in NAR and reduction in nasal minimal cross section area (MCSA) induced by intranasal spray of histamine dihydrochloride (15 µg kg⁻¹ in 200 µl of saline) were significantly attenuated by 50% following local pretreatment with 1.275 µg kg⁻¹ of TASP-V when compared with saline.

5 It is concluded that intranasal or intrabronchial pretreatment with TASP-V reduced nasal obstruction and bronchoconstriction evoked by histamine challenge in the pig. In healthy human volunteers, this agent attenuated NAR increase and MCSA reduction induced by intranasal application of histamine.

Keywords: Nasal mucosa; neuropeptide Y (NPY); Y₂ receptor analogue; TASP-V; histamine; pig; allergic rhinitis

Abbreviations: MCSA, minimum cross section area; NAR, nasal airway resistance; TASP, template-assembled synthetic peptide

Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide widely distributed in the central and peripheral nervous systems (Tatemoto, 1982; Ekblad *et al.*, 1984). It is present in most sympathetic nerves innervating the cardiovascular system and is the most abundant peptide in the brain and the heart (Tatemoto *et al.*, 1982; Lundberg *et al.*, 1982; Pernow, 1988). The bronchial tree also has a rich sympathetic distribution both in pigs (Mann, 1971) and humans (Doidge & Satchell, 1982; Partanen *et al.*, 1982; Pack & Richardson, 1984), and offers a useful experimental model. NPY coexists with noradrenaline (NA) in postganglionic perivascular sympathetic nerves in several organs, including the bronchial and nasal mucosa in various species (Ekblad *et al.*, 1984; Lacroix *et al.*, 1990). NPY is released with NA from nasal sympathetic nerve fibres and produces long-lasting vasoconstriction (Lacroix, 1989).

Intranasal administration of exogenous NPY *via* a spray in humans reduces nasal airways resistance and vascular permeability without affecting submucosal gland secretion (Baraniuk *et al.*, 1992). In healthy volunteers, intranasal pretreatment with exogenous NPY markedly reduces vasodilation and nasal secretion induced by afferent nerves stimulation

with capsaicin (Lacroix *et al.*, 1996). Putative therapeutic application of this neuropeptide has been recently suggested since allergen-evoked nasal responses in atopic patients are significantly attenuated after local pretreatment with exogenous NPY (Lacroix & Mosimann, 1996).

NPY acts *via* at least two subtypes of receptors, Y₁ and Y₂, which show differences in sensitivity to various NPY analogues *in vitro* (Wahlestedt *et al.*, 1986; Fuhlendorff *et al.*, 1990). Postjunctional NPY receptors have been called Y₁ receptors; their activation produces an increase in blood pressure and potentiates post-synaptically the action of other vasoactive substances (Wahlestedt *et al.*, 1985). Prejunctional receptors have been called Y₂ receptors (Wahlestedt *et al.*, 1986); their stimulation mediates the inhibition of neurotransmitter release (Westfall *et al.*, 1987). By means of functional experiments *in vivo* combined with binding studies *in vivo*, NPY analogues have recently been developed.

It has been recently demonstrated that sympathetic nerve stimulation, as well as exogenous NPY, attenuates the vasodilator response to subsequent parasympathetic nerve stimulation in the nasal mucosa of cats *via* non-adrenergic and non-cholinergic mechanisms (Lacroix *et al.*, 1994a). Attenuation of parasympathetic-evoked vasodilatation could also be mimicked by the NPY analogue N-acetyl [Leu²⁸, Leu³¹] NPY 24–36 (Y₂-receptor agonist) but not by exogenous Y₁ receptor

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agonist (Potter *et al.*, 1994). The Y₂-receptor agonist did not show significant vasoconstrictor action (Lacroix *et al.*, 1994b).

Recent observations obtained from *in vivo* experiments in dogs suggest that intranasal pretreatment with a Y₂-receptor agonist markedly reduced the mucous production evoked by parasympathetic nerve stimulation (Revington *et al.*, 1997). The duration of this effect was significantly prolonged after pretreatment with the endopeptidase inhibitor phosphoramidon. This observation strongly suggests that the duration of NPY analogues functional effects are regulated by an important proteolytic degradation within the nasal mucosa.

Based on molecular dynamics simulations derived from the crystal structure of avian pancreatic polypeptide, the 3D structure of NPY was elucidated. In the search for selective NPY agonists/antagonists, the concept of Template Assembled Synthetic Peptide (TASP) was used to design a compound that selectively acts at the Y₂ receptor. For this purpose, the truncated C-terminal sequence of NPY (21–36) was covalently attached *via* chemoselective ligation to a constrained cyclic peptide as topological template featuring two attachment sites, with a total molecular weight of 4967.38 (Mutter, 1988; Mutter *et al.*, 1988; 1992; 1996; Grouzmann *et al.*, 1995). The detailed synthesis of the molecule will be described in the near future (Mutter *et al.*, manuscript in preparation).

As indicated by conformational studies, the template enhances helix formation of the covalently linked NPY peptide, modulating its pharmacokinetic and biological properties. This was confirmed *in vitro* on SK-N-MC and LN319 cells that exclusively express the NPY Y₁ and NPY Y₂ receptor, respectively (Grouzmann *et al.*, 1997). Binding experiments based on displacement curves demonstrated a comparable high subnanomolar affinity of NPY and TASP-V for the Y₂ receptor (0.09 and 0.38 nM, respectively). In contrast, TASP-V exhibits a poor micromolar affinity for the Y₁ receptor (2.0 μ M). The full agonistic property of TASP-V was established by testing its ability to inhibit cyclic AMP accumulation in LN319 cells, similarly as NPY (Grouzmann *et al.*, 1997). All together, these data indicate that TASP-V acts as a selective agonist at the Y₂ receptor.

The aim of the present study was to evaluate the modulatory effect of pretreatment with TASP-V on the functional response to subsequent histamine challenge. For this purpose, the modulation of histamine-evoked nasal and bronchial responses by local pretreatment with this Y₂-receptor agonist was studied in pigs and in humans.

Methods

The study protocol was approved by the Ethic Committee of the Medical School, University of Geneva.

Domestic pigs in vivo

Experiments were performed on 16 domestic pigs of either sex (body weight from 15–25 kg). The animals were premedicated with midazolam (0.25 mg kg⁻¹), atropine (0.01 mg kg⁻¹) and anaesthetized with thiopentone (5 mg kg⁻¹) through an ear vein. A tracheostomy was performed. The lungs were mechanically ventilated (Servo Ventilator 900, Siemens-Elma, Sweden) with a tidal volume of 15 ml kg⁻¹; the respiratory rate was adjusted to obtain an end-tidal CO₂ concentration between 4 and 5% vol. An inspired oxygen concentration of 30% was used and a positive end-expiratory pressure (PEEP) of 5 cmH₂O was applied. Arterial blood gases were

periodically checked; PaO₂ was maintained between 19 and 24 kPa throughout the study in all animals, indicating that the repeated histamine administration did not produce a sufficiently important increase in pulmonary vascular permeability affecting gas exchange. At the end of each histamine challenge, a ventilation sigh was applied in order to minimize the possible histamine-induced formation of atelectasis. During surgery, continuous i.v. infusion of Ringer lactate solution (3 ml kg⁻¹ h⁻¹) was performed through the femoral vein; thiopentone (15 mg kg⁻¹ h⁻¹) and pancuronium bromide (0.5 mg kg⁻¹ h⁻¹) were continuously infused through the external jugular vein. Body temperature was maintained between 37–38°C by a heat ventilator.

Surgical procedure Catheters were introduced in the femoral artery for systemic blood pressure and heart rate monitoring, in the femoral vein for fluid administration and in the external jugular vein for drugs administration. A partial mandibulectomy was performed, allowing surgical access to the external carotid artery and its two terminal divisions: the maxillary and superficial temporal arteries. Selective recording of the nasal arterial blood flow was monitored with a Transonic flow probe (2 mm RB 143) placed around the external carotid artery and connected to a T 202 ultrasonic blood flowmeter (Transonic System Inc., Ithaca, NY, U.S.A.). All divisions of the external carotid artery distal to the flow probe, apart from the maxillary artery and its terminal nasal branch, the sphenopalatine artery, were ligated and divided. The sphenopalatine artery is responsible for most of the blood supply of the ipsilateral nasal cavity (Lacroix *et al.*, 1988). Data recordings and calculations were obtained from the same computer. Respiratory and haemodynamic variables were recorded with an AST-486 microcomputer that was interfaced *via* an analogue/digital converter at a sampling rate of 50 Hz (DaqSys, SICMU, Centre Médical Universitaire, Geneva, Switzerland) to the flow meters and the pressure transducers.

Pulmonary airways resistance and compliance Airway pressure was measured from a catheter positioned at the tip of the endotracheal tube. Transpulmonary pressure was determined by a differential pressure transducer (Hewlett-Packard ref. 267B) measuring the difference between tracheal and oesophagus pressure. Tidal volume was determined by integration of the respiratory flow signal measured with a pneumotachograph (Gould Godart ref. 17212) by means of a heated Fleisch flow transducer no. 2 connected to the endotracheal tube. Transpulmonary pressure, tidal volume and flow were continuously recorded on a 4-channel recorder (Hewlett-Packard ref. 7754B). Total airflow resistance across the lungs was determined by dividing the difference in transpulmonary pressure by inspiratory plus expiratory flow at mid-tidal volume. Dynamic pulmonary compliance (C_{dyn}) was obtained by dividing tidal volume by the difference in transpulmonary pressure at points of zero flow. Respiratory parameters were averaged for five successive tidal volumes. Data recordings and calculations were obtained from the same computer.

Stimulations (1) In early experiments ($n=7$), tachyphylaxis to histamine was investigated by three successive nasal and bronchial histamine challenges at 30 min intervals (same doses as below). (2) In the following animals ($n=9$), histamine dihydrochloride (0.25 mg kg⁻¹ in 1 ml of saline, Sigma, St Louis, U.S.A.) was sprayed with a hand held nebulizer in the left nostril under controlled conditions, and repeated 45 and 90 min after intranasal spray of TASP-V (10 μ g kg⁻¹ in 1 ml

of NaCl 0.9%). (3) Histamine dihydrochloride (0.50 mg kg⁻¹ in 3 ml of saline) was aerosolized for 3 min into the trachea with a nebulizer (nebulizer Intersurgical 8.5 mm, ref. 2507, Intersurgical, U.S.A.) fixed to the inspiratory circuit of the ventilation tube and supplemented by oxygen (4 l min⁻¹) under controlled conditions. The same procedure was repeated 15, 45 and 90 min after intratracheal nebulization of 10 and 20 µg kg⁻¹ of the Y₂-receptor agonist in 3 ml of saline.

Measurements of parameters In all animals, the nasal vascular and bronchial responses to histamine challenge were recorded under controlled condition before and after pretreatment with the Y₂-analogue. By use of a six channel pen trace recorder (Gould Electronics), the following parameters were recorded simultaneously: (1) Heart rate and systemic arterial blood pressure using the femoral artery catheter connected to a pressure transducer; (2) Pulmonary airway resistance and compliance; (3) Sphenopalatine arterial blood flow using the Ultrasonic flow meter probe.

Healthy volunteers

Eleven healthy volunteers, six males and five females, aged from 23–48, weighing from 51–82 kg, underwent a study designed on a randomized, double-blind, cross-over basis. Exclusion criteria included abnormal nasal mucosa, anatomical nasal obstruction, or use of any nasal treatment such as vasoconstrictors or corticosteroids in the last 30 days.

Pretreatment The TASP-V dilution and the placebo (saline) were prepared before each experiment by a technician not involved in the study. Each patient underwent a local pretreatment with saline or TASP-V (1.275 µg kg⁻¹ in a total of 200 µl of NaCl 0.9%) on a cross-over way. One substance was applied and tested over a period of 2 h, the other substance being applied and tested over the same duration following a break of 2 days between the two tests. In order to minimize the risk of bias produced by the cross-over, the peptide was tested in one nostril while placebo was used in the contralateral one, with a randomized and double blinded allocation. TASP-V, as tested by the authors themselves, does not have any colour, smell or taste which could interfere with the blinded protocol.

Stimulation Fifteen minutes after pretreatment with TASP-V or placebo, intranasal application of histamine dihydrochloride (15 µg kg⁻¹ in 200 µl of saline) with a spray was performed in the same nostril.

Parameters studied *Subjective symptoms* A visual analogue scale, graded from 0–5 (where 0 represented the absence of symptoms and 5 severe intensity of symptoms) was used to assess the degree of subjective nasal obstruction and rhinorrhea.

Secretions and sneezes Nasal secretions produced during 15 min after the histamine challenge were collected by nose-blowing in a pre-weighted tissue. The number of sneezes was also recorded.

Rhinomanometry NAR was recorded in each nostril by anterior rhinomanometry (Rhinotest MP 441, EVG Electronic, Vertriebs, Germany). Rhinomanometry evaluates the nasal airway resistance on the basis of an airflow-pressure curve. Transnasal pressure is approximated as the difference between pressure at the anterior occluded ipsilateral nostril

(linked to the manometer by a soft nasal probe and a flexible tube), and pressure measured within a plastic facial mask applied on the volunteer's nose and closed mouth (Broms *et al.*, 1982a,b; Schumacher, 1989). In this study, mean resistance values for each nostril were obtained after ten normal breaths and were calculated at a pressure of 150 Pa.

Acoustic rhinometry The MCSA of the nasal airways opposed to the respiratory flow was evaluated using an acoustic rhinometer (RhinoClak, Germany), the patient being seated in an examination chair with chin and forehead fixed on the same position during each MCSA recording. Acoustic rhinometry evaluates the nasal cavity geometry by the use of computerized analysis of reflected acoustic impulses. Results are expressed on a cross-sectional area per distance curve, classically presenting two to three minimal local areas (Hilberg *et al.*, 1989; Cole, 1989; Fouke & Jackson, 1992). The first local narrowest section is not modified by decongestants, and corresponds to the nasal valve (anatomical junction between alar and lateral cartilages). The second local narrowest section is associated with the head of the middle turbinate and is classically used as a reference in the evaluation of nasal resistances. It was the point evaluated in this study, prior and after all histamine challenges. The third point is highly variable and therefore rarely used as a parameter.

Measurements of parameters These were performed twice prior to the TASP-V pretreatment (T–30, T–15), then once 15 min after it. Following the histamine challenge, the same parameters were measured repeatedly at 15-min intervals during 1 h (T15, 30, 45 and 60).

Statistical analysis

Data are given as means ± s.e.mean. Statistical differences in symptom scores, NAR, MCSA and nasal secretions were estimated by using one-way analysis of variance followed by Dunnett comparison.

Results

Domestic pigs

The intranasal or intrabronchial administration of the Y₂-agonist did not induce any change in heart rate, sphenopalatine blood flow or mean arterial blood pressure; Tables 1 and 2 present absolute values of haemodynamic as well as lung mechanics variables before each histamine challenge, in order to identify a potential baseline shift over time in these recorded variables. There were no statistically significant changes over time analyzed by a one-way ANOVA for repeated measures.

The initial triple challenge with nasal histamine performed on seven animals demonstrated a reproducible increase of the sphenopalatine blood flow, as well as a decrease in vascular resistance (Figure 1A and B). Equally, no tachyphylactic phenomenon could be elicited in the bronchi, with a reproducible increase of the airway resistance and a reduction of the dynamic lung compliance (Figure 2A and B).

Nasal responses Following intranasal pretreatment with the Y₂-agonist at 10 µg kg⁻¹, the histamine-induced increase of the sphenopalatine blood flow was significantly reduced by approximately 65% over 45 and 90 min, with a maximum effect after 90 min ($P < 0.05$); the sphenopalatine vascular resistance was significantly reduced by 60% (Figure 3A and B).

Table 1 Tachyphylaxis control experiments: baseline values of vascular and respiratory variables before repeated histamine challenges in control pigs

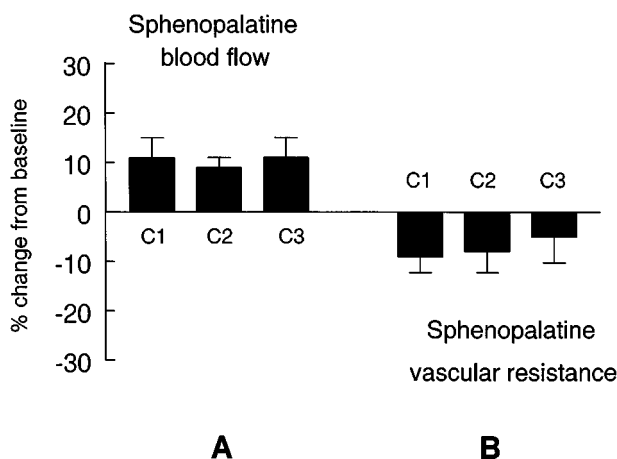
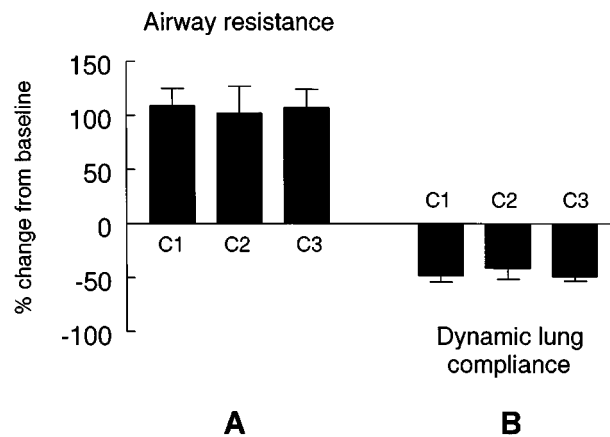
	0	Times (min) 30	60
<i>Before intranasal histamine administration</i>			
Sphenopalatine blood flow (ml min ⁻¹)	149 ± 19	126 ± 22	134 ± 18
Sphenopalatine vascular resistance (mmHg min ml ⁻¹)	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Airway resistance (cmH ₂ O s l ⁻¹)	29 ± 3	25 ± 3	27 ± 2
Dynamic lung compliance (ml cmH ₂ O ⁻¹)	14 ± 1	12 ± 1	14 ± 1
<i>Before intrabronchial histamine administration</i>			
Sphenopalatine blood flow (ml min ⁻¹)	154 ± 15	157 ± 25	167 ± 45
Sphenopalatine vascular resistance (mmHg min ml ⁻¹)	0.7 ± 0.1	0.6 ± 0.0	0.7 ± 0.1
Airway resistance (cmH ₂ O s l ⁻¹)	28 ± 1	32 ± 1	34 ± 4
Dynamic lung compliance (ml cmH ₂ O ⁻¹)	20 ± 4	16 ± 4	24 ± 1

Values are means ± s.e. mean of seven pigs. There were no statistically significant differences between time data points (one-way ANOVA for repeated measures).

Table 2 Baseline values of vascular and respiratory variables before histamine challenges in pigs given the Y₂-receptor agonist

	0	Times (min) 15	45	90
<i>Intranasal 10 µg kg⁻¹ Y₂-receptor agonist administration</i>				
Sphenopalatine blood flow (ml min ⁻¹)	107 ± 15	136 ± 4	102 ± 11	116 ± 18
Sphenopalatine vascular resistance (mmHg min ml ⁻¹)	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.2
Airway resistance (cmH ₂ O s l ⁻¹)	22 ± 3	22 ± 4	24 ± 3	24 ± 4
Dynamic lung compliance (ml cmH ₂ O ⁻¹)	24 ± 6	21 ± 4	20 ± 4	23 ± 6
<i>Intrabronchial 10 µg kg⁻¹ Y₂-receptor agonist session</i>				
Sphenopalatine blood flow (ml min ⁻¹)	128 ± 15	136 ± 4	121 ± 14	111 ± 22
Sphenopalatine vascular resistance (mmHg min ml ⁻¹)	0.9 ± 0.3	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Airway resistance (cmH ₂ O s l ⁻¹)	31 ± 2	27 ± 2	31 ± 4	35 ± 5
Dynamic lung compliance (ml cmH ₂ O ⁻¹)	19 ± 3	21 ± 4	19 ± 4	17 ± 3
<i>Intrabronchial 20 µg kg⁻¹ Y₂-receptor agonist session</i>				
Sphenopalatine blood flow (ml min ⁻¹)	122 ± 58	125 ± 63	111 ± 47	117 ± 15
Sphenopalatine vascular resistance (mmHg min ml ⁻¹)	1.2 ± 0.6	1.2 ± 0.6	1.2 ± 0.5	1.3 ± 0.3
Airway resistance (cmH ₂ O s l ⁻¹)	23 ± 3	24 ± 4	26 ± 4	27 ± 1
Dynamic lung compliance (ml cmH ₂ O ⁻¹)	20 ± 0	20 ± 1	19 ± 2	19 ± 3

Values are means ± s.e. mean of nine pigs. There were no statistically significant differences between time data points (one-way ANOVA for repeated measures).

**Figure 1** Effects of repeated intranasal spray of histamine dihydrochloride (0.25 mg kg⁻¹ in 1 ml of saline) in anaesthetized pigs on (A) the homolateral sphenopalatine blood flow and (B) the sphenopalatine vascular resistance, with 30 min rest between each challenge ($n=7$, one-way analysis of variance).**Figure 2** Effects of an intrabronchial nebulization of histamine dihydrochloride (0.50 mg kg⁻¹ in 3 ml of saline) in anaesthetized pigs on (A) the total airway resistance and (B) the dynamic lung compliance, calculated from tidal volume and transpulmonary pressure, with 30 min rest between each challenge ($n=7$, one-way analysis of variance).

Bronchial responses Two different doses of the Y₂-agonist were tested. After pretreatment with 10 µg kg⁻¹ the increase of airway resistance following the histamine challenge was attenuated by approximately 25% after 45 and 90 min, with a maximum effect observed at 90 min ($P<0.05$); the

dynamic lung compliance reduction was also significantly reduced after 45 min (Figure 4A and B). A stronger effect was observed when 20 µg kg⁻¹ were used, with an average attenuation of 55% over 15, 45 and 90 min ($P<0.05$) (Figure 5A and B).

Healthy volunteers

The effect of TASP-V against time regarding NAR was studied in an initial group of eight healthy volunteers; no significant variation of the NAR was recorded over a period of 1 h (data not shown). In the present group of 11 volunteers, the intranasal spray of the Y_2 -agonist *per se* did not produce any subjective local irritation, sneeze or increased rhinorrhea (data not shown). No significant modification of the subjective NAR was recorded (Figure 6). Rhinomanometry and acoustic rhinometry did not demonstrate any significant change of the NAR or MCSA (Figures 7 and 8).

Responses to histamine challenge In all patients, histamine challenge following placebo induced rapid onset of itching, bouts of sneezing, rhinorrhea and objective and subjective nasal obstruction (Figures 6–8). The maximum obstructive effect was observed 15 min following the histamine challenge; no significant residual effect remained after 60 min.

Pretreatment with the Y_2 -agonist did not significantly reduce subjective or objective rhinorrhea nor the number of sneezing bouts following histamine challenge (data not shown).

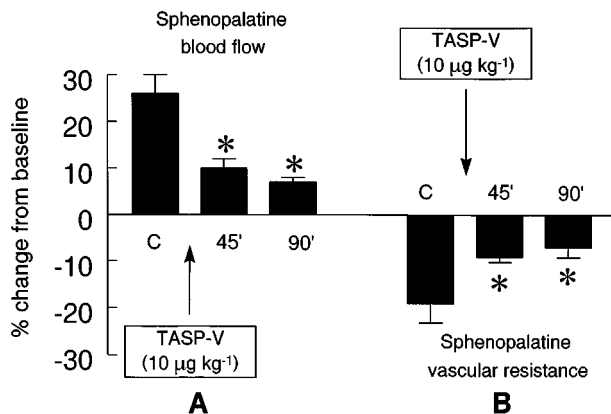


Figure 3 Effects of intranasal spray of histamine dihydrochloride (0.25 mg kg^{-1} in 1 ml of saline) in anaesthetized pigs on (A) the homolateral sphenopalatine blood flow and (B) the sphenopalatine vascular resistance, and after pretreatment with TASP-V analogue (10 µg kg^{-1} in 1 ml of saline). * $P < 0.05$ when compared with the control ($n = 9$, one-way analysis of variance).

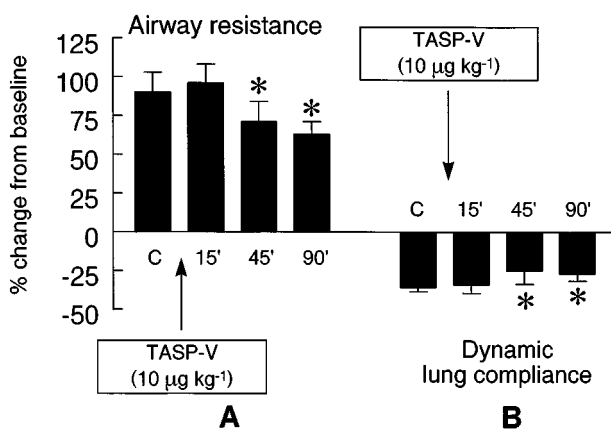


Figure 4 Effects of an intrabronchial nebulization of histamine dihydrochloride (0.50 mg kg^{-1} in 3 ml of saline) in anaesthetized pigs on (A) the total airway resistance and (B) the dynamic lung compliance, calculated from tidal volume and transpulmonary pressure, and after nebulized pretreatment with TASP-V analogue (10 µg kg^{-1} in 3 ml of saline). * $P < 0.05$ when compared with the control ($n = 9$, one-way analysis of variance).

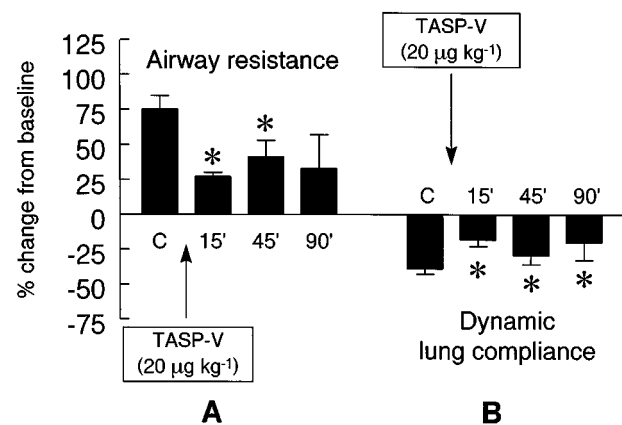


Figure 5 Effects of an intrabronchial nebulization of histamine dihydrochloride (0.50 mg kg^{-1} in 3 ml of saline) in anaesthetized pigs on (A) the total airway resistance and (B) the dynamic lung compliance, calculated from tidal volume and transpulmonary pressure, and after nebulized pretreatment with TASP-V analogue (20 µg kg^{-1} in 3 ml of saline). * $P < 0.05$ when compared with the control ($n = 9$, one-way analysis of variance).

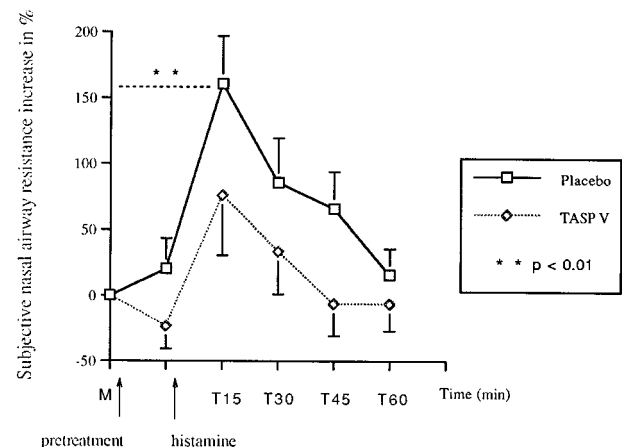


Figure 6 Time course variations of subjective nasal airway resistance (NAR, measured by a visual analogue scale graded from 0–5) in the homolateral nostril following an intranasal application of histamine dihydrochloride (15 µg kg^{-1} in 200 µl of saline) after pretreatment with placebo or TASP-V (1.275 µg kg^{-1} in 200 µl of saline) in saline spray. M = mean of T–30 and T–15. * $P < 0.05$, ** $P < 0.01$ ($n = 11$, one-way analysis of variance).

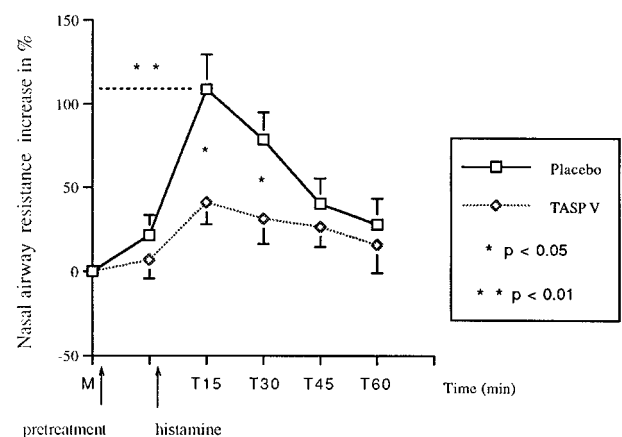


Figure 7 Time course variations of objective nasal airway resistance (NAR, measured by anterior rhinomanometry) in the homolateral nostril following an intranasal application of histamine dihydrochloride (15 µg kg^{-1} in 200 µl of saline) after pretreatment with placebo or TASP-V (1.275 µg kg^{-1} in 200 µl of saline) in saline spray. M = mean of T–30 and T–15. * $P < 0.05$, ** $P < 0.01$ ($n = 11$, one-way analysis of variance).

The increase of NAR induced by histamine, expressed in percent of the initial value (mean of T-30 and T-15), was significantly reduced by the Y₂-agonist pretreatment (Figure 7); the maximum effect was obtained after 15 and 30 min. Similarly, the reduction of the MCSA was significantly attenuated by the Y₂-agonist pretreatment with a maximum effect at 15 and 30 min (Figure 8). Though not significant, the subjective homolateral nasal obstruction produced by histamine challenge appeared to be reduced after Y₂-analogue pretreatment, again with a maximum effect after 15 and 30 min (Figure 6). None of the patients reported any side effects following the experiment.

Discussion

The present data show that intranasal or intrabronchial pretreatment with the TASP-V analogue reduced nasal obstruction or bronchoconstriction evoked by histamine challenge. The protective effect of TASP-V on both nasal and bronchial responses to exogenous histamine could be due to several mechanisms.

NPY modulates parasympathetic pathways in lower airways (Stretton & Barnes, 1988; Matran *et al.*, 1989) and sensory neuropeptides release *via* the activation of prejunctional Y₂ receptors (Westfall *et al.*, 1987; Warner & Levy, 1989; Nuki *et al.*, 1990). Not much is known about this presynaptic action. In particular, and as far as we know, there is no available data about the differential sensitivities of sensory, sympathetic and parasympathetic fibres to Y₂ agonists. Previous observations suggest that histamine released during an allergic reaction could stimulate sensory and parasympathetic nerve fibres through a naso-nasal reflex (Lundberg *et al.*, 1988; White *et al.*, 1989; Lacroix & Lundberg, 1994). Furthermore, primary sensory fibres of the nasal mucosa appear to act both as an afferent system responding to chemosensitive and mechanothermal stimuli, but also as an efferent pathway by releasing vasoactive peptides, resulting in protective reflexes such as sneezing, secretion and nasal congestion, as well as chemotactism for inflammatory cells with leukocyte migration (Lundblad, 1984; Stead *et al.*, 1987). This phenomenon has been termed neurogenic inflammation (Jancso *et al.*, 1967; Lundberg & Saria, 1983). Indeed,

stimulation of unmyelinated sensory C-fibres may induce local retrograde (or antidromic) spreading of the action potential in peripheral branches of sensory nerves (Colander & Folkow, 1953) and local release of neuropeptides, resulting in non-adrenergic non-cholinergic vasodilatation of the nasal vascular bed and an increased vascular permeability to plasma proteins (Lundblad, 1984; Stjärne, 1991). This neurogenic inflammation has been demonstrated in the skin (Stricker, 1876; Bayliss, 1901; Jancso *et al.*, 1967) and in the nasal mucosa (Lundblad, 1984). As mentioned above, atropine has been used in the animal premedication; we therefore cannot exclude an interaction with motor pathways. However, mechanisms of inflammation are based on the stimulation of sensory C-fibres. Antidromic spreading of the action potential with release of sensory neuropeptides results in non-adrenergic non-cholinergic vasodilatation of the nasal vascular bed. Moreover, predominance of parasympathetic and atropin-resistant mechanisms for vasodilation versus atropin-sensitive mechanisms for nasal secretion has already been demonstrated (Eccles & Wilson, 1973; Malm, 1973). We therefore admitted a negligible effect of atropin in this study.

Endo- or exogenous histamine stimulates sensory fibres by possible direct action on specific H₁ receptors on trigeminal nerve endings, stimulating the release of sensory neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP) (Tani & Ishikawa, 1990; Tani *et al.*, 1990). In the pig, histamine-induced skin vasodilation and bronchial constriction could be attenuated by pretreatment with capsaicin (Alving *et al.*, 1991), suggesting an attenuation of sensory C-fibres responsiveness to histamine stimulation due to depletion of sensory vasoactive peptides. In the human nasal mucosa, activation of sensory-nerve receptors with histamine initiates sneezing and increase of both nasal secretion and nasal airways resistance by vascular congestion. This response is also significantly reduced after capsaicin pretreatment (Lacroix *et al.*, 1991), confirming the importance of sensory neurones in nasal protective reflexes in humans, where histamine activates capsaicin-sensitive sensory nerves and evokes release of vasodilator sensory neuropeptides (Alving, 1991; Mosimann *et al.*, 1993).

Reversibly, SP and CGRP probably stimulate histamine release from mast cells and potentiates histamine action (Erjavec *et al.*, 1981; Piotrowski & Foreman, 1986; Lowman *et al.*, 1988; Ottosson & Edvinsson, 1997). Confirming this observation, Takeda and Imamura demonstrated that capsaicin-induced histamine release in the guinea-pig is prevented by prior destruction of sensory C-fibres by systemic capsaicin pretreatment (Takeda *et al.*, 1993; Imamura *et al.*, 1996). Similarly, histamine-induced vascular leakage is reduced by CGRP antagonist 8-37 and almost abolished in capsaicin-denervated rats, with restoration of the oedema following CGRP injection (Amann *et al.*, 1995). These observations suggest an indirect, sensory C-fibre-dependent mechanism where liberation of CGRP and SP plays an intermediate step in histamine release or action enhancement. Therefore, attenuation of sensory fibres activity may modulate histamine release, offering interesting therapeutic perspectives. Nevertheless, these observations have not been confirmed by other studies (Rangi *et al.*, 1990; Huttunen *et al.*, 1996; Sandor *et al.*, 1996) and must therefore be considered with caution.

In the present study, pretreatment with TASP-V could have reduced both nasal vascular and bronchial response to histamine through prejunctional inhibitory mechanisms on both sensory and parasympathetic nerves. This hypothesis should be confirmed by measuring the reduction in sensory and parasympathetic neuropeptide release during allergic

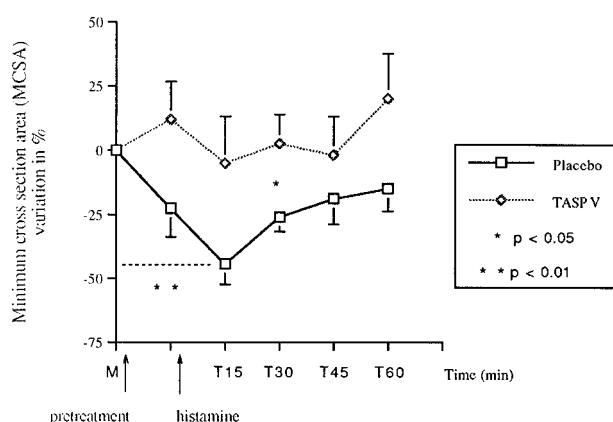


Figure 8 Time course variations of the minimal cross section area (MCSA, measured by acoustic rhinometry, see Methods) in the homolateral nostril following an intranasal application of histamine dihydrochloride ($15 \mu\text{g kg}^{-1}$ in $200 \mu\text{l}$ of saline) after pretreatment with placebo or TASP-V ($1.275 \mu\text{g kg}^{-1}$ in $200 \mu\text{l}$ of saline) in saline spray. M = mean of t-30 and T-15. * $P < 0.05$, ** $P < 0.01$ ($n = 11$, one-way analysis of variance).

reaction following TASP-V pretreatment. Bradykinin is a mediator released during allergic reaction or histamine challenge, and stimulates sensory nerves (Proud *et al.*, 1983). Since pretreatment with exogenous NPY has been shown to reduce both vascular and secretory effects of bradykinin (Baraniuk *et al.*, 1992), similar mechanisms may have occurred with TASP-V. Rhinorrhea, subjective obstruction and number of sneezes were not reduced by Y₂ analogue pretreatment. The same observation was made in previous studies (Lacroix & Mosimann, 1996; Lacroix *et al.*, 1996); we do not exclude a dose-related phenomenon where attenuation of these symptoms could appear at higher agonist concentration.

Compared with the inhibitory effect of both NPY and Y₂ agonist [Leu²⁸, Leu³¹] NPY 24–36 on parasympathetic nerve-evoked vasodilation previously described (Lacroix *et al.*,

1994a,b), TASP-V appears to have a longer-lasting effect. We hypothesize that the oxime attachment to the template may confer to NPY analogues a higher resistance towards peptidases activity.

In conclusion, intranasal or intrabronchial pretreatment with the NPY analogue TASP-V reduced nasal obstruction and bronchoconstriction evoked by histamine challenge in the pig; in healthy volunteers, this agent attenuated objective histamine-induced nasal obstruction. TASP-V analogue may have therapeutic applications in allergic rhinitis and asthma.

The present study was supported by a grant from the Swiss National Fund for Scientific Research FNSRS (number 32-050698/97). We are grateful to Mrs Michèle Brunet, Mrs Sylvie Roulet and Mr Manuel Jorge-Costa for their technical assistance.

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(Received July 27, 1998)

Revised November 12, 1998

Accepted November 23, 1998



Effect of low-dose treatment with selegiline on dopamine transporter (DAT) expression and amphetamine-induced dopamine release *in vivo*

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1 Chronic treatment with low doses of the selective monoamine oxidase (MAO) type B inhibitors selegiline [(–)-deprenyl] and rasagiline, causes elevation in extracellular level of 3,4-dihydroxyphenylethylamine (dopamine) in the rat striatum *in vivo* (Lamensdorf *et al.*, 1996). The present study was carried out to determine whether this effect of selegiline could be the result of an inhibition of the high-affinity dopamine neuronal transport process.

2 Changes in activity of the dopamine transporter (DAT) *in vivo* following selegiline treatment were evaluated indirectly by microdialysis technique in the rat, from the change in striatal dopamine extracellular concentration following systemic amphetamine administration (4 mg kg^{–1}, i.p.). Striatal levels of the DAT molecule were determined by immunoblotting. Uptake of [³H]-dopamine was determined in synaptosomes from selegiline-treated animals.

3 Amphetamine-induced increase in striatal extracellular dopamine level was attenuated by one day and by chronic (21 days) treatment with selegiline (0.25 mg kg^{–1}, s.c.).

4 Striatal levels of DAT were elevated after 1 and 21 days treatment with selegiline, but were not affected by clorgyline, rasagiline, nomifensine or amphetamine.

5 The increase in DAT expression, and attenuation of amphetamine-induced dopamine release, were not accompanied by a change in [³H]-dopamine uptake in synaptosomes of selegiline-treated animals.

6 The results suggest that a reversible inhibition of dopamine uptake occurs following chronic low dose selegiline treatment *in vivo* which may be mediated by an increase in endogenous MAO-B substrates such as 2-phenylethylamine, rather than by the inhibitor molecule or its metabolites. Increased DAT expression appears to be a special property of the selegiline molecule, since it occurs after one low dose of selegiline, and is not seen with other inhibitors of MAO-A or MAO-B. The new DAT molecules formed following selegiline treatment appear not to be functionally active.

Keywords: Selegiline; (–)-deprenyl; rasagiline; clorgyline; nomifensine; dopamine uptake; dopamine transporter; microdialysis; monoamine oxidase; amphetamine

Abbreviations: MAO, monoamine oxidase; dopamine, 3,4-dihydroxyphenylethylamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, 3-methoxy-4-hydroxyphenylacetic; DAT, dopamine transporter; EDTA, ethylenediaminetetracetic acid; HSA, heptane-sulphonic acid; PBS, phosphate-buffered saline; PEA, 2-phenylethylamine

Introduction

In the rat, striatal extracellular level of 3,4-dihydroxyphenylethylamine (dopamine) as determined by *in vivo* microdialysis is enhanced by selective inhibition of monoamine oxidase type A (MAO-A), but unaffected by inhibition of MAO-B, when the inhibitors are administered acutely (Arbuthnott *et al.*, 1990; Butcher *et al.*, 1990; Kato *et al.*, 1986). This is consistent with the fact that the isoform of MAO associated with dopaminergic neurons of the nigro-striatal tract in primates and rodent species is primarily type A (Demarest *et al.*, 1980; Fagervall & Ross, 1986; Westlund *et al.*, 1985).

In a previous microdialysis study in the rat, we observed that chronic (21 days) treatment with a low, MAO-B selective dose of selegiline [(–)-deprenyl] or rasagiline [(R(+))N-propargyl-1-aminoindan]; a selective MAO-B inhibitor which is not metabolized to amphetamine-like metabolites, increased striatal dopamine extracellular level (Lamensdorf *et al.*, 1996). The pattern of changes obtained after repeated treatment with both MAO-B inhibitors (i.e., elevation in

dopamine extracellular level without significant effect on 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylacetic acid (HVA) levels is similar to that obtained after treatment with classical uptake blockers like cocaine and nomifensine (Hurd & Ungerstedt, 1989). Chronic administration of a low dose of selegiline also enhanced dopamine release from rat striatal slices *in vitro* (Dluzen & McDermott, 1991; Knoll, 1985; 1992) and inhibited dopamine re-uptake in striatal slices (Zsilla *et al.*, 1986; Tekes *et al.*, 1988). The uptake inhibition seen after repeated selegiline administration was reversible, since the degree of inhibition was noticeably less 24 h after the last dose (Zsilla *et al.*, 1986) by contrast with the inhibition of MAO, which is irreversible.

The effect of selegiline on dopamine release could be the result of an amphetamine-like action, either of the parent molecule or metabolites, because selegiline is metabolized to (–)-methamphetamine and (–)-amphetamine (Reynolds *et al.*, 1978). However, rasagiline is not metabolized to amphetamine-like metabolites but to 1-aminoindane which does not possess amphetamine-like properties (Finberg *et al.*, 1998). As a result, we proposed (Lamensdorf *et al.*, 1996) that

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the apparent uptake-inhibitory properties of selegiline and rasagiline could be the result of the action of the MAO-B substrate 2-phenylethylamine (PEA), tissue levels of which increase markedly after selegiline treatment (Paterson *et al.*, 1990).

In the current study, we further investigated the effect of selegiline on the dopamine transporter. The activity of the dopamine transporter *in vivo* was assessed from the increase in extracellular fluid dopamine caused by systemically-administered amphetamine. In addition, the action of selegiline and rasagiline was studied on [³H]-dopamine uptake in striatal synaptosomes, and the effect of MAO inhibition and amphetamine on dopamine transporter protein levels was evaluated by immunoblotting.

Methods

Animals

Adult male Sprague-Dawley rats were housed in wire mesh cages at an environmental temperature of 19–21°C, 12 h light-dark cycle, and were fed solid rat chow and water *ad libitum* for the duration of the experiment. The rats weighed between 320–340 g at the time of dialysis probe implantation or sacrifice. All drugs were administered by s.c. or i.p. injection. Animal procedures were authorized by the Technion Animal Care and Use Committee.

Drug treatment

For comparison between the effects of chronic and acute selegiline treatment, rats received 0.25 mg kg⁻¹ selegiline daily s.c. for 21 days (chronic), or 20 days saline and 0.25 mg kg⁻¹ selegiline on day 21 (acute), or saline daily for 21 days (control). On day 21, a microdialysis probe was implanted into the left striatum (see below), and sampling of dialysate was carried out in the awake animals on day 22 (24 h after last dose of saline or drug).

Dopamine transporter (DAT) levels were determined in rats treated with selegiline (0.25 mg kg⁻¹, s.c.) or rasagiline (0.05 mg kg⁻¹, s.c.) for 1 or 21 days as above, as well as in rats which received 21 days of saline treatment and drug on day 22, 2 h before decapitation. Striatal DAT level was also determined after 21 days of repeated clorgyline administration (0.2 mg kg⁻¹, s.c.) and 1 day after nomifensine (20 mg kg⁻¹, i.p.) or (±)-amphetamine (0.25 mg kg⁻¹, s.c.).

Inhibition of [³H]-dopamine uptake by the MAO inhibitors and their metabolites was determined in striatal synaptosomes prepared from untreated rats. Kinetics of [³H]-dopamine uptake were determined in striatal synaptosomes prepared from rats which had been treated once or 21 days with selegiline and killed 24 h after the last dose as described above.

Microdialysis procedure

Dialysis probes (4 mm active length) were positioned under stereotaxic guidance in the striata of pentobarbital/chloralhydrate (12/60 mg kg⁻¹, i.p.) anaesthetized rats, using the coordinates A 1.2, L 2.5, V 7.0 (Paxinos & Watson, 1982) and were perfused continuously with artificial CSF of composition (in mM): NaCl 150, CaCl₂ 1.7, KCl 3 and MgCl₂ 0.9 at 2 µl min⁻¹. Dialysate collections (20 min) were made in miniature polyethylene vials containing 10 µl 0.1 N HClO₄. Four to five control microdialysis collections were made, and then (±)-amphetamine (4 mg kg⁻¹, i.p.) was administered. A

further six collections were then made. After the dialysis experiments, the placement of the probe was verified by visual inspection.

Microdialysis probes were fabricated from ceramic and stainless steel tubings, with a cuprophane membrane. The relative recovery for dopamine, DOPAC and HVA determined *in vitro* before implantation was 22.8 ± 2.5, 13.2 ± 1.9 and 15.2 ± 3.5% (mean ± s.e.mean of *n* = 6–7 determinations), respectively.

Analysis of dialysate

Dialysates were analysed for dopamine, DOPAC and HVA by HPLC with electrochemical detection. Separation of dopamine and metabolites was achieved using a Microsorb column (Rainin C-18, 3 µm, 4.6 mm diameter, 10 cm long) with mobile phase composed of 4.83 g NaHPO₄, 90 mg HSA, 20 ml methanol, 10 ml acetonitrile and 50 mg EDTA per 500 ml HPLC grade deionized water. The flow rate through the system was 1.5 ml min⁻¹. Compounds were detected with an ESA Coulochem model 5011 detector (ESA, U.S.A.) operated in redox mode. Column eluates were initially oxidized at a potential of +300 mV using an ESA guard cell placed before the detector, reduced to +60 mV at detector 1 and measured at -350 mV at detector 2. Chromatograms were analysed for peak height and retention time using an integrating recorder (Varian model 4270).

Determination of dopamine transporter (DAT) levels by immunoblotting

Synthetic peptides, SHGIDDLGPPR and LTNSTLINPPQ-TPVEAQE, corresponding to residues 227–237 and 42–59 of the cloned human and rat DAT respectively, were synthesized by the Department of Biological Services, Weizmann Institute, Rehovot, Israel. Conjugation and immunization procedures were identical for each of the two peptides (Simantov *et al.*, 1996). Briefly, the peptide was conjugated to keyhole limpet haemocyanin, using glutaraldehyde, mixed with an equal volume of complete Freund adjuvant, and injected into two New-Zealand White rabbits in multisite intradermal injections. Additional boosts were given at 2–3 week intervals, and serum was prepared one and a half months after the last injection.

Dissected striata were homogenized in 2% sodium dodecyl sulphate, and protein levels were determined using the Lowry assay (Lowry *et al.*, 1951). Samples of 200 µg protein were run on 7% sodium dodecyl sulphate-polyacrylamide gel, and blotted to nitrocellulose membrane. The membrane was preincubated at room temperature with 90% phosphate-buffered saline (PBS)-10% milk for 3 h, washed with PBS containing 0.05% Tween 20, and incubated with anti-DAT antibodies for 90 min. The membrane was washed, incubated for 2 h at room temperature with [¹²⁵I]-protein A, exposed to BAS IP screen (Fujifilm), scanned, and analysed using MacBAS 2.0 (Fujifilm, Fugix Bas 1000) software. The density of DAT immunoreactive band was determined by a phospho-imaging analyser, after subtracting the background level. A standard curve of 25–300 µg protein from rat striatum was blotted in parallel to the samples, and was used to convert data to absolute values.

[³H]-dopamine uptake in rat striatal synaptosomes

Measurement of IC₅₀ values for inhibition of [³H]-dopamine uptake into rat striatal synaptosomes by rasagiline, R-(+)-aminoindane, selegiline, (-)-desmethylselegiline, (-)-methamphetamine, nomifensine and GBR-12909 were carried

out according to Cooper & Carlson (1983) with slight modifications. Rats were sacrificed by decapitation, and striata homogenized in ice cold isotonic sucrose (0.32 M). Synaptosomes were incubated in Krebs'-Henseleit buffer (in mM): NaCl, 122; NaH_2PO_4 , 15.8; KCl, 4.9; CaCl_2 , 1.27; MgSO_4 , 1.19; dextrose 11; pargyline 0.09; ascorbate 1.23; adjusted to pH 7.4 for 5 min before addition of [^3H]-dopamine (100 nM). The incubation was terminated after 5 min by dilution and filtration under reduced pressure over glass fibre filters (Whatman GF/B). The filters were washed, and radioactivity determined by liquid scintillation spectrometry. Active uptake was calculated as difference between uptake at 37 and 0°C.

Data analysis

Statistical analysis of difference between drug-treated animals in microdialysis experiments was performed by one-way analysis of variance (ANOVA). Probability values (P) were calculated using Tukey-Kramer test.

The IC_{50} values for [^3H]-dopamine uptake, and the area under the amphetamine microdialysis curve values were calculated using appropriate software (Graphpad Inplot, version 4.03).

Materials

Selegiline hydrochloride, rasagiline, (–)-desmethylosegiline, (–)-methamphetamine, R-(+)-aminoindane and amphetamine sulphate were gifts of Teva (Israel). GBR-12909 and nomifensine were obtained from RBI (U.S.A.), [^3H]-dopamine (specific activity $20.34 \text{ Ci mmol}^{-1}$) from New England Nuclear-Dupont (England), and [^{125}I]-Protein A (specific activity $100 \mu\text{Ci ml}^{-1}$) from Amersham. All other reagents and chemicals were analytical grade and were purchased from either Sigma or Merck.

Results

Effects of amphetamine administration on striatal microdialysate levels of dopamine, DOPAC and HVA in rats treated with selegiline

Rats treated chronically with selegiline had a significantly higher dopamine basal extracellular level compared to the control and 1 day treated animals (see legend to Figure 1a) as we reported previously (Lamensdorf *et al.*, 1996). Chronic (21 days), and acute (1 day) treatment with selegiline (0.25 mg kg^{-1}) delayed (40 min vs 20 min, selegiline treated rats vs saline treated respectively), and significantly ($P < 0.05$) attenuated the maximum dopamine release induced by amphetamine administration (Figure 1a). In the chronically, but not in acutely treated rats, area under the dopamine curve was significantly reduced ($P < 0.05$ in comparison with acute selegiline and saline treated rats). Amphetamine-induced reduction in DOPAC and HVA microdialysate level was also attenuated 24 h after a single selegiline treatment although no changes were seen in the chronically treated rats (Figure 1b and c).

Effects of selegiline treatment on DAT levels

Selegiline increased DAT level by 154 and 145% at 24 h and 21 days treatment respectively, without effect at 2 h (Table 1). Acute or chronic treatment with rasagiline did not significantly affect DAT level, nor did acute (24 h) treatment with

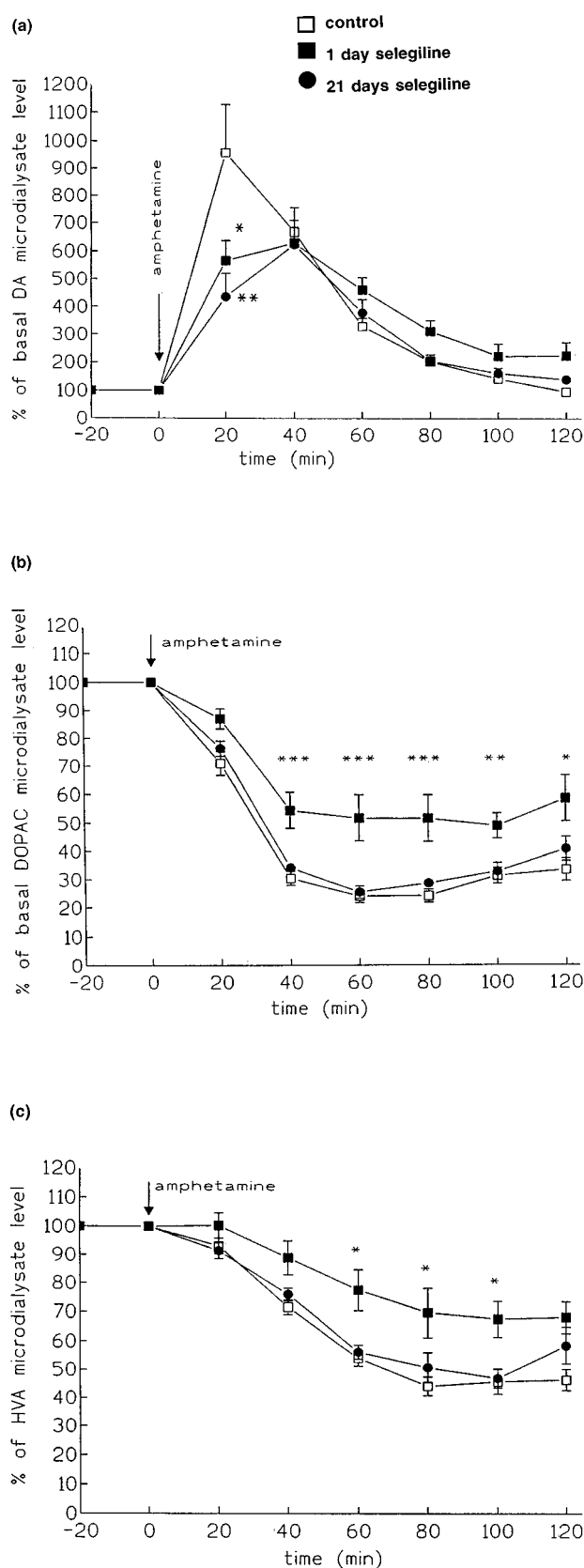


Figure 1 (a–c) Effect of acute and chronic treatment with selegiline (0.25 mg kg^{-1} , s.c.) on amphetamine (4 mg kg^{-1} , i.p.) induced changes in striatal microdialysate levels of dopamine (a), DOPAC (b) and HVA (c). Amine and metabolite levels are given as mean $\pm$ s.e. mean of per cent changes from basal levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for difference compared to saline control group. Basal microdialysate levels for dopamine, DOPAC and HVA were: control (9) 0.11 ± 0.02 , 38.3 ± 3.6 , 23.6 ± 1.3 ; acute (13) 0.11 ± 0.01 , 32.16 ± 3.76 , 23.04 ± 3.5 ; chronic (12) 0.21 ± 0.02 , 25.1 ± 1.06 , $16.7 \pm 0.9 \text{ pmol } 20 \text{ min}^{-1}$ respectively.

Table 1 Effect of *in-vivo* drug treatments on striatal DAT levels

	Control	2 h	24 h	21 days
Selegiline	210 ± 21 (11)	174 ± 50 (3)	324 ± 13 (11)*	306 ± 31 (9)*
Rasagiline	210 ± 24 (9)	268 ± 70 (3)	226 ± 39 (9)	172 ± 23 (9)
Clorgyline	210 ± 24 (9)	NT	NT	208 ± 62 (6)
Amphetamine	83 ± 4 (4)	NT	72 ± 7 (4)	NT
Nomifensine	83 ± 4 (4)	NT	65 ± 7 (4)	NT

The intensity of the immunoreactive band corresponding to DAT was determined by the phosphoimaging analyser, and a standard curve of rat striatal membranes (0–300 µg protein) was used to extrapolate the band intensity to µg protein. Data shown are mean values (µg DAT ± s.e.mean) per striatal sample. Number of rats shown in parentheses. The effects of selegiline, rasagiline and clorgyline were tested using anti-human DAT antibodies; effects of amphetamine and nomifensine were tested using anti-rat DAT antibodies. For doses of drugs see text. NT, not tested, * $P < 0.01$.

nomifensine or amphetamine, nor chronic treatment with clorgyline (Table 1).

[³H]-dopamine uptake in striatal synaptosomes

No significant changes were seen in K_M or V_{max} for [³H]-dopamine uptake in synaptosomes prepared from rats treated acutely or chronically with selegiline (Table 2). Selegiline was found to be a weak uptake blocker ($IC_{50} = 106 \pm 8.2$ µM) in comparison with its metabolites (–)-desmethylselegiline and (–)-methamphetamine (IC_{50} values 9.37 ± 0.08 and 4.51 ± 0.05 µM respectively). Rasagiline and its metabolite R-(+)-aminoindane were found to be without any significant effect on dopamine uptake (IC_{50} values 386 ± 16 ; 2950 ± 155 µM, respectively). 2-Phenylethylamine (PEA) was a potent uptake blocker ($IC_{50} = 1 \pm 0.025$ µM), and anticipated potencies were found for nomifensine (0.4 ± 0.08 µM) and GBR-12909 (1.5 ± 0.002 nM) which served as positive controls.

Discussion

We have found an inhibitory effect of selegiline on amphetamine-induced dopamine release in rat striatum *in vivo*. A possible explanation for this effect could be a reduction of dopamine high affinity transport. The dopamine-releasing property of amphetamine is complex, involving actions at both vesicular and membrane dopamine transporters. Amphetamine can enter dopaminergic neurons by both membrane transporter (Seiden *et al.*, 1993) and by lipophilic diffusion (Mack & Bonisch, 1979). Efflux of dopamine from the cytoplasmic compartment following amphetamine uptake is enabled by reverse transport *via* the membrane transporter. Amphetamine-induced dopamine release is dependent upon the membrane dopamine transporter since it is inhibited by uptake blockade with nomifensine (Butcher *et al.*, 1988). Release experiments *in vitro* also suggest the involvement of the membrane dopamine carrier, since nomifensine inhibits the effects of amphetamine on dopamine efflux from striatal slices or synaptosomes (Raiteri *et al.*, 1979). A recent study showed that genetic deletion of DAT in the mouse abolished the ability of systemic amphetamine (10 mg kg⁻¹) to induce efflux of dopamine into the extracellular space, as measured by both microdialysis and *in-vivo* voltammetry (Jones *et al.*, 1997).

Although selegiline was found to be a very weak uptake blocker, as reported by others (Fang & Yu, 1994) its metabolites (–)-methamphetamine and (–)-desmethylselegiline were more potent uptake blockers. It has been suggested that the short-lived inhibitory effect of selegiline on dopamine uptake after acute treatment, is the result of the action of (–)-methamphetamine, the major metabolite of selegiline (Knoll,

Table 2 K_M and V_{max} for [³H]-dopamine uptake in synaptosomes of rats treated acutely and chronically with selegiline

	V_{max} (pmol mg 5 min ⁻¹)	K_M (nM)
Control	6.17 ± 0.32	57.45 ± 9.8
1 day selegiline	6.85 ± 1.5	69.53 ± 20
21 days selegiline	5.85 ± 1.17	54.35 ± 18.4

Striatal tissue was pooled from six rats for each synaptosomal preparation, and three experimental runs were carried out for each determination. V_{max} and K_M values were determined by Lineweaver-Burke analysis.

1987; Fang & Yu, 1994). Although we could not exclude the possibility that selegiline decreases dopamine uptake through its active metabolites, several pieces of evidence argue against this possibility: (a) blood concentrations of (–)-desmethylselegiline and (–)-methamphetamine in the region of the IC_{50} for uptake inhibition (9.37 ± 0.08 and 4.51 ± 0.05 µM respectively) would not be achieved after a single administration of 0.25 mg kg⁻¹ s.c. selegiline; (b) Our determinations were done 24 h after the last dose of drug, and because the half-lives of amphetamines are in the region of 7 h, only very low levels of amphetamine metabolites would be expected in the blood and tissue of the rat in our studies.

Another possible explanation for the effect of selegiline to inhibit DAT activity *in vivo* is the accumulation of an endogenous factor such as PEA. The degree of MAO-B inhibition produced by a single dose of selegiline is minimal, whereas following multiple dosing, 95% of the striatal MAO-B activity is inhibited, as shown previously (Lamensdorf *et al.*, 1996). In addition, endogenous levels of PEA accumulate during chronic dosing (Paterson *et al.*, 1990). It is, therefore, apparent that marked differences exist in the brain neurochemistry between the acute and chronic situations. The mechanism whereby the acute selegiline treatment decreased amphetamine-induced dopamine release is at present not clear, and is likely to be different from that seen after chronic selegiline administration. In the animals treated chronically, endogenous PEA may mediate this effect. If PEA is responsible, it would explain why no inhibitory effect was seen in the synaptosomal preparation, since endogenous PEA would be removed during the preparation of the synaptosomes.

Amphetamine caused a reduction in DOPAC and HVA efflux into striatal microdialysate together with the increase in dopamine efflux, in agreement with findings of other workers (Zetterstrom *et al.*, 1988). This reduction in DOPAC and HVA efflux was also attenuated by the 1 day but not the 21 day

selegiline treatment. Butcher *et al.* (1988) and Zetterstrom *et al.* (1986) demonstrated that the decrease in efflux of dopamine metabolites is due to a depletion in the cytoplasmatic newly synthesized pool of dopamine. This pool is the main source for DOPAC formation and is the source of dopamine that is released by amphetamine (Zetterstrom *et al.*, 1988). However, amphetamine could also reduce the efflux of the deaminated metabolites by virtue of its ability to reversibly inhibit MAO. Since reduced efflux of the deaminated metabolites was also seen following amphetamine in mice lacking the DAT molecule (Jones *et al.*, 1997) it was inferred that the reduction in metabolites is dependent on MAO inhibition by that fraction of amphetamine which enters the neuron by the lipophilic diffusion pathway.

In the rats treated chronically with selegiline, changes in dopamine synthesis have to be considered, in addition to changes in dopamine neuronal uptake. Vrana *et al.* (1992) have shown that tyrosine hydroxylase activity was significantly reduced after chronic treatment with selegiline (0.1 mg kg⁻¹ daily for 3 and 7 days). In a previous experiment we found 30% reduction in striatal tyrosine hydroxylase activity following 21 days of selegiline treatment (0.25 mg kg⁻¹ s.c. daily; Lamensdorf *et al.*, 1997). Decrease in dopamine synthesis will deplete the newly synthesized dopamine pool, and could also explain the release of a smaller amount of dopamine by amphetamine following chronic selegiline treatment.

In our earlier study (Lamensdorf *et al.*, 1996) carried out under identical conditions to the present, the release of dopamine by a depolarizing concentration of KCl was enhanced in rats treated chronically with selegiline or rasagiline, which is compatible with inhibition of dopamine uptake (Arbutnott *et al.*, 1990). The decrease in amphetamine-induced dopamine release reported here, together with the increase in basal and KCl-induced release seen in our previous study provides strong evidence for an inhibition of dopamine reuptake by chronic selegiline treatment. The assumption that the attenuation in amphetamine induced dopamine release is due to selegiline's effect on dopamine uptake is supported by previous work by Zsilla *et al.* (1986), who found that [³H]-dopamine uptake into rat striatal slices was inhibited (by 37%) by chronic treatment with selegiline, and was transiently inhibited by acute treatment. In the synaptosomal preparation we were not able to see an effect of selegiline given to the animal previously on [³H]-dopamine uptake, possibly because the diffusion of retained small molecules such as PEA is more effective in the synaptosomal preparation than in slices.

Reduction of amphetamine-induced dopamine release was seen after both 1 day and chronic selegiline treatment, whereas increased dopamine basal extracellular level occurred only after the chronic treatment (Lamensdorf *et al.*, 1996). It is thus conceivable that the increased basal dopamine extracellular fluid level depends on both an inhibition of dopamine reuptake and a dopamine displacing effect, as is produced by PEA (Philips & Robson, 1983).

The increases in DAT levels that we observed appear to be in contradiction to the proposed reduction in functional

activity of the transporter, but activity of the transporter *in vivo* is dependent on both number of active transporter molecules as well as the presence of endogenous inhibitors, e.g. PEA. Although we found an increase in number of transporter molecules by selegiline, the newly synthesized molecules may be internalized or otherwise inactive, or their potential to enhance dopamine uptake may be inhibited by endogenous PEA. Other workers who have looked at the effect of selegiline on DAT activity have studied ligand binding. Wiener *et al.* (1989) found increased binding of [³H]-mazindol in mouse brain following long term treatment with a high dose of selegiline (10 mg kg⁻¹ i.p. daily for 4 weeks), although Yeghiayan *et al.* (1997) found no effect on [³H]-GBR-12935 binding under nearly the same conditions (10 mg kg⁻¹ for 21 days).

DAT expression was not elevated by acute treatment with a potent dopamine releaser (amphetamine) or by a potent dopamine uptake blocker (nomifensine). In addition, inhibition of MAO-B or MAO-A by rasagiline or clorgyline respectively did not affect DAT expression, and DAT was increased 1 day after a single dose of selegiline, which did not increase striatal extracellular dopamine level. Marked reductions in tyrosine hydroxylase activity by (α -methyl-p-tyrosine), or increases in dopamine content by administration of 3,4-dihydroxyphenylalanine (DOPA) did not affect the binding of ligands to DAT (Moody *et al.*, 1996). The 1 day selegiline treatment which elevated DAT content in our study would be expected to inhibit only about 60–70% of the MAO-B activity (Waldmeier *et al.*, 1981), and does not significantly affect dopamine metabolism (Lamensdorf *et al.*, 1996). Therefore, the effect of selegiline on DAT expression is apparently separate from its action on catecholamine metabolism.

One possibility is that (–)-amphetamine and (–)-methamphetamine produced *in vivo* from selegiline alter the expression of DAT, since in addition to the classical effects of amphetamine on the neuronal cell, it has been shown that amphetamine can induce up-regulation of zif/268 mRNA expression in rat forebrain (Wang & McGinty, 1995). However amphetamine given in the same dose as selegiline did not affect DAT level in our study. Selegiline has been shown to alter the expression of mRNA for a variety of proteins such as tyrosine hydroxylase, L-aromatic amino acid decarboxylase and glial fibrillary acidic protein (Li *et al.*, 1992; 1993; Vrana *et al.*, 1992) in lower dosage than is needed to influence dopamine metabolism.

In conclusion, we found functional evidence for a reduction in striatal dopamine uptake *in vivo* by selegiline, together with increased synthesis of dopamine transporter molecules. The increase in expression of transporter molecules may be a property of selegiline separate from its effect on MAO. From the present results we cannot tell whether the increase in the transporter expression is due to increase in transporter synthesis or reduction in its metabolism.

This study formed part of the PhD thesis requirements of I. Lamensdorf.

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(Received August 12, 1998

Accepted November 26, 1998)



The inhibition of nicotine-evoked relaxation of the guinea-pig isolated basilar artery by some analgesic drugs and progesterone

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1 The purpose of this study was to investigate the mechanism of nicotine-evoked relaxation of the guinea-pig isolated basilar artery and to study the effects of drugs associated with the aetiology or treatment of migraine on the nicotine response.

2 The guinea-pig isolated basilar artery, pre-contracted with prostaglandin F_{2α} (PGF_{2α}), in the presence of atropine (3 μM) and guanethidine (3 μM), relaxed on addition of nicotine (0.1 mM) in approximately 50% of preparations. The responses to nicotine were of short duration and blocked in preparations pre-treated for 10 min with capsaicin (1 μM) and are therefore probably a consequence of the stimulation of trigeminal C fibre terminals.

3 Responses to nicotine were reduced in the presence of 5-carboxamidotryptamine, 5-hydroxytryptamine and sumatriptan in that order of potency. This is consistent with a 5-HT₁ receptor mechanism. These agonists evoked small additional contractions in vessels pre-contracted with PGF_{2α}.

4 Indomethacin (0.3–10 μM), aspirin (10–30 μM), and nitro-L-arginine methyl ester (L-NAME, 0.1 mM) reduced nicotine-evoked relaxation of the basilar artery, suggesting the involvement of both nitric oxide and cyclo-oxygenase products in this response.

5 Progesterone (1 μM) markedly reduced the response to nicotine, a possible reflection of the ion channel blocking activity of high concentrations of this compound.

6 The guinea-pig basilar artery is a preparation in which the effects of drugs on responses to stimulation of trigeminal nerve terminals can be studied *in vitro* and may thus be of interest in assessing the actions of drugs used in treatment of headache.

Keywords: Basilar artery; trigeminal nerve; sumatriptan; 5-hydroxytryptamine; 5-carboxamidotryptamine; indomethacin; aspirin; L-NAME; progesterone

Introduction

O'Shaughnessy & Connor (1994) described an *in vitro* method for stimulating trigeminal terminals in the guinea-pig isolated basilar artery with nicotine. Nicotine evoked a relaxation of the basilar artery in the presence of atropine and guanethidine which was prevented by pre-treatment with capsaicin, tetrodotoxin or a neurokinin₁ receptor antagonist. This suggested that the mechanism of action of nicotine involves stimulation of capsaicin-sensitive nerve terminals, presumably of trigeminal origin, within the vessel resulting in release of substance P and subsequent relaxation of the artery. O'Shaughnessy & Connor (1994) also showed that in the presence of sumatriptan the nicotine response in the guinea-pig basilar artery was reduced, possibly due to an agonist action of sumatriptan at 5-HT₁ receptors situated on nerve terminals.

The role of trigeminal nerves in the mechanisms of headache pathogenesis and as a site of action for analgesic drugs has been a matter of great interest for some years (Moskowitz, 1992; 1993; Bruyn, 1996). An *in vivo* method for studying neurogenic plasma protein extravasation following electrical stimulation of the trigeminal ganglia of rats and guinea-pigs has been described by Markowitz *et al.* (1987). Several drugs known to be effective in treating headache including sumatriptan (Buzzi & Moskowitz, 1990) and the

non-steroidal anti-inflammatory agents indomethacin and aspirin (Buzzi *et al.*, 1989) inhibit neurogenic inflammation in this model resulting in its use in assessing the potential of new drugs for the treatment of headache. More recently progesterone and related steroids have been shown to inhibit the development of neurogenic inflammation in this *in vivo* model (Limmroth *et al.*, 1996).

Here we have investigated the effects of compounds on nicotine-evoked relaxation of the guinea-pig isolated basilar artery preparation in order to clarify the mechanism of this response and to see if similarities exist with the *in vivo* neurogenic inflammation model. We have compared sumatriptan, 5-hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) which might all be expected to inhibit the nicotine response by an inhibitory 5-HT_{1B/D} receptor agonist action on trigeminal terminals. Indeed 5-CT has been shown to be a potent inhibitor of protein extravasation in the neurogenic inflammation model but 5-HT had the opposite effect (Buzzi & Moskowitz, 1990). The non-steroidal anti-inflammatory drugs indomethacin and aspirin, both of which are active in the 'Moskowitz model' though their site of action is not clear, were also studied as was progesterone. As it seems likely that the nicotine response was mediated by substance P-induced release of nitric oxide the effects of the nitric oxide synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME) were investigated. Preliminary accounts of this work have been given in Rhodes *et al.* (1997a,b; 1998).

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Methods

Male, Hartley strain guinea-pigs (250–400 g) were killed by stunning and exsanguination. Ring segments (1.5–2.5 mm in length) of the guinea-pig basilar artery were mounted on tungsten wires (0.1 mm diameter) in 10 ml tissue baths under a tension of 0.4 g. Four preparations were normally obtained from each vessel. Tissues were maintained in Krebs' solution at 37°C, gassed with 5% CO₂ in O₂. Isometric recording of tissue tension was carried out using MacLab FSG 01 transducers and a MacLab 8 recording system.

After the tissues had stabilized for 90 min, a cumulative concentration-response curve to prostaglandin F_{2α} (PGF_{2α}, 10 nM–10 μM) was obtained. Substance P (3 or 10 nM) was added to the contracted vessels to confirm an intact endothelium. Tissues were then washed and equilibrated with guanethidine (3 μM) and atropine (3 μM). After 1 h tissues were again contracted with a submaximal concentration of PGF_{2α} (1 or 3 μM) and the relaxation evoked by nicotine (0.1 mM) was determined.

Sumatriptan, 5-HT and 5-CT

In studies with sumatriptan and related agonists tissues were washed after the initial response to nicotine and allowed 70 min to recover and then again contracted with the same concentration of PGF_{2α}. 5-HT (30 nM–1 μM), 5-CT (1 nM–1 μM), sumatriptan (0.3–30 μM) or vehicle (Krebs' solution) was added to the bath when the response to PGF_{2α} had stabilised. A response to nicotine was evoked approximately 4 min after addition of the agonist and the ratio of the nicotine-evoked relaxation in the presence of agonist (S2) with that to the first response to nicotine (S1) in the same tissue (S2:S1 ratio) was calculated. In some experiments tissue were washed for a further 70 min to allow recovery from the effect 5-HT, 5-CT or sumatriptan and the response to nicotine repeated.

In view of an increase in tension following addition of these tryptamines a group of tissues was studied in which the concentration of PGF_{2α} was increased from 1 to 2 μM at approximately 4 min before the second response to nicotine (corresponding to the time of addition of tryptamines). This was intended to give an indication of the effect of increasing tissue tension on nicotine responses.

Indomethacin, aspirin, L- and D-NAME and progesterone

In studies with indomethacin (0.3–10 μM), aspirin (3–30 μM), L- and D-NAME (0.1 mM) and progesterone (0.1 or 1.0 μM), tissue were washed for 70 min after the initial response to nicotine before again contracting with PGF_{2α} (1 or 3 μM) as above and the compounds were added to the bath 30 min (indomethacin and aspirin), 20 min (L- and D-NAME) or 1 h (progesterone) before addition of PGF_{2α}. The response to nicotine was assessed in the pre-contracted artery as above and the S2:S1 ratio calculated. The contraction to PGF_{2α} in the presence of drug was compared to the preceding contraction to PGF_{2α} in the absence of drug and S2:S1 ratios calculated. Progesterone was studied using paired preparations with ethanol vehicle compared directly with progesterone treatment.

Treatment with capsaicin

Tissues were washed for 30 min after the initial exposure to nicotine then capsaicin (1 μM) or DMSO vehicle (0.1 μl ml⁻¹)

was added to the baths for 10 min. Tissues were washed and after a further 30 min, re-contracted with PGF_{2α} (1 or 3 μM) and a response to nicotine obtained.

Effects of drugs on relaxation evoked by substance P

The method used was similar to that described above with a single, submaximal, concentration of substance P (0.3, 1.0 or 3 nM) replacing nicotine as the vasodilator. The time course of the experiments was as described for the nicotine study and S₂:S₁ ratios were calculated. The effects of 5-HT (0.3 μM), 5-CT (1 μM), indomethacin (1 μM), aspirin (30 μM) and progesterone (1 μM) were studied, the concentration used were those which had maximal effects in the experiments with nicotine.

Drugs and solutions

The composition of the Krebs' solution was as follows (mM): NaCl 118.4, KCl 4.8, NaHCO₃ 25.0, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11.1, CaCl₂ 1.25. The following compounds were purchased from Sigma Chemical Company, Poole, U.K.: nicotine hydrogen tartrate, atropine sulphate, guanethidine monosulphate, indomethacin, acetylsalicylic acid (aspirin), 8-methyl-N-vanillyl-6-nonenamide (capsaicin), 5-hydroxytryptamine hydrochloride, 5-carboxamidotryptamine maleate, substance P acetate, progesterone, dimethyl sulphoxide (DMSO), N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and N^ω-nitro-D-arginine methyl ester hydrochloride (D-NAME). Prostaglandin F_{2α} (dinoprost trimethamine) and sumatriptan maleate were gifts from Upjohn Ltd, Crawley, U.K. and from GlaxoWellcome, Stevenage, U.K. respectively.

Indomethacin and aspirin were dissolved in 1% w/v sodium carbonate solution and progesterone in ethanol to give 10 mM solutions which were further diluted in water. Capsaicin was dissolved in DMSO to give a 20 mM solution which was further diluted in water. Other compounds were dissolved directly in Krebs' solution.

Calculations and statistics

In the comparison of sumatriptan, 5-HT and 5-CT concentration-response curves were fitted to the Allfit equation (Delean *et al.*, 1978) using KaleidaGraph (Synergy Software) on an Apple Macintosh LC 475 computer. IC₅₀ values were calculated from these fitted curves. Relaxations evoked by stimulation with nicotine were compared in the presence (S2) and absence (S1) of test drug and S2:S1 ratios calculated for each tissue. Arithmetic means ± s.e.mean are shown. Treatment groups were compared with the corresponding vehicle using ANOVA and *t*-tests were appropriate. These were carried out using Microsoft Excel. Where several groups were compared with one vehicle control group Dunnett's modification to Student's *t*-test was used.

Results

Responses of control preparation to PGF_{2α}, substance P and nicotine

The mean maximum increase in tension with PGF_{2α} in vehicle control tissues (*n* = 32) was 0.58 ± 0.026 g and the initial exposure to substance P resulted in a relaxation of 0.31 ± 0.029 g. The first contraction with a single submaximal concentration of PGF_{2α} (1 μM) increased tension by 0.45 ± 0.024 g and the relaxation evoked by the first exposure

to nicotine in these tissues was 0.14 ± 0.018 g. The relaxation evoked by nicotine resembled that seen with substance P, a rapid relaxation followed by a rapid recovery (Figure 1). Approximately 50% of tissues responded to nicotine. Responses were not always consistent for preparations from the same animal with some preparations responding to nicotine and some not responding. As previously noted by O'Shaughnessy & Connor (1994) tissues which did not respond to nicotine usually had an intact endothelium as indicated by a marked relaxation to substance P. The reason for the variable sensitivity to nicotine is not clear.

The effects of sumatriptan, 5-HT and 5-CT on responses to nicotine

Concentration-related inhibition of the response to nicotine was observed with sumatriptan, 5-HT and 5-CT (Figures 1 and 2). The calculated pIC_{50} values for the agonists in inhibiting the nicotine-evoked relaxation were 5.5, 6.9 and 8.2 respectively. The S2:S1 ratios for the nicotine response at the maximum

concentration of agonist tested were 0.26 ± 0.125 (at $30 \mu\text{M}$; $n=4$), 0.27 ± 0.038 (at $1 \mu\text{M}$; $n=4$) and 0.42 ± 0.043 (at $1 \mu\text{M}$; $n=3$) for sumatriptan, 5-HT and 5-CT respectively.

All agonists evoked small additional contractions of the basilar artery pre-contracted with $\text{PGF}_{2\alpha}$. The maximum contractions to sumatriptan, 5-HT, 5-CT and vehicle respectively were 7.6 ± 3.03 (at $10 \mu\text{M}$, $n=3$), 13.1 ± 1.90 (at $0.3 \mu\text{M}$, $n=5$), 6.9 ± 1.48 (at $1 \mu\text{M}$, $n=4$) and $-0.3 \pm 0.72\%$ ($n=22$) of the initial maximum response to $\text{PGF}_{2\alpha}$. The pED_{50} values, as calculated using the Allfit equation, for sumatriptan, 5-HT and 5-CT were 5.5, 7.0 and 7.5 respectively.

The effect of increasing the tension of the tissue by increasing the $\text{PGF}_{2\alpha}$ concentration on responses to nicotine

In a group of five preparations an increase in concentration of $\text{PGF}_{2\alpha}$ from 1 to $2 \mu\text{M}$, at the time corresponding to the

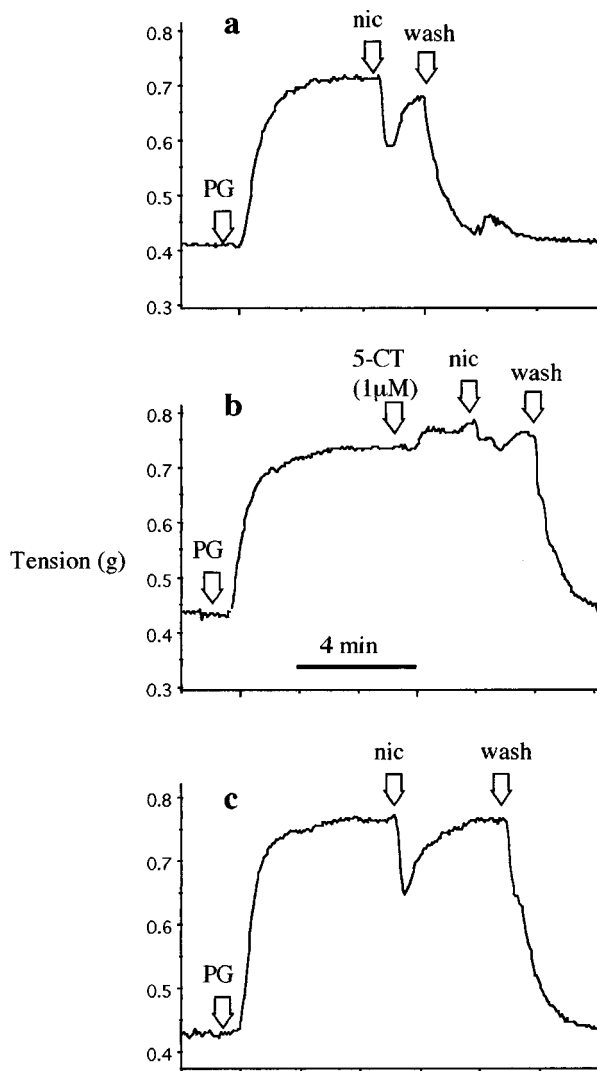


Figure 1 The effect 5-carboxamidotryptamine (5-CT, $1 \mu\text{M}$) on nicotine-evoked relaxation of the guinea-pig isolated basilar artery pre-contracted with prostaglandin $\text{F}_{2\alpha}$ (PG, $1 \mu\text{M}$). The trace shows the first response (a) of the tissue to nicotine (nic, 0.1 mM), the second response (b), following 70 min washout, to nicotine in the presence of 5-CT, and the third response (c) to nicotine following a further 70 min washout and recovery.

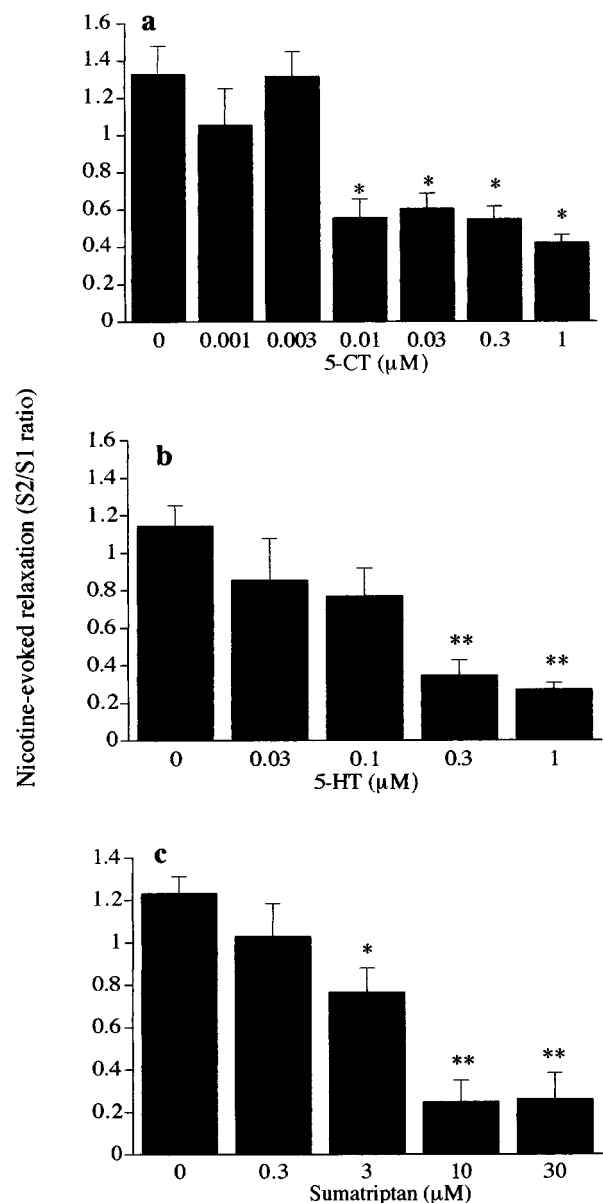


Figure 2 Inhibition of nicotine-evoked relaxation of the guinea-pig isolated basilar artery by (a) 5-hydroxytryptamine (5-HT), (b) 5-carboxamidotryptamine (5-CT) and (c) sumatriptan. Vertical bars show s.e.means of $n=3-8$. * $P<0.05$; ** $P<0.01$ using Dunnett's modified t -test for comparison with vehicle control.

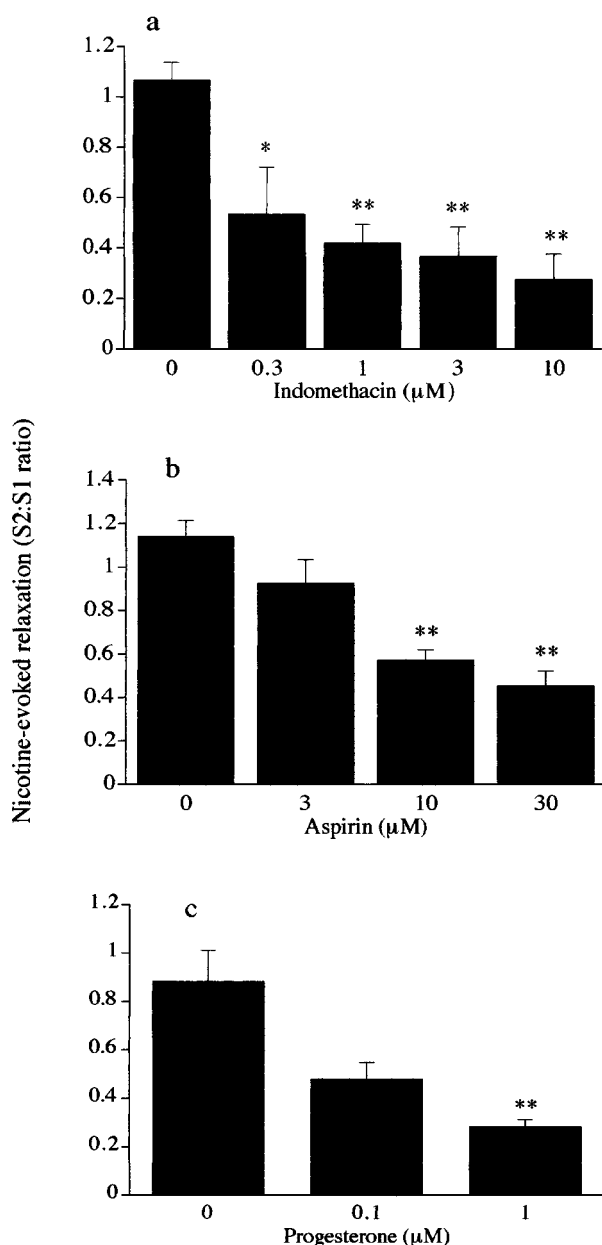


Figure 3 Inhibition of nicotine-evoked relaxation of the guinea-pig isolated basilar artery by (a) aspirin, (b) indomethacin and (c) progesterone. Vertical bars show s.e. means of $n=3-10$. * $P<0.05$; ** $P<0.01$ Dunnett's modified t -test for comparison with vehicle control.

addition of tryptamines, evoked a further increase in tension of $10.2 \pm 1.64\%$ of the initial maximum $\text{PGF}_{2\alpha}$ response. In these tissues the S2:S1 ratio for the nicotine response was 0.91 ± 0.16 . In comparison a concentration of $10 \mu\text{M}$ sumatriptan evoked an additional increase in tension of $7.6 \pm 3.03\%$ of the initial maximum $\text{PGF}_{2\alpha}$ response but was associated with a much greater inhibition of the nicotine response (S2:S1 ratio 0.25 ± 0.10 , $n=3$). It thus seems unlikely that the increase in tension following addition of sumatriptan, 5-HT or 5-CT could result in the inhibition of the subsequent response to nicotine.

The effects of indomethacin and aspirin on responses to nicotine

Concentration-related reductions in the nicotine-evoked relaxation of the basilar artery were seen with aspirin and

indomethacin (Figure 3). Indomethacin (S2:S1 ratio 0.53 ± 0.19 at $0.3 \mu\text{M}$) was approximately 30 fold more potent than aspirin (S2:S1 ratio 0.57 ± 0.05 at $10 \mu\text{M}$, Figure 3). Contractions to $\text{PGF}_{2\alpha}$ (1 or $3 \mu\text{M}$) were not changed significantly after incubation with aspirin or indomethacin (S2:S1 ratios in the presence of vehicle, indomethacin, $3 \mu\text{M}$ and aspirin, $30 \mu\text{M}$ being 1.02 ± 0.05 , $n=10$; 1.09 ± 0.03 , $n=6$ and 0.99 ± 0.03 , $n=4$ respectively).

A few preparations developed tone during the first 90 min of the tissue stabilization period in this series of experiments. In these tissues indomethacin and aspirin caused a relaxation (results not shown) indicating cyclo-oxygenase activity.

The effects of L- and D-NAME on responses to nicotine

The relaxation evoked by nicotine was much reduced following incubation with L-NAME (0.1 mM ; S2:S1 ratio 0.14 ± 0.05 ; $n=5$). In contrast little effect of D-NAME on the nicotine response was seen (S2:S1 ratio 0.83 ± 0.18 ; $n=3$). The contractions to $\text{PGF}_{2\alpha}$ were increased following exposure to L-NAME (S2:S1 ratio 1.29 ± 0.04) but not D-NAME (S2:S1 ratio 1.08 ± 0.03) suggesting that responses to $\text{PGF}_{2\alpha}$ are modulated by nitric oxide release in this tissue.

The effects of progesterone on responses to nicotine

A significant reduction in nicotine-evoked relaxation was seen in the presence of progesterone ($1 \mu\text{M}$, Figure 3). The nicotine S2:S1 ratios were 0.28 ± 0.03 and 1.06 ± 0.16 in the presence of progesterone ($1 \mu\text{M}$) and ethanol vehicle respectively ($P=0.008$, paired t -test, $n=5$). Progesterone ($0.1 \mu\text{M}$) did not significantly reduce nicotine-evoked relaxations, S2:S1 ratios being 0.48 ± 0.07 and 0.74 ± 0.19 in the presence of progesterone ($0.1 \mu\text{M}$) and ethanol vehicle respectively ($P=0.180$, paired t -test, $n=6$). Responses to $\text{PGF}_{2\alpha}$ (1 or $3 \mu\text{M}$) were not significantly different in progesterone (0.1 or $1 \mu\text{M}$) and vehicle treated groups (S2:S1 ratios 1.06 ± 0.08 , 0.97 ± 0.03 , 0.98 ± 0.02 and 0.99 ± 0.06 for progesterone $1 \mu\text{M}$, $0.1 \mu\text{M}$ and their respective ethanol vehicle controls, $n=5$). Progesterone had no effect on the tone of the basilar artery.

The effect of capsaicin on responses to nicotine

Capsaicin ($1 \mu\text{M}$) had no effect on the tone of the basilar artery in the absence of exogenous $\text{PGF}_{2\alpha}$. In preliminary experiments with tissues pre-contracted with $\text{PGF}_{2\alpha}$ a marked relaxation was seen with this concentration of capsaicin, the response being absent in tissues previously exposed to capsaicin (results not shown).

Relaxation of the basilar artery in response to nicotine was much reduced in tissues pre-treated with capsaicin (S2:S1 ratio 0.21 ± 0.07 , $n=6$). DMSO vehicle had little effect on nicotine responses (S2:S1 ratio 1.09 ± 0.08 , $n=6$). No effect of capsaicin or vehicle were seen on the $\text{PGF}_{2\alpha}$ contraction of the vessel, S2:S1 ratios being 0.99 ± 0.02 and 0.96 ± 0.02 respectively.

Effects of drugs on relaxation evoked by substance P

Results are summarized in Table 1. No effect of 5-HT, 5-CT, aspirin or progesterone was seen at the concentrations tested. Indomethacin had a significant inhibitory effect on substance P-evoked relaxation at a concentration of 10 but not $3 \mu\text{M}$.

Table 1 The effects of drugs on substance P-evoked relaxation of the guinea-pig isolated basilar artery

Drug	Concentration (μM)	$S_2:S_1$ ratio	n
5-HT	0	0.85 ± 0.080	3
	0.3	0.91 ± 0.061	3
5-CT	0	1.16 ± 0.094	3
	1.0	1.16 ± 0.108	3
Indomethacin	0	0.92 ± 0.037	8
	3.0	0.81 ± 0.118	4
	10.0	$0.53^* \pm 0.141$	4
Aspirin	0	0.94 ± 0.065	4
	30.0	0.91 ± 0.025	4
Progesterone	0	0.96 ± 0.010	2
	1.0	0.83 ± 0.283	2

The effects of drugs on the relaxation-evoked by a single submaximal concentration of substance P (0.3, 1 or 3 nM) are shown. Values of $S_2:S_1$ ratio are means $\pm$ s.e.mean.

* $P < 0.05$ Dunnett's *t*-test.

Discussion

The use of nicotine to stimulate peptide-containing nerve terminals in isolated vascular preparations is well established (O'Shaughnessy & Connor, 1994; Okamura & Toda, 1994). In the present study we have investigated the mechanism of the relaxation observed with nicotine in the guinea-pig isolated basilar artery and the effects of some drugs known to inhibit neurogenic inflammation *in vivo* by several different mechanisms (Buzzi *et al.*, 1989; Buzzi & Moskowitz, 1990; Limmroth *et al.*, 1996).

The agonist potencies (5-CT > 5-HT > sumatriptan) in inhibiting nicotine-evoked relaxation of the guinea-pig isolated basilar artery are consistent with a 5-HT₁ receptor-mediated response though sumatriptan appeared less potent than expected from the results of O'Shaughnessy & Connor (1994). This may reflect the shorter equilibration time used for agonists in the present study. The inhibitory action of 5-HT contrasts with the reported potentiation by 5-HT of neurogenic inflammation in an *in vivo* model of trigeminal nerve stimulation (Buzzi & Moskowitz, 1990) and possibly reflects multiple 5-HT receptor activation *in vivo* not seen here. Further studies to elucidate the nature of the 5-HT₁ receptor modulating the nicotine response would be of interest.

In contrast to the observations of O'Shaughnessy & Connor (1994), who saw no vasoconstrictor response to sumatriptan alone, sumatriptan consistently evoked small contractions of the guinea-pig basilar artery in the present study, as did 5-HT and 5-CT. This difference may reflect the addition of sumatriptan in the presence of PGF_{2 α} in the present study, compared to 10 min before the addition of PGF_{2 α} in the method of O'Shaughnessy & Connor, (1994). Enhanced contractile effects of sumatriptan in isolated tissues pre-contracted with a thromboxane receptor agonist have previously been reported (Bax *et al.*, 1996).

No significant reduction in the relaxation evoked by substance P was observed in tissues treated with 5-HT or 5-CT. This is in agreement with the observations of O'Shaughnessy & Connor (1994) who found no block of the

substance P response with sumatriptan in this tissue. This would suggest that the inhibitory effect of these 5-HT receptor agonists precedes the nicotine-evoked release of substance P.

The marked block of nicotine-evoked dilatation by indomethacin and aspirin and also by L-NAME suggests that both cyclo-oxygenase products and nitric oxide are involved in mediating this response in the guinea-pig basilar artery. The approximately 30 fold greater potency of indomethacin is consistent with the reported potencies of indomethacin and aspirin as inhibitors of cyclo-oxygenase 1 or 2 in intact cell assays (Mitchell *et al.*, 1994). Okamura & Toda (1994) in a study of nicotine-evoked relaxation of the dog saphenous artery attributed an indomethacin-sensitive component of the response to release of prostacyclin from vascular smooth muscle. However, in the present study, L-NAME alone reduced the nicotine response by 86% and indomethacin alone by 73%. It is possible that release of nitric oxide directly from neurones, or from endothelium following substance P release, activates cyclo-oxygenase and that prostacyclin is an important factor in mediating the response to nicotine. Salvemini *et al.* (1996) have proposed the release of prostacyclin from vascular endothelium as an important component of the nitric oxide mediated anti-platelet effects of nitrovasodilators *in vivo*. However, in the present study only a large concentration of indomethacin (10 μM) and not aspirin (30 μM) significantly reduced substance P-evoked relaxation of the basilar artery (Table 1). In the plasma protein extravasation model in the rat Buzzi *et al.* (1989) were able to show inhibitory effects of large doses of both indomethacin and aspirin in experiments where substance P was used to stimulate extravasation in the dura.

The results show that nicotine-evoked relaxation of the guinea-pig isolated basilar artery is blocked following 1 h equilibration with a high concentration of progesterone (1 μM). The mechanism of action of progesterone is unknown but high concentrations of progesterone have been reported to block the ion channel of the neuronal nicotinic receptor (Bullock *et al.*, 1997; Ke & Lucas, 1996). A GABA_A receptor agonist mechanism has been proposed for the block of neurogenic inflammation by progesterone and related neurosteroids in the rat (Limmroth *et al.*, 1996) and may contribute to the mechanism of action progesterone in the present study. Steroid hormone levels are believed to be involved in the mechanism of the migraine headaches associated with menstruation and pregnancy (Silberstein, 1996).

This study confirms the ability of sumatriptan to inhibit a neurogenic response in the guinea-pig basilar artery *in vitro* and shows similar effects of 5-HT itself and the 5-HT₁ receptor agonist 5-CT. The relaxation response to nicotine is dependent on the activity of both nitric oxide synthase and cyclo-oxygenase. The steroid hormone progesterone blocks the action of nicotine with an as yet unknown mechanism. This method may be of interest as an *in vitro* approach to the study of neurogenic cerebral vasodilatation and the effects of drugs useful in the treatment of headache.

This work was supported by the Migraine Trust.

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(Received January 8, 1998

Revised November 1, 1998

Accepted November 26, 1998)



Dual coupling of heterologously-expressed rat P2Y₆ nucleotide receptors to N-type Ca²⁺ and M-type K⁺ currents in rat sympathetic neurones

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1 The P2Y₆ receptor is a uridine nucleotide-specific G protein-linked receptor previously reported to stimulate the phosphoinositide (PI) pathway. We have investigated its effect in neurones, by micro-injecting its cRNA into dissociated rat sympathetic neurones and recording responses of N-type Ca²⁺ (I_{Ca(N)}) and M-type K⁺ (I_{K(M)}) currents.

2 In P2Y₆ cRNA-injected neurones, UDP or UTP produced a voltage-dependent inhibition of I_{Ca(N)} by ~53% in whole-cell (disrupted-patch) mode and by ~73% in perforated-patch mode; no inhibition occurred in control cells. Mean IC₅₀ values (whole-cell) were: UDP, 5.9 ± 0.3 nM; UTP, 20 ± 1 nM. ATP and ADP (1 μM) had no significant effect. *Pertussis* toxin (PTX) substantially (~60%) reduced UTP-mediated inhibition in disrupted patch mode but not in perforated-patch mode.

3 Uridine nucleotides also inhibited I_{K(M)} in P2Y₆ cRNA-injected cells (by up to 71% at 10 μM UTP; perforated-patch). Mean IC₅₀ values were: UDP, 30 ± 3 nM; UTP, 115 ± 12 nM. ATP (10 μM) again had no effect. No significant inhibition occurred in control cells. Inhibition was PTX-resistant.

4 Thus, the P2Y₆ receptor, like the P2Y₂ subtype studied in this system, couples to both of these two neuronal ion channels through at least two different G proteins. However, the P2Y₆ receptor displays a much higher sensitivity to its agonists than the P2Y₂ receptor in this expression system and higher than previously reported using other expression methods. The very high sensitivity to both UDP and UTP suggests that it might be preferentially activated by any locally released uridine nucleotides.

Keywords: Nucleotide receptors; P2Y receptors; heterologous expression; sympathetic neurones; uridine nucleotides; UTP; UDP; calcium current; potassium current; M-current

Abbreviations: ADP, adenosine 5'-diphosphate; AP₄A, P-P-di(adenosine-5') tetraphosphate; GFP, Green Fluorescent Protein; GTP, guanosine 5'-triphosphate; I_{Ca}, currents through voltage-gated Ca²⁺ channels; I_{Ca(N)}, N-type Ca²⁺ current; I_{K(M)}, M-type K⁺ current; ITP, inosine 5'-triphosphate; I/V, current/voltage; PI, phosphoinositide; PTX, *Pertussis* toxin; SCG, superior cervical sympathetic ganglion; TEA, tetraethylammonium; UDP, uridine 5'-diphosphate; UTP, uridine 5'-triphosphate

Introduction

The P2Y₆ receptor (Chang *et al.*, 1995; Communi *et al.*, 1996) is a member of the G protein-coupled P2Y family of nucleotide receptors (North & Barnard, 1997). It is a pyrimidine receptor, rather than a purine receptor (Communi & Boeynaems, 1997) – that is, it has a much higher sensitivity to uridine nucleotides than to adenosine nucleotides, and is virtually insensitive to ATP (Nicholas *et al.*, 1996). Messenger RNA for the P2Y₆ receptor has been detected in a range of peripheral tissues (Chang *et al.*, 1995; Communi *et al.*, 1996). It has also been found in whole brain (T.E. Webb, unpublished) and in some brain-derived cell lines (Maier *et al.*, 1997).

The primary intracellular signalling response usually attributed to P2Y receptor stimulation is the activation of the phosphoinositide (PI) pathway (e.g., Boarder *et al.*, 1995). The P2Y₆ receptor appears to fit this pattern. Thus, when P2Y₆ receptors were expressed in C6-15 glioma cells (Chang *et al.*, 1995) or 1321N1 astrocytoma cells (Communi *et al.*, 1996;

Nicholas *et al.*, 1996), the only responses recorded were the formation of inositol phosphates or elevation of intracellular [Ca²⁺]. Further, in C6-15 cells, this effect was not inhibited by *Pertussis* toxin (PTX), and receptor stimulation did not cause any inhibition of forskolin-stimulated adenylate cyclase, suggesting that this receptor type is incapable of activating PTX-sensitive G proteins in that expression system (Chang *et al.*, 1995).

However, in previous experiments using an alternative primary cell expression system (the rat sympathetic neurone), we found that the homologous PI-coupled uridine nucleotide-sensitive P2Y₂ receptor could induce a broader range of responses. Thus, when expressed by micro-injecting cRNA into dissociated neurones, this receptor coupled to both PTX-sensitive and insensitive G proteins, to inhibit voltage-gated Ca²⁺ and K⁺ currents with near-equal facility (Filippov *et al.*, 1997; 1998).

When compared with the usual test situation of vector transfection into a transformed cell line, the ganglion cell expression system provides the intracellular milieu of a native neurone and allows a wider range of effector responses to be monitored. Hence, in the present experiments, we have used this expression system to test whether the P2Y₆ receptor

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showed similar or different channel-coupling preferences to those previously observed for the P2Y₂ receptor.

Methods

These were essentially identical to those described previously (Filippov *et al.*, 1997; 1998). The rat P2Y₆ receptor sequence (Chang *et al.*, 1995) was excised from puc18 by digestion with *Bam*HI and was inserted into the reciprocal site of the mammalian expression vector pBK/CMV (Stratagene). Plasmid DNA was isolated, linearized with *Xho*I and capped cRNA was transcribed using a T3 transcription kit (Ambion) prior to polyadenylation and microinjected along with cRNA for jellyfish Green Fluorescent Protein (GFP) into single superior cervical sympathetic (SCG) neurones isolated from 15–19-day-old rats (killed by CO₂ asphyxiation). The usual cRNA concentration for injection was 1.25 µg µl⁻¹ (dissolved in water). Membrane currents were recorded from fluorescently-labelled neurones after 14–24 h incubation at 37°C. Recordings were made at 20°C in Krebs' solution continuously-flowing at 20–25 ml min⁻¹, using patch electrodes coupled to a discontinuous ('switching') amplifier (Axoclamp 2B) sampling voltage at 6–8 kHz. Voltage commands were generated and currents digitized and analysed using 'pClamp 6' software (Axon Instruments, Foster City, CA, U.S.A.).

Ca²⁺ channel current recording

Currents through voltage-gated Ca²⁺ channels (I_{Ca}) were usually recorded using the conventional whole-cell (disrupted-patch) method as described previously (Caulfield *et al.*, 1994). The bathing solution consisted of (in mM) tetraethylammonium (TEA) chloride 120, KCl 3, MgCl₂ 1.5, BaCl₂ 5, HEPES 10, glucose 11.1 and 0.5 µM tetrodotoxin. The pH was adjusted to 7.35 with NaOH. Patch electrodes (2–3 MΩ) were filled with a solution containing (in mM) CsCl 110, MgCl₂ 3, HEPES 40, EGTA 3, Na₂ATP 2, Na₂GTP 0.5 (pH adjusted to 7.4 with CsOH). Currents were routinely evoked every 20 s with a 100 ms depolarizing rectangular test pulse to 0 mV from a holding potential of -90 mV. Current amplitudes were measured isochronally 10 ms from the onset of the rectangular test pulse, i.e., near to the peak of the control current. To eliminate leak currents, Co²⁺ was substituted for Ba²⁺ in the external solution at the end of each experiment to block all Ca²⁺ channel currents and the residual current was digitally subtracted from the corresponding currents in Ba²⁺ solution. Currents were substantially (~67%) and irreversibly blocked by 200–300 nM ω-conotoxin GVIA but insignificantly (≤6%) by 2 µM nifedipine (Filippov *et al.*, 1997) so were largely N-type, with negligible contribution by L-type channels.

In some experiments I_{Ca} was recorded using the perforated-patch technique (Horn & Marty, 1988). Patch pipettes (2–4 MΩ) were filled by dipping the tip into a filtered solution of the same composition as used for disrupted-patch for 20–30 s. The pipette was then back-filled with the same solution containing 0.125 mg ml⁻¹ amphotericin B as the permeabilizing agent (Rae *et al.*, 1991). Access resistance after permeabilization was 8–15 MΩ. The bathing solution was the same as for disrupted-patch experiments.

M-type K⁺ current recordings

Since M-type K⁺ currents (I_{K(M)}) dissipate rapidly in disrupted whole-cell patch recordings (Brown *et al.*, 1995), we used only

the perforated-patch method for M-current recording, as described by Caulfield *et al.* (1994). Patch pipettes (2–4 MΩ) were filled by dipping the tip into a filtered solution containing (in mM) potassium acetate 90, KCl 20, MgCl₂ 3, HEPES 40, BAPTA 0.1 (adjusted to pH 7.4 by KOH), for 20–60 s. The pipette was then back-filled with the same solution containing 0.125 mg ml⁻¹ amphotericin B as the permeabilizing agent (Rae *et al.*, 1991). Access resistance after permeabilization was 8–15 MΩ. The bathing solution contained (in mM) NaCl 120, KCl 3, MgCl₂ 1.5, CaCl₂ 2.5, HEPES 10, glucose 11.1 (adjusted to pH 7.3 with NaOH). Neurones were voltage-clamped at -20 mV or -30 mV and M-currents deactivated with 1-s hyperpolarizing steps at 5-s intervals. Current/voltage (I/V) relationships were obtained using incremental voltage steps of 10 mV between -10 and -100 mV; currents were measured at the end of each hyperpolarizing step. For dose/response curves, currents were measured at -30 mV from steady-state I/V relations obtained using a ramp voltage command of 20 s from -20 to -90 mV. The leak component of current was estimated in both cases by extrapolating a linear fit to the I/V relationship from the negative potential region, where only ohmic currents were observed.

Statistical analysis

Data are presented as means ± s.e.mean as appropriate. Student's *t*-test (unpaired) was applied to determine statistical significance. The difference was considered significant if *P* ≤ 0.05. Dose-response curves were determined using concentrations added cumulatively, with 1 min exposure times. Curves were fitted (using Origin 4.1 software) to pooled data points using the equation $y = y_{max} \cdot x^{n_H} / (x^{n_H} + K^{n_H})$ where *y* = observed percentage inhibition, *y*_{max} = extrapolated maximal percentage inhibition, *x* = nucleotide concentration (µM), *K* = IC₅₀ (µM) and *n*_H = Hill coefficient.

Chemicals

Drugs were applied to the external solution by bath perfusion (bath exchange rate ≤ 5 s). Tetrodotoxin was from Calbiochem, La Jolla, CA, U.S.A. Three samples of uridine 5'-triphosphate (UTP) were used: from Sigma (St. Louis, MO, U.S.A.); molecular biology grade from Pharmacia Biotech (Uppsala, Sweden), which contained >99% UTP; and a sample of the latter purified to complete homogeneity by HPLC (HPLC-pure). All critical determinations were made with the latter two samples. ATP, adenosine 5'-diphosphate (ADP), inosine 5'-triphosphate (ITP), guanosine 5'-triphosphate (GTP), (-)-norepinephrine (noradrenaline) bitartrate, BAPTA, amphotericin B were all from Sigma; uridine 5'-diphosphate (UDP) was from Boehringer Mannheim GmbH (Germany); P-P-di(adenosine-5') tetraphosphate (AP₄A) triammonium salt was from RBI, Natick, MA, U.S.A.; Pertussis toxin (PTX) was from Porton Products, Dorset, U.K.; CoCl₂ (AnalaR grade) was from BDH, Poole, U.K.; BaCl₂ and CsCl were from Aldrich.

Results

Ca²⁺ channel currents

In initial tests, UTP at high concentrations (10–100 µM) was applied briefly to cells injected 14–24 h previously with P2Y₆ cRNA. These cells were identified by their fluorescence, from the expression of co-injected GFP cRNA. As shown in Figure

1A, such applications produced a substantial and reversible inhibition of the inward Ca²⁺ channel current (I_{Ca} – recorded using Ba²⁺ (5 mM) as charge-carrier: see Methods). After subtracting the residual ('leak') current recorded on substituting Co²⁺ (5 mM) for Ba²⁺, the mean inhibition of I_{Ca} produced by UTP (100 μ M) (measured 10 ms after current onset) was $52.7 \pm 2.0\%$ ($n=18$ cells). In contrast (and as previously reported: Filippov *et al.*, 1997), no significant inhibition ($-0.06 \pm 1.49\%$) of I_{Ca} could be detected in 16 control cells pre-injected with GFP cRNA alone (see also Boehm, 1998).

However, we also noted that, at these high concentrations (100 μ M), there was substantial desensitization during continued exposure to UTP (more so than we had experienced for expressed P2Y₂ receptors: Filippov *et al.*, 1997). This suggested to us that the P2Y₆ receptor might be more sensitive to UTP than the P2Y₂ receptor. Initial concentration – response relationships obtained using UTP from Sigma (see Methods) suggested that this was indeed the case, yielding a mean IC₅₀ for UTP of 17.2 ± 0.9 nM (as against 0.50 ± 0.05 μ M for the P2Y₂ receptor: Filippov *et al.*, 1997). However, previous observations on inositol phosphate production in 1321N1 cells expressing P2Y₆ receptors had suggested that this receptor was relatively insensitive to UTP and that the observed effect of UTP on these cells resulted largely from contamination by UDP in such samples (Nicholas *et al.*, 1996). This proved not to be the case in the present experiments, since pure (>99%) UTP yielded an IC₅₀ of 20.1 ± 1.4 nM (Figure 2A), and application of HPLC-purified UTP (100 nM) (see Methods) likewise inhibited I_{Ca} by $51.9 \pm 7.0\%$ ($n=4$ cells). (The desensitization we observed with UTP referred only to large (100 μ M) doses: there was minimal desensitization with cumulative 1 min applications of lower doses. This is clear from Figure 2, since the maxima on the graphs in Figure 2A match responses to 1 μ M concentrations of the two nucleotides given as single applications in Figure 2B.)

UDP was about three times more effective than UTP in inhibiting I_{Ca} , with an IC₅₀ of 5.90 ± 0.33 nM and extrapolated

maximal inhibition of $52.7 \pm 0.65\%$ (Figure 2A). Other nucleotides were tested at a single matched dose of 1 μ M (Figure 2B). ATP did not produce any inhibition of I_{Ca} and the recorded effect of ADP was not significantly different from the small effect of this nucleotide observed in control cells pre-injected with GFP cRNA alone. ITP produced a weak inhibition, while GTP and AP₄A were inactive.

Thus, the P2Y₆ receptor retained its selectivity for uridine over adenine nucleotides when coupled to Ca²⁺ channels, as previously described for PI-linked responses.

Voltage-dependence of Ca²⁺ channel current inhibition

As previously noted using expressed P2Y₂ receptors (Filippov *et al.*, 1998), I_{Ca} inhibition following activation of expressed P2Y₆ receptors was voltage-dependent. This is indicated by (a) the slowed current onset in Figure 1A (*c.f.* Bean, 1989) and (b) partial reversal of I_{Ca} inhibition by a 25 ms depolarizing prepulse to +120 mV (Figure 3; *c.f.* Grassi & Lux, 1989). In six experiments, such a depolarizing prepulse reduced the inhibition produced by UTP (100 μ M) from 43.2 ± 5.4 to $17.7 \pm 4.5\%$ (Figure 3A). For comparison, this depolarization reduced the inhibitory action of noradrenaline from 34.1 ± 4.2 to $11.4 \pm 1.6\%$ (Figure 3B; *c.f.* Beech *et al.*, 1992).

Effect of Pertussis toxin (PTX)

The voltage dependence of P2Y₆-mediated inhibition suggested that this receptor might couple to Ca²⁺ channels *via* a PTX sensitive G-protein of the G_i/G_o family (reviewed by Hille, 1994). In partial accordance with this, overnight pretreatment of the P2Y₆ cRNA-injected SCG neurones with PTX (500 ng ml⁻¹) significantly reduced the inhibition of I_{Ca} produced by UTP (10 μ M) in the whole-cell (disrupted-patch) experiments from 52.8 ± 4.6 to $21.0 \pm 4.9\%$ (Figure 4). In the same neurones, the inhibition produced by noradrenaline

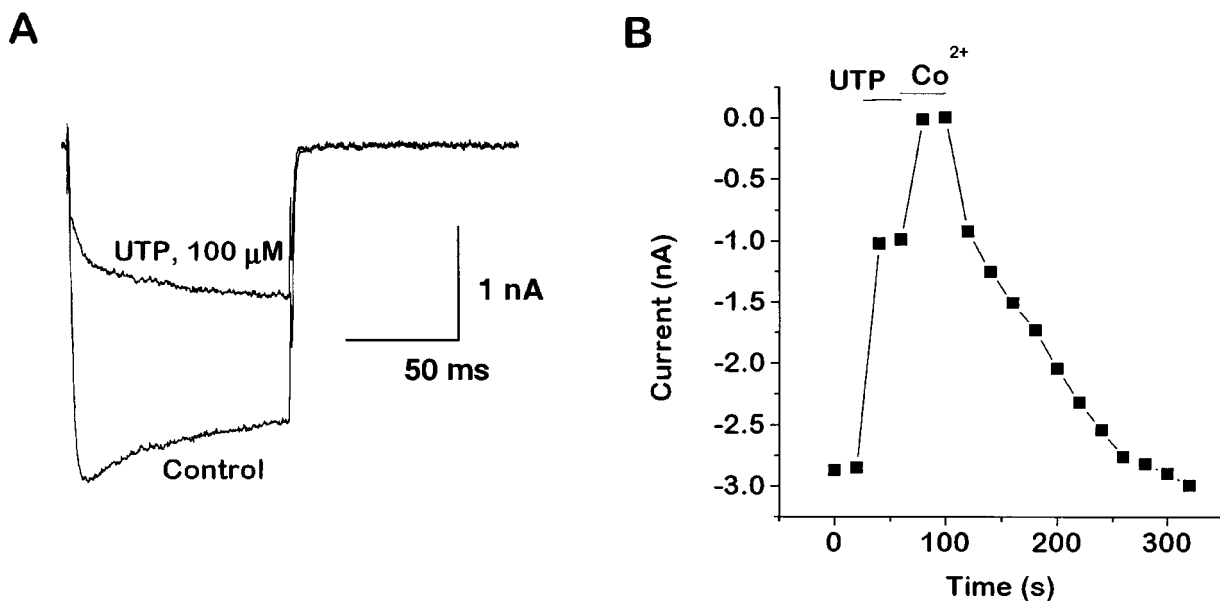


Figure 1 The heterologously expressed P2Y₆ receptor couple to Ca²⁺ channels in sympathetic neurones. Neurones were pre-injected with $1.25 \mu\text{g } \mu\text{l}^{-1}$ P2Y₆ cRNA. Records show leak-subtracted Ca²⁺ channel currents recorded at room temperature (20°C) using the whole-cell (ruptured patch) variant of the patch-clamp technique using Ba²⁺ (5 mM) as charge carrier (see Methods). Currents were recorded by stepping for 100 ms every 20 s from -90 mV to 0 mV and were measured 10 ms from the onset of the test pulse. (A) Records showing superimposed leak-subtracted currents in the absence and presence of UTP (100 μ M). (B) Time-plot of I_{Ca} amplitude (inward current downward) during the experiment shown in (A). Solid bars indicate time of exposure to UTP or Co²⁺. (Co²⁺ exposure was used to calculate the leak current for subtraction, see Methods).

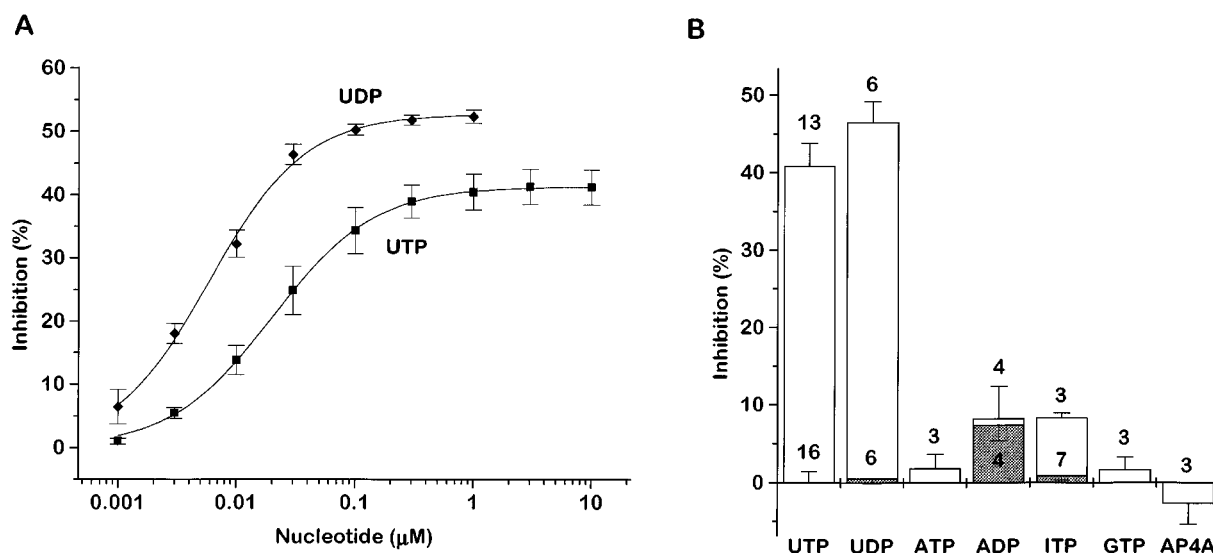


Figure 2 (A) Concentration-dependence of Ca²⁺ channel current inhibition in cells pre-injected with P2Y₆ cRNA produced by UDP (diamond) and by pure UTP (squares; see Methods). Current amplitude was measured 10 ms after the onset of the test pulse from -90 to 0 mV. Points show means $\pm$ s.e. mean of measurements in three to four cells for each nucleotide; concentrations were added cumulatively, with 1-min exposure times. Curves were fitted to pooled data points using Origin 4.1 software to the Hill equation $y = y_{max} \cdot x^{n_H} / (x^{n_H} + K^{n_H})$ where y = observed percentage inhibition, y_{max} = extrapolated maximal percentage inhibition, x = nucleotide concentration (μ M), $K = IC_{50}$ (μ M) and n_H = Hill coefficient. Values of Hill constants (mean $\pm$ s.e. mean) were as follows: UDP: $y_{max} = 52.7 \pm 0.65\%$; $K = 5.9 \pm 0.33$ nM; $n_H = 1.08 \pm 0.06$; pure UTP: $y_{max} = 41.3 \pm 0.55\%$; $K = 20.1 \pm 1.41$ nM; $n_H = 1.02 \pm 0.06$. (B) Ca²⁺ channel current inhibition produced by different nucleotides (1μ M) in cells pre-injected with 1.25μ g μ l⁻¹ P2Y₆ cRNA (open columns). Shaded columns show the effect of UTP, UDP, ITP (10μ M) and ADP (1μ M) on control cells preinjected with GFP cRNA without P2Y₆ cRNA. Current was measured as in Figure 1. Bars show s.e. mean; numbers are number of cells tested. Note that the receptor is exclusively activated by uridine nucleotides.

(10μ M) (which is mediated primarily by G_o; Caulfield *et al.*, 1994; Delmas *et al.*, 1998b) was reduced by $>90\%$ (Figure 4B), as previously reported (Beech *et al.*, 1992; Chen & Schofield, 1993; Caulfield *et al.*, 1994; Delmas *et al.*, 1998b). This indicates that the PTX pretreatment fully ADP-ribosylated G_o, and therefore that the incomplete inhibition of responses to UTP probably reflects the additional involvement of another G protein which is less sensitive or insensitive to PTX. (Unlike previous observations with the P2Y₂ receptor (Filippov *et al.*, 1998), the residual inhibition in PTX-treated cells in Figure 4 appeared to be voltage-sensitive, since the current onset was still slowed.)

We made further tests of PTX-sensitivity using perforated-patch recordings to ensure that 'diffusible intracellular messenger' pathways were well-preserved (Bernheim *et al.*, 1991). One reason for doing this is that Delmas *et al.* (1998a) found the PTX-insensitive component of Ca²⁺ current inhibition produced by activating M₁ muscarinic acetylcholine receptors was more pronounced (relative to the PTX-sensitive component mediated by M₄ receptors) with perforated-patch than with disrupted-patch recording. Indeed, in perforated-patch experiments, P2Y₆ mediated inhibition of I_{Ca} was significantly larger ($72.9 \pm 6.8\%$, $n = 5$) than that in whole-cell disrupted-patch experiments ($52.8 \pm 4.6\%$, $n = 9$). Further (and in contrast to the whole-cell disrupted-patch experiments), PTX pretreatment (500 ng ml⁻¹ overnight) did not significantly reduce this inhibition (Figure 5), whereas the effect of noradrenaline was again almost eliminated. No further inhibition of the effect of UTP was obtained on doubling the concentration of PTX ($61.1 \pm 9.7\%$ inhibition, $n = 4$, at 500 ng ml⁻¹ PTX; $63.3 \pm 7.6\%$ inhibition, $n = 4$, at 1μ g ml⁻¹ PTX). Interestingly, inhibition of I_{Ca} by UTP still appeared to be voltage-sensitive under perforated-patch recording, as judged from the slowed current onset kinetics.

M-type K⁺ channels

The M-type K⁺ current ('M-current') is a sustained, voltage-gated K⁺ current which is activated when SCG neurones are depolarized above -70 mV (see Brown, 1988, for review). It can be most readily isolated by pre-depolarizing the neurones to activate the current, then imposing hyperpolarizing steps to deactivate it (Figure 6).

UTP up to a concentration of 100μ M produced no significant M-current inhibition in cells injected with GFP cRNA alone (see Filippov *et al.*, 1998; *c.f.* Boehm, 1998 - see Discussion below). In contrast, UTP (10μ M) produced substantial inhibition of the M-current in cells pre-injected with P2Y₆ mRNA (Figure 6). This is indicated by (a) the inward shift in holding current at -20 mV (reflecting the reduction in outward K⁺ current), (b) reduced current responses to hyperpolarizing steps (reduced conductance) and loss of M-current deactivation tails, and (c) reduced outward rectification in the current/voltage curve positive to -70 mV, with no change in slope negative to -70 mV (indicating no change in 'leak' current). The mean inhibition produced by UTP (10μ M) (measured from the percentage reduction of outwardly-rectifying current at -30 mV, see Methods) was $70.9 \pm 8.6\%$ ($n = 6$). The effect of UTP on the M-current was not affected by pre-treatment with PTX. Mean inhibition after overnight incubation in 0.5μ g ml⁻¹ PTX was $71.8 \pm 8.2\%$ ($n = 5$) while that in untreated cells was $70.9 \pm 8.6\%$ ($n = 6$).

The concentration dependence of inhibition by UTP was estimated using 1 min applications of incrementing concentrations (Figure 7). The mean IC₅₀ from pooled data was 115 ± 12.6 nM and the mean extrapolated maximal inhibition was $51.7 \pm 1.38\%$ (Figure 7). (The lower maximum inhibition observed with cumulative concentrations probably reflects some degree of desensitization.) UDP was more effective, with a mean IC₅₀ of 30.3 ± 2.8 nM and a mean maximum of

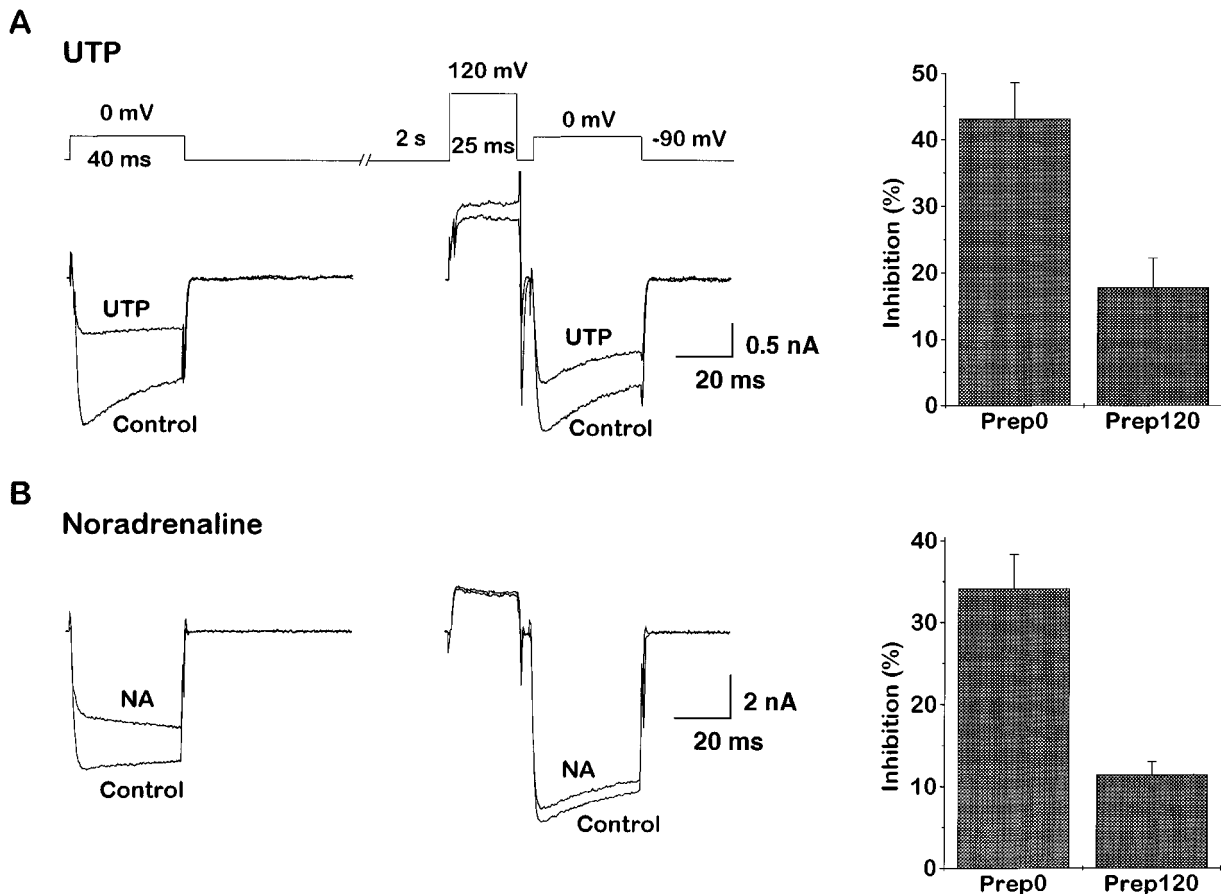


Figure 3 Inhibition of Ca²⁺ channel current produced by P2Y₆ receptor activation is voltage dependent. Records on the left show superimposed Ca²⁺ channel current records obtained with a double-pulse voltage protocol (top trace) in the absence and presence of (A) UTP (100 μ M) or (B) noradrenaline (10 μ M). The current was first recorded with a 40 ms test pulse to 0 mV; then, after a 2 s interval, a 25 ms conditioning prepulse to +120 mV was applied, followed 4 ms later by a second 40 ms test pulse to 0 mV. The bar-charts on the right show the mean percentage current inhibition (measured after 10 ms at 0 mV command potential) produced by UTP (100 μ M) (upper panel) or by noradrenaline (10 μ M) (lower panel) before (Prep0) and after (Prep120) the +120 mV prepulse. Bars show s.e.mean (six cells for each.) Note that inhibition is much less after the prepulse. Note also that the prepulse abolished the slowing of the current onset at 0 mV produced by UTP or noradrenaline.

$63.1 \pm 1.1\%$. In contrast, ATP (10 μ M) produced no significant inhibition ($2.0 \pm 2.0\%$, $n=3$). Thus, the order of potency for inhibition of $I_{K(M)}$ was the same as that for inhibition of I_{Ca} but the IC_{50} values were five to six times greater.

Discussion

Three principal points emerge from these experiments: first, the P2Y₆ receptor shows a similar dual-effector coupling to N-type Ca²⁺ channels and M-type K⁺ channels when expressed in rat sympathetic neurones as that previously observed for P2Y₂ receptors (Filippov *et al.*, 1997; 1998); second, the P2Y₆ receptor appears to be considerably more sensitive to uridine nucleotides than the P2Y₂ receptor when assessed using this expression system; and third, in this system it is about 100 fold more sensitive to UTP and about 60 fold more sensitive to UDP than previously observed from measurements of inositol phosphate production (Nicholas *et al.*, 1996).

The dual coupling appears to arise (in part, at least) from the same mechanism as that deduced for P2Y₂ receptors, namely, the parallel activation of at least two different G proteins. Thus, inhibition of $I_{K(M)}$ was completely resistant to Pertussis toxin (PTX). Like the inhibition of this current by activation of muscarinic M₁ receptors (Caulfield *et al.*, 1994; Haley *et al.*, 1998) or bradykinin B₂ receptors (Jones *et al.*,

1995), it might therefore result from activation of G_q and/or G₁₁. In contrast, in whole-cell (disrupted-patch) recording, inhibition of $I_{Ca(N)}$ was substantially (but not completely) inhibited by PTX, implying the activation of at least one additional G protein (probably G_o, Caulfield *et al.*, 1994; Delmas *et al.*, 1998a,b). Further, these two G proteins appear to be activatable at near-equivalent levels of receptor stimulation since the concentration of nucleotide required to inhibit $I_{Ca(N)}$ in disrupted-patch recording (and therefore activate G_{PTX+ve}) were no greater – indeed, less – than those that inhibited $I_{K(M)}$ (and therefore activated G_{PTX-ve}).

As noted previously (Filippov *et al.*, 1998), this form of ‘promiscuity’ in receptor-effector coupling is unusual in these cells. It is unlikely to be an artefact of receptor over-expression as such, since inhibition of neither current was complete and was generally comparable to that obtained on stimulating endogenous adrenergic or muscarinic receptors. Clearly, one cannot discount the possibility that it could be an artefact of exogenous receptor expression arising (for instance) from an inappropriate topological arrangement of exogenous receptors and endogenous G proteins. However, this does not seem very likely because the equivalent dual coupling of exogenous P2Y₂ receptors when expressed in ganglion cells parallels the effects of the endogenous receptors in the NG108-15 neural cell line (Filippov *et al.*, 1994; Filippov & Brown, 1996) and was also not changed after injecting considerably different amounts of

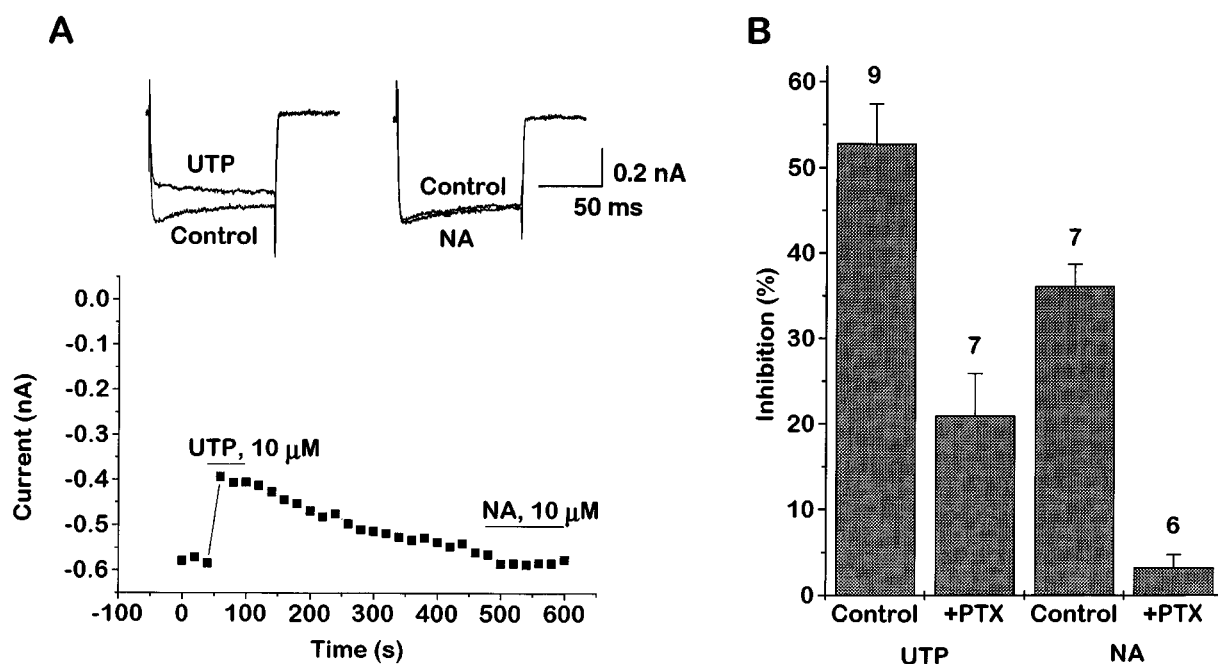


Figure 4 Effect of *Pertussis* toxin (PTX) on P2Y₆-mediated Ca²⁺ channel current inhibition recorded in whole-cell configuration. (A) Records show superimposed leak-subtracted currents in the absence and presence of UTP (10 μ M) (left panel) or noradrenaline (10 μ M) (right panel) in neurones pretreated with PTX (0.5 μ g ml⁻¹, overnight). The lower panel shows a time-plot of I_{Ca} amplitude; UTP (10 μ M) and noradrenaline (10 μ M) were added for the duration of the longitudinal bars. (B) Mean inhibition of I_{Ca} amplitude by UTP (10 μ M) or noradrenaline (NA) (10 μ M) in neurones pretreated with PTX (+PTX) and in untreated neurones. Note that PTX pretreatment did not prevent completely the effect of UTP but virtually eliminated the effect of noradrenaline.

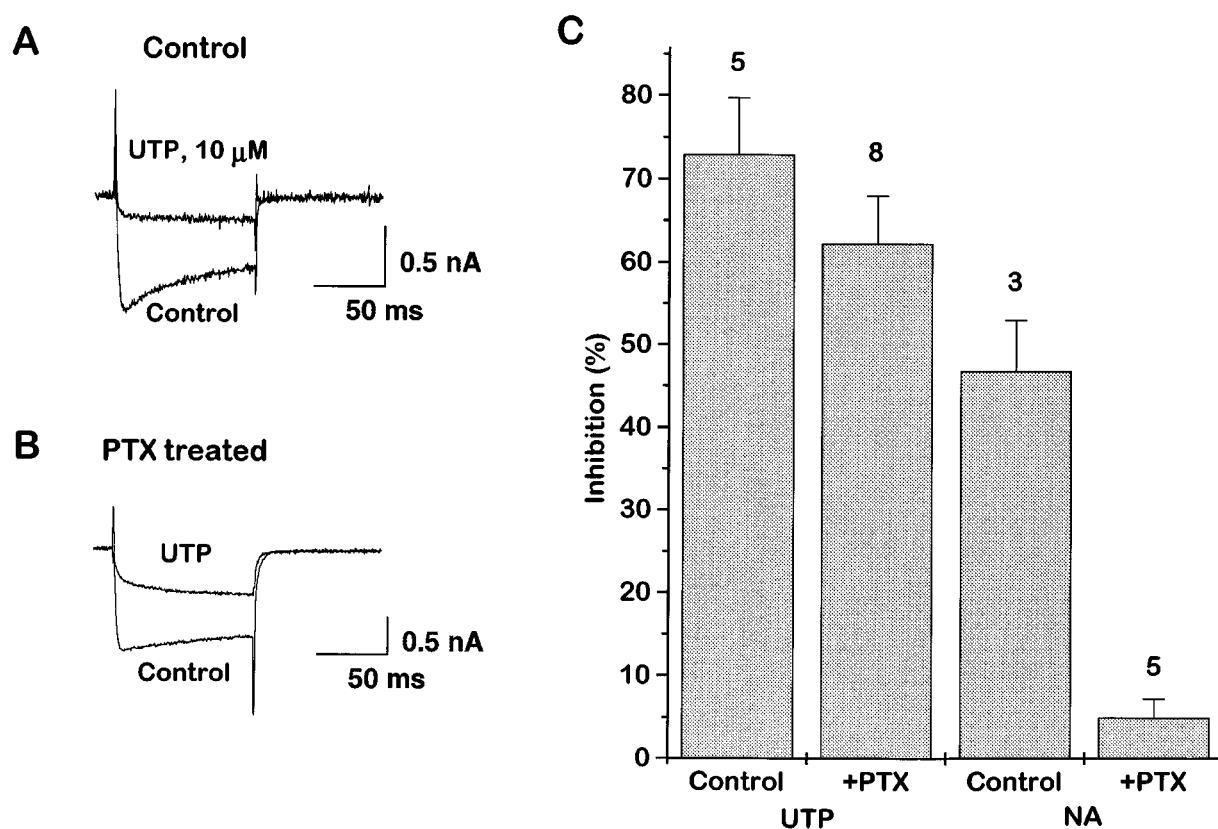


Figure 5 Effect of *Pertussis* toxin (PTX) on P2Y₆-mediated Ca²⁺ channel current inhibition recorded in perforated patch configuration. Records show superimposed leak-subtracted currents in the absence and presence of UTP (10 μ M) in control neurones (A) and in neurones pretreated with PTX, 0.5 μ g ml⁻¹, overnight (B). (C) Mean inhibition of I_{Ca} amplitude by UTP (10 μ M) or noradrenaline (NA) (10 μ M) in neurones pretreated with PTX (+PTX) and in untreated neurones. Note that PTX pretreatment did not significantly reduce the effect of UTP but virtually eliminated the effect of noradrenaline.

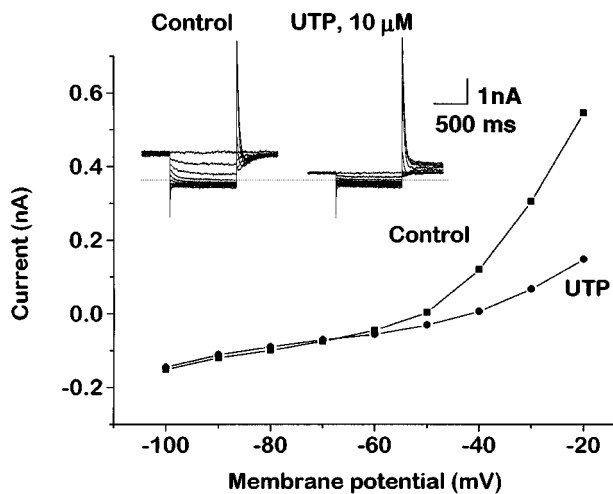


Figure 6 Activation of heterologously-expressed P2Y₆ receptor inhibits the M-type K⁺ current ('M-current', I_{K(M)}) in a rat SCG neurone. The neurone was injected 18 h beforehand with 1.25 μg μl⁻¹ P2Y₆ cRNA. M-current was recorded using a perforated-patch electrode, by pre-depolarizing the neuron to -20 mV, then deactivating the current with a series of 1-s hyperpolarizing steps in increments of 10 mV at 5-s intervals, as shown in the current records. The dotted line indicates the zero current. The graph shows the current amplitude at the end of each 1-s step measured as change from zero current. Currents were recorded before (squares, control) and after (circles) adding UTP (10 μM). Note that UTP produced an inward current at the holding potential of -20 mV, reduced the amplitude of the M-current deactivation tail-currents during the hyperpolarizing steps, and reduced the outward rectification of the current-voltage curve positive to -70 mV.

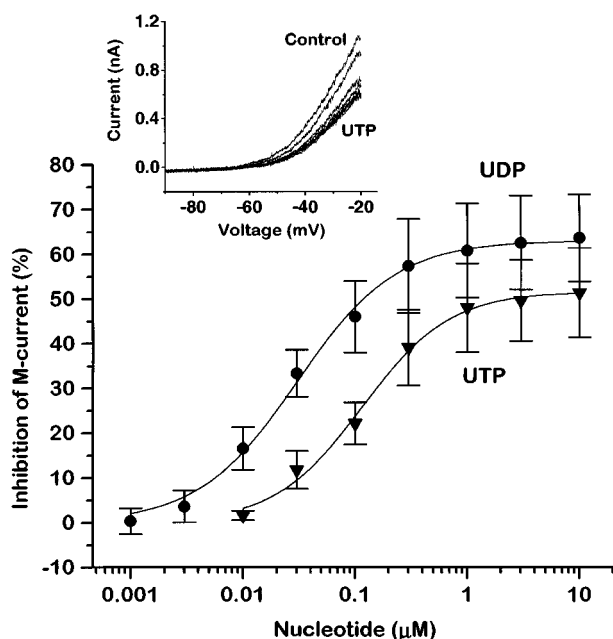


Figure 7 Concentration-dependence of M-current inhibition by UTP (triangles) and UDP (circles) in neurones pre-injected with P2Y₆ cRNA. M-current was recorded using a ramp-voltage protocol and measured at -30 mV (see Methods). Representative original current records before (control) and after adding UTP at 0.03, 0.3, 1, 3, 10 and 30 μM are shown in the inset. Points show means ± s.e. mean of measurements in four to five cells; concentrations were added cumulatively, with 1 min exposure times. Curves were fitted to pooled data points using Origin 4.1 software to the Hill equation (see legend to the Figure 2A). Values of Hill constants (mean ± s.e. mean) were as follows: UDP: $y_{max} = 63.1 \pm 1.13\%$; $K = 30.3 \pm 2.76$ nM; $n_H = 0.99 \pm 0.07$; UTP: $y_{max} = 51.7 \pm 1.38\%$; $K = 115.4 \pm 12.65$ nM; $n_H = 1.10 \pm 0.11$.

receptor cRNA (Filippov *et al.*, 1998). One small point of difference from the results obtained with the P2Y₂ receptors is that, while in both cases the concentration of UTP required to inhibit I_{K(M)} was somewhat higher than that required to inhibit I_{Ca(N)}, the difference was greater (5 fold, as opposed to 3 fold) in the case of the P2Y₆ receptor. Nevertheless, the dose-response curves overlap to a sufficient extent that, at concentrations ≥ 30 nM, significant inhibition of both currents would result.

In contrast to the results obtained with whole-cell recording, the P2Y₆-mediated inhibition of I_{Ca} in perforated patch recording was not only larger than that observed in whole-cell recording but was also very substantially (if not entirely) resistant to PTX – i.e., the PTX-resistant component of I_{Ca} inhibition observed in disrupted-patch recording was enhanced and became the predominant component in perforated-patch recording. To an extent this parallels previous observations on muscarinic receptor-mediated inhibition, where the PTX-resistant component mediated by M₁ receptors assumed a greater significance (compared with PTX-sensitive M₄ inhibition) in perforated-patch recording (Delmas *et al.*, 1998a). An obvious explanation for the latter is that, under whole-cell recording, 'diffusible messenger(s)' necessary for M₁-mediated inhibition (Bernheim *et al.*, 1991) become leached out of the cytoplasm, leaving only the more tightly-coupled M₄ inhibition. A similar explanation may, of course, apply to the effects of whole-cell recording on the response to stimulating P2Y₆ receptors, but there is one difference – namely, that the 'remote' inhibition produced by stimulating M₁ receptors is voltage-insensitive (Beech *et al.*, 1992), whereas the PTX-resistant component of P2Y₆-mediated inhibition appears to retain voltage-dependence (at least, as judged from the slowed current kinetics) – suggesting, perhaps, that no 'second messenger' other than a subunit of the G protein itself might be necessary. An alternative possibility is that the component of inhibition potentially mediated by the PTX-sensitive G protein might normally be suppressed by an ancillary molecule such as an RGS protein, which is leached out on patch-disruption. Whatever the reason, the results imply that, under normal conditions (i.e., with an undisturbed intracellular milieu), the principal effects of stimulating P2Y₆ receptors on neural ion channels (within the cell soma at least) appear to be mediated by PTX-insensitive G proteins. We have not yet tested whether this also applies to the previously-described effects of stimulating P2Y₂ receptors, but it seems quite likely since a similar proportion of the I_{Ca} inhibition in whole-cell recording produced by stimulating P2Y₂ receptors was resistant to PTX (Filippov *et al.*, 1998).

As previously reported with transfected cell lines (Chang *et al.*, 1995; Communi *et al.*, 1996; Nicholas *et al.*, 1996), the P2Y₆ receptor behaves as a uridine nucleotide-specific receptor, in that ATP and ADP were virtually ineffective at concentrations of 1 or 10 μM (implying that the IC₅₀ for these adenosine nucleotides must be at least two or three orders of magnitude greater than those for the uridine analogues). However, one interesting difference from previous studies on expressed P2Y₆ receptors is that UTP was only three to four times less effective than UDP in inhibiting I_{Ca(N)} or I_{K(M)}, whereas Nicholas *et al.* (1996) reported UTP to be more than 100 fold less effective than UDP in driving inositol phosphate production in 1321N1 cells. This stronger effect of UTP on ganglion cells can not be attributed to contamination by, or metabolism to, UDP (*c.f.* Harden *et al.*, 1997) since (a) comparable effects were obtained with purified UTP, free of detectable UDP, and (b) unlike with the transfected cell lines, our recordings were made from dissociated cells in a rapidly-

flowing medium, thus minimizing enzymatic breakdown. This difference in the relative potency of the two agonists requires further investigation. Also, the sensitivity of the P2Y₆ receptor to UDP is substantially higher here than when expressed in 132N1 cells and measured by inositol phosphate production (Nicholas *et al.*, 1996) [but see Note added at the end of this paper].

It has previously been reported that a uridine nucleotide-sensitive receptor is present in native rat SCGs (Connolly & Harrison, 1995); and Boehm (1998) has recently reported that, in SCG neurones isolated from neonatal (2–6-day-old) rats and then cultured for 7 days *in vitro*, UTP can inhibit the M-current through an endogenous P2Y receptor. However, no such effect was noted in the experiments described here, where older rats were used as a source of neurones and the cells were cultured for a much shorter period (14–24 h) in a lower concentration of added serum. Perhaps receptor expression is favoured by more prolonged culture in a serum-rich medium, which promotes differentiation of these cells by growth factors. Notwithstanding, if expressed at all, the endogenous receptor was present at too low a level to yield a response in cells injected solely with GFP cRNA.

It is not yet clear to what extent the P2Y₆ receptor is endogenously expressed in other neurones. If it were, we can make two predictions from the present results. First, we might view the effects that we have recorded in the cell soma under perforated-patch conditions as essentially representative of the sort of effect produced were a nucleotide to activate postsynaptically-located P2Y₆ receptors: here, the synergistic inhibition of I_{Ca(N)} and I_{K(M)} would result in an increased neuronal excitability (see Filippov *et al.*, 1998; Haley *et al.*, 1998). On the other hand, were the receptors located on presynaptic terminals, their effect might be inhibitory, since inhibition of I_{Ca(N)} might result in a reduction of evoked transmitter release (e.g. Hirning *et al.*, 1988; Boehm & Huck, 1996; Koh & Hille, 1997). Both effects have been reported in different neural preparations following application of exogenous uridine nucleotides. Thus, UTP inhibits the M-current and excites some sympathetic neurones (e.g., Siggins *et al.*, 1977; Adams *et al.*, 1982; Cuevas *et al.*, 1997); while Von Kugelgen *et*

al. (1994) have described inhibitory effects of UTP on evoked transmitter release from brain tissue (though the receptors responsible for these effects have not been fully characterized). On the other hand, UTP and UDP (but not ATP) have been reported to trigger transmitter release from sympathetic terminals through a P2Y receptor (Boehm *et al.*, 1995; Von Kugelgen *et al.*, 1997): this could be related to the increase in intracellular Ca²⁺ following phospholipase C stimulation. The balance between inhibitory and facilitatory effects on transmitter release might then be different in peripheral and central nerve terminals because of the complement of receptors/effectors involved.

Although there is no evidence for a true transmitter role for uridine nucleotides, UTP can be released from cells by other means, such as mechanical stimulation (Saiag *et al.*, 1995; Lazarowski *et al.*, 1997), and then easily hydrolyzed to UDP (Anderson & Parkinson, 1997). The very high sensitivity of the P2Y₆ receptors coupling to neuronal ion channels in response to UDP (e.g., IC₅₀ for I_{Ca(N)} inhibition of ~6 nM) suggests that neurones expressing this receptor would be extremely sensitive to any locally released uridine nucleotides and consequently these would have profound effects on neuronal activity.

Note added in proof

After the submission of this paper, a publication appeared (Li *et al.*, 1998) in which the results by Nicholas *et al.* (1996), cited in the Discussion above, were up-dated. The potency of UDP on the expressed rat P2Y₆ receptor is now found to be greatly increased over that reported previously. It was suggested that this change 'may be the result of higher receptor expression levels' in the clonal cell line now used (Li *et al.*, 1998). The new EC₅₀ value for UDP (6 nM) reported by Li *et al.* (1998) now agrees completely with that found by us for UDP inhibition of the Ca²⁺ current.

We thank Brenda Browning and Misbah Malik for tissue culture, Dr Kyungho Chang for the rat P2Y₆ cDNA and Professor Jaak Jaav for providing the HPLC purified UTP. This work was supported by The Wellcome Trust and the Commission of the European Union (CT grant).

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(Received July 14, 1998
Revised November 2, 1998
Accepted November 11, 1998)



Aryl propanolamines: comparison of activity at human β_3 receptors, rat β_3 receptors and rat atrial receptors mediating tachycardia

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1 The *in vitro* activity of four aryl propanolamines was compared to two prototypic β_3 receptor agonists, CGP 12177 and CL316243 at the human β_3 receptor, the rat β_3 receptor in the stomach fundus and receptors mediating atrial tachycardia.

2 L-739,574 was the most potent ($EC_{50}=9$ nM) and selective agonist at the human β_3 receptor with high maximal response (74% of the maximal response to isoproterenol).

3 A phenol-biaryl ether analogue possessed modest affinity for the human β_3 receptor ($EC_{50}=246$ nM), but was highly efficacious with a maximal response 82% of the maximal response to isoproterenol. The other derivatives were intermediate in potency with low maximal responses.

4 These agonists at the human β_3 receptor did not activate the rat β_3 receptor in the rat stomach fundus. In fact, the aryl propanolamines (10^{-6} M) inhibited CL316243-induced activation of the rat β_3 receptor. Thus, agonist activity at the human β_3 receptor translated into antagonist activity at the rat β_3 receptor.

5 L739,574 and the phenol biaryl ether increased heart rate *via* β_1 receptors.

6 Although CGP12177 produced atrial tachycardia, neither the indole sulphonamide nor biphenyl biaryl ether did, although both had high affinity for the human β_3 receptor. Thus, the atrial tachycardic receptor was not identical to the human β_3 receptor.

7 These studies (a) characterized four aryl propanolamines with high affinity at the human β_3 receptor, (b) found that they were antagonists at the rat β_3 receptor, an observation with profound implications for *in vivo* rat data, and (c) established that the rodent atrial non- β_1 , β_2 or β_3 tachycardic receptor was also unrelated to the human β_3 receptor.

Keywords: Rat atria tachycardia; rat stomach fundus; propanolamines; human β_3 receptor; 'atypical' β receptor

Abbreviations: Beta, β ; beats per minute, BPM; cyclic adenosine monophosphate, cyclic AMP; Chinese hamster ovary, CHO; [S-4-(3-((1-*t*-butyl)amino)-2-hydroxypropoxy)-1,3-dihydro-2H-benzimidazol-2-one], CGP12177; Disodium [(*R,R*)-5-(2-((2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl)-1,3-benzodioxole-2,2-dicarboxylate], CL1316243; S-N-(4-(2-((2-hydroxy-3-(4-hydroxyphenyloxy)-propyl)amino)ethyl)phenyl) benzenesulfonamide, L-739,574

Introduction

Although activation of β_1 receptors is known to increase heart rate, evidence has been accumulating to suggest that some β_3 receptor agonists including the 'nonconventional partial agonist' CGP12177 may produce a chronotropic effect in rat atria that is mediated by a non β_1 , β_2 or β_3 rodent receptor (Kaumann, 1996; Malinkowski & Schicker, 1996; Kaumann & Molenaar, 1996). In those studies, CGP12177, which has high affinity for the human β_3 receptor increased rat atrial rate whereas CL316243, a ligand with high affinity for the rat, but not human β_3 receptor, did not increase heart rate. The fact that marked differences have been reported between the pharmacology of the rat and human β_3 receptor prompted the present study to identify pharmacological tools to use to examine whether the tachycardic receptor was identical to the human β_3 receptor.

For this reason, four aryl propanolamines (Figure 1) with high affinity for the human β_3 receptor (Dow, 1997) have been synthesized to determine whether all agonists with high affinity for the human β_3 receptor will increase heart rate. Before dissecting the effect of these aryl propanolamines on atrial rate, we characterized their activity at the human β_3 receptor using

the stably expressed human β_3 receptor and at β_1 , β_2 and rat β_3 receptors. Lastly, we determined the effect of these β_3 receptor agonists on atrial rate in an effort to understand whether activation of the human β_3 receptor was linked to atrial tachycardia.

Methods

Adenylyl cyclase activation at cloned human β_3 receptor The two exon human β_3 adrenergic receptor (Granneman *et al.*, 1993) was subcloned into the BCI restriction site using a phd expression vector (Grinnell *et al.*, 1987) before transfection into the DXB-11, Chinese hamster ovary (CHO) cell line by calcium phosphate precipitation methodology. The stably transfected cells were grown to 95% confluency in 95% Dulbecco's modified Eagles Medium (DMEM), 5% foetal bovine serum, 0.01% proline. Media was removed and the cells were washed with phosphate buffered (pH 7.4) saline (without magnesium and calcium). Cells were then lifted using an enzyme free cell dissociation solution (Specialty Media, Lavallete, NJ, U.S.A.) and pelleted by centrifugation. The cells were resuspended and added (15,000 per-well) to a 96-well plate. Cells were incubated at 37°C with receptor selective compounds for 20 min in buffer (Hank's balanced salt solution, 10 mM HEPES, 0.1%

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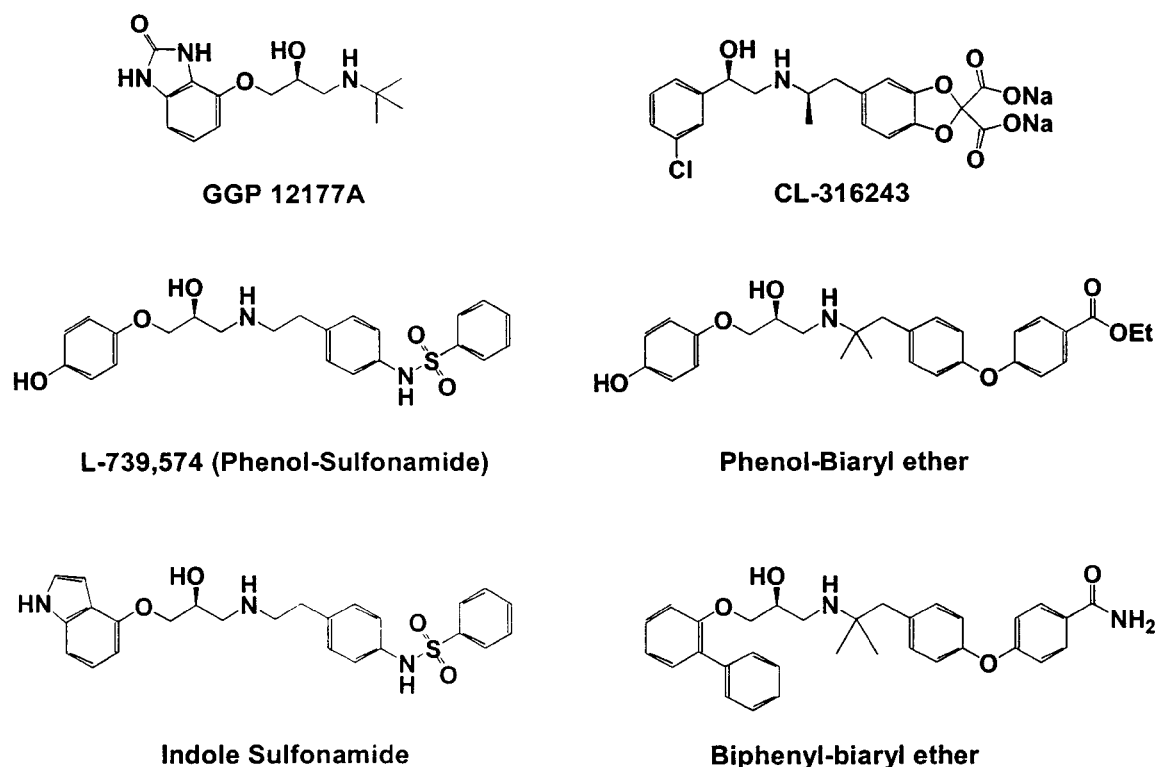


Figure 1 Chemical structures of aryl propanolamines.

BSA, 1 mM L-ascorbic acid, 0.2% dimethyl sulphoxide, 1 mM 3-isobutyl-1-methylxanthine, pH 7.4). After halting the incubation with quench buffer (50 mM Na Acetate, 0.25% Triton X-100, pH 5.8), the cyclic AMP level was quantified by scintillation proximity assay (SPA) using a modification of the commercially available cyclic AMP kit (Amersham, Arlington Heights, IL, U.S.A.) with rabbit anti-cyclic AMP antibody (ICN Biomedicals, Aurora, OH, U.S.A.).

Sigmoidal dose response curves, from the whole cell receptor coupled cyclic AMP assay were fit to a four parameter logistic equation using non linear regression: $y = (a - d) / (1 + (\text{Dose}/c)^b) + d$ where a and d are responses at zero and maximal dose, b is the slope factor and c is the EC_{50} as previously described (DeLean *et al.*, 1978). EC_{50} was assessed as the concentration producing 50% of the maximum response to each agonist. The % maximal response was assessed relative to each agonist by the maximal response to isoproterenol by dividing the maximal response to isoproterenol $\times 100$.

Beta1 and beta2 receptor binding

[^{125} I]-iodocyanopindolol binding to either human β_1 - or β_2 -adrenergic receptors was performed using transfected Sf9 cells (NEN Life Science Products, Inc., Boston MA, U.S.A.) according to the methods of Bylund & Snyder (1976). Nonspecific binding was determined in the presence of 1 μ M propranolol. Compounds were dissolved in dimethyl sulphoxide (DMSO), with final DMSO concentrations of less than 1%. Affinity constants (K_i) were determined using nonlinear least-squares regression software, Graphpad Inplot Inc., San Diego, CA, U.S.A. (Munso & Rodbard, 1980) and were calculated from IC_{50} values according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973). Affinities (K_i) were determined from eleven point concentration curves and were the means $\pm$ s.e. mean of at least three experiments.

In vitro rat stomach fundus relaxation

Male Sprague-Dawley rats (250–300 g; Harlan Sprague Dawley, Indianapolis, U.S.A.) were killed by cervical dislocation. The stomach fundus was removed and dissected free of connective tissue and two longitudinal preparations were obtained from each animal. The tissues were tied with thread at each end, the lower end being tied to a stationary metal hook and the upper end to a force transducer.

Tissues were mounted in organ baths containing 10 ml of modified Krebs' solution of the following composition (mM): NaCl 118.2, KCl 4.6, $CaCl_2 \cdot 2H_2O$ 1.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 10.0 and $NaHCO_3$ 24.8. Tissue bath solutions were maintained at 37°C and aerated with 95% O_2 -5% CO_2 . Propranolol (3×10^{-7} M) was added to the tissue baths to block β_1 and β_2 receptors without marked inhibition of β_3 receptors (Cohen *et al.*, 1995a). Tissues were placed under optimal resting force of 4 g and were allowed to equilibrate for 1 h before exposure to drugs. Isometric contractions were recorded as changes in grams of force on a Beckman Dynograph.

For relaxation studies, tissues were contracted with carbamylcholine (10^{-6} M) at an EC_{90} concentration based on previous studies (Secrest *et al.*, 1991). Once the carbamylcholine-induced contraction reached steady state, β receptor agonists were added cumulatively. Relaxation was calculated as a percentage of the initial carbamylcholine-induced force. EC_{50} values are the concentrations of β receptor agonist necessary to produce half-maximal relaxation of carbamylcholine-contracted tissues.

For studies with antagonists, tissues were preincubated with vehicle or antagonist for at least 45 min. Cumulative concentration responses to β receptor agonists were determined in the presence of antagonist or vehicle. Antagonist equilibrium dissociation constants (K_B) were determined for each concentration of antagonist according to the following equation:

$$K_B = \frac{[B]}{[DR - 1]}$$

where [B] is the concentration of the antagonist and dose ratio (DR) is the EC_{50} value of the agonist in the presence of the antagonist divided by the control EC_{50} value. These results are then expressed as the negative logarithm of the K_B (i.e., $-\log K_B$).

Rat atrial tachycardia

Male rats (150–300 g.) (Harlan Sprague Dawley, Indianapolis, Indiana, U.S.A.) were killed by cervical dislocation. Hearts were removed and the left and right atrium were dissected and mounted with thread in tissue baths containing 10 mls of modified Krebs' solution (see above). An initial optimum resting force of 1 g was applied to the atria (Cohen et al., 1982). Tissues were allowed to equilibrate approximately 30 min with vigorous oxygenation before exposure to drugs.

Agonists were added cumulatively once the spontaneous atrial rate reached a steady state from the previous addition. Agonist addition was continued until no further increase in atrial rate occurred or until a concentration of 10^{-4} M was reached. The increase in beats min^{-1} (b.p.m.) was measured for each concentration of agonist by means of a Beckmann Cardiometer.

Antagonists were evaluated by preincubation with tissue for at least 45 min. Cumulative concentration responses to the β receptor agonists were determined in the presence of antagonist or vehicle. Antagonist equilibrium dissociation constants were determined as indicated above.

Chemicals

All compounds used were reagent grade and prepared in saline, DMSO or propyleneglycol. CGP 12177A [S-4-(3-((1-*t*-butyl)amino)-2-hydroxy-propoxy)-1,3-dihydro-2H-benzimidazol-2-one], CL-316243 disodium [(*R,R*)-5-(2-((3-chlorophenyl)-2-hydroxyethyl)amino)propyl)-1,3-benzodioxole-2,2-dicarboxylate], indole sulphonamide [S-N-(4-(2-((2-hydroxy-3-(4'-indolyloxy)-propyl)amino)ethyl) phenyl)benzenesulphonamide], phenol-biaryl-ether [Ethyl-S-4-(4-(2-methyl-2-((2-hydroxy-3-(4-hydroxyphenyloxy)-propyl)amino)propyl) phenyloxy)-benzoate] and biphenyl-biaryl ether [S-4-(4-(2-methyl-2-((2-hydroxy-3-((2-phenyl)phenyloxy)-propyl)amino)propyl)phenyloxy) benzamide] were synthesized to 99% purity as determined by elemental analysis, HPLC and/or [^{13}C] spectroscopy at the Lilly Research Laboratories.

Results

Cloned human β_3 receptor agonist activity

Consistent with previous information, CL316243, a β_3 receptor agonist optimized for affinity at the rat β_3 receptor produced negligible activation of cyclic AMP formation in CHO cells stably expressing the human β_3 receptor (Table 1). In contrast, isoproterenol was a potent agonist (EC_{50} = 16 nM) with the greatest maximal activation of adenylyl cyclase of the agents studied. CGP12177 was a weak agonist at the human β_3 receptor (42% maximal response relative to isoproterenol).

All four aryl propanolamines were agonists at the human β_3 receptor as measured by their ability to increase cyclic AMP. Each of these β_3 receptor agonists was more potent than CGP12177. However, the four aryl propanolamines displayed marked diversity in agonist potency and efficacy at the human β_3 receptor (Table 1). Interestingly, both phenol bearing compounds (L-739,574 and the phenol-biaryl ether) possessed marked efficacy with maximal responses approximately 80% of the maximal response to isoproterenol at the human β_3 receptor, with L-739,574 considerably more potent than even isoproterenol. Compounds with an indole or biphenyl function were of intermediate potency. Nevertheless, although marked potency differences existed, these data document that all four

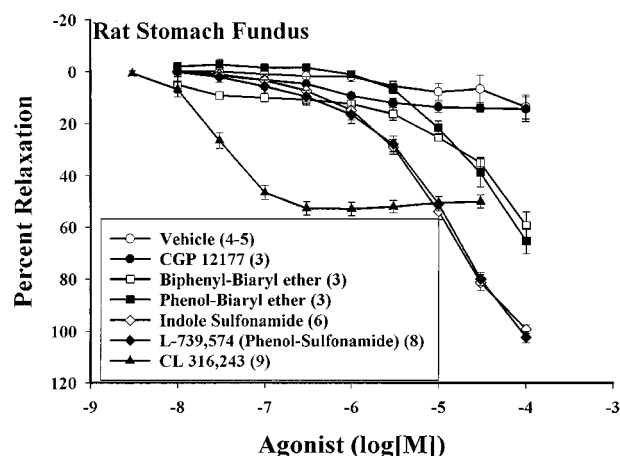


Figure 2 Effect of aryl propanolamines to relax carbamylcholine (10^{-6} M)-contracted rat stomach fundus in the presence of propranolol (3×10^{-7} M) relative to vehicle. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

Table 1 Human β receptor affinities activity of aryl propanolamines in CHO cells

Compound	β_1 K_i^a (nM $\pm$ s.e.)	β_2 K_i^a (nM $\pm$ s.e.)	EC_{50}^b (nM $\pm$ s.e.)	β_3 Maximal response ^c (percent $\pm$ s.e.)
Isoproterenol	—	—	16 $\pm$ 1	100
CGP12177	—	—	1375 $\pm$ 374	42 $\pm$ 5
CL316243	—	—	> 3160	14 $\pm$ 3
L-739,574	190 $\pm$ 60	360 $\pm$ 240	9 $\pm$ 0.5	74 $\pm$ 2
Indole sulfonamide	0.9 $\pm$ 0.2	0.6 $\pm$ 0.1	59 $\pm$ 11	55 $\pm$ 3
Biphenyl-biaryl ether	6.3 $\pm$ 2.9	2.9 $\pm$ 1.4	32 $\pm$ 4	16 $\pm$ 1
Phenol-biaryl ether	140 $\pm$ 10	17 $\pm$ 4	246 $\pm$ 46	82 $\pm$ 8

^aAffinities determined with radioligand binding as detailed in our Methods. ^b EC_{50} was the concentration producing 50% maximum response to each agonist in increasing cyclic AMP as detailed in Methods. ^cMaximal response was determined as the maximum increase in cyclic AMP relative to the maximal response to isoproterenol.

aryl propanolamines were relatively potent agonists at the human β_3 receptor.

Cloned human β_1 and β_2 receptor affinities

All four aryl propanolamines also interacted with β_1 and β_2 receptors, again with marked diversity in affinities. With the exception of L-739,574, affinities of these agents occurred in concentrations similar to or lower than concentrations that activated the β_3 receptor (Table 1). However, L-739,574 possessed only low affinity at β_1 and β_2 receptors and therefore showed high selectivity for β_3 receptors. Because of these potential β_1 and β_2 receptor interactions, subsequent studies in isolated stomach fundus and rat atria were conducted in the presence of β_1 and β_2 receptor blockade with propranolol (3×10^{-7} M), a concentration established to block β_1 and β_2 receptors (Cohen *et al.*, 1995a).

Rat stomach fundus β_3 receptor agonist activity

To examine the effect of these β_3 receptor agonists at the rat β_3 receptor, we utilized the rat stomach fundus, a preparation known to possess the rat β_3 receptor which mediates relaxation in this tissue (Cohen *et al.*, 1995a). CL316243 was a potent relaxant agonist in the rat stomach fundus ($EC_{50} = 3 \times 10^{-8}$ M) in contrast to CGP12177 (10^{-8} – 10^{-4} M) which did not relax the rat stomach fundus (less than 10% of the maximal possible relaxation which was similar to the relaxation produced by vehicle) (Figure 2).

Activity of the aryl propanolamines at the rat β_3 receptor in the stomach fundus did not parallel their activity at the human β_3 receptor. Like CGP12177, the biaryl ether derivatives did not markedly relax the rat stomach fundus in concentrations less than 10^{-5} M (Figure 2). In contrast, the sulfonamide derivatives relaxed the rat stomach fundus (in concentrations $>10^{-6}$ M) although the potency of these compounds was approximately 100 fold lower than CL316243. Cyanopindolol a known antagonist at the 'atypical' β receptor (McLaughlin & MacDonald, 1990; Blue *et al.*, 1989) was examined for its ability to inhibit the relaxation produced by these sulfonamide derivatives in the rat stomach fundus. Although cyanopindolol potently inhibited the relaxation produced by CL316243 in the rat stomach fundus [$-\log K_B = 7.62 \pm 0.20(3)$], cyanopindolol (10^{-6} M) had no effect on the relaxation produced by either the phenol or indole sulfonamide derivatives (Figure 3). Thus, the sulfonamide derivatives relaxed the rat stomach fundus by a mechanism independent of β_3 receptors.

Since these aryl propanolamine derivatives did not possess agonist activity at the rat β_3 receptor, we asked whether these agents had affinity as antagonists of the rat β_3 receptor. For these experiments, one sulphonamide and one biaryl ether derivative were examined, and both agents (10^{-6} M) dextrally shifted the relaxant concentration response curve produced by CL316243 (Figure 4). Thus, although not agonists at the rat β_3 receptor, these compounds possessed antagonist affinity for the rat β_3 receptor with the negative logarithm of the antagonist dissociation constant of $6.55 \pm 0.22(3)$ and $7.05 \pm 0.05(3)$ for the biphenyl biaryl ether and indole sulphonamide, respectively.

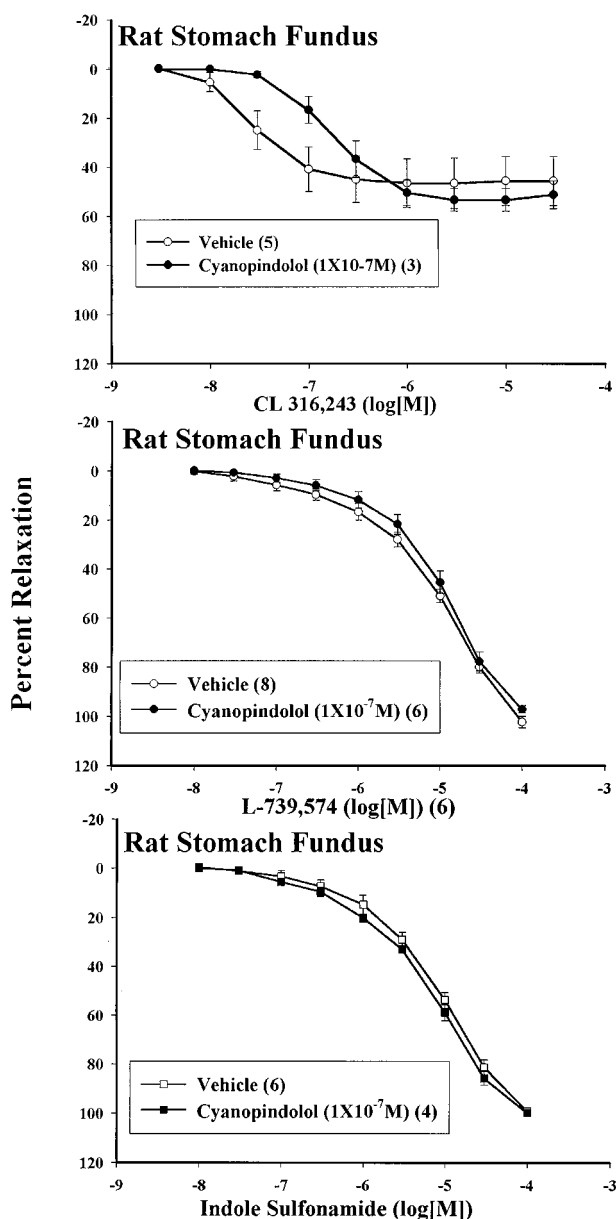


Figure 3 Effect of cyanopindolol (10^{-7} M) to inhibit relaxation of carbamylcholine (10^{-6} M) contracted rat stomach fundus strips by CL316243 (Top) L-739,574 (middle) and indole sulphonamide (bottom). Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

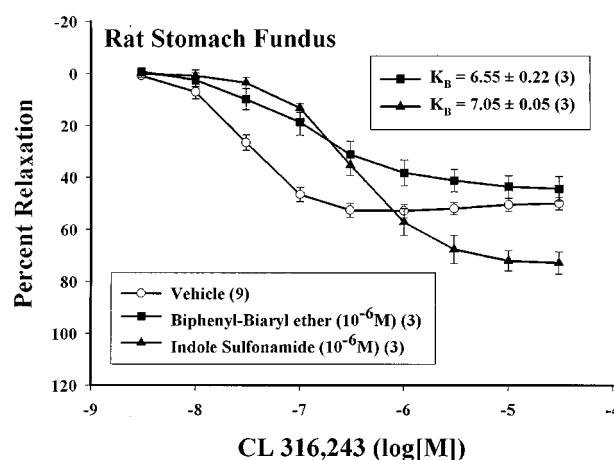


Figure 4 Effect of biphenyl-biaryl ether and indole sulfonamide (10^{-6} M) to inhibit the relaxation of carbamylcholine-induced contraction in rat stomach fundus by CL316243. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

Rat atrial tachycardic β receptors

Having characterized these aryl propanolamine derivatives as agonists at the human β_3 receptor and antagonists at the rat β_3 receptor, we determined the effect of these agents at β receptors mediating atrial tachycardia. Interestingly, of the β_3 receptor

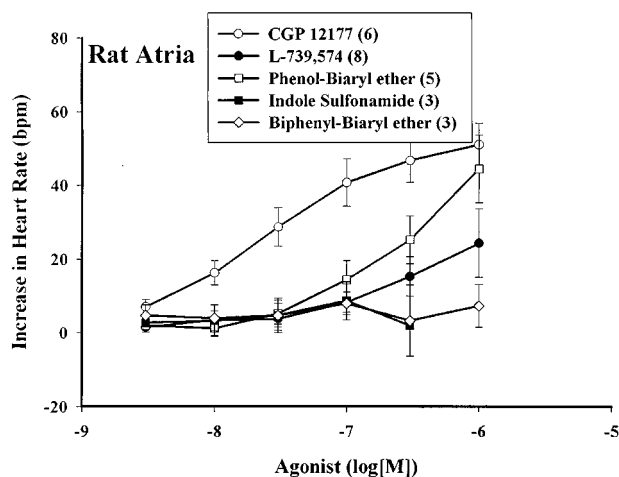


Figure 5 Effect of aryl propanolamines to increase rat atrial rate. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

agonists studied, both phenol substituted derivatives modestly increased rate in rat atria although the potencies (EC_{50} values) were less than those observed with CGP12177 (Figure 5). Neither the indole nor the biphenyl-biaryl ether derivatives increased heart rate although both agents were agonists at the human β_3 receptor. CL316243 also did not increase rat atrial rate, although it was a potent agonist at the rat β_3 receptor.

Propranolol (3×10^{-7} M), in a concentration that inhibited β_1 and β_2 but not β_3 receptors (Cohen et al., 1995a) antagonized the tachycardia induced by isoproterenol and completely abolished the tachycardia produced by the phenol substituted propanolamine derivatives (Figure 6). In contrast, the tachycardia produced by CGP12177 was unaffected by propranolol (3×10^{-7} M). These studies suggest that the tachycardia produced by the aryl propanolamine derivatives, but not by CGP12177 resulted from β_1 receptor activation.

Discussion

Aryl propanolamines are known to have high affinity at β adrenergic receptors, an effect that has been most extensively studied with regard to β_1 and β_2 receptors. However, more recently with the cloning of the rat and human β_3 receptors, efforts have been directed toward identifying high affinity agonists at the human β_3 receptor. The localization of this receptor to brown adipose tissue and the gastrointestinal

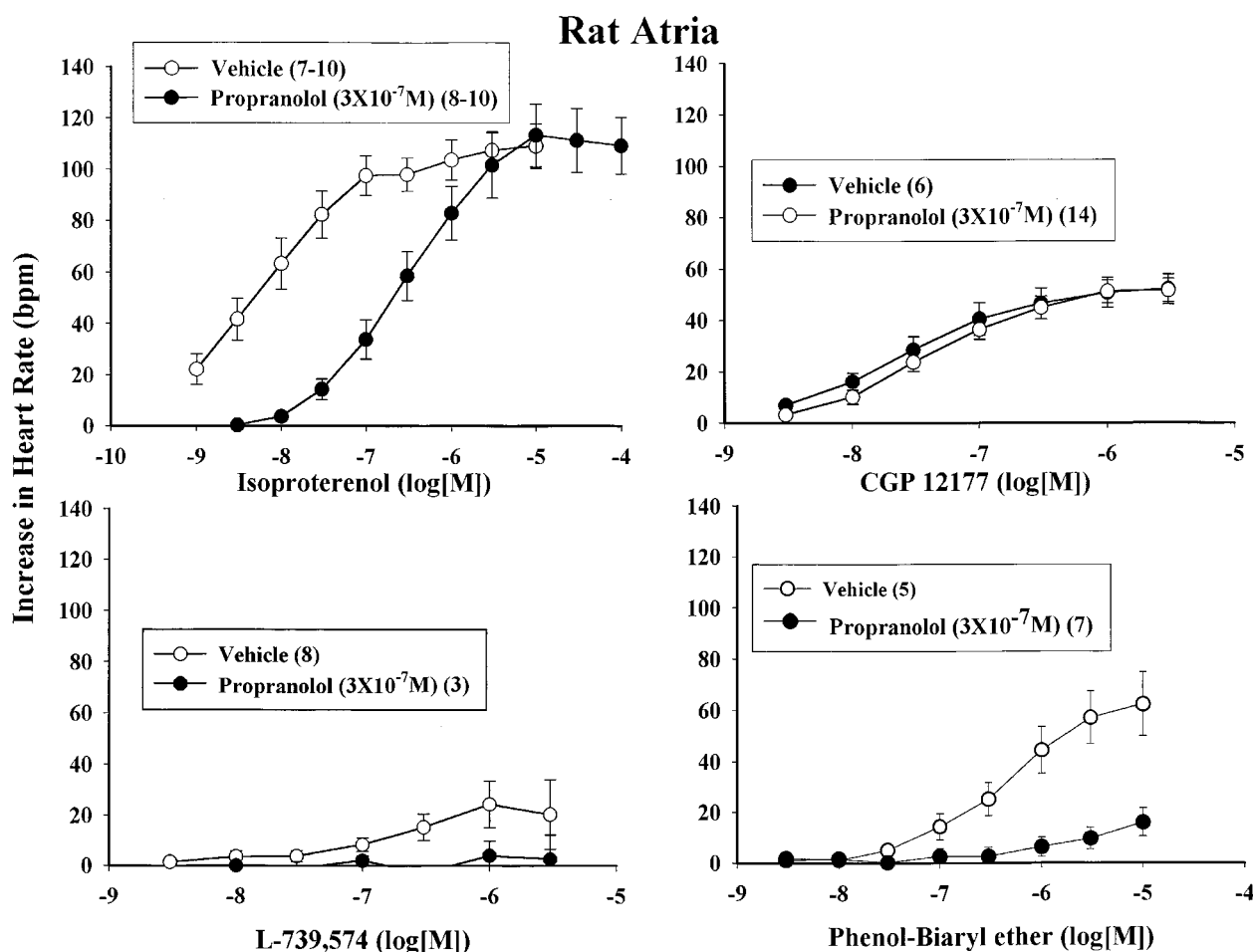


Figure 6 Effect of propranolol to inhibit the increase in rat atrial rate produced by isoproterenol, CGP12177, L-739,574, and phenol-biaryl ether. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

tract has raised the possibility that β_3 receptor agonists might be effective as anti-obesity and anti-spasmodic agents. However, one concern with regard to such agents is beta receptor activation in the heart leading to tachycardia, an undesirable property in the clinic. Recent studies have suggested that tachycardia may not only result from β_1 receptor activation, but also *via* an atypical beta receptor found in rat (Kaumann & Molenaar, 1996) and human (Kaumann, 1996) atria.

Efforts to identify high affinity β_3 receptor agonists resulted in the synthesis of L-739,574 (Dow, 1997) and three additional aryl propanolamine derivatives. The first objective of the present study was to characterize the interactions of these aryl propanolamines at the various beta receptors to establish their utility as tools to further probe β -receptor interactions in atria and other tissues. These compounds possessed relatively high affinity as human β_3 receptor agonists with marked diversity in their potency and agonist intrinsic activity at the human β_3 receptor (Table 1). Furthermore, of these agents, L-739,574 was highly selective as a β_3 agonist possessing considerably lower affinity at β_1 and β_2 receptors (see Table 1).

With regard to interactions with rat β_3 receptors, although CL316243 was a potent agonist at the rat β_3 receptor in the stomach fundus (Cohen *et al.*, 1995a; Figure 2), none of the aryl propanolamines activated the rat β_3 receptor in the stomach fundus. Furthermore, even though L-739,574 was a potent agonist at the human β_3 receptor, it only weakly relaxed the rat stomach fundus and did so by a non-beta adrenergic and possibly non-selective mechanism since cyanopindolol which inhibits β_1 , β_2 and β_3 receptors did not block the relaxation. Although not agonists at the rat β_3 receptor, both the indole sulfphonamide and the biphenyl-biaryl ether were antagonists at the rat β_3 receptor. This example of compounds interacting as an agonist in one species (human) and an antagonist in another species (rat) underscores the importance of studying drug-receptor interactions in each species rather than extrapolating information from one species to another. Furthermore, these data documenting opposing effects of some β_3 receptor agonists at human and rat β_3 receptors suggest that *in vivo* data obtained in rats with agonists possessing high affinity at human β_3 receptors may reflect antagonist rather than agonist activity.

With regard to atrial tachycardia, our studies confirm previous data documenting that CGP12177 produced atrial

tachycardia whereas CL316243 did not (Cohen *et al.*, 1995b; Kaumann 1996; Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996). Of the aryl propanolamines with high agonist activity at the human β_3 receptor, only the phenol substituted compounds produced a modest increase in atrial rate, an increase that was totally abolished by propranolol (3×10^{-7} M) in a concentration known to inhibit β_1 and β_2 receptors (Cohen *et al.*, 1995a). In contrast, as previously reported (Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996), CGP12177-induced tachycardia was not blocked by the same concentration of propranolol. The inability of propranolol to block the tachycardia produced by CGP12177 is consistent with the suggestion (Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996) that tachycardia induced by CGP12177 results from activation of a non- β_1 , β_2 , or β_3 receptor. Most importantly, the fact that these more potent agonists at the human β_3 receptor did not produce tachycardia *via* the non- β_1 , β_2 or β_3 receptor suggest that this tachycardic receptor is unrelated to and independent from the human β_3 receptor.

In summary, the present studies characterize the actions of four aryl propanolamines with high affinity for the human β_3 receptor. Although these compounds had high affinity at the human β_3 receptor, marked diversity occurred in the functional activation of the human β_3 receptor receptor with L-739574 as the most potent, selective, and efficacious agonist. Furthermore, in contrast to other β_3 receptor agonists, these aryl propanolamines were antagonists rather than agonists at the rat β_3 receptor, an observation with important implications to *in vivo* effects observed in rats. Lastly, the atrial tachycardic receptor, although previously established to be a non β_1 , β_2 or β_3 receptor (based on ligand affinities at rat β_3 receptors) has now clearly been shown to also be unrelated to the human β_3 receptor. These data support previous conclusions suggesting the presence of a fourth beta receptor associated with atrial tachycardia.

The authors are grateful to Christopher Rito for supplying chemical compounds, to Jeffrey Thrasher, Pamela Pennington, Thomas Crowell and Donald Matthews for chemical syntheses, to Carolyn Roundtree for expert technical support and to Priscilla Kirsch for excellent administrative assistance.

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(Received May 28, 1998

Revised November 13, 1998

Accepted November 17, 1998)



Effects of nicorandil as compared to mixtures of sodium nitroprusside and levcromakalim in isolated rat aorta

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1 The contribution of the relaxant mechanisms of nicorandil (NIC) were analysed by comparing its effects with those of sodium nitroprusside (SNP), levcromakalim (LEM) and mixtures (1:10, 1:30 and 1:100) of SNP:LEM in isolated endothelium-denuded rat aorta.

2 In rings precontracted with KCl (25 mM), the relative inhibitory potency of the soluble guanylate cyclase inhibitor ODQ and the K_{ATP} channel inhibitor glibenclamide (GLI) on SNP:LEM mixtures showed a good correlation with the relative proportion of SNP and LEM in the mixtures. Furthermore, the degree of the inhibition by ODQ and GLI of the effects of the 1:30 SNP:LEM mixture varied as a function of the relative potency of SNP and LEM in KCl-, noradrenaline- (NA) or NA plus nifedipine-treated arteries.

3 The inhibitory effects of ODQ, GLI and ODQ plus GLI on NIC-induced relaxation was similar to that for the 1:30 SNP:LEM mixture in NA plus nifedipine-contracted arteries, but the inhibition of GLI or ODQ plus GLI was smaller in KCl-contracted arteries.

4 In conclusion, the relative importance of activation of the cyclic GMP pathway and K_{ATP} channel opening in mixtures of SNP and LEM could be predicted by the proportion of the drugs in the mixtures and by the relative potency of SNP vs LEM in different experimental conditions. Furthermore, the present results suggest that besides these two mechanisms, a third ODQ- and GLI-insensitive mechanism, possibly involving Ca^{2+} channel blockade, also participates in the relaxant effects of NIC in KCl-induced contractions.

Keywords: Nicorandil; levcromakalim; nitroprusside; rat aorta; drug mixtures

Abbreviations: DMSO, dimethylsulphoxide; E_{max} , maximal relaxant effect; GLI, glibenclamide; IC_{30} , drug concentration producing 30% relaxation; k , factor which represents the slope of the concentration-response curve; LEM, levcromakalim; K_{ATP} , ATP-dependent K^+ channels; NA, noradrenaline; NIC, nicorandil; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; pD_2 , drug concentration exhibiting 50% of the E_{max} expressed as negative log molar; SNP, sodium nitroprusside

Introduction

Nicorandil (NIC) is an antianginal agent which causes vascular smooth muscle relaxation *via* two main mechanisms: it increases membrane K^+ conductance through the opening of ATP-dependent K^+ channels (K_{ATP}) leading to hyperpolarization and it releases nitric oxide (NO) elevating cyclic GMP levels by stimulating soluble guanylate cyclase (Furukawa *et al.*, 1981; Hester, 1985; Hamilton & Weston, 1989). Thus, NIC shares properties of K^+ channel openers and NO donors.

The relative contribution of these two mechanisms to the NIC-induced vasorelaxation varies depending on the experimental protocol and preparation studied. For example, in dog isolated mesenteric artery (Greenberg *et al.*, 1991), rat aorta contracted with endothelin-1 (Borg *et al.*, 1991) or noradrenaline (NA), (Magnon *et al.*, 1994), and rabbit and piglet pulmonary arteries contracted by NA (Magnon *et al.*, 1994; Pérez-Vizcaino *et al.*, 1998), the main mechanism of action of NIC appears to be due to stimulation of soluble guanylate cyclase. However, nicorandil increases blood flow in rat perfused heart (Borg *et al.*, 1991) and relaxes basilar arteries contracted with 5-HT (Ksoll *et al.*, 1991), mainly by K^+ channel activation and acts *via* both mechanisms in pig coronary (Borg *et al.*, 1991) and rabbit and piglet mesenteric arteries (Meisheri

et al., 1991; Magnon *et al.*, 1994; Pérez-Vizcaino *et al.*, 1998). Besides these two well known vasodilator mechanisms, an additional action of NIC involving the blockade of nifedipine-sensitive Ca^{2+} channels has been recently suggested (Fujiwara & Angus, 1996). The explanation for the varying contribution of each mechanism to the relaxant effect of NIC in different experimental conditions, i.e. the so called 'chameleon' effect of NIC (Fujiwara & Angus, 1996), remains elusive.

In the present study we have analysed the contribution of the different relaxant mechanisms of NIC by comparing its effects with those of the NO donor sodium nitroprusside (SNP), the K^+ channel opener levcromakalim (LEM) and several mixtures with different proportions (1:10, 1:30 and 1:100) of SNP:LEM in endothelium-denuded rat aorta. For this purpose we have used the specific inhibitors of K_{ATP} channels, soluble guanylate cyclase, and L-type Ca^{2+} channels, glibenclamide (GLI), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and nifedipine, respectively.

Methods

Tissue preparation

Male Wistar rats (300–350 g), were killed by a blow on the head and then exsanguinated. The descending thoracic aorta

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was rapidly dissected and placed in Krebs' solution of the following composition (in mM): NaCl 118, KCl 5, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 2, KH₂PO₄ 1.2 and glucose 11 at pH 7.4. After excess fat and connective tissue had been removed, the aorta was cut into rings (2–3 mm) and the endothelium was mechanically removed by gently rubbing the intimal surface of the rings with a metal rod. The rings were suspended horizontally by means of two parallel L-shaped stainless steel holders inserted into the lumen in 5 ml organ baths filled with Krebs' solution and bubbled with a 95% O₂–5% CO₂ gas mixture and maintained at 37°C. One holder served as anchor and the other was attached to an isometric force-displacement transducer coupled to a signal amplifier (Model PRE 206-4, Cibertec, Madrid) and connected to a computer *via* an A/D interface. Contractile tension was recorded by a REGXPC computer program (Cibertec, Madrid) as previously described (Pérez-Vizcaino *et al.*, 1993; 1998). Each ring was stretched to a resting tension of 2 g and allowed to equilibrate for 60–90 min. During this period tissues were restretched and washed every 30 min with warm Krebs' solution. The procedure of endothelium removal was tested by the lack of relaxant effects of acetylcholine (10^{−6} M) in rings precontracted with NA (10^{−6} M).

Experimental procedures

After equilibration, aortic rings were contracted by KCl (25 mM) and when the contractile response reached a stable tension, they were treated with vehicle (dimethylsulphoxide, DMSO), GLI (3 × 10^{−6} M), ODQ (10^{−6} M) or both inhibitors for 15 min. Thereafter, concentration-response curves to NIC (10^{−8}–3 × 10^{−4} M), SNP (10^{−10}–3 × 10^{−6} M), LEM (10^{−8}–3 × 10^{−5} M) or the 1:10, 1:30 and 1:100 mixtures of SNP:LEM were constructed by cumulative addition of the drugs. The proportions of the mixtures SNP:LEM were chosen because in preliminary experiments SNP was about 30 times more potent than LEM.

In another set of experiments, the aortic rings were contracted with NA (10^{−6} M) and subsequently treated with vehicle, nifedipine (10^{−7} M), GLI (3 × 10^{−6} M), ODQ (10^{−6} M) or combinations of these inhibitors for 30 min. Cumulative concentration-response curves were then constructed for SNP, LEM, the 1:30 SNP:LEM mixture or NIC.

Drugs

The following drugs were used: sodium nitroprusside, glibenclamide, acetylcholine chloride and noradrenaline (Sigma Chemical, Madrid, Spain), nicorandil (Merck Farma y Quimica, Barcelona, Spain), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Tocris Cookson, Bristol, U.K.), levromakalim (Smith Kline Beecham Pharmaceuticals) and nifedipine (Bayer AG, Leverkusen, Germany). All drugs were dissolved in distilled deionized water to prepare stock solutions (except GLI and ODQ which were dissolved in DMSO, and nifedipine in ethanol), and further dilutions were made in Krebs' solution. Ascorbic acid (10^{−4} M) was present in the stock solution of noradrenaline to prevent oxidation. The 1:10, 1:30 and 1:100 (mol:mol) mixtures of SNP:LEM were prepared by mixing the stock solutions of both drugs in the appropriate proportions just prior to use and further dilutions were made in Krebs' solution.

Analysis of the results

Results are expressed as means ± s.e. mean of measurements in *n* arteries. Individual cumulative concentration-response curves were fitted to the following logistic equation:

$$E = \frac{E_{\max}}{1 + 10^{-k \cdot ([\text{drug}] - \text{pD}_2)}}$$

where *E* is the relaxant effect of the drug, *E*_{max} is the maximal relaxant effect (an index of the efficacy of the vasodilator) expressed as a percentage of the initial contractile response, *k* is a factor which represents the slope of the curve, and *pD*₂ is the drug concentration exhibiting 50% of the *E*_{max} expressed as negative log molar (an index of the potency of the vasodilator). The results obtained with the SNP:LEM mixtures were expressed and calculated considering only the concentration of LEM. Statistically significant differences were calculated by a two way ANOVA analysis followed by a Newman Keuls test. *P* < 0.05 was considered statistically significant. The rightward shift of the concentration-response curves to the vasodilators induced by ODQ, GLI, nifedipine or their combinations was expressed as log dose-ratio. Log dose ratios were used to evaluate the potency of the inhibitors instead of the more common *pK*_B values (*pK*_B = −Log [inhibitor] + Log (dose ratio − 1)) because they allow determination of the inhibition produced by combinations of inhibitors. The log dose ratio values were calculated by subtracting the log concentrations of the vasodilator agents inducing a 30% relaxation (logIC₃₀) in the presence and absence of the inhibitors. The level of 30% relaxation was chosen because ODQ reduced the maximal relaxant effect of SNP to less than 40%.

Results

Relaxant effects of SNP, LEM, 1:10, 1:30 and 1:100 SNP:LEM mixtures and NIC on 25 mM KCl-induced contractions

SNP, LEM and NIC relaxed in a concentration-dependent manner (*pD*₂ values of 8.45 ± 0.13, *n* = 9, 7.12 ± 0.06, *n* = 8 and 5.10 ± 0.11, *n* = 10 respectively) the contractile responses induced by 25 mM KCl (1308 ± 40 mg, *n* = 162) with a similar efficacy (*E*_{max} values of 96 ± 6, 91 ± 3 and 99 ± 4%, respectively)

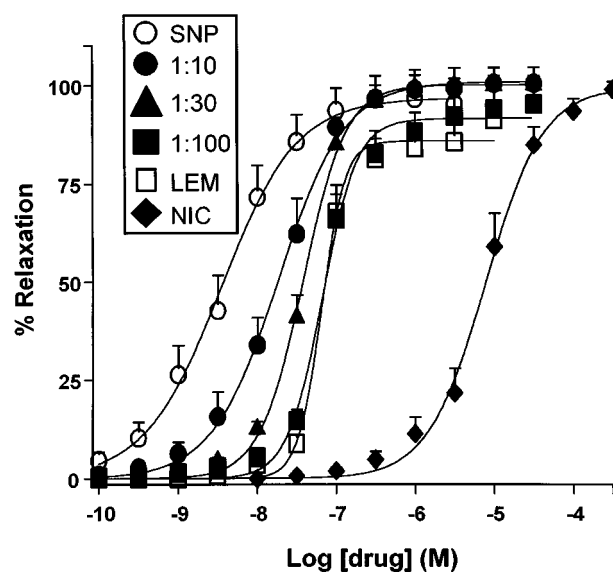


Figure 1 Concentration-response curves to sodium nitroprusside (SNP), levromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM). The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean ± s.e. mean of 7–10 experiments.

(Figure 1). The slope (k) of the concentration-response curve to LEM was significantly steeper than that to SNP (6.3 ± 1.4 vs 2.9 ± 0.4 , respectively, $P < 0.05$) and the slope of the curve to NIC was in between of both values (3.6 ± 0.3). The 1:10, 1:30 and 1:100 SNP:LEM mixtures also induced monophasic relaxant effects on 25 mM KCl-induced contractions. The pD_2 values, expressed in terms of LEM, were 7.75 ± 0.16 , $n=8$, 7.43 ± 0.04 , $n=8$ and 7.15 ± 0.04 , $n=7$, respectively, and the E_{max} values were 100 ± 2 , 100 ± 5 and $93 \pm 5\%$, respectively.

The slope of these curves increased as the proportion of LEM increased in the mixture (3.1 ± 0.3 , 4.5 ± 0.3 and 5.2 ± 0.8 , respectively).

ODQ (10^{-6} M), GLI (3×10^{-6} M) or their combination, when added after the 25 mM KCl-induced contractions reached steady-state, had no effect on arterial tone (2.3 ± 1.2 , 2.9 ± 1.2 and $4.4 \pm 1.7\%$ relaxation, respectively) as compared to vehicle (DMSO, $3.5 \pm 0.8\%$). ODQ strongly shifted rightwards the curve to SNP ($pD_2 = 7.06 \pm 0.18$, $n=6$), reduced the

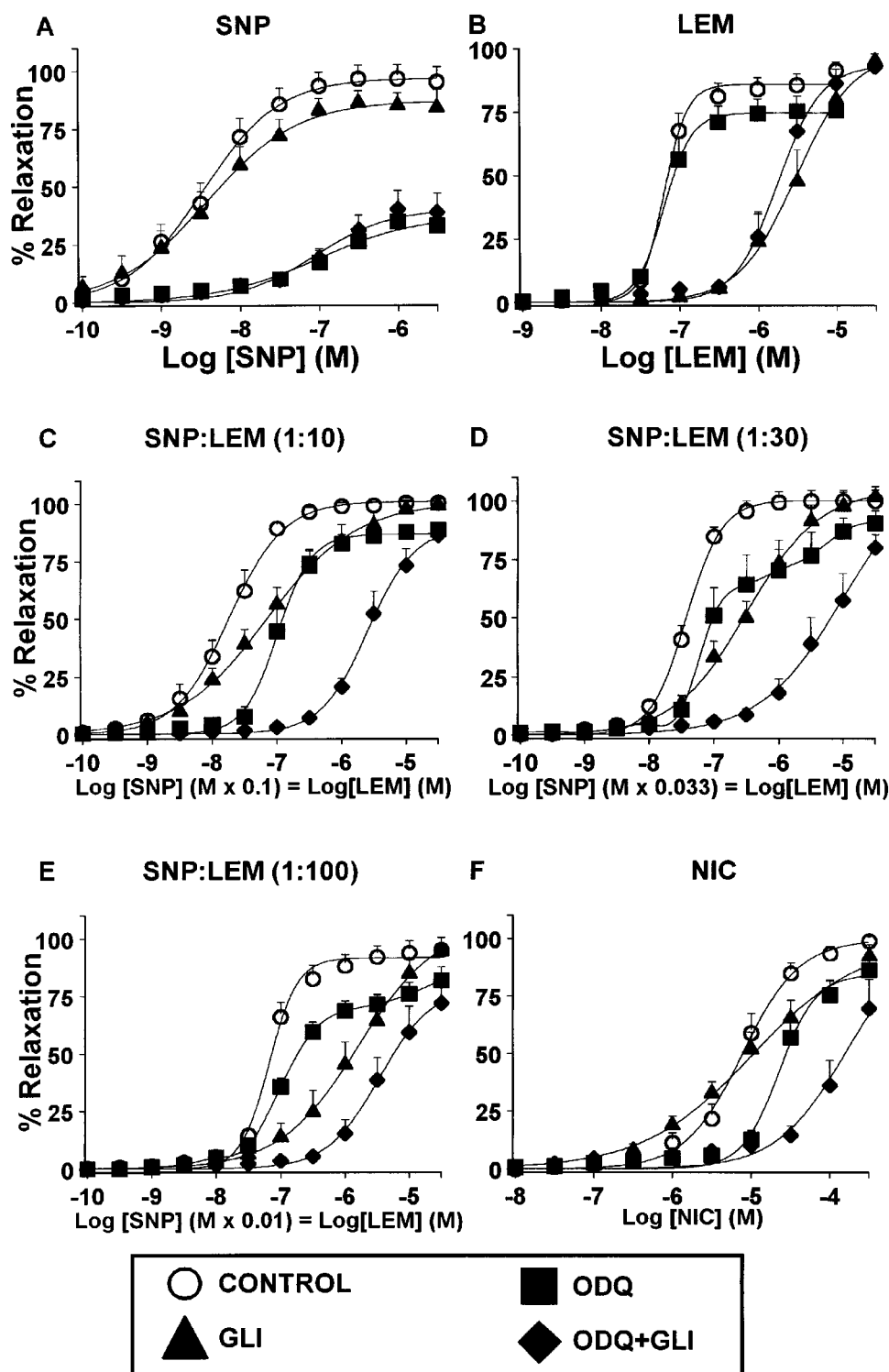


Figure 2 Concentration-response curves to sodium nitroprusside (SNP), levromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM) in the absence (control) or in the presence of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors. The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean $\pm$ s.e. mean of 5–10 experiments.

$E_{\max}$ ($36 \pm 7\%$) and had no effect on the slope, whereas GLI, either in the absence or presence of ODQ, had no significant effect on any parameter (Figure 2A, $n=6$). In contrast, GLI produced a strong rightward shift of the curve to LEM ($pD_2=5.52 \pm 0.15$, $n=8$) whereas ODQ, either in the absence ($n=6$) or presence of ODQ ($n=9$), had no effect (Figure 2B). Neither ODQ nor GLI had any significant effect on the $E_{\max}$ or the slopes of the curves to LEM.

Both ODQ and GLI significantly shifted rightwards the concentration-response curves for the SNP:LEM mixtures (Figure 2C–E $n=6-10$). ODQ reduced the $E_{\max}$ ($87 \pm 3\%$) and increased the slope (5.5 ± 1.0) of the 1:10 mixture. The curves for the 1:30 and 1:100 mixtures appeared to be biphasic in the presence of ODQ, with a high affinity component which accounted for about 70–80% of the $E_{\max}$. GLI had no effect on the $E_{\max}$ but significantly reduced the slopes of the concentration-response of the 1:10, 1:30 and 1:100 mixtures (1.7 ± 0.2 , 2.2 ± 0.3 and 2.7 ± 0.6 , respectively). The inhibitory potency of ODQ and GLI decreased and increased, respectively, as the proportion of LEM increased in the mixture (Figure 3). The combination of ODQ plus GLI produced a

greater inhibition on the SNP:LEM mixtures ($n=5-8$) than any of the inhibitors alone (Figures 2 and 3). ODQ produced a significant rightward shift of the curve to NIC ($pD_2=4.53 \pm 0.12$, $n=6$, Figure 2F), increased its slope (5.3 ± 0.8) but it had no significant effect on the $E_{\max}$ value ($91 \pm 5\%$). GLI had no effect on the pD_2 or $E_{\max}$ values of the curve to NIC but significantly reduced its slope (1.7 ± 0.1 , $n=5$). Moreover, the combination of ODQ plus GLI produced a greater shift of the concentration-response curves to NIC than ODQ alone (Figures 2 and 3).

Relaxant effects of SNP, LEM, 1:30 SNP:LEM mixture and NIC on NA-induced contractions

NA (10^{-6} M) induced sustained contractile responses which averaged 1717 ± 52 mg ($n=164$) which was partially relaxed by 10^{-7} M nifedipine ($24 \pm 1\%$, $n=84$). SNP was six times less potent in relaxing NA- ($pD_2=7.65 \pm 0.14$, $n=7$) than 25 mM KCl-induced contractions, whereas LEM was equipotent in relaxing both contractile responses (Figure 4, pD_2 for NA-induced contractions = 7.03 ± 0.06 , $n=8$). No differences were found in the slope or $E_{\max}$ values in SNP- or LEM-induced relaxant response in NA- as compared to KCl-contracted arteries (not shown). Nifedipine did not modify the effect of SNP ($pD_2=7.72 \pm 0.11$, $n=8$) but shifted rightwards the curve to LEM ($pD_2=6.29 \pm 0.09$) and reduced its slope (2.8 ± 0.4 , Figure 4). ODQ and GLI produced rightward shifts of the curves to SNP and LEM, respectively, which were similar in KCl-, NA- and NA plus nifedipine-contracted arteries (Figure 4).

The 1:30 SNP:LEM mixture was three times less potent in relaxing NA-contracted vessels ($pD_2=6.96 \pm 0.14$, $n=8$) than 25 mM KCl-induced contractions and nifedipine did not produce any further change ($pD_2=7.10 \pm 0.16$, $n=9$, Figure 5). NIC was equipotent in relaxing the contractile responses in the three experimental conditions (pD_2 in NA- and NA plus nifedipine-contracted arteries 5.18 ± 0.04 , $n=7$, and 4.97 ± 0.16 , $n=7$, respectively, Figure 5). No differences were found in the slope or $E_{\max}$ values in the curves to the 1:30 mixture (4.9 ± 1.0 and $94 \pm 4\%$, respectively) or NIC (3.1 ± 0.5 and $98 \pm 3\%$, respectively) in NA- as compared to KCl-contracted arteries whereas nifedipine tended to reduce the slope of both the curves (3.0 ± 0.3 and 2.0 ± 0.4 , respectively, $P<0.05$) but had no effect on the $E_{\max}$ values.

In ODQ-treated arteries, either in the absence or presence of nifedipine, the curves for the 1:30 mixture were shifted to the right and appeared to be biphasic, the high affinity component accounting for 60–70% of the $E_{\max}$ (Figure 5A and B, respectively). GLI had no significant effect on the $E_{\max}$ values but reduced the slope (4.1 ± 0.6 , not significant and 2.2 ± 0.2 , $P<0.05$, in nifedipine-untreated and treated arteries, respectively) and shifted the curve to the 1:30 mixture to the right ($pD_2=6.15 \pm 0.11$ and 6.52 ± 0.14 , respectively, $P<0.05$ nifedipine-treated vs untreated arteries). ODQ shifted rightwards to a similar extent the curve to NIC in the absence and presence of nifedipine ($pD_2=4.54 \pm 0.07$ and 4.55 ± 0.15 , respectively) and reduced the $E_{\max}$ ($87 \pm 4\%$ not significant and 86 ± 4 , $P<0.05$, respectively) but did not significantly change the slopes of the curves (Figure 5C and D). GLI produced a weak rightward shift of the curves to NIC which was only significant in the absence of nifedipine ($pD_2=4.78 \pm 0.13$), reduced the slopes (1.9 ± 0.3 , $P<0.05$ and 1.5 ± 0.2 , $P>0.05$, in the absence and presence of nifedipine, respectively) and reduced the $E_{\max}$ only in the presence of nifedipine ($83 \pm 7\%$). However, despite the statistical differences, as can be observed in Figure 5C and D, the behaviour of

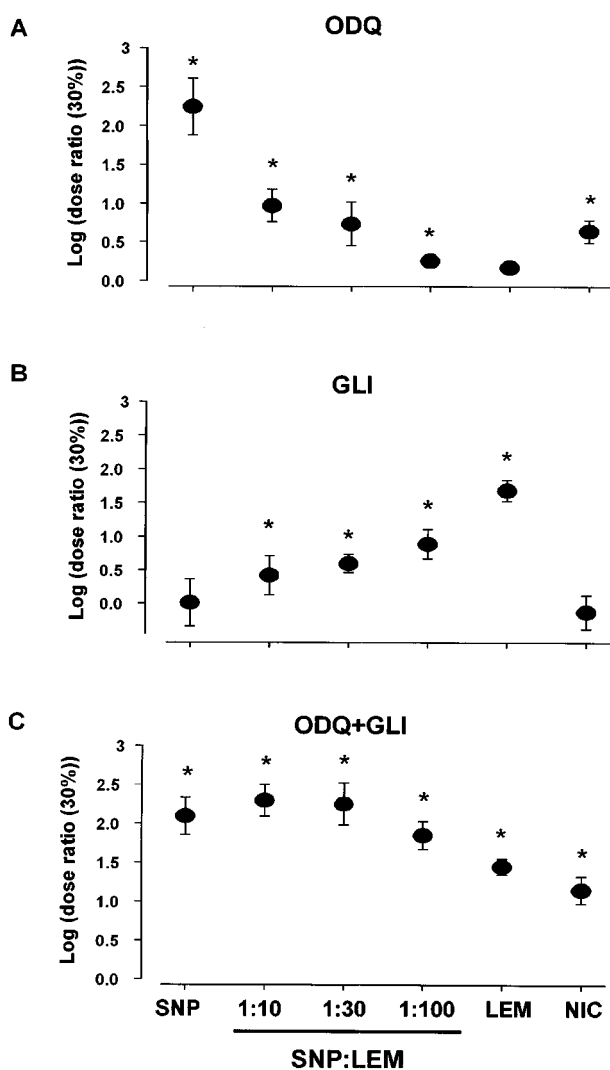


Figure 3 Inhibitory effects (expressed as log [dose ratio]) of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors on the relaxant effects induced by sodium nitroprusside (SNP), levcromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM). The log dose ratios were calculated from Figure 2 at the level of 30% relaxation. * $P<0.05$ vs 0.

ODQ and GLI on NIC-induced relaxations were quite similar in untreated and nifedipine-treated arteries.

The combination of ODQ plus GLI induced a shift of the concentration-response to the relaxant effects of the 1:30 SNP:LEM mixture and NIC which was significantly greater than that induced by any of the inhibitors alone (Figure 5). The combination was equally potent to shift the curve to the SNP:LEM mixture in KCl-, NA- or NA plus nifedipine-treated arteries whereas it was significantly more potent in inhibiting NIC-induced relaxations in NA- or NA plus nifedipine- than in KCl-contracted arteries (Figure 5). Thus, GLI and GLI plus ODQ produced a significantly greater inhibition on the effects of the 1:30 mixture than on those of NIC in KCl contracted arteries, but no differences were observed in the potencies of the antagonists in NA or NA plus nifedipine contracted arteries (Figure 6).

Figure 7 shows the correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of the 1:1, 1:10, 1:30, 1:100 and 1:1000 SNP:LEM mixtures and the relative potency of SNP vs LEM to relax KCl-contracted arteries. The figure also shows the correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of the 1:30 SNP:LEM mixture and the relative potency of SNP vs LEM in KCl-, NA- and NA plus nifedipine-treated arteries. Both correlations followed a similar trend so that the data with different proportions and different experimental conditions were pooled for the regression analysis yielding a regression

line (slope = 1.1 ± 0.1 and y intercept = -0.6 ± 0.1) with a very good correlation ($r = 0.98$, $P < 0.01$). In contrast, there was no correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of NIC and the relative potency of SNP vs LEM to relax KCl-, NA- and NA plus nifedipine-treated arteries.

Discussion

In the present study we have analysed the mechanism of NIC-induced relaxation in isolated endothelium-denuded rat aorta under several experimental conditions. Nicorandil is known to relax vascular smooth muscle mainly by releasing NO and increasing the intracellular concentrations of cyclic GMP and by opening K_{ATP} channels (Furukawa *et al.*, 1981; Hester, 1985; Hamilton & Weston, 1989). The contribution of guanylate cyclase activation and K_{ATP} channel opening in the relaxant effects of NIC has been analysed by means of their specific inhibitors ODQ and GLI, respectively and by comparison with the specific stimulator of guanylate cyclase SNP, the specific K_{ATP} channel opener LEM and mixtures of both drugs. The analysis of the mechanisms of NIC-induced relaxation is complicated by several factors: (a) the possibility that the pharmacological tools employed are not specific enough; (b) the ability of a given mechanism to induce a relaxant response may vary in the different experimental

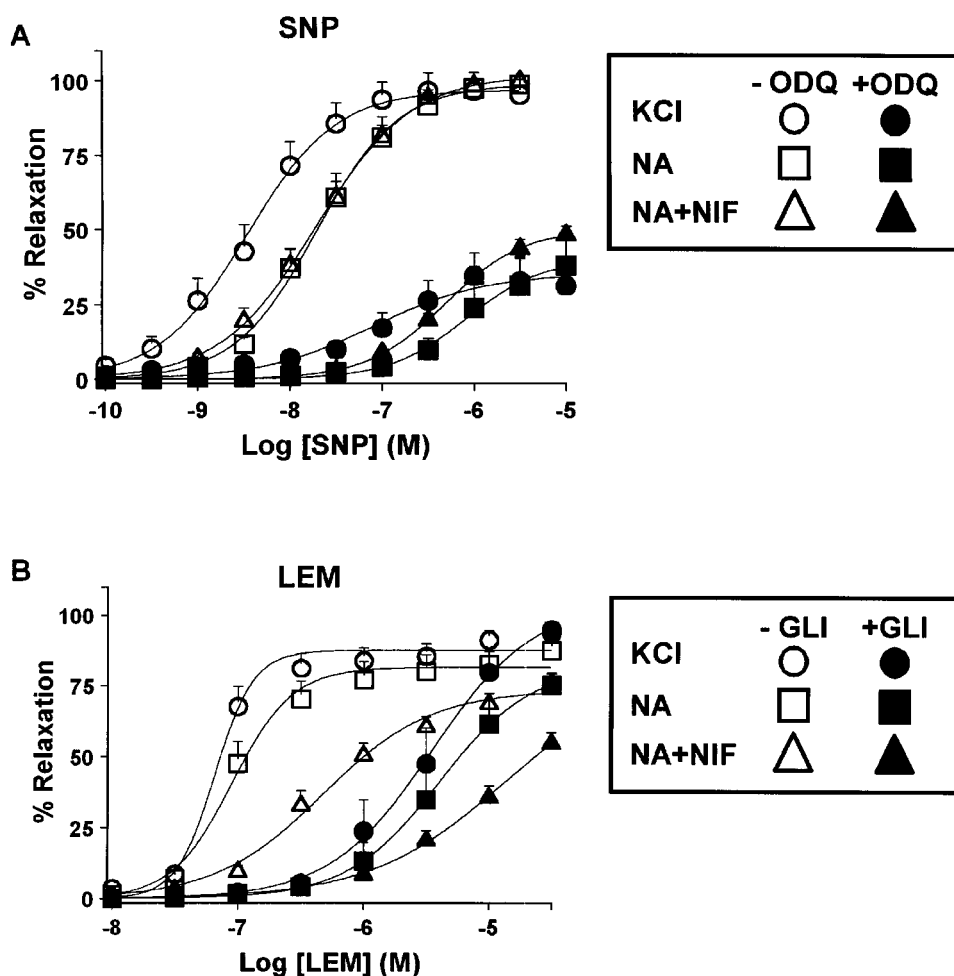


Figure 4 Concentration-response curves to sodium nitroprusside (SNP) and levromakalim (LEM) in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM), NA (10^{-6} M) or the combination of NA plus 10^{-7} M nifedipine (NA + NIF) in the absence or in the presence of ODQ (10^{-6} M) or glibenclamide (GLI, 3×10^{-6} M). Each symbol represents the mean $\pm$ s.e. mean of 6–9 experiments.

conditions; (c) the possible interactions between the different mechanisms; and (d) finally, the existence of other 'unexpected' mechanisms.

Specificity of SNP, LEM, ODQ and GLI

Inhibitors of guanylate cyclase such as methylene blue have been widely used to analyse the possible involvement of cyclic GMP in drug-induced relaxation. However, this drug is relatively unspecific and conclusions drawn may be inadequate (Gryglewski *et al.*, 1992). Thus, we have employed ODQ, a much more specific tool to inhibit guanylate cyclase. ODQ has been reported to be both a competitive and a non competitive

inhibitor of guanylate cyclase (Schrammel *et al.*, 1996). Likewise, in our experiments ODQ produced a rightward shift of the concentration-response to SNP (typical of a competitive antagonist) but also reduced the maximal relaxant effect of SNP (typical of a non competitive antagonist). Furthermore, ODQ did not interfere with LEM-induced relaxation. In functional studies, GLI behaves as an apparent competitive antagonist of LEM (Pérez-Vizcaino *et al.*, 1993; Edwards & Weston, 1993). Likewise, in the present study, GLI produced a parallel shift of the concentration-response curves to LEM but had no effect on SNP-induced relaxation. Furthermore, ODQ did not modify the inhibitory action of GLI on LEM-induced relaxation and, conversely, GLI did not affect the inhibitory

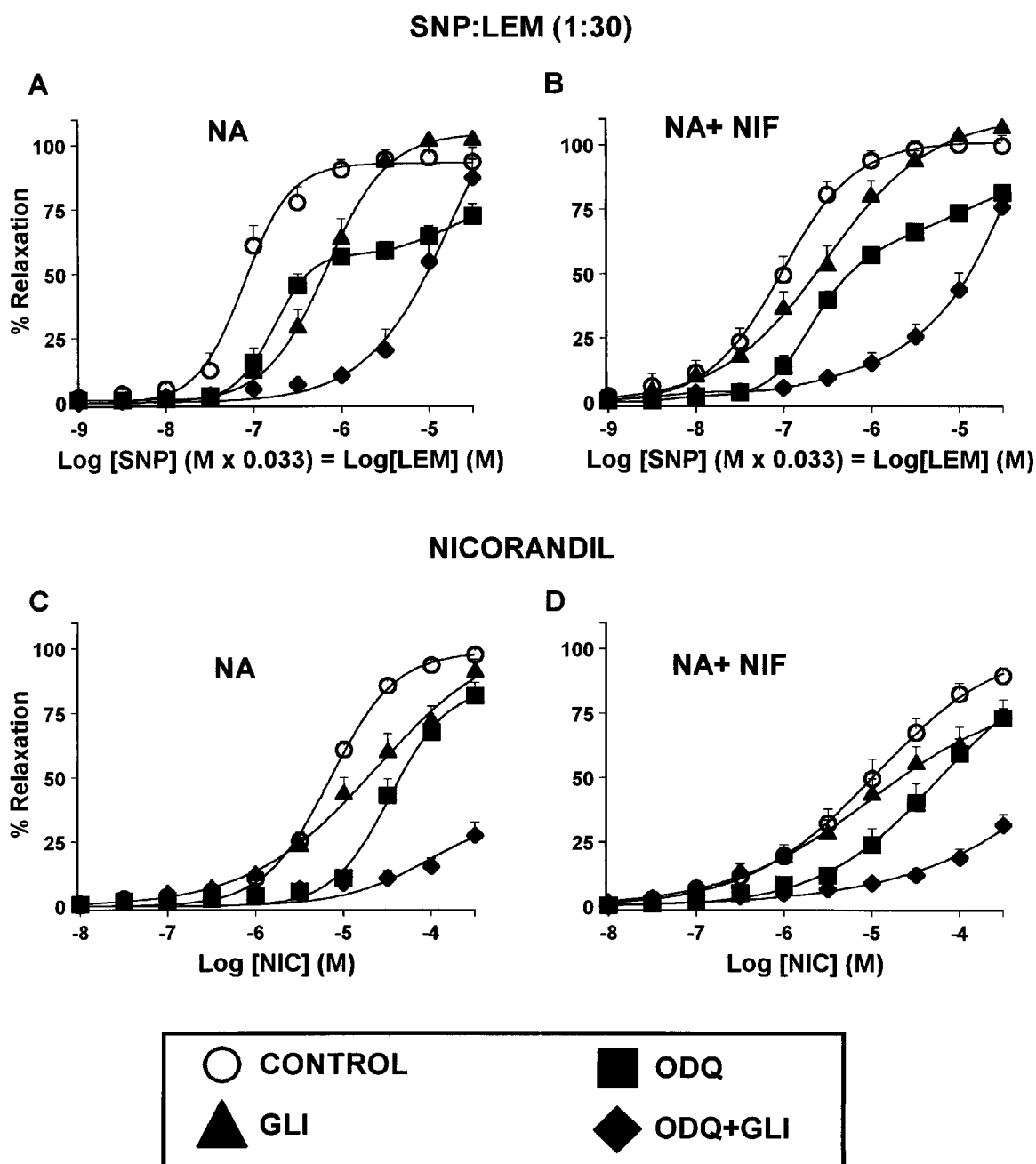


Figure 5 Concentration-response curves to the 1:30 sodium nitroprusside:levromakalim (SNP:LEM) mixture and nicorandil (NIC) in endothelium-denuded rat aortic rings pre-contracted with NA (10^{-6} M) or the combination of NA plus 10^{-7} M nifedipine (NA+NIF) in the absence (control) or in the presence of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors. The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean $\pm$ s.e.mean of 6–10 experiments.

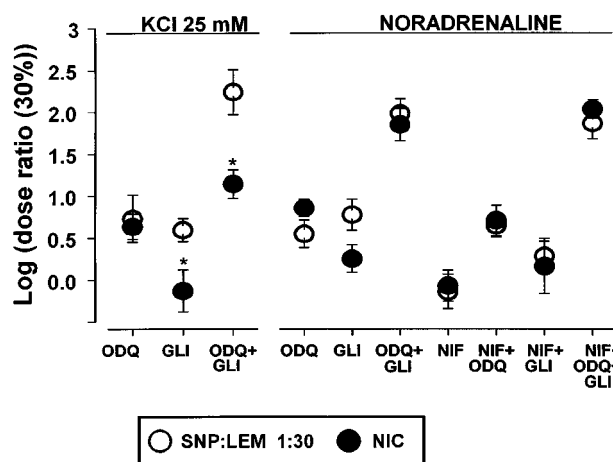


Figure 6 Inhibitory effects (expressed as log [dose ratio]) of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M), nifedipine (NIF, 10^{-7} M) or combinations of these inhibitors on the relaxant effects induced by nicorandil (NIC) and the 1:30 SNP:LEM mixture in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM), or NA (10^{-6} M). The log dose ratios were calculated from data in Figures 2 and 5 at the level of 30% relaxation. * $P < 0.05$ SNP:LEM vs NIC.

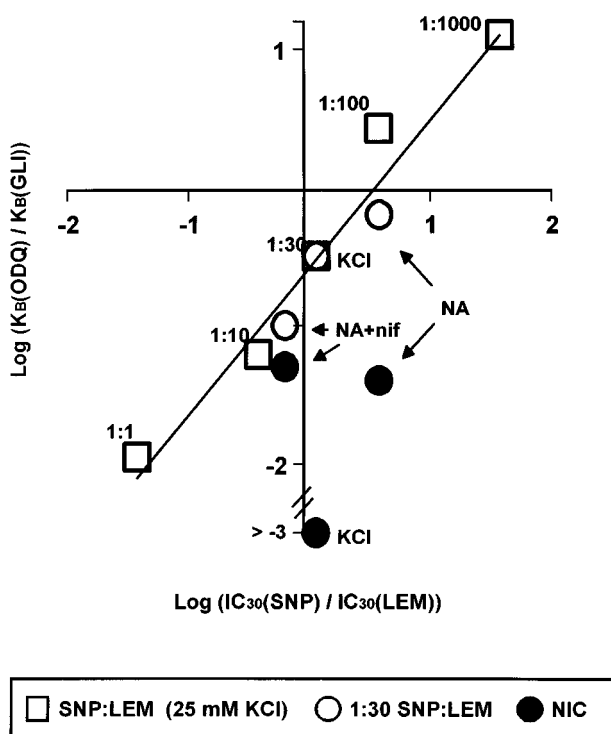


Figure 7 Correlation between the relative potency of ODQ vs glibenclamide (GLI) to inhibit the relaxant effects of SNP:LEM mixtures and nicorandil (NIC) and the relative potency of SNP vs LEM in different experimental conditions. Ordinate: logarithm of the ratio of the potency of ODQ and GLI expressed as apparent K_B . The apparent K_B were calculated at the level of 30% relaxation. Abscissa: logarithm of the ratio of the potency of SNP and LEM expressed as IC_{30} . The relative potencies ($\text{IC}_{30}(\text{SNP})/\text{IC}_{30}(\text{LEM})$) were 0.038, 0.125 and 0.021 for KCl, NA and NA plus nifedipine, respectively, and these values were corrected for the proportion factor in the mixtures, for the data with NIC these values were artificially set to those of the 1:30 SNP:LEM mixture. The data for the 1:10, 1:30 and 1:100 SNP:LEM mixtures were calculated from previous figures and the data for the 1:1 and 1:1000 are not shown. Since open circles and squares followed a similar trend they were pooled for the regression analysis. Note that data for the 1:30 SNP:LEM mixture fell into the line, while those for NIC did not follow any correlation. Since GLI had no inhibitory action on NIC-induced relaxation in KCl-stimulated arteries, the $\text{Log}(K_B(\text{ODQ})/K_B(\text{GLI}))$ cannot be calculated but has to be smaller than -3.

action of ODQ on SNP-induced relaxation. In spite of the differences in potency of SNP and LEM under different experimental conditions (arteries treated with KCl, NA or NA plus nifedipine) no apparent changes in the mechanism of action of these drugs were observed since ODQ and GLI produced similar inhibitory actions. In summary, in our experimental conditions, both the agonists (SNP and LEM) and the antagonists (ODQ and GLI) appeared to be very specific tools.

Effects of the SNP:LEM mixtures

The concentration-response curves to the SNP:LEM mixtures in the absence of inhibitors were monophasic. This can be explained because both drugs produced a nearly full relaxant effect and the combination of both drugs did not produce a maximal relaxation greater than that induced by the individual drugs. It could be expected that the contribution of K_{ATP} channel opening would increase and that the contribution of the cyclic GMP pathway in the relaxant effects of the mixtures would decrease with increasing proportions of LEM in the mixture. In fact, the potency of GLI and ODQ to inhibit the relaxant effects of the mixtures increased and decreased, respectively, as the proportion of LEM increased in the mixture (Figure 3). Additionally, since the concentration-response curves to LEM were steeper than those to SNP, the slopes of the curves to the mixtures increased as the proportion of LEM increased (Figure 1). Furthermore, due to this difference in the slope observed for the individual drugs, the contribution of cyclic GMP should predominate at low concentrations, while the contribution of K_{ATP} channel opening should be more important at high concentrations of the mixtures. Indeed, GLI produced a greater inhibition at high concentrations, decreasing the slopes of the curves, whereas ODQ produced a greater inhibition at low concentrations of the mixture (Figure 2). The biphasic nature of the curves to the SNP:LEM mixtures in the presence of ODQ may be explained because this drug reduced the maximal relaxant effect of the SNP component.

We hypothesized that under the different experimental conditions (i.e. KCl-, NA- and NA plus nifedipine-treated arteries) the differences in the relative potency of SNP compared to LEM (26.3, 8.0 and 46.8, respectively, when calculated at 30% relaxation) should affect the relative contribution of cyclic GMP and K_{ATP} channel opening of the relaxant effects of the 1:30 SNP:LEM mixture. As shown in Figure 7, when the relative potency of SNP vs LEM is varied by using mixtures with different proportions of SNP:LEM, the relative potency of the inhibitors ODQ vs GLI changed accordingly with a very good correlation. When the relative potency of ODQ vs GLI to inhibit the effect of 1:30 SNP:LEM mixture was plotted against the relative potency of SNP and LEM under the different experimental conditions (KCl-, NA- and NA plus nifedipine-treated arteries), the points fell in the same regression line as for the different mixtures in KCl-contracted arteries. Thus, the relative importance of a mechanism contributing to the final effect of drug mixtures depends not only on the relative proportion of the individual drugs in the mixture but also on the relative potency of the two drugs, and this relative potency may vary depending on the tissue and experimental conditions. By extrapolation, for a given drug exerting its effects through two independent pathways (e.g. NIC), which can be considered as a drug mixture in a fixed proportion, the relative contribution of a single mechanism should depend on the relative potency of that component.

Effects of NIC

The concentration-response curves to NIC were monophasic showing a slope which was intermediate between that of SNP and LEM. The relaxant effects of NIC on 25 mM KCl-induced contractions were inhibited by ODQ indicating that activation of the cyclic GMP pathway is an important mechanism by which NIC induces its relaxant effect in the rat aorta. In contrast, GLI had a weak effect on NIC-induced relaxation which was only observed at high concentrations of NIC, i.e. it reduced the slope of the curve. Thus, as occurred for SNP:LEM mixtures, the contribution of K_{ATP} channel opening is more important at high concentrations of NIC. However, the combination of ODQ and GLI induced a greater inhibitory action than ODQ alone, indicating that K_{ATP} channel opening may also contribute when the cyclic GMP pathway is already inhibited. Since the two pathways (K_{ATP} channels and cyclic GMP) may act simultaneously in NIC-induced relaxation and interactions between them might occur, the relative contribution of each mechanism would be unpredictable based only on the effects of SNP and LEM. Therefore, the effects of NIC were compared with those of SNP:LEM mixtures in different proportions. Based on the inhibition induced by ODQ, NIC behaved as the 1:30 SNP:LEM mixture, and since at this proportion SNP and LEM are approximately equipotent, the relaxant effects of NIC might be attributed equally to the cyclic GMP pathway and K_{ATP} channel opening. Based on the effects of GLI, NIC behaved as SNP, so that the effects of NIC might be attributed solely to the stimulation of cyclic GMP pathway. Finally, the inhibition induced by the combination of ODQ plus GLI was similar for LEM and NIC which could be interpreted if NIC exerted its relaxant effect only by activation of K_{ATP} channels. This apparent paradox can be explained by a third ODQ- and GLI-insensitive mechanism of action of NIC. In fact, it has been recently reported that in rat mesenteric arteries stimulated by the thromboxane A_2 mimetic U46619, nifedipine inhibited the relaxant action of NIC, whereas it had no effect on cromakalim-induced relaxation suggesting an additional action of NIC involving the blockade of nifedipine-sensitive Ca^{2+} channels (Fujiwara & Angus, 1996). We could not test this on KCl-induced contractions because at 10^{-7} M nifedipine fully relaxed 25 mM KCl-induced contractions. However, in our experiments, nifedipine did not modify the relaxant effects of NIC on NA-induced contractions, whereas it reduced the relaxant effect of LEM, i.e. opposite results than those reported by Fujiwara & Angus (1996). Nevertheless, these results do not exclude that NIC may relax rat aorta contracted by 25 mM KCl-induced contractions through Ca^{2+} channel blockade since this mechanism is much more effective in relaxing KCl- than NA-induced contractions. The discrepancy between our results and those of Fujiwara & Angus (1996) may be explained because agonist-induced contractions are more sensitive to the blockade of Ca^{2+} channels in resistance arteries than in the aorta (Cauvin *et al.*, 1983). Therefore, Ca^{2+} channel blockade might participate in NIC-induced relaxant effects on KCl- but not on NA-induced contractions in the rat aorta. According to this proposal, the

combination of ODQ plus GLI produced a similar inhibition of the relaxation induced by NIC and the 1:30 SNP:LEM mixture on NA-induced contractions indicating that the third relaxant mechanism of NIC is absent on NA-induced contractions. As discussed above, Ca^{2+} channel blockade modifies the relative contribution of the cyclic GMP pathway and K_{ATP} channel opening to the effects of the 1:30 SNP:LEM mixture. However, nifedipine did not modify the inhibitory action of ODQ or GLI on NIC-induced relaxation which can be explained assuming that NIC is already exerting a nifedipine-like effect. Thus, in the presence of nifedipine the relative contribution of the cyclic GMP pathway and K_{ATP} channel opening was similar for NIC and for the 1:30 mixture. In the present study, the lack of correlation (Figure 7) between the relative inhibitory action of ODQ vs GLI on NIC-induced relaxations and the relative potency of SNP and LEM under the different experimental conditions may be explained by the third, ODQ- and GLI-insensitive mechanism of relaxation. In a previous paper we suggested that the relative contribution of the mechanisms involved in the relaxant effects of NIC under different experimental conditions (piglet mesenteric and pulmonary arteries) could only be partly explained by the relative efficacy and potency of SNP vs LEM (Pérez-Vizcáino *et al.*, 1998). In these experiments, it can be observed that the experimental conditions which were relatively less sensitive to the Ca^{2+} channel blocker verapamil (pulmonary arteries or arteries stimulated by U46619) were more sensitive to inhibition by the combination of ODQ plus GLI suggesting that a third mechanism involving Ca^{2+} channel blockade may also participate in the relaxant effects of NIC. Nevertheless, the evidences for the Ca^{2+} channel blocking properties of NIC in the present paper and that of Fujiwara & Angus (1996) are only indirect so that this conclusion has to be taken with caution.

In conclusion, mixtures of SNP and LEM produced relaxant effects in isolated rat aorta through the activation of the cyclic GMP pathway and K_{ATP} channel opening. The relative importance of each mechanism, as determined by the inhibitory action of ODQ and GLI, could be predicted by the proportion of the drugs and by their relative potency. Both mechanisms were also involved in the relaxant effects of NIC. However, our results suggest that a third, ODQ- and GLI-insensitive mechanism, possibly involving Ca^{2+} channel blockade, may also participate in the relaxant effects of NIC. Therefore, NIC induces a relaxant effect on KCl-induced contractions which is mediated by activation of the cyclic GMP pathway, K_{ATP} channel opening and possibly Ca^{2+} channel blockade. NIC relaxes NA-induced contractions by activation of the cyclic GMP pathway and K_{ATP} channel opening; the possible Ca^{2+} channel blockade of NIC, although not contributing to its relaxant effects, may alter the relative potency of activation of the cyclic GMP pathway and K_{ATP} channel opening.

The authors are grateful to C. Rivas and R. Vara for excellent technical assistance. This work was supported by a CICYT (SAF 96/0042) Grant. A.L. Cogolludo and M. Ibarra are supported by grants from Comunidad Autónoma de Madrid and Universidad de Alcalá, respectively.

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(Received July 24, 1998

Revised November 19, 1998

Accepted November 20, 1998)



Investigation of the inhibitory effects of homocysteine and copper on nitric oxide-mediated relaxation of rat isolated aorta

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1 Elevated plasma levels of homocysteine (HC) and copper have both been associated with the development of inflammatory vascular diseases, such as atherosclerosis. In this study, the effects of a combination of HC and copper on nitric oxide (NO)-mediated relaxation of isolated rat aortic rings were investigated.

2 Exposure to HC (10–100 μ M; 30 min) had no effect on relaxation to acetylcholine (ACh; 0.01–10 μ M, $n=4$). Pre-incubation of aortic rings with a higher concentration of HC for an extended period (1 mM; 180 min) significantly inhibited endothelium-dependent relaxation ($n=4$), but this inhibition was prevented by the presence of the copper chelator bathocuprione (10 μ M, 180 min, $n=6$).

3 Exposure to HC (100 μ M) and copper (10–100 μ M; 30 min) caused a copper concentration-dependent inhibition of endothelium-dependent relaxation ($n=4$). This inhibitory effect was reduced in the presence of either superoxide dismutase (SOD; 100 u ml⁻¹; $n=4$) or catalase (100 u ml⁻¹; $n=4$), and further reduced by the presence of both enzymes ($n=5$).

4 HC and copper (100 μ M; 30 min) significantly inhibited endothelium-independent relaxation to glyceryl trinitrate (0.01–10 μ M; $n=8$). In contrast, HC (1 mM), alone or in combination with copper (100 μ M), did not inhibit relaxation to the endothelium-independent relaxant sodium nitroprusside (0.01–10 μ M; $n=4$).

5 These data indicate that the presence of copper greatly enhances the inhibitory actions of HC on NO-mediated relaxation of isolated aortic rings. The reduction of inhibition by catalase and SOD indicates a possible role for copper-catalyzed generation of superoxide and hydrogen peroxide leading to an increased inactivation or decreased production of endothelium-derived NO.

Keywords: Homocysteine; copper; nitric oxide

Abbreviations: ACh, Acetylcholine; GTN, glyceryl trinitrate; H₂O₂, hydrogen peroxide; OH, hydroxyl radical; HC, homocysteine; NO, nitric oxide; ONOO⁻, peroxynitrite; S-NO-HC, S-nitroso-homocysteine; SNP, sodium nitroprusside; O₂⁻, superoxide; SOD, superoxide dismutase

Introduction

Elevated plasma levels of the sulphur-containing amino acid homocysteine (HC) are suggested to be a powerful independent risk factor for premature atherosclerosis and other inflammatory vascular diseases (Berwanger *et al.*, 1995; McCully, 1996). Vascular endothelial cell dysfunction is considered to be a major factor in the development of atherosclerosis (Ross, 1993), and recent studies have shown that mild to moderate elevations in plasma levels of HC (20–35 μ M) are associated with impaired endothelium-dependent arterial dilatation in both humans (Tawakol *et al.*, 1997; Woo *et al.*, 1997) and monkeys (Lentz *et al.*, 1996) *in vivo*.

A number of studies have demonstrated that HC can cause endothelial cell dysfunction (Harker *et al.*, 1976; Stamler *et al.*, 1993). HC-induced inhibition of the synthesis of endothelium-derived nitric oxide (NO), increased loss of NO *via* the generation of superoxide anion (O₂⁻) and the formation of peroxynitrite (ONOO⁻), have been suggested to contribute to the attenuation of endothelium-dependent dilatation observed in the presence of homocysteinaemia (Upchurch *et al.*, 1996). However, the mechanisms by which HC alters vascular reactivity have not been clearly defined.

Raised plasma levels of the essential metalloid element copper have also been reported in patients with homocysteinaemia (Dudman & Wilcken, 1983) and may be a contributory factor

in atherogenesis (Aalbers & Houtman, 1985; Iskra *et al.*, 1993). Copper can catalyze the oxidation of thiols leading to the reduction of oxygen and the generation of hydrogen peroxide (H₂O₂; Cavallini *et al.*, 1969). Furthermore, HC-induced endothelial cell injury *in vitro* can be reduced by catalase, a scavenger of H₂O₂, indicating that H₂O₂ may be the mediator of HC-induced cellular damage (Wall *et al.*, 1980; Starkebaum & Harlan, 1986).

However, the elevation of plasma copper levels associated with inflammatory disease has been suggested to be an important component of the physiological response to overcome inflammation (reviewed by Sorenson, 1989). Copper can convert H₂O₂ to water and oxygen, and reduce the toxic hydroxyl radical (OH) to hydroxide (Farhataziz & Ross, 1977).

We have recently demonstrated that copper augments NO-mediated smooth muscle relaxation (Plane *et al.*, 1997). In the present study, we investigated the effect of the interaction between HC and copper on NO-mediated relaxation in the rat isolated aorta. A preliminary account of this study has been presented to the British Pharmacological Society (Emsley *et al.*, 1997).

Methods

Male Wistar-Kyoto rats (250–350 g) were stunned and decapitated. The descending aorta was rapidly removed,

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cleared of adhering fat and connective tissue and cut into cylindrical segments approximately 2 mm in length.

Tension studies

Aortic segments were mounted in organ baths containing Krebs solution, maintained at 37°C and gassed with 95% O₂/5% CO₂. Isometric tension was measured with Grass FTO3 force displacement transducers and data recorded on disc (MacLab). Tissues were set to a pre-determined optimal tension of 1.5 g and left to equilibrate for 30 min, after which time the tension was reset.

Aortic rings were pre-contracted with phenylephrine (1–3 µM). The concentration of phenylephrine was adjusted to give a similar level of tone in all experiments (approximately 1 g). The tissues were then relaxed with a bolus dose of acetylcholine (ACh; 5 µM) to assess the integrity of the endothelium. Following washout and equilibration, the rings were pre-contracted and relaxed with cumulative concentrations of the endothelium-dependent vasodilator, ACh (0.01–10 µM) or the endothelium-independent vasodilators, sodium nitroprusside (SNP; 0.01–10 µM) and glyceryl trinitrate (GTN; 0.01–10 µM) in the presence or absence of HC and/or copper (30–180 min). The responses to SNP and GTN were slower to develop than those to ACh taking approximately 18 and 16 min respectively to attain full relaxation as compared to around 13 min for ACh. In experiments carried out to investigate the role of O₂^{•-} or H₂O₂ in the observed inhibitory effect of HC and copper, superoxide dismutase (SOD) and/or catalase were added together with the HC and copper.

Solutions and drugs

For tension studies, aortic rings were maintained in Krebs buffer (pH 7.4) of the following composition (mM): NaCl 119, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 2.5 and glucose 11, and containing 2.8 µM indomethacin. Acetylcholine chloride, bathocuproinedisulphonic acid (disodium salt), catalase (bovine liver), copper (II) chloride, D-L homocysteine, L-phenylephrine hydrochloride, sodium nitroprusside, superoxide dismutase (bovine erythrocytes) were all obtained from Sigma (Poole, Dorset, U.K.). Glyceryl trinitrate was obtained from Schwarz Pharma Ltd (Chesham, Bucks, U.K.).

Analysis of data

Relaxation of isolated aortic rings is expressed as per cent reversal of phenylephrine-induced tone. All results are expressed as means ± s.e.mean for *n* separate experiments. Comparisons were made using Student's *t*-test or ANOVA followed by Dunnett's *post hoc* test for multiple comparisons with the control, with rejection of the null hypothesis at the 5% level.

Results

Effect of copper on endothelium-dependent relaxation of isolated rat aortic rings

ACh (0.01–10 µM) evoked endothelium-dependent relaxation of isolated rat aortic rings pre-contracted with phenylephrine (1–3 µM). Pre-incubation of aortic rings with copper (100 µM; 30 min) significantly potentiated relaxation to the endothelium-dependent vasodilator, ACh. Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of copper (100 µM; 30 min) are shown in Figure 1.

Effect of HC on endothelium-dependent relaxation of isolated rat aortic rings

Pre-incubation of arterial rings with HC (10–100 µM) for 30 min had no significant effect on relaxation to ACh. Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC (100 µM; 30 min) are shown in Figure 2a.

Exposure of aortic rings to a higher concentration of HC (1 mM) for 30 or 180 min significantly attenuated relaxation to ACh, reducing the maximum response from 96 ± 4 and 92 ± 6%, to 68 ± 9 and 46 ± 7% (*n* = 4 in each case; *P* < 0.01), respectively.

Pre-incubation with superoxide dismutase (SOD; 100 u ml⁻¹) or catalase (100 u ml⁻¹) alone, or in combination for 3 h had no significant effect on relaxation to ACh (*n* = 4 in each case). However, the inhibitory effect of HC (1 mM; 180 min) was significantly reduced by the presence of either superoxide dismutase (SOD; 100 u ml⁻¹; *n* = 4) or catalase (100 u ml⁻¹; *n* = 4) alone, or in combination (*n* = 4). Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC, SOD and catalase are shown in Figure 2b and c.

The presence of the copper chelator, bathocuprione (10 µM) in the Krebs buffer had no significant effect on relaxation to ACh (*n* = 6). Pre-incubation for 180 min with HC (1 mM) in the presence of bathocuprione (10 µM) had no significant effect on relaxation to ACh (*n* = 6). Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC (1 mM) and bathocuprione (10 µM) are shown in Figure 2d.

Effect of a combination of HC and copper on endothelium-dependent relaxation of rat isolated aortic rings

In the presence of copper (10–100 µM), pre-incubation of aortic rings with HC (100 µM; 30 min), which alone had

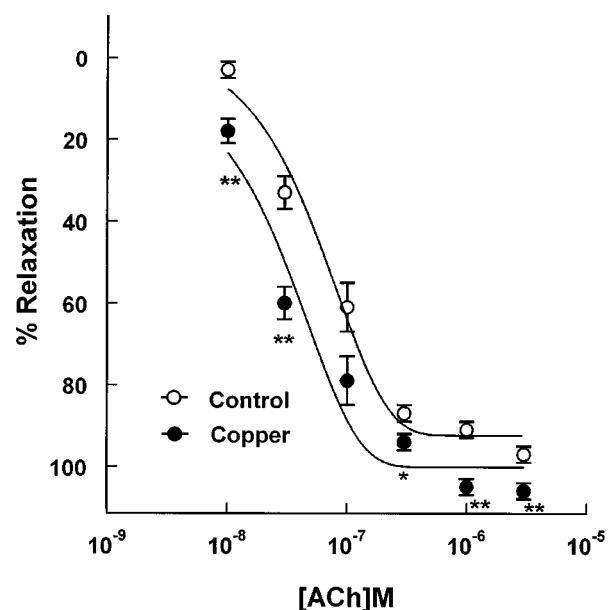


Figure 1 Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the presence and absence of copper (100 µM; 30 min). All mean values are from four experiments with s.e.means shown by vertical bars. ***P* < 0.01, **P* < 0.05 compared with control.

no effect on endothelium-dependent relaxation, caused a copper concentration-dependent inhibition of relaxation to ACh. In the presence of HC and 10 μM copper relaxation to ACh was unaltered ($n=4$). However, in the presence of HC and 20 μM copper, relaxation to ACh was significantly inhibited and the maximum response reduced from 95 ± 2 to $51.3 \pm 6.0\%$ ($n=4$, $P<0.01$). In the presence of HC and 100 μM copper, the maximal response to ACh

was reduced from 91 ± 5 to $27.9 \pm 13.1\%$ ($n=4$, $P<0.01$). Representative traces showing ACh-evoked relaxation of an aortic ring in the absence and presence of HC, alone and in combination with copper (100 μM) are shown in Figure 3a. Mean concentration-response curves for ACh-evoked relaxation in the presence of HC (100 μM) and copper (10, 20 and 100 μM ; 30 min) are shown in Figure 3b.

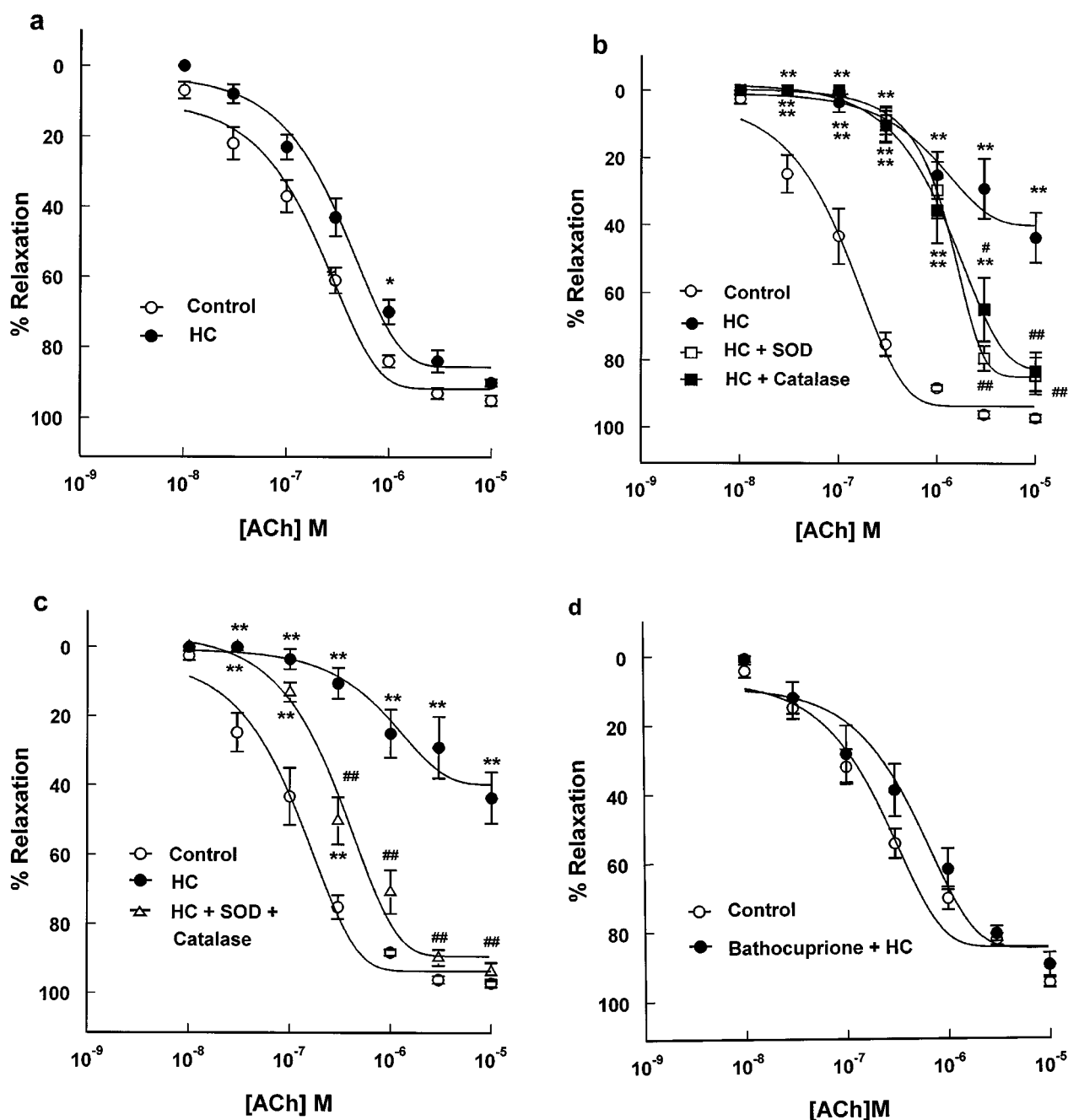


Figure 2 (a) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (100 μM ; 30 min). $*P<0.05$ compared with control. (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM; 180 min), together with catalase (100 u ml^{-1}) or SOD (100 u ml^{-1}). (c) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM; 180 min), together with catalase and SOD (100 u ml^{-1}). $**P<0.01$ compared with control, $*P<0.05$ compared with control, $\#P<0.05$ compared to relaxation in the presence of HC (100 μM ; 30 min), $##P<0.01$ compared to relaxation in the presence of HC (100 μM ; 30 min). All values are means from four experiments with s.e.means shown by vertical bars. (d) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings in the presence of HC (1 mM) and bathocuprione (10 μM ; 180 min). All values are means from six experiments with s.e.means shown by vertical bars.

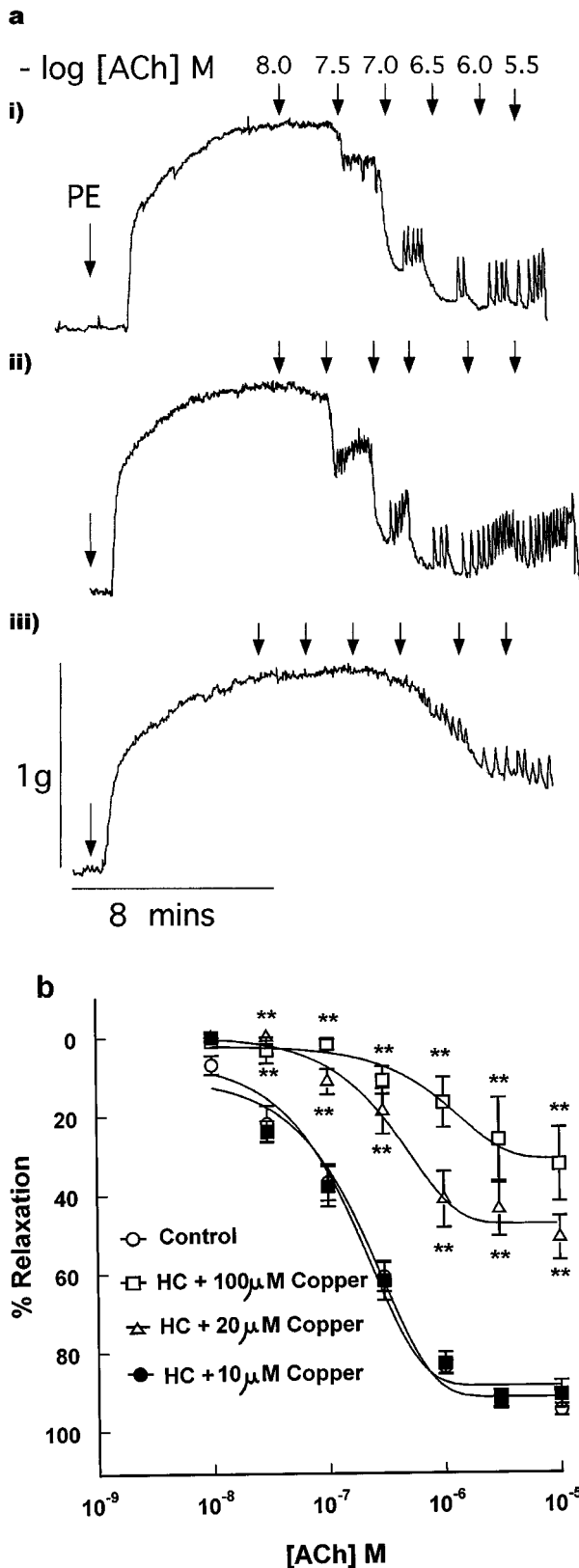


Figure 3 (a) Representative traces showing changes in tension elicited by ACh (0.01–10 μM) in a rat isolated aortic ring pre-contracted with phenylephrine (3 μM). (i) control (ii) in the presence of HC (100 μM) alone (iii) in the presence of HC together with copper (both 100 μM). (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (100 μM) and increasing concentrations of copper (10–100 μM). All values are means from four experiments with s.e. means shown by vertical bars. ** $P < 0.01$ compared with control.

Effect of HC and copper on endothelium-independent relaxation

Exposure to HC (10 μM –1 mM; 30 min) alone did not alter relaxation to the endothelium-independent relaxant SNP ($n=4$). Exposure of aortic rings to copper (100 μM ; 30 min) significantly potentiated relaxation to SNP and the combination of HC (1 mM) and copper (100 μM) caused a similar potentiation of relaxation to SNP as seen in the presence of copper alone. Concentration-relaxation curves for SNP-evoked relaxation in the presence and absence of HC and copper are shown in Figure 4a.

GTN evoked concentration-dependent relaxation of endothelium-denuded aortic rings. Pre-incubation with HC (100 μM) or copper (100 μM) for 30 min had no significant effect on relaxation to GTN ($n=4$). However, exposure of aortic rings to HC and copper together (both 100 μM ; 30 min) significantly inhibited relaxation to GTN reducing the maximum relaxation from 96 ± 2 to $32 \pm 5\%$ ($n=8$; $P < 0.01$). Concentration-relaxation curves for GTN-evoked relaxation in the presence and absence of HC and copper are shown in Figure 4b.

Effect of catalase and SOD on the inhibition of endothelium-dependent relaxation by HC and copper

The inhibition of ACh-stimulated relaxation observed in the presence of HC and copper (both 100 μM) together was significantly reduced by the presence of either catalase (100 u ml^{-1} ; $n=4$) or SOD (100 u ml^{-1} ; $n=4$). In the presence of both catalase and SOD, the inhibitory effect of the combination HC and copper was further reduced and relaxations to ACh were not significantly different from controls ($n=4$). Concentration-response curves for ACh-evoked relaxation in the presence and absence of HC and copper, SOD and catalase are shown in Figure 5a and b.

Discussion

The results of the present study demonstrate that copper causes a concentration-dependent enhancement of the inhibition of NO-mediated relaxation of isolated vascular tissue by HC. In the presence of copper, a short exposure to HC, which alone had no effect, significantly inhibited endothelium-dependent relaxation to ACh and endothelium-independent relaxation to GTN, without inhibiting relaxation to the endothelium-independent relaxant SNP.

Normal plasma levels of HC are in the range of 1–10 μM (Berwanger *et al.*, 1995) but in homocysteinaemic patients plasma HC levels of 100–500 μM have been detected (Malinow, 1994). In the present study, addition of HC alone, at a concentration of 100 μM , had no significant effect on endothelium-dependent relaxation. Similarly, a previous study demonstrated that brief exposure of endothelial cells to low concentrations of HC did not alter NO-mediated inhibition of platelet aggregation but in fact resulted in the formation of S-nitroso-homocysteine (S-NO-HC), a vasodilator and an inhibitor of platelet aggregation, which does not generate H_2O_2 (Stamler *et al.*, 1993). Thus, the formation of S-NO-HC may attenuate the potentially adverse effects of this amino acid and could contribute to the lack of inhibition of endothelium-dependent relaxation observed following a short exposure to HC in our experiments.

In contrast, exposure of isolated tissues to a higher concentration of HC (1 mM for 30–180 min) did cause an

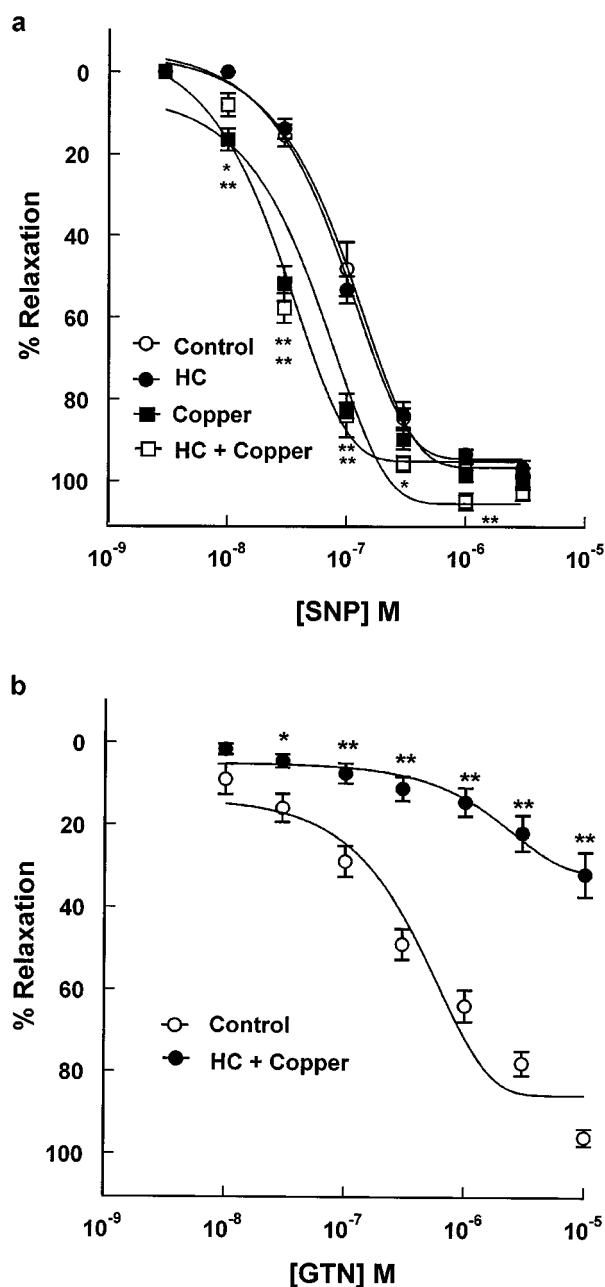


Figure 4 (a) Mean concentration-response curves for SNP-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM) and copper (100 μ M). All values are means from four experiments with s.e.means shown by vertical bars. $**P < 0.01$ and $*P < 0.05$ compared with control. (b) Mean concentration-response curves for GTN-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min). All values are means from eight experiments with s.e.means shown by vertical bars. $**P < 0.01$ and $*P < 0.05$ compared with control.

attenuation of endothelium-dependent relaxation. Similar results have recently been reported in other isolated vascular preparations (Lang *et al.*, 1997; Quéré *et al.*, 1997), and prolonged exposure of cultured endothelial cells to high concentrations of HC have been shown to progressively impair their ability to inhibit platelet aggregation (Stamler *et al.*, 1993). However, the inhibition of relaxation by 1 mM HC was prevented by the presence of the copper chelator, bathocuprione indicating that the effects of HC observed in this study and in previous investigations (Lang *et al.*, 1997) may in fact be due to the interaction of HC with trace amounts of

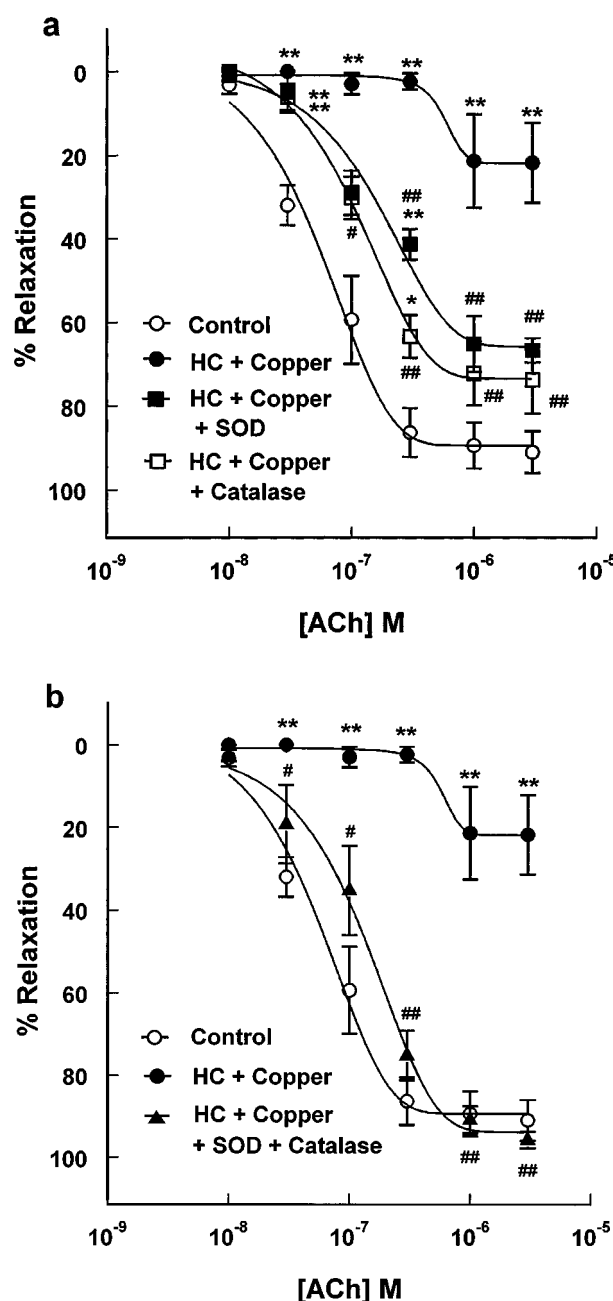


Figure 5 (a) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min), together with catalase (100 u ml^{-1}) or SOD (100 u ml^{-1}). (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min), together with catalase and SOD (both 100 u ml^{-1}). All values are means from 4–5 experiments with s.e.means shown by vertical bars. $**P < 0.01$ compared with control. $\#P < 0.05$ compared to relaxation in the presence of HC and copper (both 100 μ M; 30 min), $###P < 0.01$ compared to relaxation in the presence of HC and copper (both 100 μ M; 30 min).

copper present in the Krebs buffer used during the experiments. This observation raises the possibility that during prolonged exposure of arteries to HC, as occurs *in vivo*, only very low concentrations of copper may be required to interact with HC and cause vascular dysfunction.

In the present study, the inhibitory effects of high concentrations of HC on endothelium-dependent relaxation were reduced by SOD and/or catalase. Oxidation of thiols can lead to the generation of both O_2^- and H_2O_2 , (Loscalzo, 1996)

and HC inhibits glutathione peroxidase, which normally removes H_2O_2 (Upchurch *et al.*, 1997). Thus, the attenuation of inhibition by SOD and catalase implicates both O_2^- and H_2O_2 in HC-induced endothelial dysfunction as previously suggested (Starkebaum & Harlan, 1986; Upchurch *et al.*, 1996). These reactive species can directly damage endothelial cells to decrease the production of NO and the reaction of NO with O_2^- leading to the formation of ONOO^- may contribute to a decreased NO-mediated relaxation (Beckman & Koppenol, 1996).

Exposure to copper alone potentiates NO-mediated endothelium-dependent relaxation in the rat isolated aorta, indicating that copper may play a role in the modulation of NO production (Plane *et al.*, 1997). In the present investigation copper caused a concentration-dependent enhancement of the inhibition of NO-mediated relaxation caused by HC. Thus, under pathophysiological conditions, the interaction between HC and essential metalloelements such as copper, may contribute to the vascular dysfunction in homocysteinaemic patients (Lentz *et al.*, 1996; Tawakol *et al.*, 1997; Woo *et al.*, 1997).

The inhibitory effect of the combination of HC and copper on endothelium-dependent relaxation was partially reduced by catalase or SOD, and further reduced by the two enzymes together, indicating a role for the generation of H_2O_2 and O_2^- in this process. Similarly, a previous study has shown that in the presence of low levels of copper (1–10 μM), HC causes lysis of cultured endothelial cells, an effect which is blocked by catalase (Starkebaum & Harlan, 1986). *In vivo*, almost all plasma copper is bound to caeruloplasmin and the levels of ionically bonded or salt forms of copper in plasma are too small to be measured accurately (May *et al.*, 1976). However, a number of studies have indicated that protein-bound copper can catalyse reactions in a similar manner to free copper (Starkebaum & Harlan, 1986; Ehrenwald *et al.*, 1993) and the reaction between O_2^- and NO leads to the formation of ONOO^- which can release of copper ions from caeruloplasmin (Swain *et al.*, 1994).

These results indicate a role for both O_2^- and H_2O_2 in the inhibition by HC and copper of NO-induced relaxation. The

mechanisms of action of these reactive oxygen species are quite distinct. O_2^- destroys NO in the extracellular space, and thus reduces the amount of available NO to cause relaxation (Gryglewski *et al.*, 1986; Rubanyi & Vanhouette, 1986). This action can be reversibly inhibited by SOD. In contrast H_2O_2 is known to stimulate NO production (Schimizu *et al.*, 1994) but subsequently causes irreversible endothelial damage leading to reduced NO production. The action of H_2O_2 can be prevented by catalase but cannot be reversed once endothelial damage has occurred. As SOD and catalase cannot enter cells, but reduce the inhibition of ACh mediated relaxation by HC and copper, the reactive oxygen species generated from the interaction between HC and copper appear to inhibit NO-mediated relaxation in the extracellular space.

The combination of HC and copper significantly inhibited NO-mediated relaxation to the endothelium-independent relaxant, GTN. However, in the presence of copper, alone or with HC, relaxation to the endothelium-independent relaxant SNP was not inhibited but was slightly, but significantly potentiated. This observation may reflect the ability of copper to enhance the release of NO from SNP, analogous to the release of NO from nitrosothiols (McAninly *et al.*, 1993) which may mask the inhibitory effect of the combination of HC and copper.

In conclusion, this study indicates that although ionically bonded copper does not inhibit NO-mediated responses, the interaction between copper and HC inhibits endothelium-dependent arterial dilatation which may in turn contribute to the pro-atherogenic effects of this amino acid. At present, the only treatment for homocysteinaemia is the administration of folic acid, alone or with vitamin B_6 or B_{12} . The clinical benefit of this therapy is unproven and thus the development of future strategies could focus on the possible use of copper chelators and antioxidants for the treatment of this condition.

A.M. Emsley is supported by a British Heart Foundation studentship and F. Plane is a Wellcome Trust Career Development Fellow. We would like to thank Prof John R.J. Sorenson of the University of Arkansas, U.S.A., for helpful criticism and discussion during the preparation of this manuscript.

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(Received July 24, 1998

Revised November 9, 1998

Accepted November 19, 1998)



Cell-specific coupling of PGE₂ to different transduction pathways in arginine vasopressin- and glucagon-sensitive segments of the rat renal tubule

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1 The aim of the present study was to investigate the transduction pathways elicited by prostaglandin E₂ (PGE₂) to inhibit hormone-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in the outer medullary collecting duct (OMCD) and medullary thick ascending limb (MTAL) microdissected from the rat nephron.

2 In the OMCD, 0.3 μ M PGE₂ and low concentrations of Ca²⁺ ionophores (10 nM ionomycin or 50 nM A23187) inhibited by about 50% a same pool of arginine vasopressin (AVP)-stimulated cyclic AMP content through a same process insensitive to *Bordetella pertussis* toxin (PTX).

3 Sulprostone, an agonist of the EP₁/EP₃ subtypes of the PGE₂ receptor, decreased AVP-dependent cyclic AMP accumulation in OMCD and MTAL samples. The concentration eliciting half-maximal inhibition was of about 50 nM in OMCD and 0.1 nM in MTAL.

4 In MTAL, 1 nM sulprostone and PGE₂ inhibited by about 90% a same pool of AVP-dependent cyclic AMP content through a PTX-sensitive, Ca²⁺-independent pathway.

5 In the OMCD, PGE₂ decreased by about 50% glucagon-dependent cyclic AMP synthesis by a process sensitive to PTX and Ca²⁺-independent. Sulprostone 1 nM induced the same level of inhibition.

6 These results demonstrate that PGE₂ decrease hormone-dependent cyclic AMP accumulation through a G α i-mediated inhibition of adenylyl cyclase activity in MTAL cells and glucagon-sensitive cells of the OMCD or through a PTX-insensitive increase of intracellular Ca²⁺ concentration in AVP-sensitive cells of the OMCD.

Keywords: Cyclic AMP accumulation; arginine vasopressin; glucagon; Ca²⁺ ionophores; prostaglandin E₂; sulprostone; microdissected segments; rat kidney

Abbreviations: AVP, arginine vasopressin; [Ca²⁺]_i, intracellular free concentration of Ca²⁺; CCD, cortical collecting duct; cyclic AMP, adenosine 3':5'-cyclic monophosphate; MTAL, medullary thick ascending limb; OMCD, outer medullary collecting duct; PDE, phosphodiesterase; PGE₂, prostaglandin E₂; PTX, *Bordetella pertussis* toxin

Introduction

The urinary concentration process is regulated by arginine vasopressin (AVP) which increases intracellular adenosine 3':5' cyclic monophosphate (cyclic AMP) in two well-defined segments of the rat renal tubule, the thick ascending limb and the collecting tubule (Morel & Doucet, 1986). Previous studies on rat microdissected segments have established that the AVP-dependent cyclic AMP accumulation can be decreased by prostaglandin E₂ (Torikai & Kurokawa, 1983; Chabardès *et al.*, 1990; Aarab *et al.*, 1993; Firsov *et al.*, 1995; De Jesus Ferreira *et al.*, 1998). However, depending on the segment studied, the transduction pathway involved in prostaglandin E₂ (PGE₂)-mediated regulation of AVP-dependent cyclic AMP accumulation appears different. In the medullary portion of the thick ascending limb (MTAL), PGE₂ inhibits cyclic AMP synthesis (Torikai & Kurokawa, 1983; Firsov *et al.*, 1995) by a process sensitive to *Bordetella pertussis* toxin (PTX) and thus presumably through interactions with GTP-dependent G α i proteins. In contrast, in the outer medullary portion of the rat

collecting duct (OMCD), PGE₂-induced inhibition of AVP-dependent cyclic AMP accumulation appears mediated mainly by an increase of the cyclic AMP breakdown (Chabardès *et al.*, 1990) by a process insensitive to PTX (Aarab *et al.*, 1993). This increase of cyclic AMP catabolism might be linked to a rise of intracellular free concentration of Ca²⁺ ([Ca²⁺]_i) since PGE₂ increases [Ca²⁺]_i in the OMCD and that the inhibitory effect of PGE₂ on AVP-dependent cyclic AMP accumulation is not observed in Ca²⁺-depleted tubule samples (Aarab *et al.*, 1993). In agreement with this hypothesis, it has been observed that the phosphodiesterase activity measured in isolated rat OMCD is stimulated by the addition of Ca²⁺ (Kusano *et al.*, 1985).

AVP-dependent cyclic AMP accumulation in rat OMCD and MTAL is also inhibited by Ca²⁺ ionophores. However, the mechanism of action elicited by Ca²⁺ ionophores depends on the segment studied and/or on the concentration tested. In MTAL, Ca²⁺ ionophores decrease cyclic AMP accumulation by an effect restricted to the catabolism of cyclic AMP (Kusano *et al.*, 1985). In the OMCD, high concentrations of Ca²⁺ ionophores (in the micromolar range) inhibit cyclic AMP synthesis whereas low concentrations are effective only on cyclic AMP catabolism (Kusano *et al.*, 1985; Chabardès *et al.*, 1996). These observations and the results obtained with PGE₂ recalled above suggest that low concentrations of Ca²⁺ ionophores and PGE₂ might have a same mechanism of action

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to inhibit AVP-dependent cyclic AMP accumulation in the rat OMCD.

Morphological studies have established that the rat OMCD contains mainly two cell types, the principal cell and the type A intercalated cell (Madsen & Tisher, 1986). As previously discussed, different experimental results support the conclusion that adenylyl cyclase activity is stimulated by AVP and glucagon in principal cells and intercalated cells, respectively (Morel & Doucet, 1986; Chabardès *et al.*, 1996). In rat MTAL, there is only one morphological cell type (Madsen & Tisher, 1986) and different hormones including AVP and glucagon stimulate the same catalytic units of adenylyl cyclase (Morel *et al.*, 1987). Thus, the different effects elicited by PGE₂ in MTAL and AVP-sensitive cells of the OMCD reflect regulations present in two different well-defined cell types. To our knowledge, a possible action of PGE₂ in glucagon-sensitive cells of the rat OMCD has not yet been described.

The present studies were designed to define the cell types sensitive to PGE₂ and the mechanism of action of PGE₂ in the rat OMCD and MTAL. Different approaches were used. The first approach was to compare the properties of regulation elicited by PGE₂ and low concentrations of Ca²⁺ ionophores in the AVP-sensitive cells of the OMCD in order to study if these compounds inhibit hormone-dependent cyclic AMP accumulation by a same mechanism of action. The second approach was to compare the transduction pathway elicited by PGE₂ and sulprostone, a selective agonist of the EP₁/EP₃ subtypes of the PGE₂ receptor (Coleman *et al.*, 1994) in the different cell types studied. Finally, we investigated a possible action of PGE₂ in the glucagon-sensitive cells of the rat OMCD. The results establish that PGE₂-mediated inhibition of hormone-dependent cyclic AMP accumulation is the consequence of a cell-specific coupling of PGE₂ to either a PTX-sensitive inhibition of adenylyl cyclase activity or through an increase of [Ca²⁺]_i insensitive to PTX.

Methods

Measurement of AVP-dependent cyclic AMP accumulation

The experimental conditions used to measure hormone-dependent cyclic AMP accumulation on an intact single segment have been detailed previously (Chabardès *et al.*, 1990; Aarab *et al.*, 1993; Firsov *et al.*, 1995) and will be recalled briefly here. Experiments were performed on anaesthetized (pentobarbital, 6 mg 100 g⁻¹ body weight) male Wistar rats (140–180 g of body weight, Iffa-Credo, France). After perfusion and hydrolysis of the left kidney with microdissection medium containing collagenase (collagenase A from *Clostridium histolyticum*, 0.406 U mg⁻¹), single pieces (0.3–1.8 mm length) of collecting duct or thick ascending limb were microdissected at 4°C from the outer medulla. The standard microdissection medium was composed of (mM): NaCl 137; KCl 5; MgSO₄ 0.8; Na₂HPO₄ 0.33; KH₂PO₄ 0.44; MgCl₂ 1; NaHCO₃ 4; CH₃COONa 10; CaCl₂ 1.0; glucose 5; 1,2, N-2-hydroxyethylpiperazine *N'*-2-ethanesulphonic acid (HEPES) 20, pH 7.4 and 0.1% (w/v) bovine serum albumin (fraction V). The medium was supplemented with 5 µM indomethacin and 0.5 U ml⁻¹ adenosine deaminase. The effect of extracellular Ca²⁺ on cyclic AMP accumulation was investigated in tubule samples microdissected and incubated in media without CaCl₂ and containing 0.1 mM [ethylene bis (oxyethylenenitrilo)] N, N'-tetraacetic acid (EGTA). This long duration in a Ca²⁺-free medium induces a marked depletion of

intracellular Ca²⁺ pools as indicated by the lack of agonist-induced [Ca²⁺]_i variations in such tubule samples. For long preincubation periods, in experiments performed with *Bordetella pertussis* toxin (PTX) or bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetracetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM), all media contained essential and nonessential amino acids as well as vitamins (minimum Eagle's medium).

Due to the small number of cells per tubule sample (from about 200–600 cells), cyclic AMP accumulation can be measured only in the presence of a phosphodiesterase (PDE) inhibitor. The incubation medium had the same composition as the microdissection medium but included either 1 mM 3-isobutyl-1-methylxanthine (IBMX), which blocks all PDE types in the rat kidney (Hoey & Houslay, 1990), or 50 µM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), a specific inhibitor of the low Km cyclic AMP specific PDE family IV. PDE family IV is not regulated by Ca²⁺ (Beavo, 1995). Ro 20-1724 therefore allows to measure cyclic AMP accumulation resulting from effects on both cyclic AMP synthesis and partial cyclic AMP breakdown and was used in all experiments performed to study regulations of the response to AVP. Unless otherwise specified, each sample was preincubated 10 min at 30°C and then, after addition of incubation medium containing the agonists to be tested, incubated 4 min at 35°C. The amounts of cyclic AMP were measured on acetylated samples by radioimmunoassay. In our conditions, the basal level of cyclic AMP present in one single piece of tubule is close to, or below, the sensitivity threshold of the assay (2 fmol cyclic AMP per reaction tube) and thus only hormone-dependent cyclic AMP accumulation can be measured. AVP was used at concentrations which induce maximal stimulation of adenylyl cyclase activity, i.e., 1 and 10 nM in collecting duct and thick ascending limb tubule samples, respectively. PGE₂, Ca²⁺ ionophores and sulprostone had no intrinsic agonist activity in our experiments.

The results were calculated in femtomoles of cyclic AMP accumulated per millimetre of segment per 4 min incubation time (fmol mm⁻¹ 4 min⁻¹). In each experiment, different experimental conditions were tested on replicate tubule samples microdissected from a same rat kidney (6–10 samples per condition). The mean of the cyclic AMP values obtained in each condition was taken as one single data point and the results were expressed in absolute value or in percentage of the response to AVP or in percentage of inhibition calculated from the corresponding AVP value. Results were calculated from *n* different experiments or from *n* tubule samples in individual experiments.

Measurement of intracellular Ca²⁺ concentration

Intracellular free concentration of Ca²⁺ ([Ca²⁺]_i) was measured in single tubule samples microdissected from collagenase-treated kidneys by using the calcium-sensitive fluorescence probe acetoxymethyl ester of fura-2 (fura-2 AM) as previously described (Aarab *et al.*, 1993; Champigneulle *et al.*, 1993). Briefly, the samples were loaded for 60 min with 10 µM fura-2 AM. Each loaded tubule was then transferred to a superfusion chamber fixed on the stage of an inverted fluorescence microscope (model IM 35; Zeiss, Oberkochen, Germany). The tubule was superfused at 37°C at a rate of 10–12 ml min⁻¹, corresponding to about ten exchanges per min. The composition of the superfusion medium was similar to that used in cyclic AMP experiments, except that serum albumin, indomethacin and adenosine deaminase were not added since the superfusion medium was flushed continuously. After a 5- to 10-min equilibration period, agonists were added

to the medium and superfused over tubule. An area of 60 μm diameter was selected over the tubule image and the fluorescence intensity emitted (during brief excitation periods at 340 and 380 nm alternatively, at a maximal rate of 30 cycles min^{-1}) was recorded each 2 s. Calculations of $[\text{Ca}^{2+}]_i$ was performed as previously described (Champigneulle *et al.*, 1993). The results were obtained from different tubules (n) microdissected from several rats.

Statistical analysis

Data are presented as the means $\pm$ s.e.mean. The statistical evaluation of the data was assessed either by unpaired Student's *t*-test or, for multiple comparison, by one-way analysis of variance on weighted means followed by LSD Fisher's *t*-test.

Materials

Unless otherwise specified, the compounds were from Merck (Darmstadt, Germany), Sigma (St Louis, MO, U.S.A.) And from Calbiochem (San Diego, CA, U.S.A.). Collagenase and adenosine deaminase were obtained from Boehringer (Mannheim, Germany). Amino acids and vitamins were obtained from Eurobio (Les Ulis, France) and bovine serum albumin was obtained from Pentex Miles Inc. (Kankakee, IL, U.S.A.). Glucagon (human) was purchased from Neosystem Laboratoire (Strasbourg, France). Sulprostone (16-phenoxy- ω -17,18,19,20-tetranor-PGE₂-methylsulfonylamide) was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Specific cyclic AMP antibody and ¹²⁵I-labelled cyclic AMP succinyl-tyrosine-methyl ester were purchased from either Sanofi Diagnostics Pasteur (Marnes-La-Coquette, France) or NEN Life Sciences Products (Le Blanc Mesnil, France).

Results

Effect of Ca^{2+} ionophores on AVP-dependent cyclic AMP accumulation and on $[\text{Ca}^{2+}]_i$ in the outer medullary collecting duct

Ca^{2+} ionophores induced a dose-dependent inhibition of AVP-stimulated cyclic AMP accumulation (Table 1). For a same concentration of Ca^{2+} ionophore, the level of inhibition was

more pronounced with ionomycin than with A23187. The response to 1 nM AVP was inhibited to a similar extent by 50 nM A23187 or 10 nM ionomycin (Table 1) and the percentage of inhibition observed in individual experiments ranged from about 40–70%. These values were similar to those previously observed with 0.3 μM PGE₂ (Chabardès *et al.*, 1990; Aarab *et al.*, 1993). Thus, concentrations of 10 nM ionomycin or 50 nM A23187 were used in the further experiments and compared to the effect elicited by 0.3 μM PGE₂.

The $[\text{Ca}^{2+}]_i$ variation induced by 10 nM ionomycin was characterized by a slow and progressive increase up to a maximal value which was maintained even after the end of ionomycin superfusion (Figure 1). The mean increase over basal value of $[\text{Ca}^{2+}]_i$ calculated at the end of a 3 min perfusion period with 10 nM ionomycin was of 50.4 ± 7.1 nM $[\text{Ca}^{2+}]_i$ ($n=11$ single OMCD microdissected from five kidneys). As previously described (Aarab *et al.*, 1993), a different pattern of $[\text{Ca}^{2+}]_i$ was elicited by PGE₂ which was characterized by a peak followed by a plateau (Figure 1). In a same OMCD sample, the response to ionomycin was not modified by a first superfusion with PGE₂ (Figure 1 and data not shown).

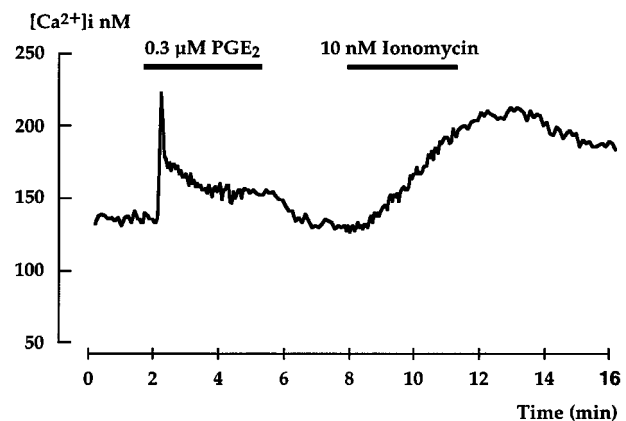


Figure 1 $[\text{Ca}^{2+}]_i$ variations induced by PGE₂ and ionomycin in the outer medullary collecting duct. Representative traces of $[\text{Ca}^{2+}]_i$ variations elicited by adding 0.3 μM PGE₂ and then 10 nM ionomycin in the superfusion medium. The superfusion period for each agent is indicated by the bar.

Table 1 Ca^{2+} ionophore-induced inhibition of AVP-dependent cyclic AMP accumulation in the rat OMCD

Experimental series	Cyclic AMP accumulation (fmol mm^{-1} 4 min^{-1})	P	% Inhibition of the response to AVP
1. $n=10$, AVP 1 nM	74.2 ± 6.1		
+ ionomycin 10 nM	31.3 ± 4.6	<0.001	58.0 ± 4.8
2. $n=5$, AVP 1 nM	42.0 ± 5.8		
+ ionomycin 50 nM	7.4 ± 0.8	<0.001	80.5 ± 4.1
3. $n=7$, AVP 1 nM	55.0 ± 12.9		
+ ionomycin 100 nM	10.5 ± 2.7	<0.01	81.5 ± 4.2
4. $n=10$, AVP 1 nM	61.0 ± 7.4		
+ A23187 50 nM	31.0 ± 5.9	<0.025	51.7 ± 4.1
5. $n=3$, AVP 1 nM	47.1 ± 4.1		
+ A23187 100 nM	15.3 ± 1.6	<0.005	69.0 ± 1.9

Each experimental series corresponds to different concentrations of either ionomycin or A23187. The incubation medium contained 50 μM Ro 20-1724. Data are the absolute mean values $\pm$ s.e.mean calculated from different experiments (n); *P* value compares the mean value obtained with either ionomycin or A23187 to the corresponding AVP mean value. In each experiment, the percentage of inhibition was calculated from the corresponding AVP mean value and the last column gives the mean values $\pm$ s.e.mean obtained from the different experiments.

Increases in $[Ca^{2+}]_i$ by ionomycin may induce activation of phospholipase A₂ and thus the formation of metabolites which might account for the observed inhibition. However, indomethacin, which prevents endogenous synthesis of PGE₂ by isolated segments, was present in all experiments (see Methods section). The further addition of quinacrine, a phospholipase A₂ inhibitor, did not affect the inhibition induced by 10 nM ionomycin ($57.6 \pm 8.9\%$ of inhibition and $57.6 \pm 12.2\%$ without and with the addition of 20 μ M quinacrine, respectively, $n=3$). These results suggest that the inhibitory effect observed with 10 nM ionomycin was not due to an indirect regulation mediated by arachidonic acid metabolites.

Comparison of the inhibitory properties induced by Ca²⁺ ionophores and PGE₂ on AVP-dependent cyclic AMP accumulation in the OMCD

The inhibition elicited by Ca²⁺ ionophores was no longer observed in OMCD studied in Ca²⁺-free medium. A23187 elicited $45.2 \pm 6.1\%$ of the response to AVP in 1 mM Ca²⁺ medium ($P < 0.005$ versus AVP) and $114.4 \pm 8.5\%$ (NS) in Ca²⁺-free medium, $n=6$; with ionomycin, the corresponding values observed were of $55.7 \pm 9.4\%$ ($P < 0.05$) and 88.0 ± 1.0 (NS), $n=3$. In the same experiments, PGE₂-induced inhibition was also dependent from extracellular Ca²⁺ ($31.0 \pm 2.2\%$ of the response to AVP ($P < 0.001$ and 98.3 ± 6.2 (NS) in 1 mM Ca²⁺ and Ca²⁺-free medium, respectively, $n=9$).

A potential role of the GTP-dependent protein G α_i was investigated by using PTX. In each experiment, OMCD samples were microdissected from the same rat kidney, then pre-incubated (2 h at 30°C) in the presence or not (control) of PTX and incubated as usual. This long pre-incubation period decreased AVP-stimulated cyclic AMP accumulation in control OMCD but PTX improved this response (Figure

2A). The inhibition induced by 50 nM A23187 was of $49.2 \pm 6.8\%$ and $58.2 \pm 10.8\%$ in PTX-treated and control OMCD, respectively ($n=3$, Figure 2B). Studied in parallel, PGE₂-induced inhibition was also slightly reduced but was still highly significant in each of the experiments performed ($73.3 \pm 3.3\%$ of inhibition and $63.0 \pm 4.9\%$ in control and PTX-treated OMCD, respectively, Figure 2B). In another experimental series, 10 nM ionomycin led to a comparable observation with an inhibition of $61.5 \pm 11.0\%$ and $66.7 \pm 15.9\%$ in control and PTX-treated OMCD, respectively ($n=3$). In contrast, the inhibitory effect induced by the α_2 -adrenergic agonist clonidine was relieved by pertussis toxin ($70.4 \pm 3.9\%$ of inhibition and $14.8 \pm 8.9\%$ in control and PTX-treated OMCD, respectively, $n=3$, Figure 2B).

Experiments of multiple combined inhibition were performed to compare the inhibition induced by either PGE₂ or a low concentration of A23187 to the inhibition elicited by the simultaneous addition of both agents. Although the concentration of Ca²⁺ ionophore used in these experiments did not induce a maximal inhibition (see Table 1), the validity of the summation experiments is fulfilled by the use of 0.3 μ M PGE₂, a concentration which elicits a maximal PGE₂-induced inhibition of AVP-dependent cyclic AMP accumulation in the rat OMCD (Chabardès *et al.*, 1990; Aarab *et al.*, 1993). Table 2 gives the detailed results obtained in a typical experiment with A23187 and PGE₂. The inhibition of AVP-dependent cyclic AMP accumulation induced by A23187 and PGE₂ alone was not different from the inhibition observed with the simultaneous addition of both agents. In five different experiments, both agents inhibited by $61.1 \pm 2.6\%$ the response to glucagon, an inhibition similar to the effect of A23187 alone ($49.8 \pm 5.5\%$) or PGE₂ alone ($60.0 \pm 5.4\%$). The theoretical value calculated assuming a cumulative inhibition hypothesis was different from the mean value obtained with the addition of both A23187 and PGE₂ (12.6 ± 2.13 fmol mm⁻¹ 4 min⁻¹

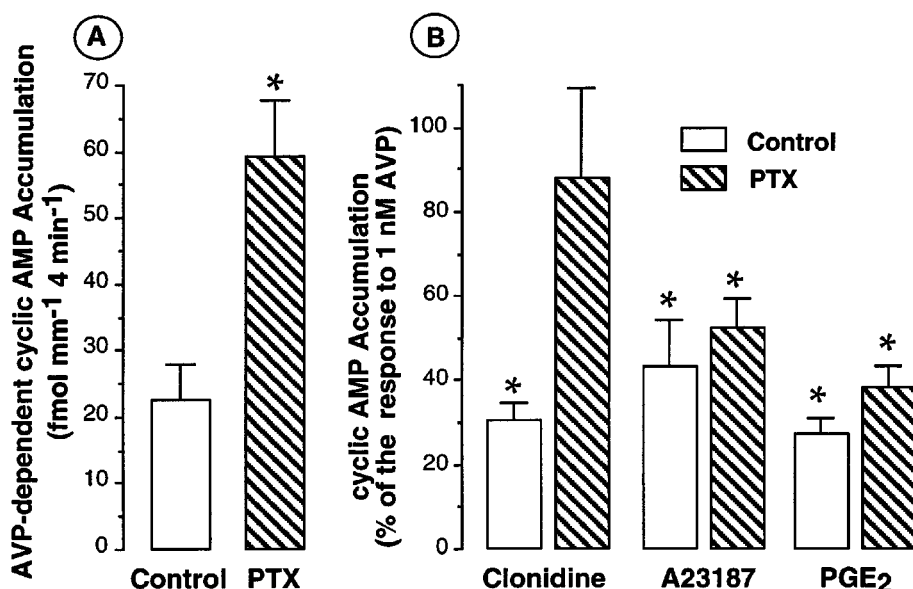


Figure 2 Effect of pertussis toxin on AVP-dependent cyclic AMP accumulation and on the inhibitory effect elicited by either clonidine, A23187 or PGE₂ in the rat OMCD. (A) Absolute values of cyclic AMP accumulation induced by 1 nM AVP in the presence of 50 μ M Ro 20-1724 in control and PTX-treated OMCD. Tubule samples were microdissected from the same kidney and preincubated 2 h at 30°C either in the control medium or in the presence of 500 ng ml⁻¹ PTX. Data are the mean values $\pm$ s.e. mean calculated from three experiments. * $P < 0.005$ or 0.001 in individual experiments when compared to the corresponding response obtained with AVP in control tubule samples. (B) In experiments presented in (A), the effect elicited by 1 μ M clonidine, 50 nM A23187 or 0.3 μ M PGE₂ on AVP-dependent cyclic AMP accumulation was tested in control and PTX-treated OMCD. Data are expressed in percentage of the corresponding response to 1 nM AVP and are given as the mean values $\pm$ s.e. mean; * $P < 0.005$ or 0.001 in individual experiments when compared to the corresponding response to AVP.

and 24.2 ± 1.7 , respectively, $n = 5$, $P < 0.01$). A23187 and PGE₂ therefore were effective by a same mechanism to decrease AVP-dependent cyclic AMP accumulation.

The role of $[Ca^{2+}]_i$ increases to account for PGE₂-induced inhibition was further studied in OMCD preincubated 30 min in the presence or not (control) of BAPTA (cell permeant form), a Ca^{2+} chelator which prevents agonists-stimulated increases in $[Ca^{2+}]_i$. PGE₂ induced $49.5 \pm 7.8\%$ of the response to 1 nM AVP ($P < 0.05$) and 104.2 ± 6.4 (NS) in control and 30 μ M BAPTA-AM-treated OMCD, respectively, $n = 5$.

Regulation of AVP-dependent cyclic AMP accumulation by the EP₁/EP₃ agonist of the PGE₂ receptor, sulprostone

Tested at different concentrations, sulprostone had a higher efficiency to inhibit AVP-dependent cyclic AMP accumulation in MTAL than in OMCD samples. Indeed, 1 nM sulprostone induced a maximal inhibition in the MTAL but had no effect in the OMCD (Figure 3). As estimated from these results, the concentration eliciting half-maximal inhibition of AVP-dependent cyclic AMP accumulation was of about 0.1 nM in the MTAL and 50 nM in the OMCD (Figure 3). In the same experiments, the addition of 0.3 μ M PGE₂ decreased cyclic AMP values to $8.4 \pm 1.2\%$ of the response to AVP ($n = 3$) and to $58.1 \pm 3.7\%$ ($n = 4$) in MTAL and OMCD, respectively. These values are similar to those induced by 0.1 μ M sulprostone ($10.7 \pm 3.3\%$ of the response to AVP and $60.9 \pm 6.7\%$ in MTAL and OMCD, respectively, Figure 3).

The pattern of $[Ca^{2+}]_i$ increase induced by 0.1 μ M Sulprostone in the OMCD was comparable to that observed with PGE₂. The mean increase over basal value was of 42.6 ± 5.3 nM $[Ca^{2+}]_i$ and 21.2 ± 1.7 nM at the peak and plateau phase, respectively ($n =$ five single tubules microdissected from two kidneys). The superfusion of 1 nM sulprostone induced a barely detectable peak of $[Ca^{2+}]_i$ (stimulated minus basal value = 14.3 ± 3.7 , $n = 5$) in the OMCD and had no effect on $[Ca^{2+}]_i$ variations in the MTAL.

Different experimental protocols were performed in the MTAL in order to define the properties of inhibition elicited by sulprostone and PGE₂: (i) the presence or not of Ca^{2+} in the incubation medium did not impair PGE₂-induced inhibition of the response to 10 nM AVP ($84.3 \pm 2.0\%$ of inhibition and $82.1 \pm 6.1\%$, in 1 mM Ca^{2+} and Ca^{2+} -free medium, respectively, $n = 5$). A similar result was obtained with 1 nM

sulprostone tested in some of these experiments ($83.4 \pm 2.3\%$ of inhibition and 77.8 ± 1.8 in 1 mM Ca^{2+} and Ca^{2+} -free medium, respectively, $n = 3$); (ii) a preincubation of MTAL samples during 4 h at 35°C with 500 ng ml⁻¹ PTX relieved the inhibition elicited by 1 nM sulprostone (Figure 4); (iii) in experiments of multiple combined inhibition, the simultaneous addition of 0.3 μ M PGE₂ and 1 nM sulprostone induced an inhibition similar to that elicited by PGE₂ alone (Table 3).

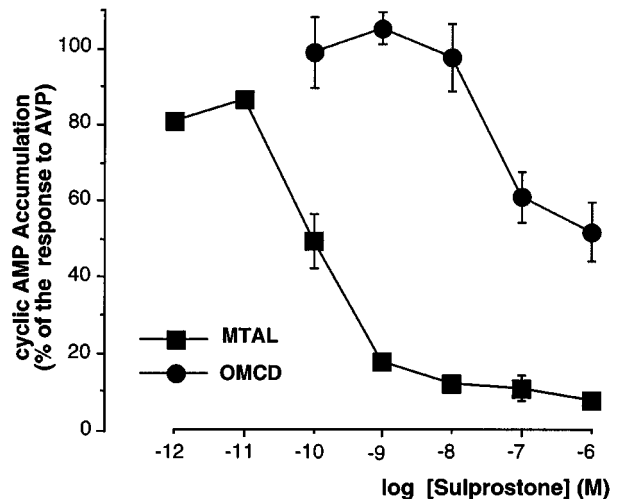


Figure 3 Dose-response curves of sulprostone-induced inhibition of AVP-dependent cyclic AMP accumulation in the rat MTAL and OMCD. Different concentrations of sulprostone were tested on cyclic AMP accumulation induced by 1 nM (OMCD) or 10 nM (MTAL) AVP in the presence of 50 μ M Ro 20-1724. The results are expressed in percentage of the corresponding response obtained with AVP. The data are the mean values $\pm$ s.e.mean calculated from three experiments in the MTAL and from four experiments in the OMCD. In each experiment, a statistical significant inhibition was observed from a concentration of either 0.1 nM in the MTAL ($P < 0.005$ – 0.001 versus the corresponding AVP value) or 0.1 μ M in the OMCD ($P < 0.025$ – 0.005).

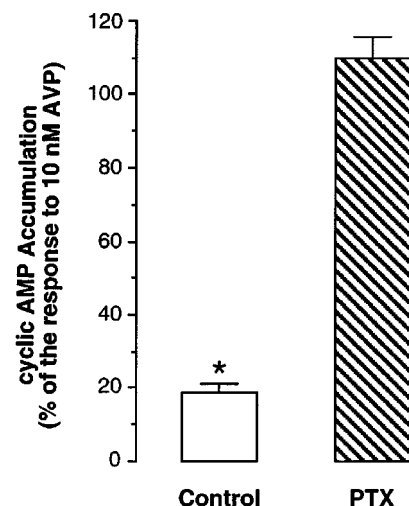


Figure 4 Effect of pertussis toxin on sulprostone-induced inhibition of AVP-dependent cyclic AMP accumulation in the MTAL. MTAL samples microdissected from the same kidney were preincubated 6 h at 35°C in the control medium or in the presence of 500 ng ml⁻¹ PTX. The effect elicited by 1 μ M sulprostone is expressed in percentage of the corresponding response to 10 nM AVP. Data are the mean values $\pm$ s.e.mean calculated from four experiments performed in the presence of Ro 20-1724. * $P < 0.01$ – 0.001 in individual experiments when compared to the corresponding response to AVP.

Table 2 Multiple combined inhibition of A23187 and PGE₂ on AVP-dependent cyclic AMP accumulation in the rat OMCD

Conditions	Cyclic AMP accumulation (fmol mm ⁻¹ 4 min ⁻¹)	% Inhibition of the response to AVP
AVP 1 nM	49.8 $\pm$ 3.6 (10)	
AVP + A23187 50 nM	23.4 $\pm$ 4.3 ^a (7)	53.0
AVP + PGE ₂ 0.3 μ M	27.2 $\pm$ 3.3 ^a (8)	45.4
AVP + A23187 + PGE ₂	23.1 $\pm$ 4.6 ^{a,b} (8)	53.6

Data are the means $\pm$ s.e.mean calculated from replicate samples (n) measured in a same experiment performed in the presence of 50 μ M Ro 20-1724. a: $P < 0.01$ when compared to the cyclic AMP level obtained with 1 nM AVP, b: not significant from the inhibition obtained with either A23187 or PGE₂. Assuming the hypothesis of a cumulative inhibition induced by the simultaneous addition of both agents (Aarab et al., 1993), the calculated theoretical value of the residual cyclic AMP and of the percentage of inhibition was of 12.8 fmol mm⁻¹ 4 min⁻¹ and 74.3%, respectively.

Effect of PGE₂ on glucagon dependent cyclic AMP synthesis in the outer medullary collecting duct

These experiments were performed in the presence of 1 mM IBMX so that the variations of intracellular cyclic AMP content reflected regulations prevailing only on cyclic AMP synthesis.

Glucagon-dependent cyclic AMP synthesis was measured in OMCD samples incubated in the presence of different extracellular Ca²⁺ concentrations (Table 4). The adenylyl cyclase sensitive to glucagon was lower in 2 mM Ca²⁺ medium as compared to 1 mM Ca²⁺ or Ca²⁺-free medium. In contrast to that observed in AVP-sensitive cells of the OMCD, glucagon-dependent cyclic AMP synthesis was inhibited with a similar efficiency by PGE₂ whatever the Ca²⁺ concentration present in microdissection and incubation media (Table 4). Tested in 1 mM Ca²⁺ medium, 1 nM sulprostone inhibited the response to glucagon to the same extent than 0.3 µM PGE₂ (57.7 ± 2.6% of inhibition versus 1 µM glucagon, *P* < 0.01, *n* = 3).

The transduction pathway mediated by PGE₂ was further studied on OMCD samples preincubated or not (control) with 500 ng ml⁻¹ PTX during 2 h at 30°C. The inhibition elicited by PGE₂ was markedly decreased in PTX-treated tubule samples (61.9 ± 5.4% of inhibition and 19.4 ± 6.0 in control and PTX-treated OMCD, respectively, Figure 5).

Table 3 Multiple combined inhibition with sulprostone and PGE₂ on AVP-dependent cyclic AMP accumulation in the rat MTAL

Conditions	Cyclic AMP accumulation (fmol mm ⁻¹ 4 min ⁻¹)	% Inhibition of the response to AVP
AVP 10 nM	41.9 ± 1.6	
AVP + Sulprostone 1 nM	4.9 ± 0.7 ^a	88.3 ± 3.6
AVP + PGE ₂ 0.3 µM	2.5 ± 0.2 ^a	94.1 ± 0.4
AVP + Sulprostone + PGE ₂	2.4 ± 0.2 ^{a,b}	94.2 ± 1.0 ^b

Data are the means ± s.e.mean calculated from three experiments performed in the presence of Ro 20-1724. The percentage of inhibition was calculated in each experiment from the corresponding AVP value and expressed as the means ± s.e.mean obtained from three experiments. ^a*P* < 0.001 when compared to the mean response obtained with 10 nM AVP; ^bassuming a hypothesis of cumulative inhibition induced by the simultaneous addition of both agents, the calculated theoretical value of the residual cyclic AMP and of the percentage of inhibition was of 0.3 ± 0.03 fmol mm⁻¹ 4 min⁻¹ and 99.3 ± 0.2%, respectively (*P* < 0.05 versus measured mean values).

Table 4 Regulation of glucagon-dependent cyclic AMP synthesis of PGE₂ in the outer medullary collecting tubule

Experimental conditions	Cyclic AMP (fmol mm ⁻¹ 4 min ⁻¹)		P	% Inhibition
	Glucagon	Glucagon + PGE ₂		
2 mM Ca ²⁺ (7)	20.2 ± 2.6*	9.4 ± 1.1	< 0.005	51.1 ± 4.8
1 mM Ca ²⁺ (4)	45.5 ± 10.1	16.1 ± 2.1	< 0.05	61.9 ± 5.4
Ca ²⁺ -free medium (4)	41.8 ± 4.4	21.7 ± 2.6	< 0.01	47.3 ± 6.2

OMCD samples were microdissected and incubated in either 2 mM Ca²⁺, 1 mM Ca²⁺ or Ca²⁺-free medium (0 Ca²⁺ + 0.1 mM EGTA) in the presence of 1 mM IBMX. The values obtained in Ca²⁺-free medium were obtained in OMCD samples studied in parallel in four of the seven experiments performed in 2 mM Ca²⁺ medium. In each experiment, the percentage of inhibition was calculated from the corresponding response obtained with glucagon. Data are the mean values ± s.e.mean calculated from different experiments (*n*). *P* refers to the comparison of the absolute values obtained with glucagon and glucagon + PGE₂; **P* < 0.01 and < 0.05 when compared to 1 mM Ca²⁺ and Ca²⁺-free medium, respectively.

Discussion

The different results presented in this study allow the further characterization of two different transduction pathways elicited by PGE₂ to inhibit hormone-dependent cyclic AMP accumulation along the rat renal tubule. The different properties of inhibition observed in this study and those previously obtained with PGE₂ in the rat OMCD and MTAL are summarized in Table 5.

In the AVP-sensitive cells of OMCD, PGE₂ and a low concentration of Ca²⁺ ionophore exhibited similar properties to inhibit a same pool of intracellular cyclic AMP content. Previous studies in the OMCD have shown that the response to forskolin is also inhibited by PGE₂ and that PGE₂-induced [Ca²⁺]_i increase consists of two phases, a release of intracellular Ca²⁺ and an entry of extracellular Ca²⁺ (Chabardès *et al.*, 1990; Aarab *et al.*, 1993). The different observations made support the conclusion that PGE₂-induced inhibition is linked to its capability to increase [Ca²⁺]_i in the AVP-sensitive cells of OMCD and is due mainly to cyclic AMP hydrolysis. In the glucagon-sensitive cells of OMCD and in the MTAL cells, PGE₂ decreased cyclic AMP synthesis through a process sensitive to PTX and independent from extracellular

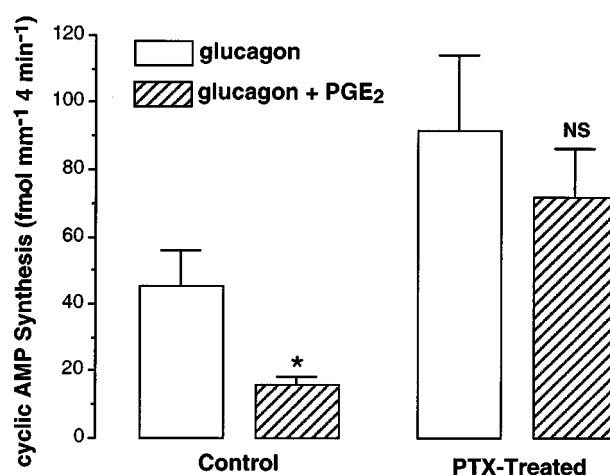


Figure 5 Effect of pertussis toxin on PGE₂-mediated inhibition of glucagon-dependent cyclic AMP synthesis. In each experiment, OMCD samples were microdissected from the same rat kidney, preincubated (2 h at 30°C) and incubated (4 min at 35°C) in 1 mM Ca²⁺ medium without (control) or with 500 ng ml⁻¹ PTX. Data are the mean values calculated from four experiments performed in the presence of 1 mM IBMX. *Refers to the effect of 0.3 µM PGE₂ on the cyclic AMP synthesis induced by 1 µM glucagon, *P* < 0.001 in each experiment; NS indicates that the effect of PGE₂ was not significantly different from the response to glucagon.

Table 5 Properties of inhibition of PGE₂ and/or sulprostone on hormone-dependent cyclic AMP content in AVP- and glucagon-sensitive segments

Properties of inhibition	OMCD: AVP-sensitive cells	OMCD: GLU-sensitive cells	MTAL cells
PGE ₂ -elicited inhibition	42–73% ^{a,b,c}	62% ^a	82–93% ^{a,d,e}
SUL-elicited inhibition	1 nM: no effect ^a 100 nM: 39% ^a	1 nM: 58% ^a 100 nM: NT	1 nM: 82% ^a 100 nM: 89% ^a
Ca ²⁺ -dependent inhibition	Yes ^{a,c}	No ^a	No ^a
PTX-sensitive inhibition	No ^{a,c}	Yes ^a	Yes ^{a,c}
PDEs-mediated inhibition	Yes ^b	No ^a	No ^d

The percentages of inhibition given in the table correspond to those found in the present study in 1 nM Ca²⁺ medium. These values are in the range of those previously published (see Ref. b, c, d and e listed below). The letters given refer to the following results: ^afrom the present study; ^bfrom Chabardès *et al.*, 1990; ^cfrom Aarab *et al.*, 1993; ^dfrom Torikai & Kurokawa, 1983; ^efrom Firsov *et al.*, 1995. OMCD: outer medullary collecting duct; MTAL: medullary thick ascending limb; GLU: glucagon; SUL: sulprostone; PTX: pertussis toxin; PDEs: phosphodiesterases; NT: not tested.

Ca²⁺ variations. The same properties were observed with sulprostone in MTAL and, in this segment, sulprostone and PGE₂ decreased a same pool of cyclic AMP content. These data therefore support an interaction of PGE₂ receptor with a GTP-dependent G α protein in the MTAL and the glucagon-sensitive cells of the OMCD.

The sensitivity of sulprostone to inhibit the response to AVP is much higher in MTAL than in OMCD. The concentration of sulprostone eliciting half-maximal inhibition is similar to that previously observed with PGE₂ in the MTAL (Torikai & Kurokawa, 1983; Firsov *et al.*, 1995) and the OMCD (Chabardès *et al.*, 1990). These observations suggest that, in the rat nephron, sulprostone is as effective as PGE₂ to decrease AVP-dependent cyclic AMP accumulation whatever the transduction pathway accounted for this regulation. An equipotent action of PGE₂ and sulprostone to inhibit AVP-induced cyclic AMP synthesis through a pertussis toxin-sensitive process has already been described in rabbit kidney cells (Sonnenburg & Smith, 1988). However, the higher sensitivity of inhibition induced by sulprostone and PGE₂ in MTAL than in OMCD suggests that these compounds have a higher potency to inhibit adenylyl cyclase activity than to elicit [Ca²⁺]_i increases in renal segments. Although different concentrations of sulprostone were not tested in our experiments, the potency of sulprostone to inhibit glucagon-stimulated cyclic AMP synthesis in the OMCD appeared comparable to that observed in the MTAL cells.

The different transduction pathways elicited by PGE₂ in MTAL and OMCD may result from the interaction of PGE₂ to different subtypes of PGE₂ receptor (Coleman *et al.*, 1994). In different species, both EP₁ and EP₃ subtype mRNAs have been detected in the collecting duct and only EP₃ subtype mRNA in the thick ascending limb (Breyer *et al.*, 1993; 1996; Sugimoto *et al.*, 1994; Takeuchi *et al.*, 1994; Taniguchi *et al.*, 1994). The expression of EP₁ subtype mRNA in transfected cells or *Xenopus* oocytes results in [Ca²⁺]_i increases but not stimulation or inhibition of adenylyl cyclase activity (Funk *et al.*, 1993; Watabe *et al.*, 1993). Multiple isoforms of the PGE₂ receptor EP₃ subtype mRNA have been cloned and one given isoform can be coupled to multiple second messenger systems (Coleman *et al.*, 1994; An *et al.*, 1994; Kotani *et al.*, 1995; Schmid *et al.*, 1995; Boie *et al.*, 1997). Most EP₃ isoforms decrease cyclic AMP content through a pertussis toxin-sensitive inhibition of adenylyl cyclase activity (Coleman *et al.*, 1994; An *et al.*, 1994; Kotani *et al.*, 1995; Schmid *et al.*, 1995). PGE₂ and Sulprostone have a higher affinity to bind to EP₃ than to EP₁ subtype (Coleman *et al.*, 1994; Boie *et al.*, 1997; Kiriya *et al.*, 1997). In addition, tested on transduction pathways, a higher potency of sulprostone and

PGE₂ is observed in cells transfected with the rat EP₃ subtype as compared to the EP₁ subtype transfected cells (Boie *et al.*, 1997). Thus, the localization of EP subtypes in the kidney, the signalling properties of PGE₂ observed in our experiments and their comparison with the functional expression of EP₁ or EP₃ subtype in transfected cells suggest a different coupling of PGE₂ along the rat renal tubule, i.e. to an EP₁ subtype in the AVP-sensitive cells of the OMCD, and to an EP₃ subtype in the glucagon-sensitive cells of the OMCD and the MTAL cells. The observations made with sulprostone and PGE₂ in MTAL suggest that these agents interact with an EP₃ isoform not associated to [Ca²⁺]_i increases. In agreement with this hypothesis, PGE₂ inhibits adenylyl cyclase activity and does not increase [Ca²⁺]_i in the cortical portion of the rat thick ascending limb (De Jesus Ferreira *et al.*, 1998).

The regulation elicited by PGE₂ on the response to AVP in the rat collecting duct differs from the observations made in the rabbit collecting duct. Indeed, in the cortical portion (CCD) of the rabbit collecting duct, PGE₂ stimulates a pertussis toxin-sensitive pathway which inhibits AVP-stimulated cyclic AMP production (Griffiths *et al.*, 1993) and AVP-dependent permeability to water (Hébert *et al.*, 1993). In the same segment, PGE₂ decreases sodium reabsorption through a second pathway, Ca²⁺-dependent and pertussis toxin-insensitive (Hébert *et al.*, 1993) but the precise cell type involved in this regulation remains to be defined. In the rat CCD, PGE₂ does not regulate either AVP-dependent cyclic AMP content (Chabardès *et al.*, 1990) or AVP-stimulated transepithelial water permeability or sodium transport (Chen *et al.*, 1991). In the rat therefore, PGE₂ inhibits the response to AVP in more terminal portions of collecting duct and this action is not mediated through a G α i-dependent inhibition of adenylyl cyclase activity. In contrast to the species differences observed in the AVP-sensitive cells of the collecting duct, PGE₂-mediated pertussis toxin-sensitive inhibition of adenylyl cyclase activity is observed in MTAL isolated from all species studied up to now, i.e. the rat, the mouse (Takaichi & Kurokawa, 1988) and the rabbit (Griffiths *et al.*, 1993).

PGE₂ and a low concentration of sulprostone also inhibited adenylyl cyclase activity in glucagon-sensitive cells of rat OMCD. A similar effect of PGE₂ has been previously observed in the isoproterenol-sensitive cells of rat CCD (Chabardès *et al.*, 1990). Glucagon- and isoproterenol-stimulated cyclic AMP synthesis are located in intercalated cells which are involved in proton secretion and/or bicarbonate reabsorption (Madsen & Tisher, 1986; Morel & Doucet, 1986). These observations therefore support a regulatory role of PGE₂ in the control of acid-base balance, in addition to its well known inhibitory effect on the urinary concentration processes (Stokes, 1981).

In summary, this study demonstrates a differential cell-specific localization along the rat nephron of the transduction pathways elicited by PGE₂ to inhibit hormone-dependent cyclic AMP accumulation. An interaction of PGE₂ with G α i-coupled inhibition of adenylyl cyclase is observed in MTAL cells and the glucagon-sensitive cells of the OMCD. In AVP-sensitive cells of the OMCD, the decrease of cyclic AMP content results from PGE₂-mediated [Ca²⁺]_i increases through a process insensitive to PTX. Compared to the low sensitivity of PGE₂ in the OMCD (Chabardès *et al.*, 1990), the high sensitivity of PGE₂ (Torikai & Kurokawa, 1983; Firsov *et al.*, 1995) and sulprostone (this study) to inhibit hormone-

dependent cyclic AMP synthesis in the MTAL suggest that low concentrations of PGE₂ can be very effective to regulate physiological functions of the rat thick ascending limb.

We are grateful to N. Griffiths and A. Doucet for critical advice and careful review of this manuscript and to M. Imbert-Teboul for her valuable advice in intracellular Ca²⁺ measurement. This work was supported by grants from the Centre Nationale de la Recherche Scientifique (URA 1859) and from the CEA (DBCM, SBCe). L. Aarab was supported in part by a grant from the Commissariat à l'Energie Atomique.

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(Received August 31, 1998

Revised October 23, 1998

Accepted November 26, 1998)



Characterization of high affinity neurotensin receptor NTR1 in HL-60 cells and its down regulation during granulocytic differentiation

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- 1 We investigated responses to neurotensin in human promyelocytic leukaemia HL-60 cells.
- 2 Neurotensin increased the cytosolic calcium concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner and also produced inositol 1,4,5-trisphosphate ($InsP_3$).
- 3 Among the tested neurotensin analogues, neurotensin 8-13, neuromedin-N, and xenopsin also increased $[Ca^{2+}]_i$, whereas neurotensin 1–11 and neurotensin 1–8 did not elicit detectable responses.
- 4 SR48692, an antagonist of NTR1 neurotensin receptors, blocked the neurotensin-induced $[Ca^{2+}]_i$ increase, whereas levocabastine, which is known as an NTR2 neurotensin receptor antagonist, did not attenuate the neurotensin-evoked effect.
- 5 The expression of NTR1 neurotensin receptors was confirmed by Northern blot analysis and reverse transcriptase-polymerase chain reaction (RT–PCR).
- 6 During 1.25% dimethylsulfoxide (DMSO)-triggered granulocytic differentiation of HL-60 cells, the neurotensin-induced $[Ca^{2+}]_i$ rise became gradually smaller and completely disappeared 4 days after treatment with DMSO. The mRNA level for neurotensin receptors was also decreased after differentiation.
- 7 The results show that HL-60 cells express NTR1 neurotensin receptors and suggest that granulocytic differentiation involves transcriptional regulation of the receptors resulting in down-regulation of the neurotensin-induced signalling.

Keywords: Neurotensin; cytosolic Ca^{2+} ; phospholipase C; human hemopoietic cells; differentiation

Introduction

Neurotensin, the tridecapeptide ELYENKPRRPYIL, has been studied as a modulator in neuronal and non-neuronal systems (Kitabgi *et al.*, 1985; Vincent, 1995). Neurotensin reveals its modulatory effect in almost every region of the nervous system including the cerebral cortex, terminals of striatal nucleus, hypothalamus, midbrain, cerebellar purkinje cells, retina, and spinal cord (Shi & Bunney, 1992a). In the central nervous system neurotensin is known to modulate the signals of dopaminergic neurons by regulating D_2 receptors. Therefore, neurotensin displays activities similar to antipsychotics in clinical trials involving schizophrenia, Parkinson's disease, and Alzheimer's disease (Nemeroff *et al.*, 1992). It has been also reported that neurotensin potentiates the barbiturate- and ethanol-induced sedation and that it induces antinociception, catalepsy, and hypothermia.

For non-neuronal systems, major roles of neurotensin have been demonstrated in the gastrointestinal and cardiovascular system. Neurotensin is localized at the intestinal mucosa (endocrine N-cells) where it is thought to be involved in intestinal contraction (Buhner & Ehrlein, 1989). Intravenous injection of neurotensin changes the concentration of insulin, glucagon, and pituitary hormones including thyroid releasing hormone (Kitabgi *et al.*, 1985; Schimpff *et al.*, 1995). It also induces vasodilatation and affects vascular permeability by way of vascular, portal vein, and smooth muscle contraction (Bachelard *et al.*, 1992). The

neurotensin effect on the cardiovascular system somewhat depends on the species, because neurotensin causes hypotension in dogs but hypertension in guinea pigs. It has been reported that endothelial cells of the umbilical vein also express the neurotensin receptor which is involved in the increase of intracellular Ca^{2+} (Schaeffer *et al.*, 1995) and the production of prostacyclin (Schaeffer *et al.*, 1997). However, it is not yet known what role neurotensin plays in hemopoietic or immune cells.

Neurotensin evokes two major actions at the cellular level. It causes inositol 1,4,5-trisphosphate ($InsP_3$) production and increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) through the activation of phospholipase C (PLC) (Goedert *et al.*, 1984; Bozou *et al.*, 1989; Hermans *et al.*, 1992). In another signalling pathway, neurotensin modulates the intracellular cyclic nucleotide level including cyclic GMP (Gilbert & Richelson, 1984; Amar *et al.*, 1985) and cyclic AMP (Bozou *et al.*, 1986; Yamada *et al.*, 1994).

HL-60 cells have served as a good model in which to study signal transduction of various pharmacological receptors before and after differentiation of the hemopoietic cells (Klinker *et al.*, 1996). HL-60 cells can be forced to terminally differentiate into neutrophil-like cells by dibutyl cyclic AMP, DMSO, or retinoic acid treatment and into adherent macrophage-like cells by 1, 25-dihydroxy vitamin D_3 and phorbol esters (Klinker *et al.*, 1996; Collins *et al.*, 1978).

Here we report the expression of PLC-coupled neurotensin receptors in HL-60 human promyelocytic leukaemia cells and the down-regulation of neurotensin-induced signalling during granulocytic differentiation into neutrophil-like cells.

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Methods

Cell culture

HL-60, U937, K562, Jurkat T, H9, and U266 cells were maintained at 37°C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated bovine calf serum and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed daily. We induced differentiation of HL-60 cells by incubating them in 1.25% DMSO for 6 days. We observed the morphology of the cells and monitored fMLP-induced [Ca²⁺]_i increase, which was only detectable in differentiated HL-60, as indication of differentiation into neutrophil-like cells. We also counted viable cells by the Trypan-blue exclusion method.

Isolation of human neutrophils

Human neutrophils were isolated from the peripheral blood of healthy donors as previously described (Pember *et al.*, 1983). Isolated cells were suspended in phosphate buffered saline/glucose (in mM: KCl 2.6, KH₂PO₄ 1.5, MgCl₂ 0.5, NaCl 136, Na₂PO₄ 8 and glucose 5.5, pH 7.4) and used immediately.

Measurement of [Ca²⁺]_i

The level of intracellular Ca²⁺ was determined using fura-2 tetraacetoxymethyl ester (fura-2/AM) as we have previously described (Choi & Kim, 1997). Briefly, the cell suspension was incubated in fresh serum-free RPMI 1640 medium with 3 µM fura-2/AM at 37°C for 60 min with continuous stirring. Sulphinpyrazone (250 µM) was added to prevent dye leakage. Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and 380 nm and emission wavelength of 500 nm. The calibration of the fluorescence signal in terms of [Ca²⁺]_i was performed according to Grynkiewicz *et al.* (1985).

Measurement of InsP₃ production

InsP₃ production was determined by competition assay with [³H]-InsP₃ as described previously (Lee *et al.*, 1997). To determine InsP₃ production, 1 × 10⁶ cells were stimulated with drugs. The reaction was terminated by addition of ice-cold 5% trichloroacetic acid containing 10 mM EGTA. The supernatant of the cell lysate was saved and the trichloroacetic acid was removed by extraction with diethyl ether. The aqueous fraction was neutralized with 200 mM Trizma base and adjusted to pH 7.4. 20 µl of extract was added to 20 µl of assay buffer (100 µM Tris buffer containing 4 mM EDTA) and 20 µl [³H]-InsP₃ (0.1 µCi ml⁻¹). The mixture was incubated for 15 min on ice and then centrifuged at 2,000 × *g* for 10 min. One hundred µl water and 1 ml liquid scintillation cocktail were added to the pellet to measure the radioactivity. The InsP₃ concentration in the sample was determined by comparison to a standard curve and expressed as pmol mg⁻¹ of protein. The total cellular protein concentration was measured by the Bradford method (Bradford, 1976) after sonication of 1 × 10⁶ cells.

Measurement of cyclic AMP

Intracellular cyclic AMP was determined by measuring the formation of [³H]-cyclic AMP from [³H]-adenine nucleotide pools as previously described (Choi & Kim, 1997). Briefly, cells were loaded with [³H]-adenine (1 µCi ml⁻¹) in complete

medium for 24 h. After the loading, the cells were washed two times with Locke's solution (in mM: NaCl 154, KCl 5.6, MgCl₂ 1.2, CaCl₂ 2.2, HEPES 5, and glucose 10, pH 7.3). 1 × 10⁶ cells were aliquoted into Eppendorf tubes for stimulation. The stimulation reaction was stopped by addition of 1 ml ice-cold 5% (v/v) trichloroacetic acid containing 1 µM unlabelled cyclic AMP. [³H]-cyclic AMP and [³H]-ATP were separated using sequential chromatography on Dowex AG50W-X4 (200–400 mesh) cation exchanger and a neutral alumina column. The increase in intracellular cyclic AMP concentration was presented as [³H]-cyclic AMP/([³H]-ATP + [³H]-cyclic AMP) × 10³.

RNA isolation and RT-PCR

Total cellular RNA was prepared from undifferentiated and DMSO-induced differentiated HL-60 cells by the method of Chomczynski & Sacchi (1987). Poly(A)⁺-RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography (Boehringer Mannheim). One µg of poly(A)⁺ mRNA was used as template for the first-strand cDNA synthesis using SUPERScript II reverse transcriptase (GIBCO-BRL) in a final volume of 40 µl supplemented with 50 ng of random hexamer and 500 ng of oligo(dT)12-18. Three µl of the reverse transcriptase reaction mixture was amplified with 20 pmol of subtype specific oligonucleotide primers (human NTR1: 1090-1732, 25mer; 5-aaCACCTTCATGTCCTTCATATTCC; 3-gcggtatccTCTGCCTGACCCCCATC; Vita *et al.*, 1993) using Pfu polymerase. The cycle profile was as follows: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 35 cycles, and finally, a 10-min extension at 72°C. The PCR products and 1 Kb ladder (GIBCO-BRL) were electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide. The DNA fragments were inserted into the *Hinc*II site of the pBluescript II SK(+) vector and sequenced to confirm the authenticity of the neurotensin receptor gene.

Northern blot analysis

Poly (A)⁺-RNA (10 µg per lane) was resolved by electrophoresis through 1% agarose gel containing 660 mM formaldehyde and transferred to a nylon membrane (ICN, East Hills, NY). The blot was hybridized with each human neurotensin receptor cDNA probe (listed above) labelled by random primer extension with [α -³²P]dCTP and Klenow enzyme. The hybridization was performed at 65°C in a solution containing 10% polyethylene glycol, 7% SDS, EDTA 10 mM, NaCl 250 mM, Na₂HPO₄ 85 mM (pH 7.2), denatured salmon sperm DNA (100 µg ml⁻¹) and the radiolabelled probes (5 × 10⁵ c.p.m. ml⁻¹). After hybridization, the blot was washed twice in 1 × saline-sodium citrate (SSC; 300 mM NaCl and 30 mM sodium citrate) solution containing 0.1% SDS at room temperature, three times in 0.2 × SSC with 0.1% SDS at 65°C, twice in 0.1 × SSC at room temperature. The membrane was later reprobbed with the cDNA of the human ribosomal large subunit protein 32 (a gift from Dr H.S. Shin) as a control.

Materials

RPMI 1640 and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, U.S.A.). Bovine calf serum was obtained from HyClone Laboratories (Logan, UT, U.S.A.). Neurotensin, neurotensin fragments 1–8, 1–11 and 8–13, neuromedin N, xenopsin, cyclic AMP, EGTA, trichloroacetic acid, isobutyl methyl xanthine (IBMX), fMLP, DMSO, and sulphinpyrazone were purchased from Sigma Chemical Co.

(St. Louis, MO, U.S.A.). Ionomycin and PMA were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). [^3H]-adenine and [^3H]-InsP₃ were purchased from NEN (Boston, MA, U.S.A.). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). Levocabastine (Schotte *et al.*, 1986) was a gift from Dr Marcel Janssen (Janssen Research Foundation) and SR48692 (Gully *et al.*, 1993) was a gift from Dr Danielle Gully (Sanofi Recherche).

Analysis of data

All quantitative data are expressed as the means $\pm$ s.e.mean. The results were analysed for differences using unpaired Student's *t*-test. We calculated half maximal effective concentration (EC₅₀) and half maximal inhibitory concentration (IC₅₀) with the AllFit program (De Lean *et al.*, 1978).

Results

We examined the neurotensin-induced responses in HL-60 cells. Neurotensin (300 nM) triggered the [Ca^{2+}]_i rise in the presence or absence of extracellular Ca^{2+} in fura-2-loaded undifferentiated cells (Figure 1A). Neurotensin elevated the

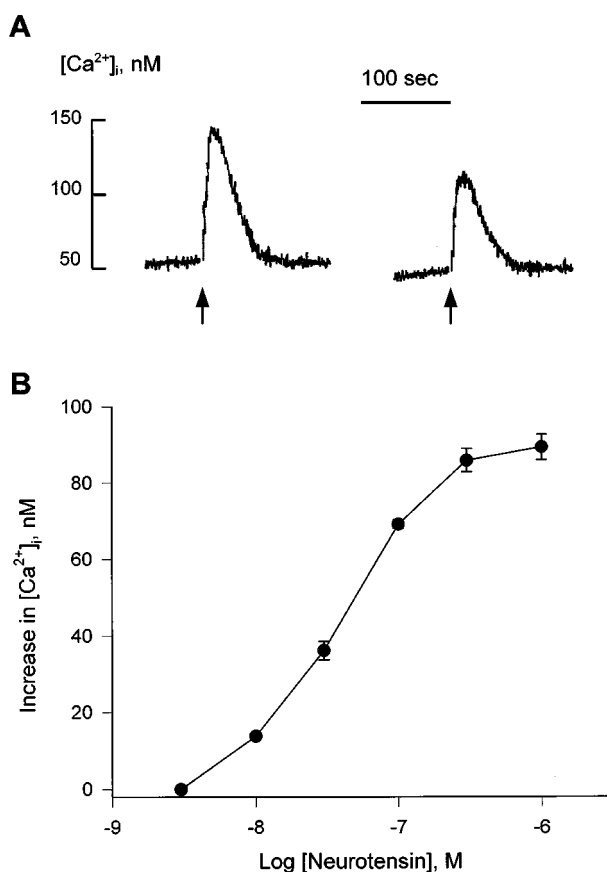


Figure 1 Neurotensin-induced increase in cytosolic Ca^{2+} in undifferentiated HL-60 cells. (A) Fura-2/AM-loaded HL-60 cells were challenged with 300 nM neurotensin with (left) or without (right) 2.2 mM extracellular Ca^{2+} . The level of cytosolic Ca^{2+} was measured as described in Methods. A typical trace from more than 20 separate experiments is shown. (B) Fura-2/AM-loaded HL-60 cells were treated with the indicated concentrations of neurotensin. The peak height of each stimulation was monitored. Each result is the means $\pm$ s.e.mean from triplicate assays. The result is representative of five separate experiments. The results were reproducible.

[Ca^{2+}]_i in a concentration-dependent manner at maximal and half-maximal effective concentrations (EC₅₀) of 300 and 38.5 ± 3.0 nM, respectively (Figure 1B). We tested the production of InsP₃ upon neurotensin treatment, because neurotensin elicited [Ca^{2+}]_i rise in the absence of extracellular Ca^{2+} . Figure 2 shows that treatment with 300 nM neurotensin evoked production of InsP₃ with the maximum effect seen 15 s after treatment.

To compare the potency of neurotensin analogues in elevating [Ca^{2+}]_i, we used several neurotensin peptide fragments and related peptide hormones. As shown in Figure 3, neurotensin fragment 8-13, neuromedin N, and xenopsin increased the [Ca^{2+}]_i with an EC₅₀ of 9.9 ± 3.2 nM, 40.2 ± 8.3 nM and 37.5 ± 4.8 nM, respectively. However, neurotensin fragments 1-8 and 1-11 did not elicit an increase in [Ca^{2+}]_i at concentrations up to 10 μM . We further tested whether the neurotensin response overlapped the responses elicited by other peptide hormones. Among the tested peptide hormones bradykinin, substance P, angiotensin II, somatostatin, galanin, vasoactive intestinal peptide (VIP), cholecystokinin, and endothelin did not change the [Ca^{2+}]_i, but thrombin did increase the [Ca^{2+}]_i in HL-60 cells (Table 1). However, thrombin pretreatment did not block a subsequent response to neurotensin. In addition, 300 μM extracellular ATP, 300 μM histamine, 3 μM platelet-activating factor, and 100 nM small peptide WKYMVm (Seo *et al.*, 1997), which are all known to elicit [Ca^{2+}]_i rise in HL-60 cells, also did not overlap with neurotensin in its [Ca^{2+}]_i elevating effect (data not shown). The results suggest that neurotensin has its own receptor on HL-60 cells and elevates [Ca^{2+}]_i through activation of PLC.

To identify the neurotensin receptor subtype expressed on HL-60 cells, subtype-specific antagonists were tested. It is generally accepted that SR48692 (Gully *et al.*, 1993) and levocabastine (Schotte *et al.*, 1986) block the neurotensin receptors NTR1 and NTR2, respectively. SR48692 inhibited the neurotensin-induced [Ca^{2+}]_i increase in a concentration-

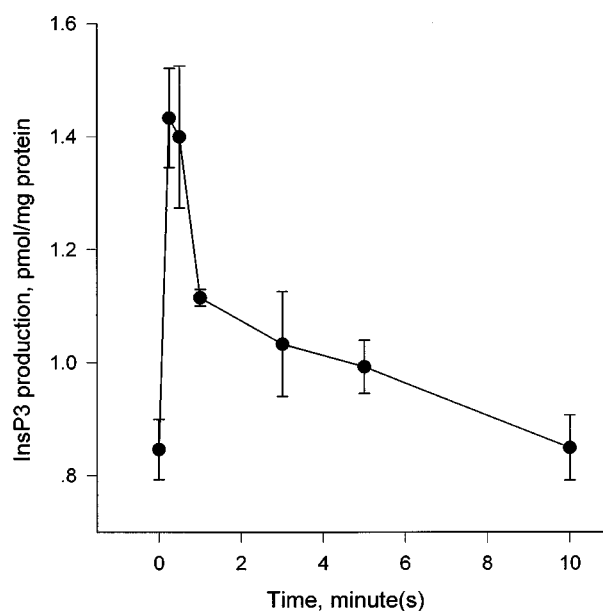


Figure 2 Neurotensin-induced InsP₃ generation in undifferentiated HL-60 cells. Cells were stimulated with 300 nM neurotensin for the indicated times. The levels of InsP₃ produced were measured as described in Methods. Each point is the means $\pm$ s.e.mean of triplicate measurements. The result is representative of six separate experiments and were reproducible.

dependent manner with an IC_{50} of 14.0 ± 0.2 nM (Figure 4). However, levocabastine did not inhibit the neurotensin-induced effect even at millimolar concentrations. The results suggest that HL-60 cells express NTR1 neurotensin receptors.

It has been reported that neurotensin receptors are also coupled to adenylyl cyclase (Yamada *et al.*, 1994). So we tested whether neurotensin regulates cyclic AMP signalling in HL-60 cells. 300 nM neurotensin did not have any effect on cyclic AMP production, whereas prostaglandin E_2 did increase the cyclic AMP level (Figure 5A). In addition, simultaneous treatment or pretreatment with neurotensin did not affect the prostaglandin E_2 -induced cyclic AMP formation (Figure 5B).

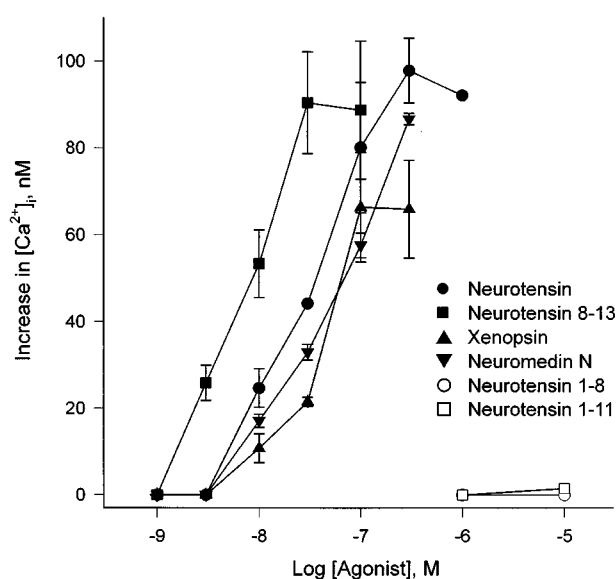


Figure 3 Concentration dependent increases in cytosolic Ca^{2+} by neurotensin and its analogues. Undifferentiated HL-60 cells were treated with various concentrations of neurotensin, neurotensin 8–13 fragments, 1–11 fragments, 1–8 fragments, neuromedin N, and xenopsin. The peak height of each stimulation was monitored. Each result is the means $\pm$ s.e. mean from triplicate assays. The result is representative of four separate experiments. The results were reproducible.

Table 1 Cytosolic Ca^{2+} -mobilizing activity of major peptide agonists in HL-60 cells

Peptide (concentration)	Changes in $[Ca^{2+}]_i$	Cross-reactivity with neurotensin (300 nM)
Bradykinin (10 μ M)	ND	ND
Substance P (10 μ M)	ND	ND
fMLP (1 μ M)	ND*	ND
Thrombin (50 U ml $^{-1}$)	Increase	ND
Angiotensin II (10 μ M)	ND	ND
Somatostatin (1 μ M)	ND	ND
Galanin (10 nM)	ND	ND
VIP (100 nM)	ND	ND
Cholecystokinin (300 nM)	ND	ND
Endothelin (20 μ M)	ND	ND

Fura-2/AM-loaded undifferentiated HL-60 cells were challenged with the indicated concentrations of peptide agonist. The experiments were performed more than four times independently and the results were reproducible. VIP, vasointestinal peptide; ND, not detected. *Increase in $[Ca^{2+}]_i$ was detected in the DMSO-triggered differentiated HL-60 cells.

We further analysed changes in the neurotensin-induced effect during DMSO-induced differentiation. HL-60 cells had morphologically and physiologically differentiated into neutrophil-like cells after incubation with 1.25% DMSO for 6 days (so called granulocytic differentiation; Collins *et al.*,

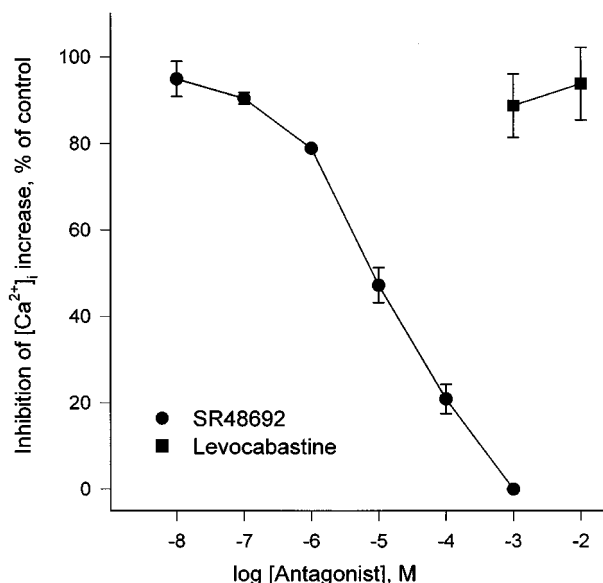


Figure 4 The effects of SR48692 and levocabastine on neurotensin-induced $[Ca^{2+}]_i$ rise in HL-60 cells. SR48692 or levocabastine were added to fura-2-loaded cells at the indicated concentrations. After 3 min the cells were challenged with 300 nM neurotensin. The peak heights of neurotensin-induced $[Ca^{2+}]_i$ rise were monitored. The experiments were performed three times independently, and the results were reproducible. Each result is the means $\pm$ s.e. mean from triplicate assays.

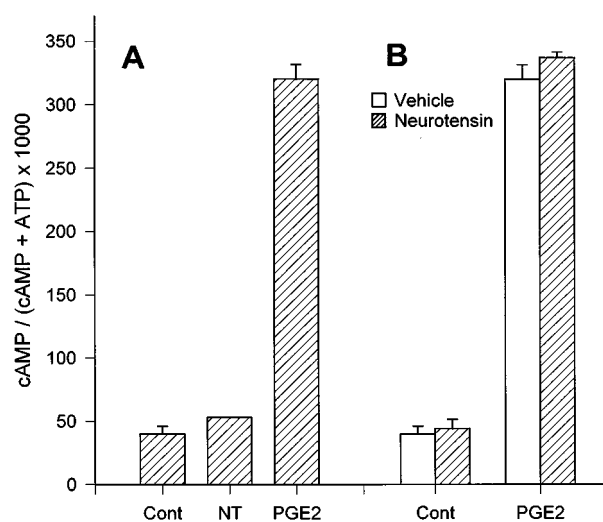


Figure 5 Lack of neurotensin-induced changes in cyclic AMP level in HL-60 cells. (A) $[^3H]$ -adenine-loaded cells were preincubated with 1 mM IBMX for 15 min, then stimulated for 15 min with 300 nM neurotensin (NT) or 10 μ M prostaglandin E_2 (PGE2) in the presence of 1 mM IBMX. (B) Cells were preincubated with or without 300 nM neurotensin, then challenged with 10 μ M prostaglandin E_2 in the presence of 1 mM IBMX. The levels of cyclic AMP produced were measured as described in Methods. We expressed the cyclic AMP level as cyclic AMP/(cyclic AMP + ATP) $\times$ 1000. Each point is the means $\pm$ s.e. mean of triplicate measurements. The result is representative of six separate experiments. The results were reproducible.

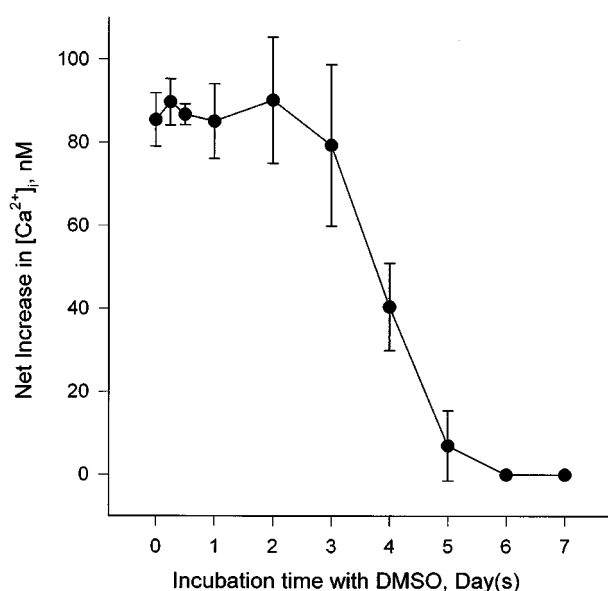


Figure 6 Changes in neurotensin-induced $[Ca^{2+}]_i$ increase in HL-60 cells during DMSO-induced differentiation. Undifferentiated HL-60 cells were cultured with 1.25% DMSO containing medium for the indicated times, then 300 nM neurotensin-induced $[Ca^{2+}]_i$ rise was examined. The peak height of the $[Ca^{2+}]_i$ increase was recorded. The experiments were performed three times independently, and the results were reproducible. Each result is the means $\pm$ s.e. mean of triplicate assays.

1978). In the neutrophil-like HL-60 cells a neurotensin-induced $[Ca^{2+}]_i$ increase was not detected. During the time course of the DMSO incubation the extent of the neurotensin-induced $[Ca^{2+}]_i$ increase became gradually smaller and disappeared completely after 4 days of incubation with DMSO (Figure 6).

To confirm the expression and down-regulation of the neurotensin receptor in differentiated cells, PCR with specific probes (Vita *et al.*, 1993) after a reverse transcriptase reaction with purified mRNA of HL-60 cells and Northern blot analysis were carried out. A specific DNA band was obtained, and the DNA fragment was confirmed by the digestion patterns obtained after reactions with *HinfI* (358 and 284 bp) and *XhoI* (467 and 175 bp) and by direct sequencing. The results clearly indicate that HL-60 cells express NTR1 neurotensin receptors (Figure 7A). The Northern blot analysis showed that the amount of neurotensin receptor mRNA in 6-day differentiated cells dramatically decreased in comparison to untreated control cells (Figure 7B). The result suggests that the signalling of neurotensin is down-regulated *via* the reduction of receptor expression during granulocytic differentiation.

Furthermore, we tested the neurotensin signalling in hemopoietic cell lineages of various types of human lymphocytes or cell lines. As shown in Table 2, we could detect a neurotensin-evoked $[Ca^{2+}]_i$ rise in human histiocytic leukaemia U937 cells which are closely related to HL-60 cells in their differentiation phylogeny. However, there was no detectable Ca^{2+} mobilization in isolated human neutrophils which are the final form of granulocytic differentiation. In addition, human myelogenous leukaemia K562 cells, human T cell leukaemia Jurkat T cells and H9 cells, and human B lymphoma U266 cells, which are all of a distinct differentiation lineage, did not exhibit response to neurotensin treatment.

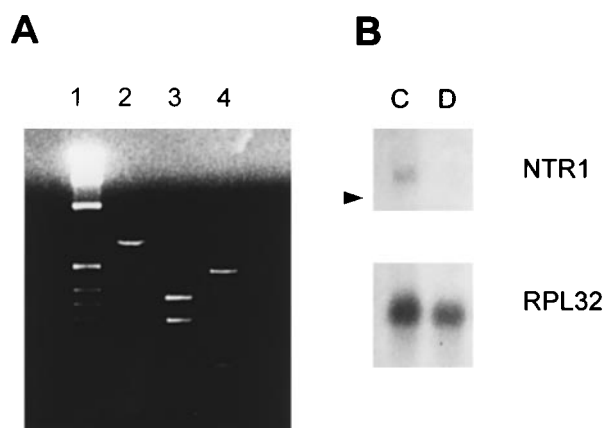


Figure 7 RT-PCR and Northern blot analysis of the expression of neurotensin receptors in undifferentiated and DMSO-induced differentiated HL-60 cells. (A) RNA extracted from undifferentiated HL-60 cells were reverse-transcribed into cDNA. Then amplification was performed using NTR1 neurotensin receptor specific primers and Pfu polymerase as described in Methods. Lane 1, 1 kb ladder; lane 2, PCR product (642 bp); lane 3, PCR product digested with *HinfI* (358 and 284 bp); lane 4, PCR product digested with *XhoI* (467 and 175 bp). (B) Poly(A)⁺-RNA (10 μ g per lane) from control (lane C) and DMSO-induced differentiated HL-60 cells (lane D) was electrophoresed, transferred to a membrane, and hybridized with the human NTR1 neurotensin receptor cDNA (NTR1) for Northern blot analysis. The position of the 28S ribosomal RNA is indicated by an arrowhead. The human ribosomal large subunit protein 32 (RPL32) was used as a marker for RNA level.

Table 2 The neurotensin-triggered Ca^{2+} responses of cells in hemopoietic lineage

Cells in hemopoietic lineage	Changes in $[Ca^{2+}]_i$
HL-60 (human promyelocytic leukaemia cells)	Increase
U937 (human histiocytic leukaemia cells)	Increase
Human neutrophils	ND
K562 (human myelogenous leukaemia cells)	ND
Jurkat T (human T cell leukaemia cells)	ND
H9 (human T cell leukaemia cells)	ND
U266 (human B lymphoma cells)	ND

Fura-2/AM-loaded cells were challenged with 300 nM neurotensin, and changes in $[Ca^{2+}]_i$ were monitored. The experiments were performed more than three times independently and the results were reproducible. ND, not detected.

Discussion

Neurotensin is one of the important peptide hormones for both neuronal and non-neuronal systems that remain to be studied with regard to its mechanism of action. Here we demonstrate that human myeloid leukaemia cells, HL-60, express NTR1 neurotensin receptors. This is the first evidence that the NTR1 neurotensin receptor is expressed in hemopoietic cells.

At the present time, neurotensin receptors are believed to exist in three types: a high affinity brain-type neurotensin receptor (NTRH or NTR1), a low affinity brain-type receptor (NTRL or NTR2), and a low affinity mastocyte or macrophage neurotensin binding site. Since the NTR1 neurotensin receptor had been purified from rat brain (Mazella *et al.*, 1985), it was cloned from rat brain tissue (Tanaka *et al.*, 1990) and human colon adenocarcinoma HT-29 cells (Vita *et*

al., 1993). Mazella *et al.* (1988) purified two distinct neurotensin-binding sites from an adult mouse brain homogenate, each of which differed in binding affinity and sensitivity to levocabastine. Based on the above results, NTR2 was then cloned from mouse brain (Mazella *et al.*, 1996) and rat brain (Chalon *et al.*, 1996). In addition, a third type of receptor was also identified as a low affinity binding site on mastocytes/macrophages; not only neurotensin but also bradykinin or substance P could bind to this receptor (Lazarus *et al.*, 1977; Goldman & Bar-Shavit, 1983). It remains unclear whether this low affinity binding site's function is solely directed at neurotensin exactly because of its very low binding affinity for neurotensin and its cross-reactivity with bradykinin and substance P.

Schaeffer *et al.* (1995) have suggested major characteristics for the NTR1 neurotensin receptors: (i) a more potent effect by neurotensin fragments 8–13 than by neurotensin, (ii) lack of inhibition by levocabastine, (iii) lack of cross-binding other peptide hormones such as bradykinin and substance P and (iv) mRNA detection with specific NTR1 neurotensin receptor probes. Our results meet the above criteria for the expression of NTR1 neurotensin receptors in HL-60 cells. Neurotensin produced InsP_3 and increased $[\text{Ca}^{2+}]_i$ at nanomolar concentrations in HL-60 cells (Figure 1). Neurotensin fragments 8–13 was more potent in elevating $[\text{Ca}^{2+}]_i$ than neurotensin (Figure 2). Levocabastine did not inhibit the neurotensin-evoked response, but NTR1 receptor antagonist SR48692 completely blocked it (Figure 3). The receptor did not cross-react with bradykinin and substance P (Table 1). Finally, the expression of the NTR1 receptor was detected by PCR and confirmed by sequencing (Figure 5).

Though the neurotensin receptor in HL-60 cells is clearly distinguishable from the mastocyte/macrophage neurotensin binding sites, it is of value to exclude the possibility that neurotensin may act on other receptors. HL-60 cells have several receptors which are coupled to Ca^{2+} mobilization such as receptors for extracellular ATP, histamine, platelet-activating factor, and WKYMVm. However, neurotensin independently elicited $[\text{Ca}^{2+}]_i$ rise when cells were sequentially treated with the above stimulants (data not shown).

It has been suggested that neurotensin performs its role not only by elevating $[\text{Ca}^{2+}]_i$ but also by regulating cyclic AMP levels. The neurotensin-induced increase in cyclic AMP and the subsequent activation of protein kinase A inhibit the dopaminergic signalling in the midbrain (Shi & Bunney, 1992b). In CHO-K1 cells expressing rat NTR1 neurotensin receptors, neurotensin increased the intracellular cyclic AMP level (Yamada *et al.*, 1994). In N1E115 cells, neurotensin

decreased the basal and the G-protein-mediated elevated cyclic AMP level (Bozou *et al.*, 1986). However, neurotensin-induced changes in the cyclic AMP level were not detected in HL-60 cells (Figure 4). The result suggests that neurotensin may use different signalling pathways in different cells and tissues.

In addition, HL-60 cells are of hemopoietic lineage and can be triggered to differentiate into neutrophils or macrophages. It has been reported that the neurotensin receptors were undetectable in undifferentiated primary cultured neurons but did increase during differentiation (Chabry *et al.*, 1990). Because the level of expression of the receptor is closely linked to the size of the physiological effect, it was valuable for us to test the expression of the neurotensin receptor in HL-60 cells during differentiation to neutrophils. We show here clearly that the signalling of neurotensin decreases during the DMSO-induced granulocytic differentiation. The down-regulation of the Ca^{2+} signalling correlated with a gradual decrease in the expression of neurotensin receptor mRNA. In contrast, the fMLP-induced $[\text{Ca}^{2+}]_i$ elevation increased during the differentiation process (data not shown). Whenever there is a bacterial infection, the fMLP receptor is activated evoking superoxide production in granulocytes and macrophages. It has been generally accepted that PLC-coupled receptors are involved in defence mechanisms of the immune system such as chemotaxis, phagocytosis, and secretion of cytokines. However, it is still unclear what the physiological function of the down-regulation of the neurotensin receptor during differentiation may be. Neurotensin presumably plays a critical role in the early stages of hemopoietic differentiation and loses its physiological effect after differentiation. Cytokines including IL-3, granulocyte/macrophage-CSF, and macrophage-CSF, and granulocyte-CSF, play important roles in the early stages of differentiation from bone marrow cells. It is generally assumed that Ca^{2+} signalling in bone marrow cells regulates the effect of the cytokines described above (Cook *et al.*, 1989). Moore *et al.* (1989) reported that neurotensin regulates the macrophage colony stimulating factor-induced myelopoiesis. Thus, it is possible to propose the interesting hypothesis that neurotensin modulates other differentiation signals such as are sent by cytokines or growth factors during differentiation.

We wish to thank Dr J.K. Seo for providing human neutrophils and valuable discussions. We are grateful to Ms G. Hoschek for editing this manuscript. This study was supported by grants from the Basic Science Research Institute Program (Project BSRI-98-4435) from the Ministry of Education. S.Y. Choi was supported by research fund for young scientists from Korea Research Foundation (1998).

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(Received September 2, 1998

Revised November 11, 1998

Accepted November 20, 1998)



Distinction between surmountable and insurmountable selective AT₁ receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT₁ receptors

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1 CHO-K1 cells that were stably transfected with the gene for the human AT₁ receptor (CHO-AT₁ cells) were used for pharmacological studies of non-peptide AT₁ receptor antagonists.

2 In the presence of 10 mM LiCl, angiotensin II caused a concentration-dependent and long-lasting increase of inositol phosphates accumulation with an EC₅₀ of 3.4 nM. No angiotensin II responses are seen in wild-type CHO-K1 cells.

3 [³H]-Angiotensin II bound to cell surface AT₁ receptors (dissociates under mild acidic conditions) and is subject to rapid internalization.

4 Non-peptide selective AT₁ antagonists inhibited the angiotensin II (0.1 μM) induced IP accumulation and the binding of [³H]-angiotensin II (1 nM) with the potency order: candesartan > EXP3174 > irbesartan > losartan. Their potencies are lower in the presence of bovine serum albumin.

5 Preincubation with the insurmountable antagonist candesartan decreased the maximal angiotensin II induced inositol phosphate accumulation up to 94% and, concomitantly, decreased the maximal binding capacity of the cell surface receptors. These inhibitory effects were half-maximal for 0.6 nM candesartan and were attenuated by simultaneous preincubation with 1 μM losartan indicating a syntopic action of both antagonists.

6 Losartan caused a parallel rightward shift of the angiotensin II concentration-response curves and did not affect the maximal binding capacity. EXP3174 (the active metabolite of losartan) and irbesartan showed a mixed-type behavior in both functional and binding studies.

7 Reversal of the inhibitory effect was slower for candesartan as compared with EXP3174 and irbesartan and it was almost instantaneous for losartan, suggesting that the insurmountable nature of selective AT₁ receptor antagonists in functional studies was related to their long-lasting inhibition.

Keywords: Angiotensin II; AT₁ receptors; CHO cells; calcium transients; extracellular acidification; inositol trisphosphate; antagonist; candesartan; surmountable; insurmountable

Abbreviations: [Ca⁺⁺]_i, intracellular free calcium concentration; candesartan, 2-ethoxy-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazole-7-carboxylic acid; CHO-AT₁ cells, Chinese hamster ovary cells expressing human AT₁ receptors; CHO-K1 cells, Wild-type Chinese hamster ovary cells; DMEM, Dulbecco's Modified Essential Medium; EXP 3174, 2-n-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid; IP, inositol mono-, bis- and trisphosphates; irbesartan, 2-n-butyl-4-spirocyclopentane-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one; losartan, 2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole

Introduction

Angiotensin II, the effector peptide of the renin-angiotensin system, is an important regulator of the cardiovascular system which stimulates contraction of vascular smooth muscle cells and promotes their growth (Timmermans *et al.*, 1992). These biological actions of angiotensin II are mediated by receptors of the AT₁ subtype (Hodges *et al.*, 1992).

The effect of antagonists on AT₁ receptor function is traditionally studied by measuring their ability to antagonize angiotensin II-induced contraction of rabbit aorta rings/strips, a system with very small receptor reserve (Zhang *et al.*, 1993). These studies include a preincubation step, in which the tissue is equilibrated with the antagonist, and an incubation step

which comprises the consecutive addition of increasing concentrations of angiotensin II to generate a concentration-response curve. These experiments have shed light on the existence of different categories of selective AT₁ receptor antagonists. Most antagonists produce parallel rightward shifts of the angiotensin II concentration-response curve, without affecting the maximal response. These are classified as surmountable antagonists (Gaddum *et al.*, 1955) and losartan is a typical example (Mochizuki *et al.*, 1995). Insurmountable antagonists are able to depress the maximal response. The extent of this decline varies considerably from one antagonist to another and it seems to be unrelated to the potency of the antagonist to produce rightward shifts of the concentration-response curve (Liu *et al.* 1992; Cazaubon *et al.*, 1993; Noda *et al.*, 1993; Mochizuki *et al.*, 1995). The molecular basis for insurmountable antagonism is still a matter of debate. Although the longevity of the antagonist-receptor complex is

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retained as the cause in many studies, insurmountable antagonism has also been explained by the presence of allosteric binding sites on the receptor (Timmermans *et al.*, 1991); slowly interconverting receptor conformations (de Chaffoy de Courcelles *et al.*, 1986; Robertson *et al.*, 1994); slow removal of the antagonist from tissue compartments, cells or matrix surrounding the receptor (Robertson *et al.*, 1992; Panek *et al.*, 1995) and even by the ability of the antagonist to modulate the amount of internalized receptors (Liu *et al.*, 1992).

Compared to contraction studies with rabbit aortic strips, cell lines expressing the transfected human AT₁ receptor may offer additional advantages for the investigation of the fundamental properties of antagonist-AT₁ receptor interactions. First, radioligand binding and functional experiments can be performed on a single, intact cell system and under the same experimental conditions. Second, the presence of the human AT₁ receptor also minimizes potential species-related differences when comparing the *in vitro* effects of antagonists with their clinical action. Third, the untransfected parent cells may serve as useful controls for the detection of receptor-unrelated phenomena and, finally, experiments are possible with various receptor mutants which lack typical properties such as activation and internalization.

The aim of the present study is to use CHO-K1 cells transfected with the gene coding for the human AT₁ receptor (CHO-AT₁ cells) to distinguish between surmountable and full or partially insurmountable antagonists by functional assays and radioligand binding.

Methods

Drugs used

Candesartan (CV-11974; 2-ethoxy-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazole-7-carboxylic acid) (Shibouta *et al.*, 1993; Noda *et al.*, 1993), EXP 3174 (2-n-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid) (Wong *et al.*, 1990), losartan (DuP 753; 2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) (Wong *et al.*, 1990) and irbesartan (SR 47436; 2-n-butyl-4-spirocyclopentane-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one) (Cazaubon *et al.*, 1993) were obtained from Astra Hässle (Mölnådal, Sweden). Unlabelled angiotensin II, bovine serum albumin (BSA, Fraction V), (±)-sulphinpyrazone and ionomycin were obtained from Sigma (St. Louis, MO, U.S.A.). [³H]-Angiotensin II (50 Ci mmol⁻¹) was obtained from New England Nuclear (Boston, U.S.A.) and myo-[³H]-inositol (20 Ci mmol⁻¹) was from Pharmacia/Amersham/Biotech (Buckinghamshire, U.K.). Fura-2-acetoxymethylester and pluronic acid were from Molecular Probes Inc. (Eugene, U.S.A.). All other chemicals were of the highest grade commercially available.

Cell culture

Wild-type Chinese Hamster Ovary cells (CHO-K1) were kindly donated by Dr H. Verschuere (Pasteur Institute, Brussels, Belgium). CHO-K1 cells were stably transfected with the cDNA for the human angiotensin II AT₁ receptor (denoted as CHO-AT₁ cells) as follows. Primers corresponding to the 5'-(ATGATTCTCAACTCTTCTACT) and 3'-(TCACTCAACCTCAAAACATGG) ends of the AT₁ receptor sequence (Furuta *et al.*, 1992) were used to perform PCR on

cDNA from human adrenal gland (Clontech, U.S.A.). The PCR was done in a final volume of 50 µl and contained 1 ng cDNA, 1 × AmpliTaq DNA polymerase buffer, 200 µM of each deoxynucleotide, 1 µM of each primer and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, U.S.A.). The PCR thermal profile used was 93°C for 45 s, 60°C for 20 s and 72°C for 60 s for a total of 30 cycles. Resulting PCR product, containing the entire coding region of human AT₁ receptor was cloned in PCR3 vector (Invitrogen) and sequenced using the sequenase kit (Amersham, U.K.). The entire experiment starting with PCR was done on three occasions, and the clone containing the exact sequence as earlier published (Furuta *et al.*, 1992) was chosen for further study. The cloned human AT₁ receptor DNA was transfected in CHO-K1 cells using Lipofectin (Life Technologies, U.S.A.) according to the manufacturer's instructions. After 72 h of transfection, cells were harvested and replated in medium containing 1 mg ml⁻¹ geneticin. Individual clones were collected, grown and analysed for binding to [¹²⁵I]-angiotensin II. The clone with maximum binding was grown further in medium containing 0.5 mg ml⁻¹ geneticin, and used in present studies.

Cells were cultivated in 75 cm² flasks in Dulbecco's Modified Essential Medium (DMEM) which was supplemented with 2 mM L-glutamine, 2% of a stock solution containing 5000 I.U. ml⁻¹ penicilline and 5000 µg ml⁻¹ streptomycin (Life Technologies, Merelbeke, Belgium) and 10% foetal bovine serum (Life Technologies, Merelbeke, Belgium). The cells were grown in 5% CO₂ at 37°C until confluent.

[³H]-Angiotensin II binding

Cells were plated in 24 well plates and cultured until confluent. Before the experiment, the cells were washed three times with 0.5 ml per well of DMEM at room temperature and left for 15 min 37°C in 400 µl DMEM. Preincubations were initiated by addition of 50 µl DMEM either alone (total binding) or containing 1 µM unlabelled angiotensin II (non-specific binding) or unlabelled competitor and proceeded at 37°C for 30 min or the indicated periods of time (kinetic experiment, Figure 9). Incubations were started by adding 50 µl of DMEM containing [³H]-angiotensin II (final concentration: 0.5–10 nM for saturation binding as in Figure 8 and 1 nM for other studies) was added and the plates incubated at 37°C for 30 min or for the indicated time periods (kinetic experiment, Figure 6). At the end of the incubation, the cells were placed on ice and washed three times with ice-cold PBS containing (in g l⁻¹) CaCl₂·2H₂O, 0.132, KCl 0.2, KH₂PO₄ 0.2, MgCl₂·6H₂O 0.1, NaCl 8 and Na₂HPO₄·2H₂O 1.44). The cell surface binding of [³H]-angiotensin II was extracted by mild acid treatment: i.e. a 5 min incubation with 0.5 ml ice cold 50 mM glycine buffer (pH 3) containing 125 mM NaCl. This step was repeated and the radioactivity in the pooled fractions was counted after addition of 7 ml of scintillation liquid (Optisafe of Wallac, Turku, Finland) for 3 min in a liquid scintillation counter. To measure internalized radioligand, 0.5 ml 1 M NaOH was added to the acid-treated cells and the solubilized radioactivity counted as above.

IP accumulation

The cells were plated in 24 well plates and cultured until confluent. The medium was replaced by DMEM containing 1 µCi ml⁻¹ myo-[³H]-inositol and the cells were grown further for 20 h in 5% CO₂ at 37°C. Just before the incubation, the cells were washed two times with DMEM (0.5 ml per well), subsequently 400 µl of DMEM containing 10 mM LiCl added

to each well and the cells left for 15 min at 37°C. Preincubations were initiated by addition of 50 μ l DMEM containing 10 mM LiCl without (controls) or with antagonists and proceed at 37°C for 30 min or the indicated periods of time (kinetic experiment, Figure 9). When candesartan was tested in the presence of 1 μ M losartan (Figure 4b) both antagonists were added simultaneously. Subsequent incubations were initiated by adding 50 μ l of DMEM containing 10 mM LiCl alone (basal accumulation) or containing the indicated concentration angiotensin II and the plates incubated at 37°C for 5 min (or for 30 min in Figure 4a) or for the indicated time periods (kinetic experiment, Figure 1c).

For the recovery experiments (Figure 6), cells were preincubated with 500 μ l DMEM either alone (controls) or containing antagonist at 37°C for 30 min. Then the cells were washed three times with 500 μ l DMEM, left for the indicated periods of time at 37°C, washed three times again and left for 15 minutes at 37°C in 400 μ l DMEM containing 10 mM LiCl. Incubations were started by adding 50 μ l of DMEM either alone (basal accumulation) or containing 0.1 μ M angiotensin II and the plates incubated at 37°C for 5 min.

The accumulation of IP was determined using a method modified from Seeuwen *et al.* (1988). Briefly, at the end of the incubation, the 24 well plates were placed on ice and IP formation stopped by aspiration of the medium and by addition of 750 μ l of 10 mM formic acid at 4°C. After 30 min this solution was applied to a column containing 1 ml Bio-Rad anion exchange resin (AG 1 $\times$ 8, formate form, 200–400 mesh) and free inositol was eluted by 3 ml of 0.1 M formic acid. The inositol mono-, bis- and trisphosphates (denoted as IP accumulation) were eluted with 3 ml 0.1 M formic acid containing 0.8 M ammonium formate and the radioactivity measured in a liquid scintillation counter after addition of 10 ml of scintillation liquid (Ultima-flo-AF, Packard, Groningen, The Netherlands).

Measurement of $[Ca^{2+}]_i$ in CHO-AT₁ cells

Cells were plated on round glass coverslips (13 mm diameter), placed in 12 well plates and cultured until confluent. Then, the coverslips were washed four times with 2 ml per well of loading buffer containing (mM): HEPES 10, MgSO₄ 1, KCl 5, glucose 10, NaCl 145, CaCl₂ 1. ($\pm$)Sulphinpyrazone (0.25 mM) was added to the buffer to block the leakage of fura-2 through non-selective anion channels. After the last wash step, the coverslips were placed in loading buffer containing 3 μ M fura-2-acetoxymethylester and 0.06 % (w v⁻¹) pluronic acid and incubated for 2 h in the dark at room temperature. The coverslips were then washed three times with loading buffer and kept in the dark at room temperature for at least 20 min. Just before measurement the coverslips were washed again two times and placed diagonally in 5 ml polymethacrylate cuvettes (Kartell, Milano, Italy) containing 1.5 ml loading buffer in a thermostated holder at 37°C in a fluorimeter (Photon Technology International Inc., Ontario, Canada), with the coverslip 45° with respect to the excitation light beam. The light source was a 75 W Power ArcTM Xenon lamp (Photon Technology International Inc., Ontario, Canada). After 5 min of equilibration fura-2, fluorescence was excited alternatively at 340 and 380 nm via a monochromator and the intensity of the light emitted through a 510 nm bandpass filter was measured using a SX-R928PT photomultiplier tube (Photon Technology International Inc., Ontario, Canada). The ratio of 340 and 380 nm excitation was calculated each 2 s with FelixTM software version 1.1 (Photon Technology International Inc., Ontario, Canada). The intracellular free calcium

concentration was calculated according to Grynkiewicz *et al.* (1985) and the compounds were applied directly into the cuvette by injection of 50 μ l from a stock solution in loading buffer. Compensation for background fluorescence was performed by subtracting the autofluorescence measured in cells that were not loaded with fura-2-acetoxymethylester. After the measurements, the fluorescence values at saturating $[Ca^{2+}]_i$ were determined for each coverslip by application of 10 μ M ionomycin. Subsequently, minimal calcium levels were determined by application of 8 mM EGTA and 0.2 M NaOH.

Extracellular acidification rate

Extracellular acidification rates were measured using a Cytosensor microphysiometer (Molecular Devices Corp., Menlo Park, CA, U.S.A.). Cells were seeded 16–18 h before the experiment into 12 mm diameter disposable polycarbonate cell capsule cups (Molecular Devices Corp.) at 3×10^5 cells in each cup and incubated at 37°C in 5% CO₂ in growth medium (DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin). The capsule cups were loaded into the sensor chambers of the microphysiometer and perfused with running medium (growth medium without serum and without NaHCO₃) either alone (basal acidification levels) or containing the indicated concentrations of angiotensin II. Flow rate was set to 100 ml min⁻¹ pump cycles were 90 s. All experimental conditions were controlled by the Cytosoft software running on a Macintosh computer. For each cycle, cells were perfused with medium for the first 60 s and the pump was switched off for the remaining 30 s. The pH of the medium was recorded during the 65–90 s interval. The acidification rate of the buffer was calculated by the Cytosoft programme as derived from pH-measurement of the last 30 s of the cycle and rates were reported as percent of control; i.e. as per cent of the average acidification rate during the four cycles immediately preceding the addition of angiotensin II.

Data analysis

All experimental values represented the means $\pm$ s.e.mean of at least three independent experiments (each performed in duplicate or triplicate). IC₅₀ values from competition binding experiments, the K_D and the Bmax values from saturation binding curves, EC₅₀ values and corresponding maximal effects from concentration-response curves and kinetic constants from time curves were calculated by non-regression analysis by GraphPad Prism (San Diego, CA, U.S.A.) based on a one-site bimolecular reaction obeying the mass action law (the corresponding equation is $[L-R] = [R_{tot}].[L].([L] + K_D)^{-1}$) and for functional assays as described by Limbird (1996). The pK_B value of losartan ($-\log K_D$) was calculated by Schild regression analysis according to Arunlakshana & Schild (1959). Statistical significance was assessed by a non-paired Mann-Whitney test with two-tailed *P*-values.

Results

Angiotensin II mediated functional responses in CHO-AT₁ cells

Angiotensin II increased the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the extracellular acidification rate in CHO-AT₁ cells but not in the untransfected CHO-K1 cells (data not shown). These effects of angiotensin II were

concentration dependent with EC_{50} values of approximately 3 nM. The rise in $[Ca^{2+}]_i$ (up to 1000 nM) was rapid and was already declined to basal values (40–150 nM) after 1 min. The increase in extracellular acidification rate (up to 44% above baseline values) reached a maximum within less than 90 s and decreased rapidly thereafter.

In the presence of 10 mM LiCl, 0.1 μ M angiotensin II caused a time-dependent accumulation of IP in *myo*-[^{3} H]-inositol loaded CHO-AT $_1$ cells (Figure 1, insert) but not in the untransfected CHO-K1 cells (data not shown). The accumulation increased linearly with the incubation time for the first 10 min and then gradually levelled off to attain a plateau value at 30 min. At this time point, cells still contained about 70% of the original amount of incorporated *myo*-[^{3} H]-inositol. Unless stated otherwise, incubations for 5 min were adopted for all subsequent experiments. As shown in Figure 1, angiotensin II elicited a concentration-dependent increase of the IP accumulation with an EC_{50} of 3.4 ± 0.7 nM ($n = 11$). Maximal stimulation was produced at 0.1 μ M angiotensin II and corresponded to a 6.6 ± 0.12 fold stimulation over the basal IP level. This assay allowed the most accurate, reproducible and time-efficient determinations and was adopted for the measurement of angiotensin II-mediated responses in subsequent experiments.

Effect of selective AT $_1$ receptor antagonists on IP accumulation

As shown in Figure 2, preincubation of the cells for 30 min with selective AT $_1$ receptor antagonists caused a concentration-dependent inhibition of angiotensin II (0.1 μ M) induced IP accumulation. The order of potency of the tested antagonists was: candesartan > EXP3174 > irbesartan > losartan (Table 1). The concentration-response curve of angiotensin II was not affected when adding 0.1% BSA in the medium (data not shown) but the inhibition curve of some of the antagonists was shifted to the right (Table 1). In the subsequent experiments BSA was omitted.

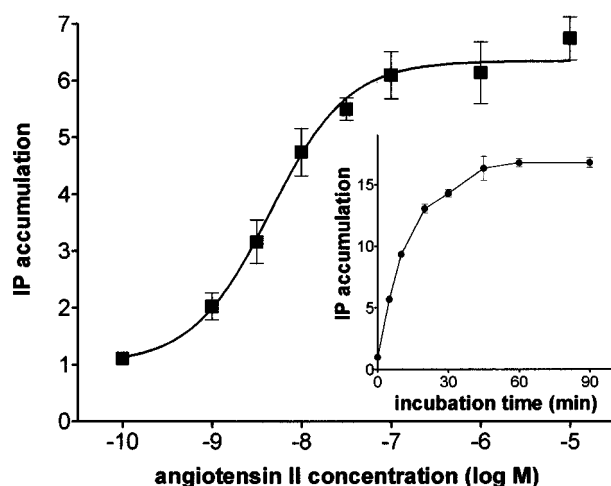


Figure 1 Concentration-response curve of angiotensin II mediated increase of IP accumulation in CHO-AT $_1$ cells. Each data point is the average $\pm$ s.e. mean of 11 independent experiments (each performed in duplicate) and are given as fold stimulation i.e. d.p.m after addition of the indicated angiotensin II concentration/d.p.m without angiotensin II. Basal and angiotensin II (10 μ M) induced accumulation correspond to 318 ± 24 and 2002 ± 168 d.p.m respectively. Inset: Fold stimulation of basal IP accumulation in CHO-AT $_1$ cells by addition of 0.1 μ M angiotensin II and incubation for different periods of time.

The effect of these antagonists on angiotensin II concentration-response curves was depicted in Figure 3 and the corresponding quantitative parameters were given in Table 2. The maximal response to angiotensin II declined by half when the cells were preincubated with 0.5 nM candesartan (Figure 3a). The decline was near to complete at 1.5 and 5 nM candesartan. Preincubation of the cells with 0.5–5 nM EXP3174 produced a partial reduction in the maximal response, along with a rightward shift of the angiotensin II concentration-response curve (Figure 3b). At concentrations between 10 and 100 nM, irbesartan produced a concentration-dependent, rightward shift of the concentration-response curve but a similar ($\pm 27\%$) reduction of the maximal response (Figure 3c). In contrast, preincubation of the cells with 100–1000 nM losartan only caused a progressive rightward shift of the concentration response curve without altering the maximal response (Figure 3d). Schild regression analysis of these data allowed to calculate the pK_B for losartan was 7.90 ± 0.06 with a slope of 0.92 ± 0.06 . As shown in Figure 4a for losartan and candesartan, the pattern of inhibition was retained when the incubation time with angiotensin II was increased to 30 min.

An angiotensin II concentration-response curve was obtained after preincubation of the cells with both candesartan

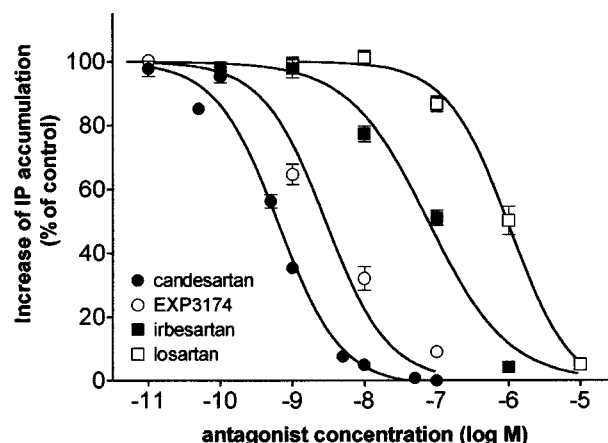


Figure 2 Inhibition curves of angiotensin II (0.1 μ M, 5 min incubation) IP accumulation in CHO-AT $_1$ cells after preincubating the cells for 30 min with increasing concentrations of candesartan, EXP3174, irbesartan or losartan. Data points are the average $\pm$ s.e. mean of three independent experiments (duplicate) and are expressed as per cent of the control angiotensin II effect by preincubation with medium alone.

Table 1 Potency of antagonist inhibition of [^{3} H]-angiotensin II binding and IP accumulation

Assay Compound	[^{3} H]-Angiotensin II binding		IP accumulation	
	Control IC $_{50}$ (nM)	BSA IC $_{50}$ (nM)	Control IC $_{50}$ (nM)	BSA IC $_{50}$ (nM)
Candesartan	0.26 ± 0.05	0.69 ± 0.14	0.64 ± 0.03	3.43 ± 1.44
EXP3174	0.45 ± 0.1	2.10 ± 0.60	2.11 ± 0.54	4.80 ± 0.23
Irbesartan	1.9 ± 0.19	2.7 ± 0.4	127 ± 32	107 ± 28
Losartan	33.9 ± 7.6	102 ± 21	1168 ± 335	2590 ± 599
Angiotensin II	0.97 ± 0.12	1.05 ± 0.35	–	–

Cells are pretreated for 30 min with the antagonists either in the absence or presence of 0.1% BSA, after which they are incubated with 1 nM [^{3} H]-angiotensin II or the angiotensin II (0.1 μ M) induced IP accumulation is measured. IC $_{50}$ values are the means $\pm$ s.e. mean of three independent experiments (duplicate) and are calculated by non-linear regression analysis using Graphpad Prism software.

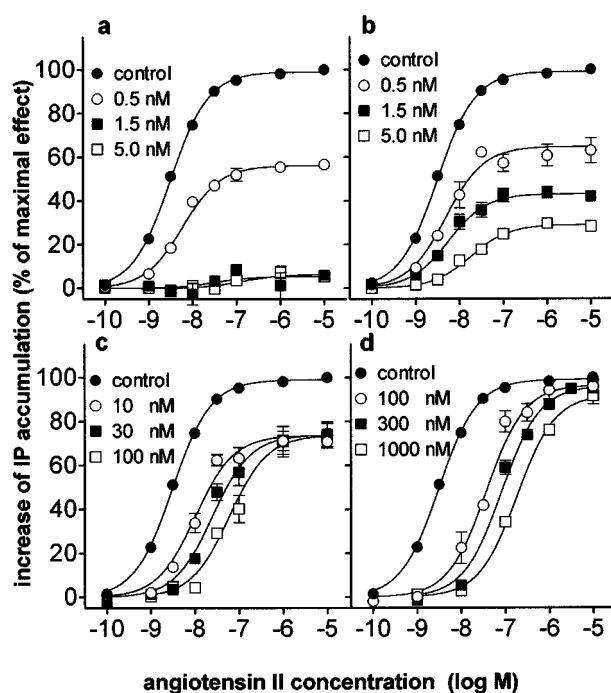


Figure 3 Concentration-response curves of angiotensin II mediated IP accumulation after preincubating CHO-AT₁ cells for 30 min with increasing concentrations of candesartan (a), EXP3174 (b), irbesartan (c) or losartan (d). Data are the average $\pm$ s.e. mean of at least three independent experiments (each performed in duplicate) and are expressed as per cent of the maximal angiotensin II response after preincubation with medium alone (control). The corresponding EC₅₀ values are given in Table 2.

Table 2 Angiotensin II concentration-response curves of IP accumulation: effect of antagonists

Antagonist	Concentration (nM)	EC ₅₀ value (nM)	E _{max} (% of control)
Medium only	0	3.4 $\pm$ 0.7	100 $\pm$ 1.8
Candesartan	0.5	5.3 $\pm$ 0.7	57 $\pm$ 2.6†
	1.5	*	6 $\pm$ 2.1†
	5	*	7 $\pm$ 1.3†
EXP3174	0.5	5.6 $\pm$ 1.2	64 $\pm$ 6.6†
	1.5	5.3 $\pm$ 1.1	46 $\pm$ 4.8†
	5	13.8 $\pm$ 1.9†	29 $\pm$ 2.4†
Irbesartan	10	15.1 $\pm$ 4.7	71 $\pm$ 5.2†
	30	29.4 $\pm$ 10.8	73.4 $\pm$ 9.4†
	100	111 $\pm$ 36.2†	73.2 $\pm$ 9.6†
Losartan	100	25.4 $\pm$ 7.6†	98 $\pm$ 5.1
	300	61.3 $\pm$ 15.0†	95 $\pm$ 3.6
	1000	181.7 $\pm$ 26.1†	92 $\pm$ 5.3

The EC₅₀ and E_{max} (maximal stimulation) values are the average $\pm$ s.e. mean of at least three independent experiments (duplicate determinations). The E_{max} values are expressed as per cent of the maximal angiotensin II response after preincubation with medium only. *No accurate calculation of EC₅₀ values is possible, †Statistical significant ($P < 0.05$) difference from control values.

(1.5 nM) and losartan (1000 nM) and compared with the curve with candesartan (1.5 nM) or losartan (1000 nM) pretreatment alone (Figure 4b). The maximal angiotensin II effect (E_{max} values) were 96 $\pm$ 6% for losartan and 85 $\pm$ 9% (extrapolated values) for losartan and candesartan and 7 $\pm$ 1.5 (stimulation at 10 μ M angiotensin II) for candesartan alone. The corresponding $-\log EC_{50}$ values were 8.50 $\pm$ 0.04 nM for the

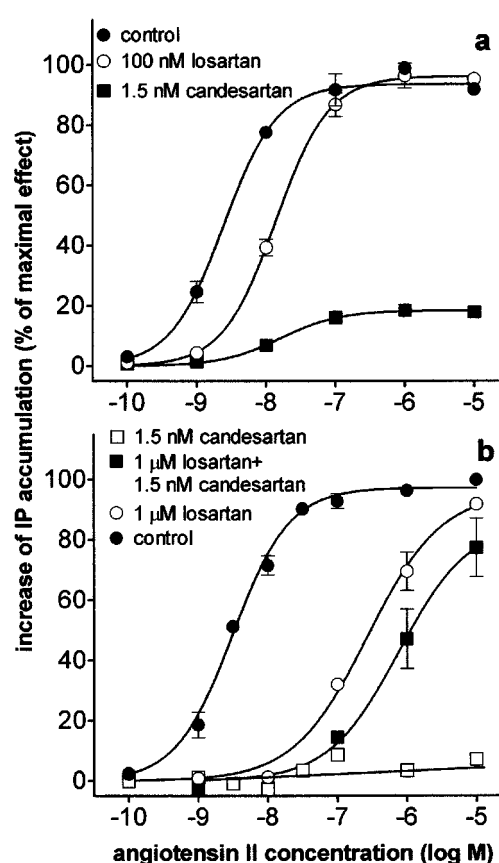


Figure 4 Concentration-response curves of angiotensin II mediated IP accumulation in CHO-AT₁ cells pretreated with antagonists. Data are the average $\pm$ s.e. mean of three independent experiments and expressed as outlined in Figure 3. (a) Incubation with angiotensin II for 30 min (control) or after pretreatment losartan or candesartan. (b) Incubation with angiotensin II for 5 min (control) or after pretreatment with losartan, candesartan or both.

control curve, 6.57 $\pm$ 0.12 nM with losartan alone and 6.11 $\pm$ 0.16 nM for the combination of losartan and candesartan.

Washout experiments were performed to compare the duration of the inhibitory effect of the different antagonists. For this purpose, cells were incubated for 30 min with antagonist, washed and left in fresh medium for the desired periods of time before measuring the (0.1 μ M) angiotensin II-mediated response. As shown in Figure 5a 0.5 and 5 nM candesartan produced a 64 $\pm$ 6 and 93 $\pm$ 2% decrease of the response before washout. This decrease was minimally affected by the washing step (of 5 min) and was only slowly alleviated during the subsequent incubation step in fresh medium. After 4 h of incubation the response to angiotensin II was still attenuated by 36 $\pm$ 3 and 60 $\pm$ 3%, respectively. In contrast, the recovery of the angiotensin II response was much faster for EXP3174-, irbesartan- and losartan-pretreated cells; the inhibitory effect of EXP3174- and irbesartan was completely reversed after 4 and 1.5 h incubation in fresh medium, respectively (Figure 5b,c), and the inhibition by losartan completely reversed within less than 30 min (Figure 5d).

Effect of selective AT₁ receptor antagonists on the binding of [³H]-angiotensin II

Whereas control CHO-K1 cells did not display any specific [³H]-angiotensin II binding (data not shown) its binding to

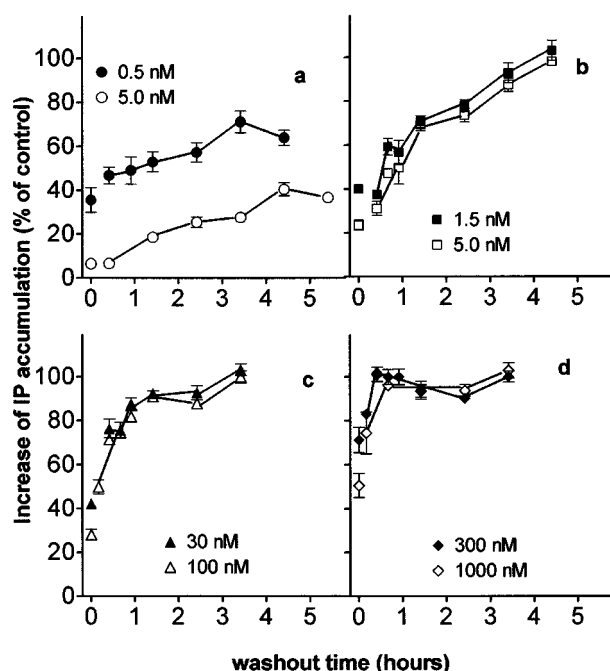


Figure 5 Recovery of angiotensin II ($0.1 \mu\text{M}$, 5 min incubation) induced IP accumulation from CHO-AT₁ cells preincubated with candesartan (a), EXP3174 (b), irbesartan (c) or losartan (d). Cells are preincubated with antagonists for 30 min, washed and incubated for the indicated periods of time (washout time) in fresh medium before addition of angiotensin II. Data are the average $\pm$ s.e. mean of at least three independent determinations (performed in triplicate) and are expressed as per cent of the control angiotensin II effect after treating the cells with medium alone under corresponding conditions.

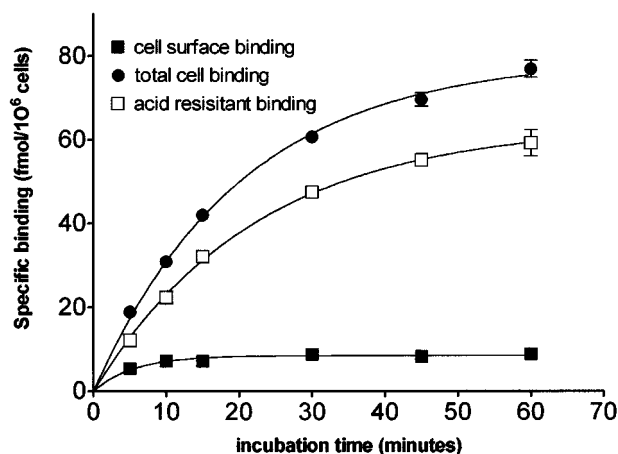


Figure 6 Distinction between cell surface binding of [³H]-angiotensin II and internalization of the radioligand in CHO-AT₁ cells. Binding is measured after incubation of the cells with 1 nM radioligand for the indicated periods of time and removal of the free radioligand by washing with PBS at 4°C (see Methods section). Cell surface binding is determined by mild acid treatment after which internalized radioligand is determined by NaOH treatment. Total cell binding is measured in cells treated with NaOH only.

intact CHO-AT₁ cells was shown in Figure 6. [³H]-angiotensin II bound initially to the cell surface receptors, followed by rapid internalization of the angiotensin II-receptor complexes. Surface-bound and internalized radioligand could be separated from each other: the former was readily released by mild acid treatment (see Methods) of the cells and the latter, acid-resistant binding, could be solubilized using 0.1 M NaOH

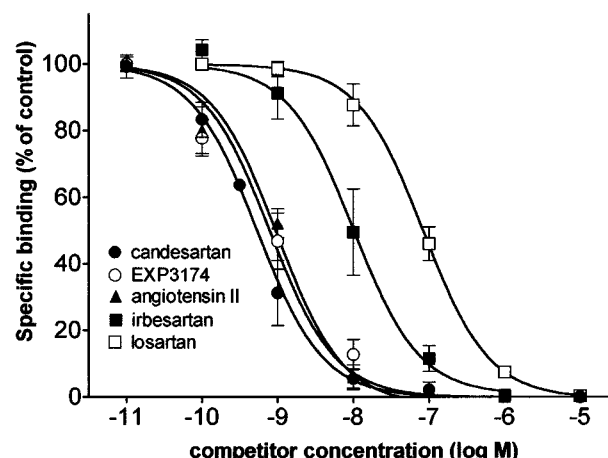


Figure 7 Inhibition of cell surface binding of 1 nM [³H]-angiotensin II by preincubation of the cells with candesartan, angiotensin II, EXP3174, irbesartan or losartan. Cell surface binding is measured by mild acid treatment and each data point represents the average $\pm$ s.e. mean of three independent experiments (performed in duplicate) and is expressed as per cent of control binding (i.e. preincubation with medium only).

(Croizat *et al.*, 1986; Conchon *et al.*, 1994; Hein *et al.*, 1997). When CHO-AT₁ cells were incubated at 37°C with 1 nM [³H]-angiotensin II, there was a steady increase of the binding with the incubation time (between 5 and 90 min). Although acid-resistant radioligand accounted for most of the binding at all the incubation times (90% after 5 min to 95% after 90 min), it was almost exclusively responsible for the time-dependent increase in total binding. Only acid-displaceable (cell surface) binding was considered in the subsequent experiments and the incubation time was set at 30 min.

Preincubation of the cells for 30 min with selective AT₁ receptor antagonists caused a concentration-dependent inhibition of the cell-surface binding of 1 nM [³H]-angiotensin II (Figure 7). The order of potency of the antagonists was the same as for the functional experiments: i.e. candesartan > EXP3174 > irbesartan > losartan. The antagonists displaced the binding to the same extent as unlabelled angiotensin II and, as shown in Table 1, addition of 0.1% BSA in the incubation medium decreased the IC₅₀ values of candesartan (2.7 fold), EXP3174 (4.7 fold) and losartan (3.0 fold) but not of irbesartan and angiotensin II.

Figure 8 and Table 3 show the effect of the antagonists on the maximal binding capacity (B_{max}) of [³H]-angiotensin II. In these experiments, cells were preincubated for 30 min with the antagonist, after which the binding of increasing concentrations of [³H]-angiotensin II to cell surface receptors was measured. Non-linear regression analysis of the saturation curves (Figure 8 and Table 3) revealed that 0.25 nM candesartan decreased the B_{max} without significantly affecting the apparent K_D . EXP3174 (0.5 nM) also decreased the B_{max} and slightly increased the apparent K_D . In contrast, 30 nM losartan and 5 nM irbesartan increased the apparent K_D and left the B_{max} value unaffected.

Time-dependency of the inhibitory effect of candesartan

In Figure 9 the time-dependency of the inhibition by candesartan of (a) the angiotensin II-mediated IP accumulation and (b) the binding of [³H]-angiotensin II to the cell surface receptors is shown. In these experiments, CHO-AT₁

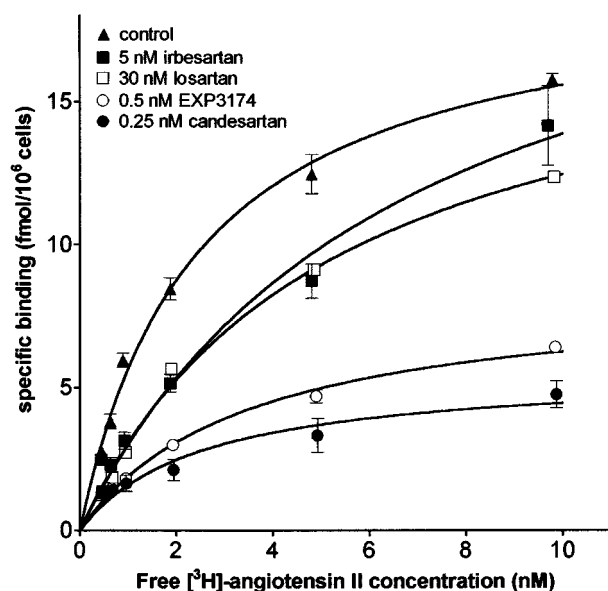


Figure 8 Cell surface saturation binding of [^{3} H]-angiotensin II after pretreatment of the cells with buffer (control), candesartan, EXP3174, irbesartan or losartan. Cell surface binding is measured by mild acid treatment. Data are the average $\pm$ s.e. mean of triplicate determinations of one representative experiment.

Table 3 Effect of antagonists on [^{3} H]-angiotensin II saturation binding

Condition	Apparent K_D (nM)	B_{max} (fmol/ 10^6 cells)
Control	2.12 ± 0.16	19.7 ± 2.3
0.25 nM Candesartan	2.83 ± 0.57	$6.2 \pm 0.7^*$
0.5 nM EXP3174	$3.10 \pm 0.06^*$	$8.0 \pm 0.2^*$
5 nM Irbesartan	$5.20 \pm 0.94^*$	19.1 ± 1.2
30 nM Losartan	$6.7 \pm 1.03^*$	23.2 ± 5.9

The apparent K_D and B_{max} values are the average $\pm$ s.e. mean of three independent experiments (triplicate determinations each) and are calculated by non-linear regression analysis using Graphpad Prism software. *Statistical significant ($P < 0.05$) difference from control values.

cells were preincubated with 1.5 nM candesartan for increasing periods of time up to 60 min, after which the response to 0.1 μ M angiotensin II and the binding of 1 nM [^{3} H]-angiotensin II were measured. Both were inhibited by candesartan in a matching, time-dependent fashion. The inhibition was almost complete after 60 min preincubation and the calculated apparent first-order rate constants for this process were $0.118 \pm 0.007 \text{ min}^{-1}$ for the angiotensin II-mediated IP accumulation and $0.094 \pm 0.019 \text{ min}^{-1}$ for the binding of [^{3} H]-angiotensin II to the cell surface receptors.

Discussion

Cell lines which have been transfected with the gene encoding for wild-type and mutant AT_1 receptors have proven to be particularly useful for investigating phenomena such as receptor-desensitization and internalization. Their utility in pharmacological studies was initially evoked by Perlman *et al.* (1995) and is further supported by the observations in the present report.

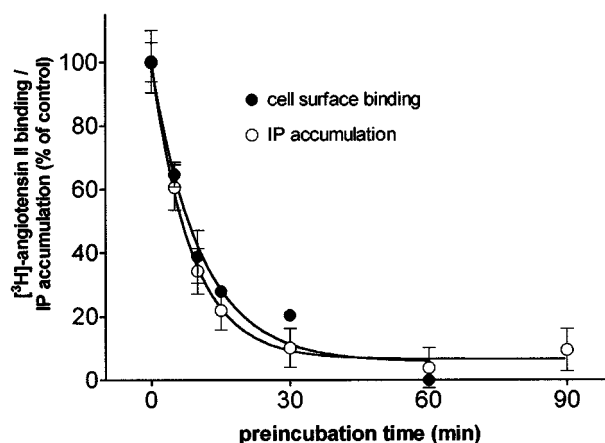


Figure 9 Comparison of the effect of candesartan on cell surface binding of [^{3} H]-angiotensin II and IP accumulation. Cells are pretreated for 30 min with 1.5 nM candesartan for the indicated periods of time. Then 1 nM [^{3} H]-angiotensin II is added and the incubation is continued for 30 min before measuring its cell surface binding. Alternatively, 0.1 μ M angiotensin II is added and the incubation is continued for 5 min before measuring IP accumulation. Data are the average $\pm$ s.e. mean of three independent experiments (triplicate) and are expressed as per cent of control binding or IP accumulation (i.e. with no candesartan pretreatment).

AT_1 receptors are a member of the seven transmembrane guanyl nucleotide-binding protein (G-protein)-coupled receptor superfamily and biochemical studies have established that, in primary cultures of vascular smooth muscle cells, they stimulate the phosphoinositide signalling system leading to an elevation of the intracellular free calcium concentration ($[Ca^{2+}]_i$). This, in turn, triggers cell growth and constriction (Vincentini & Villereal, 1986). The initial AT_1 receptor-evoked responses can conveniently be measured in primary cell cultures and in cell lines (Dickinson *et al.*, 1994; Koh *et al.*, 1994; Panek *et al.*, 1995; Perlman *et al.*, 1995; Balmforth *et al.*, 1997) and appear to be common to the biological actions of angiotensin II. In addition, it was also observed by Dickinson *et al.* (1994) that angiotensin II increases the rate at which rabbit aortic smooth muscle cells acidify their environment with products of their energy metabolism. This latter response is commonly observed for G-protein coupled receptors and it is claimed to occur irrespective of the initial cellular events (McConnell *et al.*, 1992).

In the present study, CHO-K1 cells were permanently transfected with the gene coding for the human AT_1 receptor (CHO- AT_1 cells). In this transfected cell line, angiotensin II concentration-response curves yielded comparable potencies for the IP production, rise in $[Ca^{2+}]_i$ and increase in extracellular acidification rate. The two latter responses were transient and completely faded within less than 1–2 min. On the other hand, IP accumulation increased almost linearly during the first 10 min, after which it gradually levelled off to come to a standstill after about 30 min. Since only 30% of the cell's original amount of [^{3} H]-phosphatidyl-inositol had been consumed, it is likely that the discontinued response is related to the well-known desensitization of the AT_1 receptor. As the data from the phosphatidyl inositol turnover assay showed the least variability, this assay was selected for the ensuing investigation of selective AT_1 receptor antagonists. As in rabbit aortic strip contraction studies, CHO- AT_1 cells were preincubated with the investigated antagonist (30 min), and then challenged with angiotensin II.

Under these conditions, the selective AT₁ receptor antagonists varied in the type of inhibition from almost completely insurmountable (candesartan, producing an over 90% decline of the maximal response) to completely surmountable (losartan, only producing a leftward shift of the agonist concentration-response curve). The same pattern for candesartan and losartan was observed when the IP accumulation was measured after a 5 or 30 min period, an observation in agreement with earlier rabbit aortic strip contraction studies (Cazaubon *et al.*, 1993; Noda *et al.*, 1993; Mochizuki *et al.*, 1995). Kinetic experiments (Figure 9 and Noda *et al.*, 1993) indicate that the inhibitory effect of candesartan increases with the preincubation time and that optimal inhibition is obtained after prolonged incubation. Since the preincubation times vary considerably from one report to another, particular attention should be paid to this factor when comparing with data in the literature. Yet, under comparable experimental conditions (i.e. also after a preincubation of 30 min), candesartan reduced the maximal response to angiotensin II by half at about 0.5 nM in the present study on CHO-AT₁ cells, and at about 0.3 nM in rabbit aortic strip contraction experiments (Noda *et al.*, 1993). On the other hand, the inhibitory effect antagonists such as losartan was the same regardless of the preincubation time (data not shown). This suggests that the equilibrium binding between this antagonist and the receptors is already attained after a very short incubation time. In agreement, the slope of the Schild regression analysis for losartan is close to unity (0.92 ± 0.05) and the pK_B-value (7.90 ± 0.06) is comparable to those (8.2–8.5) reported in earlier contraction studies (Robertson *et al.*, 1992; Renzetti *et al.*, 1995; Mochizuki *et al.*, 1995).

EXP3174, the active metabolite of losartan and irbesartan exhibited intermediary properties with only a partial suppression of the maximal response curve (respectively about 70 and 27%) along with a leftward shift of the concentration-response curve. Comparable values have been reported earlier for rabbit aortic strip contraction studies (Cazaubon *et al.*, 1993; Mochizuki *et al.*, 1995).

In agreement with the functional experiments, surmountable and insurmountable AT₁ antagonists could also be discriminated by binding studies with [³H]-angiotensin II. Pretreatment of the CHO-AT₁ cells with candesartan produced a net decrease in the number of [³H]-angiotensin II-labelled receptors at the cell surface. Kinetic experiments, involving different preincubation times, pointed out that this decrease in receptor number coincides with the decline in the angiotensin II-mediated IP accumulation. In contrast, losartan was only capable of increasing the K_D of [³H]-angiotensin II without affecting the total number of AT₁ receptors at the cell surface. Taken together, these data suggest that, at least for CHO-AT₁ cells, there is a close relationship between the number of cell surface receptors that are available for [³H]-angiotensin II binding and the extent of the angiotensin II-mediated IP accumulation.

The degree of insurmountable antagonism was also reflected by the difference between the antagonist IC₅₀ values for inhibiting the angiotensin II-mediated IP accumulation and [³H]-angiotensin II binding (Table 1). This is related to the larger agonist concentration in the functional experiment as in radioligand binding (100 versus 1 nM) and to the fact that inhibition curves for surmountable/competitive antagonists are shifted to the right when the angiotensin II concentration increases, whereas the curves for insurmountable antagonists

are unaffected by this (data not shown). BSA, which is well known to bind selective AT₁ receptor antagonists (Beauchamp *et al.*, 1994), also produced rightward shifts of the inhibition curves (Table 1) but no distinction could be made between surmountable and insurmountable antagonists.

Although the molecular basis for insurmountable antagonism is still a matter of debate, it has been advanced that the longevity of the antagonist-receptor complex is responsible for the insurmountable effect of candesartan and several other selective AT₁ receptor antagonists (Olins *et al.*, 1994; Cirillo *et al.*, 1995; Ojima *et al.*, 1997). This explanation was based on the very slow recovery of the angiotensin II-mediated contraction in candesartan-treated rabbit aortic strips. In agreement, the angiotensin II-mediated IP accumulation in CHO-AT₁ cells recovered only slowly after the removal of candesartan. Half-maximal recovery occurred after about 6 h. In contrast, the recovery was appreciably faster for EXP3174 (half-maximal after about 90 min) and still faster in the case of irbesartan and losartan.

The present findings are compatible with a model in which insurmountable antagonists dissociate so slowly from the receptors that they cannot be replaced by subsequently added angiotensin II. However, it cannot be excluded at the present level of investigation that such antagonists produce a long-lasting effect without remaining bound to the receptor. Such situations, in which the interconversion between inactive and active receptor conformations is much slower than the ligand binding, has been proposed in the two-state model by Gero (1983) and Robertson *et al.* (1994) and in the related coupling model of de Chaffoy de Courcelles *et al.* (1986). In addition, it was suggested by Panek *et al.* (1995) that antagonists may be insurmountable in tissues because of their slow removal from tissue compartments, cells or matrix surrounding the receptor, which allows the antagonist to rebind to the receptor. Direct radioligand binding studies with insurmountable antagonists such as candesartan should provide more insight in these remaining issues.

Allosteric modulation of AT₁ receptors has been advanced as an alternative explanation for insurmountable antagonism (Wienen *et al.*, 1992). Yet, similar to the experiments on rabbit aortic strips by Liu *et al.* (1992) and Ojima *et al.* (1997), we found that in CHO-AT₁ cells the insurmountable antagonism by candesartan of angiotensin II-mediated IP accumulation is attenuated in the presence of a high concentration of the surmountable antagonist losartan. This result rather suggests that candesartan antagonism is due to a syntopic action at the AT₁ receptor.

In conclusion, [³H]-angiotensin II binding and IP accumulation in intact CHO-AT₁ cells can be used to discriminate between insurmountable (e.g. candesartan) and surmountable (e.g. losartan) selective AT₁ receptor antagonists. In agreement with previous functional studies in isolated organs, our data suggest that the insurmountable action of candesartan is related to the slow recovery of angiotensin II responses after antagonist pretreatment.

G.V. is Onderzoeksdirecteur of the Nationaal Fonds voor Wetenschappelijk Onderzoek, Belgium. We are most obliged to Astra-Hässel Sweden, Astra Belgium and the Queen Elisabeth Foundation Belgium for their kind support. This text presents research results of the Belgian program on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. The scientific responsibility is assumed by its authors.

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(Received September 28, 1998

Revised November 26, 1998

Accepted December 2, 1998)



Evidence for novel caffeine and Ca^{2+} binding sites on the lobster skeletal ryanodine receptor

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1 The effects of Ca^{2+} , ATP and caffeine on the gating of lobster skeletal muscle ryanodine receptors (RyR) was investigated after reconstitution of the channels into planar phospholipid bilayers and by using [^3H]-ryanodine binding studies.

2 The single channel studies reveal that the EC_{50} (60 μM) for activation of the lobster skeletal RyR by Ca^{2+} as the sole ligand is higher than for any other isoform of RyR studied.

3 Inactivation of the channel by Ca^{2+} (EC_{50} = 1 mM) occurs at concentrations slightly higher than those required to inactivate mammalian skeletal RyR (RyR1) but lower than those required to inactivate mammalian cardiac RyR (RyR2).

4 Lifetime analysis demonstrates that cytosolic Ca^{2+} , as the sole activating ligand, cannot fully open the lobster skeletal RyR (maximum Po approximately 0.2). The mechanism for the increase in open probability (Po) is an increase in both the frequency and the duration of the open events.

5 ATP is a very effective activator of the lobster RyR and can almost fully open the channel in the presence of activating cytosolic [Ca^{2+}]. In the presence of 700 μM Ca^{2+} , 1 mM ATP increased Po to approximately 0.8.

6 Caffeine, often used as a tool to identify the presence of RyR channels, is relatively ineffective and cannot increase Po above the level that can be attained with Ca^{2+} alone.

7 The results reveal that caffeine increases Po by a different mechanism to that of cytosolic Ca^{2+} demonstrating that the mechanism for channel activation by caffeine is not 'sensitization' to cytosolic Ca^{2+} .

8 By studying the mechanisms involved in the activation of the lobster RyR we have demonstrated that the channel responds in a unique manner to Ca^{2+} and to caffeine. The results strongly indicate that these ligand binding sites on the channel are different to those on mammalian isoforms of RyR.

Keywords: Ryanodine receptor; lobster; ATP; caffeine; Ca^{2+} ; sarcoplasmic reticulum; Ca^{2+} -release

Abbreviations: CICR, Ca^{2+} -induced Ca^{2+} -release; DAT, Digital Audio Tape; EGTA, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PIPES, potassium piperazine-N,N'-bis-2-ethanesulphonic acid; Po, open probability; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)-methylamine; VDCR, voltage-dependent Ca^{2+} -release

Introduction

Excitation-contraction (EC) coupling in mammalian striated muscle is thought to occur by at least two very different mechanisms. In cardiac muscle, Ca^{2+} -release from the sarcoplasmic reticulum (SR) is triggered by Ca^{2+} entering the cell via L-type sarcolemmal Ca^{2+} channels and is termed Ca^{2+} -induced Ca^{2+} -release (CICR) (Fabiato, 1985; Beuckelmann & Wier, 1988; Nabauer *et al.*, 1989; Bers, 1991). In mammalian skeletal muscle, depolarization of the T-tubule membrane leads to SR Ca^{2+} -release without the need for Ca^{2+} entry and is termed voltage-dependent Ca^{2+} -release (VDCR) or directly-coupled Ca^{2+} -release (Schneider & Chandler, 1973; Ashley *et al.*, 1991; Rios & Pizarro, 1991; Schneider, 1994). In both types of muscle, the SR Ca^{2+} -release/ryanodine receptor channels (RyR) act as the pathway for Ca^{2+} release into the cytoplasm.

In crustacean skeletal muscle, unlike mammalian skeletal muscle, Ca^{2+} entry into the cell is required to stimulate SR Ca^{2+} -release and activate tension development (Goblet & Mounier, 1987; Lea & Ashley, 1989; 1990) and evidence suggests that CICR is the mechanism for EC-coupling (Palade & Györke, 1993) as proposed for mammalian cardiac muscle. This is very interesting given that the resting intracellular [Ca^{2+}] in crustacean muscle is estimated to be much higher

(300–350 nM) (Ridgway & Gordon, 1995) than in cardiac muscle and that large fluxes of Ca^{2+} have been calculated to occur during the action potential (Atwood, 1972; Atwater *et al.*, 1974). These results suggest that ligands typically thought of as activators of RyR channels, may interact very differently with crustacean RyR. We have therefore investigated the mechanisms involved in ligand activation of the lobster skeletal RyR, specifically, the effects of Ca^{2+} , ATP and caffeine. Our results demonstrate that the mechanisms involved in Ca^{2+} -activation of the lobster skeletal muscle RyR exhibit unique characteristics. In addition, the effects of caffeine on the gating of lobster RyR channels indicate that the caffeine binding sites on lobster RyR may be different to those on mammalian isoforms of RyR.

Methods

Membrane preparation

Live Maine lobsters were obtained from Harrods (Harrods Ltd., London, U.K.) and transported to the laboratory on ice. Lobsters were killed by decapitation and SR membrane fractions were prepared as previously described (Sitsapesan *et al.*, 1991) with slight modifications. The abdominal muscle was

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rapidly excised and minced prior to homogenization in a solution containing phenylmethyl-sulphonide fluoride (PMSF) (1 mM), aprotinin (100 nM), leupeptin (1 μ M), sucrose (300 mM) and potassium piperazine-N'-N'-bis-2-ethanesulphonic acid (PIPES) (20 mM), pH 7.4. The homogenate was centrifuged at 8000 r.p.m. in a Sorvall GSA rotor for 22 min. The supernatant was then centrifuged at 36,000 r.p.m. for 50 min in a Sorvall T647.5 rotor. The mixed membrane population sedimented by this step was resuspended in a solution containing (in mM): KCl 400, MgCl₂ 0.5, CaCl₂ 0.5, ethylene glycol-bis(β -aminoethyl ether)-N,N,N,N'-tetraacetic acid (EGTA 0.5), PIPES 25, pH 7.0 plus 10% sucrose w/v. The membrane suspension was layered over identical salt solutions containing 20, 30, and 40% sucrose w/v and sedimented at 28,000 r.p.m. for 150 min in a Sorvall AH629 rotor. Membrane vesicles were collected at the 10–20, 20–30, 30–40% interfaces. [³H]-ryanodine binding was used to assess the distribution of RyR on the sucrose gradient. The amount of [³H]-ryanodine binding in the membrane fraction from the 20–30% interface was not significantly lower than that obtained from the 30–40% interface and therefore both fractions were used in the experiments. Membrane vesicles were diluted in KCl (400 mM) and pelleted by centrifugation at 36,000 r.p.m. for 50 min in a Sorvall T647.5 rotor before resuspension in a solution containing sucrose (400 mM), N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (5 mM) titrated to pH 7.2 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were stored in liquid nitrogen.

Planar lipid bilayer methods

Planar phospholipid bilayers, composed of phosphatidylethanolamine (Avanti Polar Lipid, Birmingham, AL) dispersed in decane (35 mg ml⁻¹) were painted across a 200 μ m diameter hole in a polystyrene partition separating two fluid-filled compartments, the *cis* and *trans* chambers, of volumes 500 and 700 μ l respectively. The *trans* chamber was held at ground and the *cis* chamber was clamped at various potentials relative to ground. Current flow through the bilayer was measured using an operational amplifier as a current-voltage converter as described by Miller (1982). During bilayer formation both the *cis* and *trans* chambers contained choline chloride (50 mM), CaCl₂ (5 mM) and HEPES/Tris, pH 7.2 (10 mM). An osmotic gradient was established by the addition of 100 μ l of KCl (3 M) or CsCl (3 M) to the *cis* chamber. SR vesicles were added to the *cis* chamber and stirred to induce fusion of vesicles with the bilayer. After fusion of vesicles containing large conductance cation channels the *cis* chamber was perfused with a solution containing HEPES (250 mM), Tris (125 mM) and free Ca²⁺ (10 μ M), pH 7.2 and the *trans* chamber was perfused with glutamic acid (250 mM), HEPES (10 mM), titrated to pH 7.2 with Ca(OH)₂ (free [Ca²⁺] approximately 50 mM). The *cis* chamber was assumed to be the cytosolic side of the RyR channels as ATP activated the channels from this side. The cytosolic free [Ca²⁺] on the *cis* side of the bilayer was altered by adding CaCl₂ and EGTA and the free [Ca²⁺] of the solutions was measured using a Ca²⁺ electrode (Orion 93-20) as previously described (Sitsapesan *et al.*, 1991). Unless otherwise stated, drugs were added to the cytosolic side of the bilayer. Experiments were performed at room temperature (23 $\pm$ 1°C).

Data acquisition and analysis

Channel data were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). Current recordings were

replayed, low-pass filtered at 1.0 kHz and digitized at 2.0 kHz using the Satori program (Intracel, Cambridge). Channel open probability (Po) and the lifetimes of open and closed events were monitored by 50% threshold analysis at a holding potential of 0 mV. Lifetimes accumulated from 3 min steady-state recordings, were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes < 1 ms were not fully resolved under the conditions of data acquisition described here and were therefore excluded from the fitting procedure. A missed-events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to up to three exponentials (Blatz & Magleby, 1986).

[³H]-ryanodine binding

Membrane vesicles were diluted to 50–100 μ g protein/ml and incubated at 37°C in a buffered medium containing KCl (1 M), PMSF (5 μ M), PIPES-KOH (25 mM), pH 7.2. For solutions containing ATP or where the free [Ca²⁺] $\leq$ 10 μ M, EGTA (1 mM) and various additions of CaCl₂ were added. The free [Ca²⁺] of all solutions was measured at 37°C using a Ca²⁺ electrode (Orion 93-20). Incubation time was 60 min with [³H]-ryanodine (5 nM). This incubation time was chosen because no further increase in binding was achieved with longer incubation periods at various free [Ca²⁺]. At the completion of incubation, the medium was diluted with 5 ml of ice-cold buffer and filtered through Whatman GF-B filters, presoaked in buffer. Filters were washed with 3 $\times$ 5 ml aliquots of buffer and counted in 10 ml aqueous counting scintillant the following day. Non-specific binding was determined from incubations to which unlabelled ryanodine (5 μ M) had been added. All incubations were performed in triplicate.

The mean $\pm$ s.e.mean is given where $n \geq 4$. For $n = 3$, standard deviation (s.d.) is given. Where appropriate, Student's *t*-test was used to assess the difference between mean values. A *P* value of < 0.05 was taken as significant.

Drugs

[³H]-ryanodine was purchased from New England Nuclear Ltd. Unlabelled ryanodine was purchased from Calbiochem (Nottingham, U.K.). All other chemicals were of AnalaR or best available grade from BDH (Poole, U.K.) or Sigma (Poole, U.K.). Aqueous counting scintillant was purchased from Amersham International (Amersham, U.K.).

Results

[³H]-ryanodine binding studies

Figure 1 illustrates the relationship between ryanodine binding and free [Ca²⁺]. In the absence of other ligands [³H]-ryanodine binding was negligible at [Ca²⁺] $\leq$ 1 μ M ($\leq 0.05 \pm 0.02$ pmol mg⁻¹ protein; s.e.m., $n = 5$). Maximum binding (4.37 ± 1.51 pmol mg⁻¹ protein; s.e.m., $n = 5$) occurred at approximately 300 μ M free Ca²⁺ and began to decrease at concentrations above 1 mM Ca²⁺. In the presence of ATP (1 mM) (Figure 1), ryanodine binding was increased at virtually all free [Ca²⁺] and this was especially apparent at [Ca²⁺] ≥ 10 μ M. Optimal binding was increased to 9.5 ± 0.79 pmol mg⁻¹ protein (s.e.m.; $n = 6$). In contrast to ATP, caffeine (40 mM) shifted the Ca²⁺ activation curve to the

left and did not significantly increase the maximum level of binding above that observed with Ca^{2+} as the sole activator of RyR.

Single channel experiments

Conductance measurements Figure 2A shows examples of the current fluctuations at different voltages that were obtained after activation of the channel by cytosolic Ca^{2+} ($30\ \mu\text{M}$) and ATP ($1\ \text{mM}$) under the ionic conditions described in the Methods. Figure 2C illustrates the current-voltage relationship of a representative single lobster RyR channel. The conductance, measured by linear regression, between holding potentials of $+20$ and $-40\ \text{mV}$ was $97.7 \pm 2.1\ \text{pS}$ (s.e.m.; $n=5$). This value is similar to those previously reported for mammalian isoforms of RyR (Smith *et al.*, 1986; Rousseau & Meissner, 1989; Sitsapasan & Williams, 1990) under the same ionic conditions. Our preliminary data therefore suggests that the ion handling properties of the lobster RyR may be very similar to those observed in other RyR isoforms.

The effect of ryanodine on a typical lobster RyR channel is illustrated in Figure 2B. Ryanodine modified the channels into a reduced conductance long open state $36.0 \pm 2.2\%$ (s.d.; $n=3$) of the full current amplitude. This effect of ryanodine is similar to that reported for mammalian skeletal and cardiac isoforms of RyR (Rousseau *et al.*, 1987; Williams, 1992) and identifies the channels we are studying in the bilayer as ryanodine receptors.

Modification of lobster RyR gating by cytosolic Ca^{2+}

Figure 3 illustrates the effect of increasing the cytosolic $[\text{Ca}^{2+}]$ on the gating of a typical single lobster RyR at the holding potential of $0\ \text{mV}$. At $10\ \mu\text{M}$ cytosolic Ca^{2+} the Po was very low (0.008 ± 0.005) and only a few brief openings were observed with a mean open lifetime of $0.67 \pm 0.07\ \text{ms}$ (s.e.m.; $n=8$). Increasing the $[\text{Ca}^{2+}]$ to $300\ \mu\text{M}$ increased Po to 0.2 ± 0.1 (s.e.m.; $n=6$) and the mean open lifetime to $11.55 \pm 8.6\ \text{ms}$ (s.e.m.; $n=6$). Concentrations of Ca^{2+} above $700\ \mu\text{M}$ tended to reduce Po . For example at $1\ \text{mM}\ \text{Ca}^{2+}$, the Po and mean open lifetime were 0.041 ± 0.05 and $0.64 \pm 0.24\ \text{ms}$ (s.e.m.; $n=5$) respectively. Figure 4 demon-

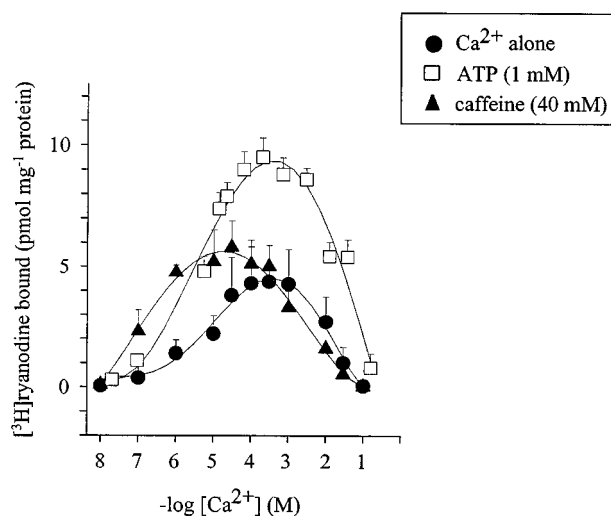


Figure 1 Stimulation of $[\text{H}^3]$ -ryanodine binding to lobster SR skeletal vesicles by Ca^{2+} in the absence of other ligands and in the presence of ATP ($1\ \text{mM}$) or caffeine ($40\ \text{mM}$). Each point is the mean $\pm$ s.e. mean for 4–6 different experiments.

strates the relationship between Po , mean open and closed lifetimes and cytosolic $[\text{Ca}^{2+}]$. The figure shows that Ca^{2+} as the sole cytosolic activator cannot fully activate the channel and that Po does not usually increase above 0.3. Peak Po values occurred at $300\text{--}700\ \mu\text{M}$. The EC_{50} values for activation and inhibition by Ca^{2+} were $60.0\ \mu\text{M}$ and $1.0\ \text{mM}$ respectively. The figure illustrates that as Po is increased by increasing cytosolic $[\text{Ca}^{2+}]$, mean open time tends to increase

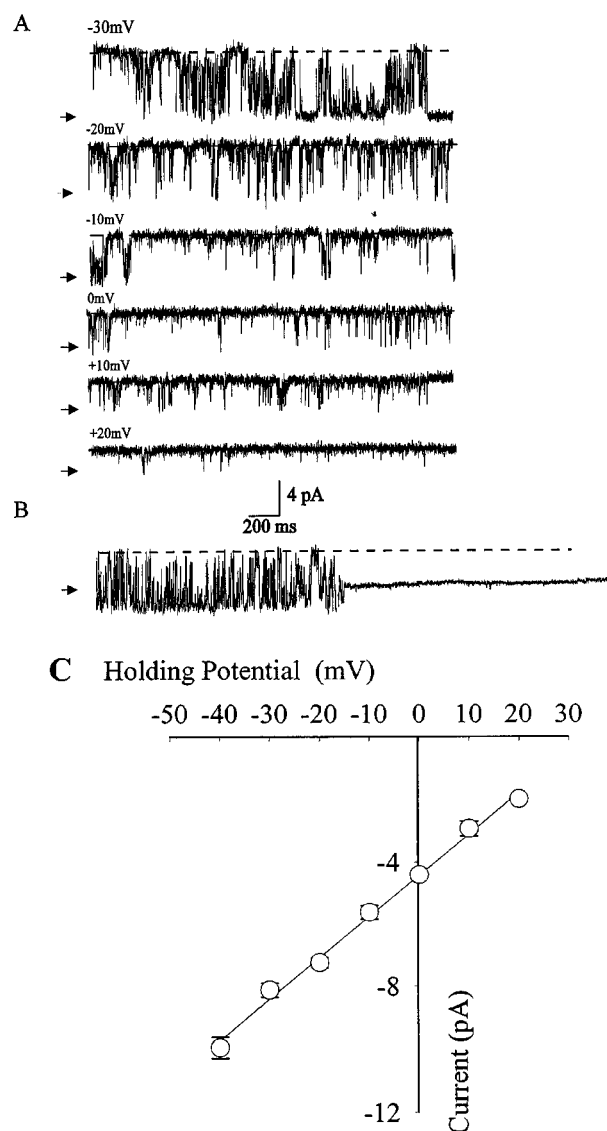


Figure 2 (A) Representative single channel fluctuations at a range of indicated holding potentials. The dotted lines and arrows indicate the fully open and closed channel levels respectively. Under the ionic conditions used, Ca^{2+} flows through the RyR channel from the *trans* to the *cis* chamber at all holding potentials displayed and channel openings are shown as upward deflections. In agreement with published work on other isoforms of RyR (Sitsapasan & Williams, 1994; 1995a; Percival *et al.*, 1994) the lobster RyR appears to be more open at positive potentials however this was not scientifically tested. (B) Single channel current fluctuations approximately 3 min after the addition of ryanodine ($1\ \mu\text{M}$) to the solution bathing the cytosolic face of the membrane. The channel enters a characteristic irreversible low-conductance open state following the binding of ryanodine. The holding potential was $0\ \text{mV}$. (C) Single channel conductance of the lobster skeletal RyR. The current-voltage relationship was plotted from five similar single channel currents as those shown in (A). The standard error bars are shown or are within the symbols. Linear regression gave a conductance of $97.7 \pm 2.1\ \text{pS}$ (s.e.m.; $n=5$) over the entire range of applied voltages (-40 to $+20\ \text{mV}$).

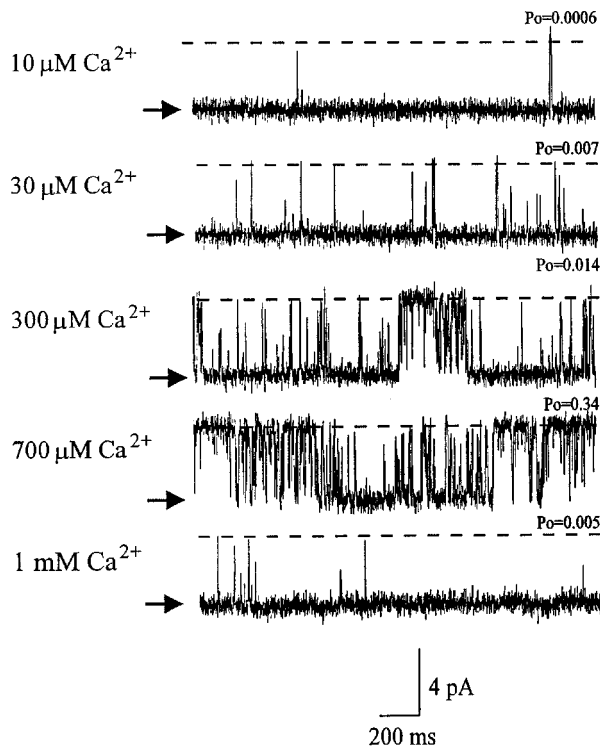


Figure 3 The effects of increasing cytosolic $[Ca^{2+}]$ on the gating of a typical lobster RyR channel held at 0 mV. Channel openings are shown as upward deflections. The arrows indicate the zero current level and the dotted lines indicate the open channel levels. Cytosolic free $[Ca^{2+}]$ was raised sequentially from 10 μM to 30 μM , 300 μM , 700 μM and 1 mM (E).

and mean closed time decreases. As P_o declines at high $[Ca^{2+}]$, open lifetime durations tend to decrease and closed times increase. Cytosolic Ca^{2+} , therefore, appears to cause changes in P_o by modulating both open and closed times. Analysis of the open and closed lifetime distributions supports this assumption. Figure 5 illustrates the open and closed lifetime distributions of a typical lobster RyR activated by 30 μM , 300 μM and 709 μM Ca^{2+} . Even at 30 μM Ca^{2+} (Figure 5A) when P_o is low, the open lifetime distribution is best fit by a probability density function (pdf) with three exponentials indicating at least three open states. The figure demonstrates that as cytosolic Ca^{2+} is increased, the duration of all three open states is increased (Figure 5B). At higher $[Ca^{2+}]$ when P_o declines, the duration of all three open states is reduced (Figure 5C). The effect of Ca^{2+} on closed lifetime durations is even more obvious. At 30 μM Ca^{2+} three closed states can be distinguished (Figure 5A). At 300 μM Ca^{2+} the closed lifetime durations are markedly reduced and only two brief closed states can be observed (Figure 5B). More than 90% of the events now occur to the shortest closed state. As P_o is reduced at higher $[Ca^{2+}]$, the duration of the closed lifetimes is increased and three closed states can again be resolved (Figure 5C). Clearly, Ca^{2+} controls P_o by altering both the duration and the frequency of channel openings but the effect on frequency is more marked. It is reasonable to conclude that Ca^{2+} binds to both open and closed states of the channel to cause activation and inactivation of the lobster RyR.

Effect of ATP and caffeine

The $[^3H]$ -ryanodine binding studies indicate that the maximum effect of caffeine would be observed at approximately

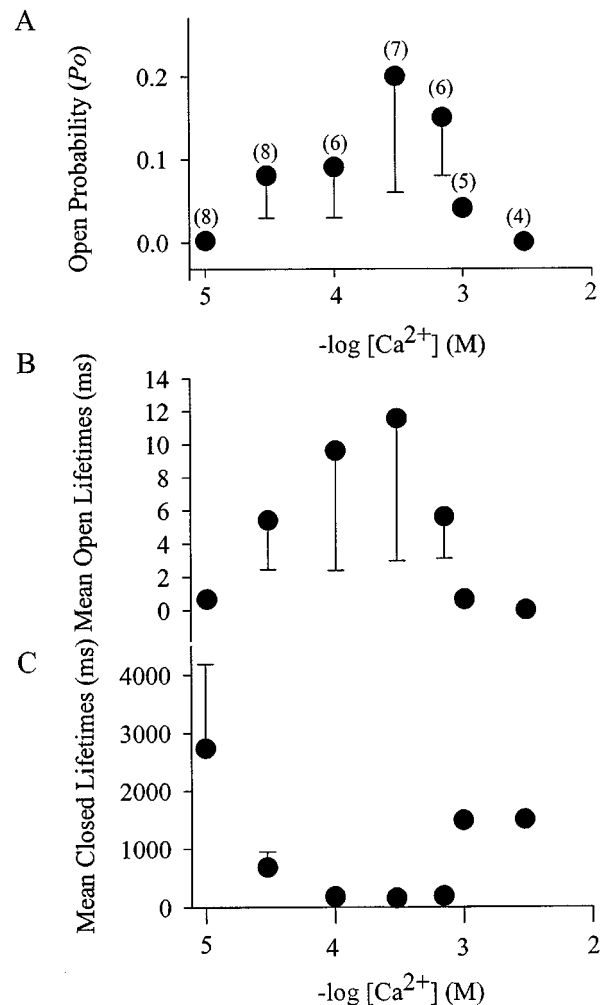


Figure 4 The relationship between cytosolic $[Ca^{2+}]$ and P_o (A), mean open lifetimes (B) and mean closed lifetimes (C) in the lobster RyR channels. In A, the number above each symbol is the number of experiments included. The s.e.m. is shown by the error bars or included within the symbol.

10 μM Ca^{2+} but that very little increase in P_o would be observed at optimal $[Ca^{2+}]$. In contrast, ATP would be expected to be most effective at $[Ca^{2+}]$ in the range 100 μM –1 mM. We therefore investigated the effects of caffeine and ATP on the gating of channels at 10 and 700 μM Ca^{2+} . Figures 3 and 4 show that 700 μM Ca^{2+} causes optimal activation of single channels incorporated into bilayers but that 10 μM Ca^{2+} is on the threshold for activation by Ca^{2+} (occasional openings can be observed but P_o is essentially zero). Figure 6 illustrates the effect of ATP (1 mM) on the gating of representative lobster skeletal RyR channels in the presence of 10 μM Ca^{2+} (Figure 6A) and in the presence of 700 μM Ca^{2+} (Figure 6B). In the presence of cytosolic 10 μM Ca^{2+} , very occasional brief openings were observed but P_o was effectively 0 ($n=5$). After adding ATP (1 mM), P_o only increased to 0.005 ± 0.003 (s.e.m.; $n=5$). In contrast, in the presence of 700 μM Ca^{2+} , ATP (1 mM) increased P_o from 0.15 ± 0.07 (s.e.m.; $n=6$) to 0.78 ± 0.3 ($P \geq 0.01$; s.e.m.; $n=4$). The increase in P_o was caused largely by an increase in the duration of open lifetimes which increased from 0.56 ± 0.25 ms in the absence to 77.7 ± 24.5 ms (s.e.m.; $n=4$) in the presence of ATP. Mean closed lifetimes were 29.8 ± 10.3 ms and 6.36 ± 4.74 ms (s.e.m.; $n=4$) before and after the addition of ATP (1 mM).

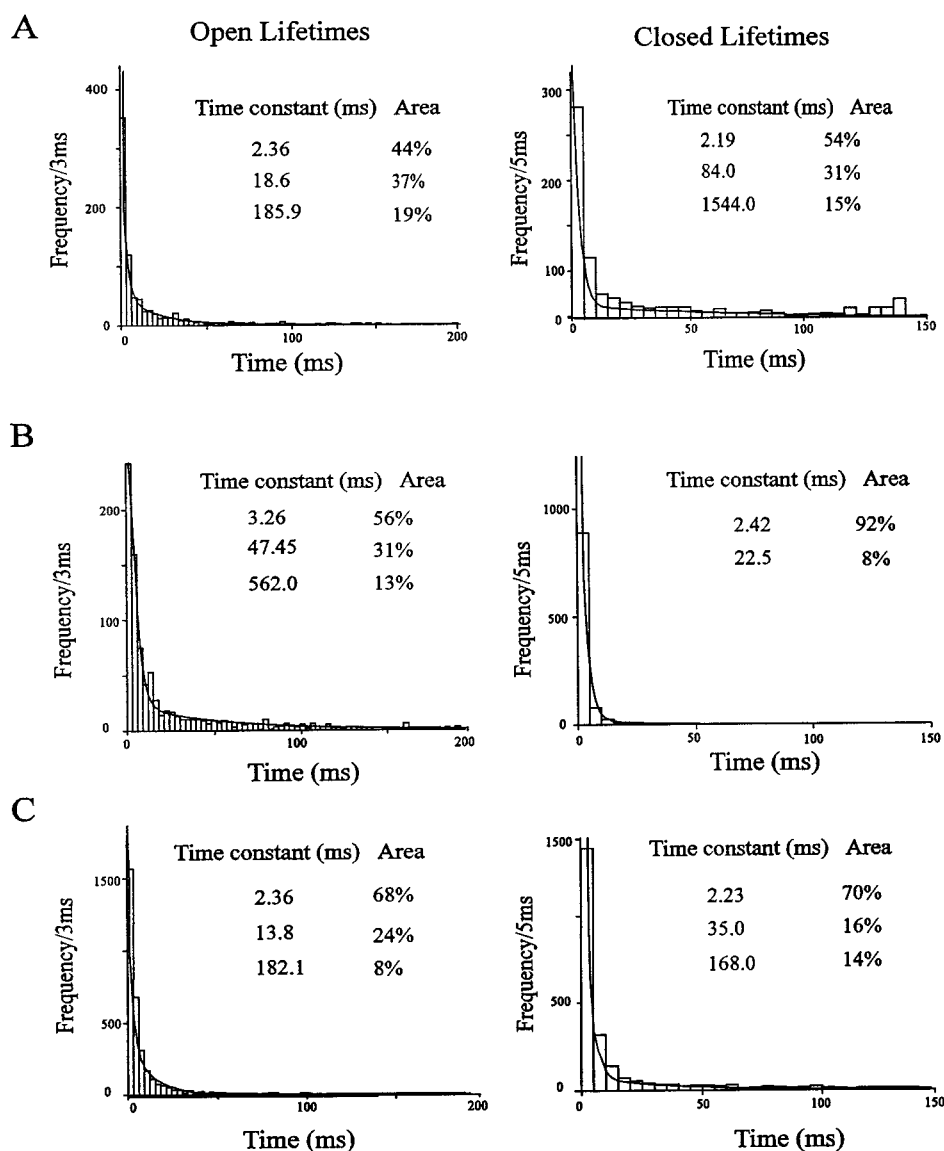


Figure 5 Effects of increasing cytosolic $[Ca^{2+}]$ on the open and closed lifetime distributions of a representative single lobster RyR. Open and closed lifetime distributions and pdfs were constructed from channel recordings at 30 μM (A), 300 μM (B) and 700 μM cytosolic Ca^{2+} (C). Time constants and percentage areas from the pdf's are shown. Lifetimes were determined as described in Methods. pdfs were fitted by the method of maximum likelihood according to the equation: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \dots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas a and time constants τ .

Figure 7 illustrates the effect of caffeine on the gating of typical single RyR channels in the presence of cytosolic 10 μM Ca^{2+} (Figure 7A) and 700 μM Ca^{2+} (Figure 7B). Addition of caffeine (10 mM) in the presence of 10 μM cytosolic $[Ca^{2+}]$ increased P_o from 0 to 0.032 ± 0.02 (s.e.m.; $n=4$). Caffeine appeared to elicit only brief channel openings. The mean open lifetime was 0.98 ± 0.03 ms (s.e.m.; $n=4$) after addition of caffeine (10 mM). In the presence of 700 μM Ca^{2+} , caffeine caused no change in P_o which was 0.060 ± 0.040 (s.e.m.; $n=4$) before and 0.059 ± 0.025 (s.e.m.; $n=4$) after addition of caffeine (10 mM). Figure 8 summarizes and compares the effects of ATP and caffeine on the P_o of lobster RyR at the two different $[Ca^{2+}]$. While caffeine can significantly increase P_o in the presence of 10 μM Ca^{2+} , caffeine (10 mM) does not even increase P_o to the maximal level observed with Ca^{2+} as the sole channel activator (see Figure 4) and at 700 μM Ca^{2+} , caffeine does not cause any further increases in P_o . Thus the effect of caffeine on the gating of the lobster RyR is very different to its effect on the gating of mammalian isoforms of RyR where the

channels can be fully opened by caffeine at activating $[Ca^{2+}]$ and can be activated to a lower level at sub-activating $[Ca^{2+}]$ (Rousseau *et al.*, 1988; Rousseau & Meissner, 1989; Sitsapasan & Williams, 1990).

Discussion

The effects of cytosolic Ca^{2+} on lobster RyR channel function

The $[^3H]$ -ryanodine binding experiments (Figure 1) demonstrate that stimulation of ryanodine binding occurs over a wide range of free $[Ca^{2+}]$. Stimulation of binding starts at sub-micromolar $[Ca^{2+}]$ and peaks at approximately 300 μM with high $[Ca^{2+}]$ causing a reduction in ryanodine binding. The single channel studies reveal that peak P_o values occur within the same $[Ca^{2+}]$ range between 300–700 μM . Single channel studies (see Figure 4) also demonstrate that cytosolic Ca^{2+} , in

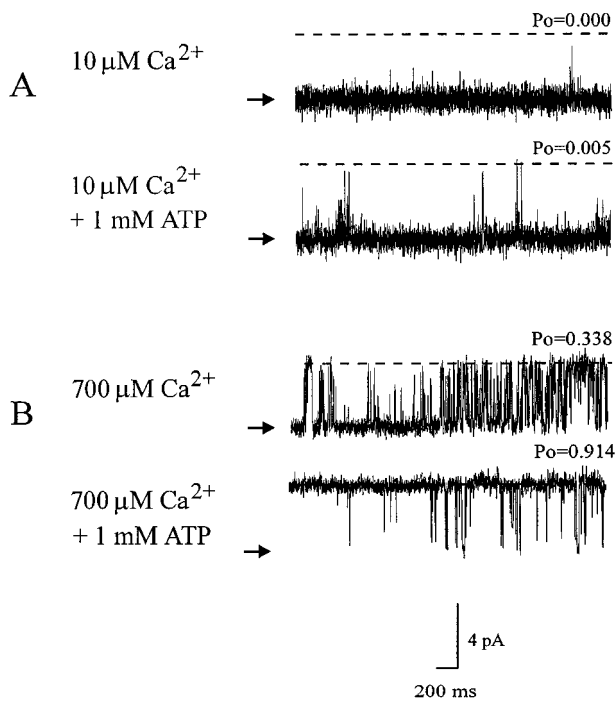


Figure 6 The effects of ATP on the gating of typical single lobster RyR channels. In (A) the free $[Ca^{2+}]$ is $10\ \mu M$. The upper and lower traces illustrate the current fluctuations through the channel before and after addition of ATP (1 mM) respectively. In (B) the free $[Ca^{2+}]$ is $700\ \mu M$. The upper and lower traces illustrate the current fluctuations through the channel before and after addition of ATP (1 mM) respectively. The holding potential was 0 mV. The arrows and the dotted lines indicate the closed and open channel levels respectively. The P_o values are shown above each trace.

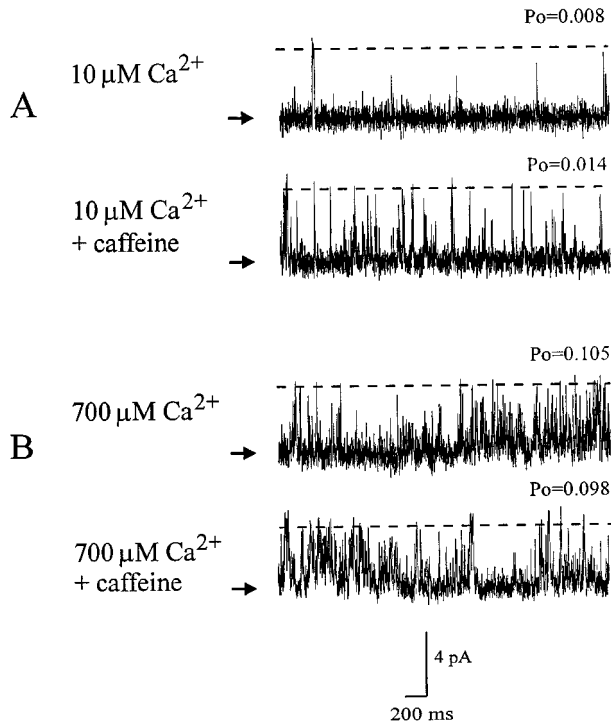


Figure 7 The effects of caffeine on the gating of typical single lobster RyR channels. In (A) the free $[Ca^{2+}]$ is $10\ \mu M$. The upper and lower traces illustrate the current fluctuations through the channel before and after addition of caffeine (10 mM) respectively. In (B) the free $[Ca^{2+}]$ is $700\ \mu M$. The upper and lower traces illustrate the current fluctuations through the channel before and after addition of caffeine (10 mM) respectively. The holding potential was 0 mV. The arrows and the dotted lines indicate the closed and open channel levels respectively. The P_o values are shown above each trace.

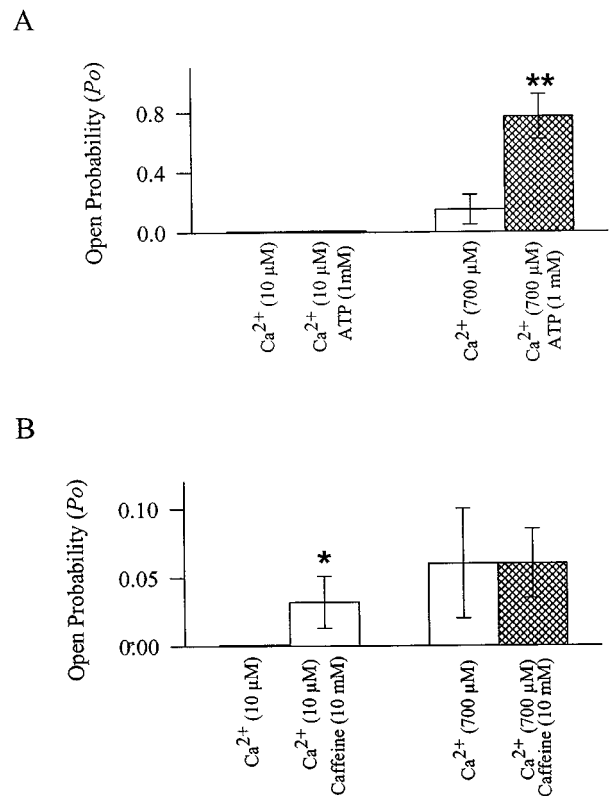


Figure 8 Comparison of the effects of ATP and caffeine on P_o at 10 and $700\ \mu M$ Ca^{2+} . In (A) the effect of ATP (1 mM) in the presence of free Ca^{2+} (10 and $700\ \mu M$) is shown. In (B) the effect of caffeine (10 mM) in the presence of free Ca^{2+} (10 and $700\ \mu M$) is shown. In each case, $n \geq 4$ and s.e.m. is shown by the bars. ** indicates P value < 0.01 , and * $P < 0.05$.

the absence of other ligands, cannot fully activate the lobster RyR and maximum steady-state P_o values are generally < 0.5 . This is also the case for other mammalian skeletal isoforms of RyR (Smith *et al.*, 1986; Sitsapesan & Williams, 1995a; Copello *et al.*, 1997) and the sheep cardiac RyR (Ashley & Williams, 1990). In contrast, cytosolic Ca^{2+} can activate dog cardiac RyR to P_o values between 0.75 and 1. The EC_{50} values for the activation and inactivation of lobster skeletal RyR channels by Ca^{2+} are $60\ \mu M$ and 1 mM respectively. Ca^{2+} activation of the lobster RyR therefore requires higher $[Ca^{2+}]$ than any other isoform of RyR studied at the single channel level (Smith *et al.*, 1986; Ashley & Williams, 1990; Bull & Marengo, 1993; Percival *et al.*, 1994; Copello *et al.*, 1997; Chen *et al.*, 1997). The single channel studies demonstrate that inactivation of the lobster RyR by Ca^{2+} occurs at only slightly higher levels than are generally required for inactivation of mammalian skeletal channels (Chu *et al.*, 1993; Laver *et al.*, 1995; Copello *et al.*, 1997) but at lower levels to those required for inactivation of the mammalian cardiac RyR (Chu *et al.*, 1993; Laver *et al.*, 1995; Copello *et al.*, 1997).

How do our results compare with other reports of the effects of cytosolic Ca^{2+} on lobster skeletal RyR gating? Seok *et al.* (1992) reported that micromolar Ca^{2+} was ineffective and that maximal increases in the P_o of purified lobster RyR channels were only observed at very high $[Ca^{2+}]$ (≥ 10 mM). These results could reflect the loss of accessory proteins, essential for fully functional channels, during the purification procedure. In contrast, Quinn *et al.* (1998) report maximal P_o values for native lobster skeletal RyR at approximately $1\ \mu M$ Ca^{2+} and P_o levels remain above 0.1 even at $[Ca^{2+}]$ as low as 10 nM. As the conductance of the channels reported by Quinn *et al.*

(1998) is approximately half (49 pS) that reported as the full conductance level for all other isoforms of RyR (including the lobster RyR in the present study (98 pS)) it is reasonable to conclude that they are observing RyR channels gating in a sub-conductance level and therefore the mechanisms of channel activation by various ligands may be affected.

Figures 3, 4 and 5 demonstrate that cytosolic Ca^{2+} increases P_o by increasing both the frequency of channel opening and the duration of the open events. Lifetime analysis also highlights that at $\geq 30 \mu\text{M}$ Ca^{2+} , three open states and three closed states can be resolved (Figure 5). In mammalian skeletal RyR, under similar ionic conditions and even under conditions where the resolution of the events is much greater, only one or two open states can be resolved (Smith *et al.*, 1986; Sitsapasan & Williams, 1995a,b) at any cytosolic $[\text{Ca}^{2+}]$ and 75–100% of the open events occur with the shortest time constant which is close to 1 ms. In contrast, the lobster RyR at $\geq 30 \mu\text{M}$ cytosolic Ca^{2+} exhibits openings to at least three time constants which are of longer duration than has been reported for any skeletal muscle isoform of RyR at comparable P_o values when activated solely by cytosolic Ca^{2+} (Smith *et al.*, 1986; Percival *et al.*, 1994; Sitsapasan & Williams, 1995a,b). The lobster RyR also exhibits openings that are fairly well distributed between the three open states with only 40–70% of the events occurring to the shortest time constant. Thus the basic gating scheme for the Ca^{2+} -activated lobster skeletal RyR deviates significantly from that reported for mammalian skeletal isoforms of RyR. The mechanism for Ca^{2+} activation appears to be more similar to that reported for dog cardiac RyR (Rousseau & Meissner, 1989; Chu *et al.*, 1993) where the open events are generally longer and Ca^{2+} as the sole activator causes large increases in the open lifetime duration in addition to an increase in the frequency of channel opening.

ATP activation

Both the $[\text{H}^3]$ -ryanodine binding and the single channel studies demonstrate that ATP is most effective at optimal concentrations of Ca^{2+} . The single channel studies demonstrate that ATP has little effect on RyR gating at a $[\text{Ca}^{2+}]$ where P_o is very low but can cause almost full activation of the channel at the optimal $[\text{Ca}^{2+}]$ of $700 \mu\text{M}$. The effects of ATP on lobster RyR are therefore highly dependent on cytosolic $[\text{Ca}^{2+}]$. The results suggest that the effects of ATP on the lobster RyR are very similar to the reported effects of ATP on the gating of mammalian isoforms of RyR (Smith *et al.*, 1986; Kermode *et al.*, 1995; Chen *et al.*, 1997; Copello *et al.*, 1997; Meissner *et al.*, 1997). ATP appears to interact synergistically with Ca^{2+} to increase P_o to levels greater than the sum of the effects of Ca^{2+} alone and ATP alone. Seok *et al.* (1992) found that ATP did not activate purified lobster RyR but as the purified channels were not sensitive to micromolar cytosolic Ca^{2+} and as ATP and Ca^{2+} act in synergy to fully activate lobster RyR, any change in the Ca^{2+} sensitivity of the channels will necessarily alter the effects of ATP.

Caffeine activation

Caffeine, at concentrations (10–40 mM) that will virtually fully open mammalian isoforms of RyR (Rousseau *et al.*, 1988; Rousseau & Meissner, 1989; Sitsapasan & Williams, 1990; Chen *et al.*, 1997) in the presence of activating cytosolic $[\text{Ca}^{2+}]$, did not cause very large changes in the P_o of lobster skeletal RyR (see Figures 7 and 8). It has been suggested that low concentrations (0.5–2 mM) of agents acting *via* the caffeine binding sites on sheep cardiac RyR sensitize the channel to

Ca^{2+} (Sitsapasan & Williams, 1990; Williams & Holmberg, 1990) but that high concentrations of caffeine or related compounds can, in the presence of activating cytosolic $[\text{Ca}^{2+}]$, fully activate the channel by invoking a different gating scheme (Sitsapasan & Williams, 1990; Williams & Holmberg, 1990; McGarry & Williams, 1994). With the lobster RyR, however, even in the presence of activating levels of cytosolic Ca^{2+} , caffeine cannot maximally activate the channel. Caffeine increases the frequency of channel opening but has little effect on the duration of channel openings. Therefore, although the ryanodine binding studies indicate that caffeine is sensitizing RyR to Ca^{2+} because the activation portion of the $[\text{H}^3]$ -ryanodine bound– $[\text{Ca}^{2+}]$ relationship is shifted to the left, the single channel studies indicate a different mechanism of action. If caffeine was sensitizing the channel to Ca^{2+} , then it would be expected that caffeine would increase P_o by increasing both the frequency and the duration of open events since this is the mechanism by which Ca^{2+} activates the lobster RyR. Therefore, while the $[\text{H}^3]$ -ryanodine binding studies indicate that the channels appear to be more sensitive to cytosolic Ca^{2+} , the mechanism for the increase in P_o may not be simply 'sensitization' to Ca^{2+} although it may involve a change in the mechanism of Ca^{2+} binding to RyR and/or a change in the effect of Ca^{2+} once bound to the channel. High levels of caffeine (>10 mM) are required to cause slight activation of the lobster RyR. In comparison, 10 fold lower [caffeine] can increase the P_o of mammalian cardiac (Rousseau & Meissner, 1989; Sitsapasan & Williams, 1990), skeletal (Rousseau *et al.*, 1988) and recombinant rabbit uterus RyR3 (Chen *et al.*, 1997) to 0.5–1. The caffeine binding sites on the lobster RyR may be significantly different to those on mammalian isoforms of RyR so far studied. Recently it has been reported that caffeine does not stimulate $[\text{H}^3]$ -ryanodine binding to an insect muscle membrane fraction whereas Ca^{2+} and ATP do (Scott-Ward *et al.*, 1997). It is therefore, reasonable to postulate that the caffeine binding sites on subtypes of RyR from arthropods may be very different (or even absent in some species) to those on mammalian isoforms of RyR.

Physiological relevance

Evidence suggests that the mechanism for EC-coupling in crustacean skeletal muscle is CICR (Rojas *et al.*, 1987; Vergara & Verdugo, 1988; Lea & Ashley, 1989; 1990; Rojas *et al.*, 1992; Palade & Györke, 1993) rather than VDCR which occurs in mammalian skeletal muscle. Our experiments have demonstrated that the lobster RyR responds in a novel manner to cytosolic Ca^{2+} and to caffeine. The concentration range over which Ca^{2+} activates and inactivates the lobster RyR may be of particular importance. Higher levels of trigger $[\text{Ca}^{2+}]$ may be required to cause SR Ca^{2+} -release in crustacean skeletal muscle than is required in cardiac muscle since the activation portion of the relationship between lobster RyR P_o and $[\text{Ca}^{2+}]$ lies to the right of that for RyR2. This is not unreasonable since the resting intracellular free $[\text{Ca}^{2+}]$ in crustacean muscle is thought to be relatively high (300–350 nM) (Ridgway & Gordon, 1995). The gating of the lobster RyR is undoubtedly very sensitive to regulation by other agents, for example ATP, which act synergistically with Ca^{2+} to alter the degree of channel activation and the apparent sensitivity of the channel for Ca^{2+} .

In conclusion, we have demonstrated that RyRs from lobster skeletal muscle have close functional similarities with vertebrate RyR in terms of the single channel conductance and the effects of ryanodine and ATP. The mechanism for Ca^{2+} -activation of lobster RyR, while showing similarities to that of

Ca^{2+} -activation of other isoforms of RyR, has distinct characteristics. Our experiments also suggest that the caffeine sites on lobster RyR are different to those on mammalian isoforms of RyR.

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This work was supported by the Wellcome Trust and the British Heart Foundation.

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(Received October 5, 1998
Revised November 19, 1998
Accepted December 2, 1998)