EP₄ prostanoid receptor-mediated vasodilatation of human middle cerebral arteries

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1 Dilatation of the cerebral vasculature is recognised to be involved in the pathophysiology of migraine. Furthermore, elevated levels of prostaglandin E₂ (PGE₂) occur in the blood, plasma and saliva of migraineurs during an attack, suggestive of a contributory role. In the present study, we have characterised the prostanoid receptors involved in the relaxation and contraction of human middle cerebral arteries *in vitro*.

2 In the presence of indomethacin (3 μ M) and the TP receptor antagonist GR32191 (1 μ M), PGE₂ was found to relax phenylephrine precontracted cerebral arterial rings in a concentration-dependent manner (mean pEC₅₀ 8.0 $\pm$ 0.1, n = 5).

3 Establishment of a rank order of potency using the EP₄ > EP₂ agonist 11-deoxy PGE₁, and the EP₂ > EP₄ agonist PGE₁-OH (mean pEC₅₀ of 7.6 $\pm$ 0.1 (n = 6) and 6.4 $\pm$ 0.1 (n = 4), respectively), suggested the presence of functional EP₄ receptors. Furthermore, the selective EP₂ receptor agonist butaprost at concentrations < 1 μ M failed to relax the tissues.

4 Blockade of EP₄ receptors with the EP₄ receptor antagonists AH23848 and EP₄A caused significant rightward displacements in PGE₂ concentration–response curves, exhibiting pA₂ and pK_B values of 5.7 $\pm$ 0.1, n = 3, and 8.4, n = 3, respectively.

5 The IP receptor agonists iloprost and cicaprost relaxed phenylephrine precontracted cerebral arterial rings (mean pEC₅₀ values 8.3 $\pm$ 0.1 (n = 4) and 8.1 $\pm$ 0.1 (n = 9), respectively). In contrast, the DP and FP receptor agonists PGD₂ and PGF_{2 α} failed to cause appreciable relaxation or contraction at concentrations of up to 30 μ M. In the absence of phenylephrine contraction and GR32191, the TP receptor agonist U46619 caused concentration-dependent contraction of cerebral artery (mean pEC₅₀ 7.4 $\pm$ 0.3, n = 3).

6 These data demonstrate the presence of prostanoid EP₄ receptors mediating PGE₂ vasodilatation of human middle cerebral artery. IP receptors mediating relaxation and TP receptors mediating contraction were also functionally demonstrated.

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Abbreviations: AH23848, [1 α (Z),2 β ,5 α]-(+/-)-7-[5-[[[1,1'-biphenyl]-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; GR32191, 1,11 α ,15S-trihydroxy-prost-13E-en-9-one;9-oxo-15S-hydroxy-prost-13E-en-1-oic acid; EP₄A, (4'-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[1,2,4]triazol-4-ylmethyl]-biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide]; PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂; PGE₁-OH, prostaglandin E₁ alcohol; PGF_{2 α} , prostaglandin F_{2 α} ; PGI₂, prostaglandin I₂

Introduction

The actions of the five naturally occurring prostanoid metabolites of arachidonic acid (PGD₂, PGE₂, PGF_{2 α} , PGI₂ and thromboxane A₂) are mediated *via* interaction with specific plasma membrane, G-protein-coupled receptors. Five major subdivisions of the prostanoid receptor family, termed DP, EP, FP, IP and TP, have been defined on the basis of their pharmacological sensitivity and molecular identity (Coleman *et al.*, 1994a). In smooth muscle, FP and TP receptors are functionally associated with contractile responses, while DP

and IP receptors mediate relaxation. EP receptors have been pharmacologically classified further into EP₁, EP₂, EP₃ and EP₄ subtypes, on the basis of their relative sensitivities to a range of naturally occurring and synthetic agonists and antagonists. While EP₁ and EP₃ receptors are coupled to Ca²⁺ mobilisation and the inhibition of cAMP *via* Gq/Gi G-proteins and mediate smooth muscle contraction, EP₂ and EP₄ receptors are coupled to the stimulation of adenylyl cyclase *via* Gs G-proteins, and have previously been shown to exert relaxant effects on vascular smooth muscle (Negishi *et al.*, 1995).

Prostaglandins are important mediators of pain and inflammation, and considerable evidence implicates their

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involvement in the pathogenesis of migraine headache. Clinically, intravenous administration of the cyclooxygenase inhibitor aspirin is effective in treating acute migraine (Diener, 1999). Furthermore, non-steroidal anti-inflammatory drugs such as paracetamol, ibuprofen, ketoprofen and diclofenac, which can inhibit prostaglandin synthesis, have been shown to be 2–3-fold more effective than placebo in treating migraine and tension headache (The Diclofenac-K/Sumatriptan Migraine Study Group, 1999; Kellstein *et al.*, 2000; Codispoti *et al.*, 2001; Dib *et al.*, 2002; Prior *et al.*, 2002). Levels of PGE₂ are elevated in the plasma and saliva of migrainers during an attack (Nattero *et al.*, 1989; Obach Tuca *et al.*, 1989), and in venous blood it reaches a sustained peak response within 2 h of migraine onset (Sarchielli *et al.*, 2000). Prostaglandins have also been implicated in alcohol-induced migraine attacks and hangover, where low concentrations of ethanol can enhance PGE₂-stimulated cAMP formation, stimulate prostaglandin biosynthesis and block prostaglandin metabolism (Parantainen, 1983). Furthermore, migraine-like symptoms can be induced in migrainers by the exogenous administration of prostaglandins of the E series (Carlson *et al.*, 1968; Peatfield *et al.*, 1981). In addition, a well-observed, dominant adverse side effect seen on oral administration of the IP receptor agonist iloprost is headache (Hildebrand, 1997; Gao *et al.*, 2002).

Clinically effective treatments such as the 5-HT_{1B/1D} receptor agonists, known as triptans, are supposed to derive their antimigraine benefit *via* inhibition of neuropeptide-mediated activation of trigeminovascular afferents (Goadsby & Edvinsson, 1993) and vasoconstriction of the cerebral and meningeal vasculature (Humphrey & Feniuk, 1991; Moskowitz, 1993), but not the cerebral microcirculation (Kobari *et al.*, 1993). Of these two interlinked components, electrical or inflammatory mediated stimulation of rat trigeminal ganglia *in vitro* has been demonstrated to cause a significant release of the neuropeptide calcitonin gene-related peptide (CGRP) from sensory nerve fibres, and a delayed synthesis and release of PGE₂ from dura mater (Rich *et al.*, 1996; Ebersberger *et al.*, 1999). This, in turn, can lead to the activation of pain-stimulating trigeminovascular afferents that innervate and cause vasodilatation of the cranial and cerebral vasculature (Williamson *et al.*, 1997). cAMP-coupled, functional EP prostanoid receptors have recently been demonstrated to be present on cultured rat trigeminal neurones, where stimulation mediates Ca²⁺-dependent CGRP release (Jenkins *et al.*, 2001).

The effects of prostanoids on isolated cerebral blood vessels have been examined previously, but considerable species differences have been reported. PGE₂ has been demonstrated to weakly relax 5-HT precontracted feline basilar and middle cerebral arteries (Whalley *et al.*, 1989), but to contract canine, rabbit and human basilar arteries (Nakagomi *et al.*, 1988; Parsons & Whalley, 1989). Despite potential experimental protocol and species differences, these data may suggest that distinct populations of prostanoid receptors are found in different vascular regions, as illustrated by differences in vasoconstrictor 5-HT receptors present in the macro and microcirculation. Given the potential involvement of PGE₂ in the pathophysiology of migraine, in the present study, we have pharmacologically classified prostanoid receptors mediating responses to PGE₂ on human middle cerebral artery.

Methods

Human tissues

All samples of human tissue were obtained through medically qualified intermediaries at the Netherlands Brain Bank with the informed consent of the donor or donor's next of kin, and with approval of the local research ethics committee. Tissues were rapidly removed at autopsy, transported on wet ice in phosphate-buffered saline (PBS), and stored in Krebs buffer at 4°C until the experiment, which was carried out within 72 h of tissue removal from the patient. Viable cerebral arteries were obtained from 19 donors (11 male, eight female), age range 59–88 (mean age $\pm$ s.e.m. of 76 ± 4 years).

Pharmacology

Sections of middle cerebral artery were carefully removed from samples of human cerebral vasculature containing an intact circle of Willis using sharp dissection scissors. Visibly, atherosclerotic regions of tissue were not used. Intact rings of middle cerebral arteries, ~2–3 mm in length and 1–2 mm internal diameter, were suspended between stainless steel hooks in 10 ml organ baths containing oxygenated (95% O₂/5% CO₂) Krebs' buffer containing (in mM): NaCl 118.2, KCl 4.69, MgSO₄·7H₂O 1.18, KH₂PO₄ 1.19, glucose 11.1, NaHCO₃ 25.0, CaCl₂·6H₂O 2.5, indomethacin 0.003, pH 7.4 at 37°C. Tissues were placed under a tension equivalent to 5 mN and left to equilibrate for a period of at least 60 min. Responses were recorded using isometric transducers coupled to an Apple Macintosh computer *via* a MacLab interface. Following the equilibration period, a cumulative contractile concentration–response curve to phenylephrine (minimum of 5 min contact time of each concentration of agonist) was constructed in all tissues. After washout, an approximate EC₅₀ concentration of phenylephrine (1 μ M) was added to obtain a stable contraction. Tissues were subsequently exposed to increasing concentrations of a single prostanoid receptor agonist, in the absence or presence of receptor antagonists (incubated for 60 min prior to exposure to agonist), to pharmacologically characterise and functionally determine the role of prostanoid receptors in controlling arterial tone. Untreated tissues were run in parallel to agonist-treated tissue, to serve as time-matched controls. Experiments on the relaxant effects of prostanoids were performed in the presence of the TP receptor antagonist GR32191 (1 μ M) (Lumley *et al.*, 1989); as in the absence of GR32191, PGE₂ caused vasodilatation at low concentrations, but vasoconstriction at concentrations greater than 0.1 μ M ($n=3$ data not shown). The maximal relaxatory responses of tissues were determined at the end of each experiment, by the addition of prostacyclin (1 μ M).

Materials

The following compounds were used in this study: PGE₂, PG1₂, PGD₂, PGE₁-OH and 11-deoxy PGE₁, PGF_{2 α} (Alexis Corp, U.K.), [1*R*-[1 α (Z),2 β ,3 β ,5 α]]-(+)-7-[5-[[1,1'-biphenyl]-4-ylmethoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptonic acid (GR32191) was a kind gift from Glaxo SmithKline, U.K., iloprost and cicaprost were gifts from Schering AG, Germany, 1 α (Z),2 β ,5 α -(+/-)-7-[5-[[1,1'-biphenyl]-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid (AH-23848)

(Coleman *et al.*, 1994b) and 4'-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[1,2,4]triazol-4-ylmethyl] biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide (EP₄A) (Machwate *et al.*, 2001; 2003) were synthesised in-house.

Data analysis

To calculate a pK_B value for EP₄A, the mean CR was plotted as log (CR-1) against log antagonist molar concentration, according to the method of Arunlakshana & Schild (1959). If the slope of the plot did not differ significantly from unity, it was constrained to unity to calculate an apparent pK_B value. As only one concentration of AH23848 was tested, a slope of unity was assumed and pA₂ estimated using the Gaddum-Schild equation (where pA₂=log[concentration ratio-1]-log[antagonist]).

Results

Addition of phenylephrine (10⁻⁸–10⁻⁴ M at half-log increments) caused reproducible, concentration-dependent contractile responses of isolated preparations of the human middle cerebral artery, exhibiting a mean pEC₅₀ of 5.8±0.1 (*n*=7). Contractions observed at all phenylephrine concentrations were well maintained. Application of increasing concentrations of PGE₂ to cerebral artery rings precontracted with an approximate pEC₅₀ concentration of phenylephrine (1 µM) produced concentration-dependent relaxations to basal levels (Figure 1), exhibiting a mean pEC₅₀ for PGE₂ of 8.0±0.1 (*n*=5) (Figure 2). Mechanical denudation of the arterial endothelium or the addition of the nitric oxide synthase inhibitor L-NAME (100 µM) had no effect on PGE₂ relaxations (*n*=3, data not shown), suggestive that these responses are mediated *via* direct action on the vascular smooth muscle and not *via* nitric oxide generation in the endothelium.

Of the four known members of the EP prostanoid receptor family, only EP₂ and EP₄ receptors potentially mediate relaxatory responses of smooth muscle to PGE₂. PGE₂-induced relaxant responses were characterised in three ways: by establishment of a rank order of agonist potencies of prostanoid ligands, *via* the use of a selective EP₂ agonist, and finally with the use of two known EP₄ receptor antagonists.

A range of PGE₂-related analogues was shown to cause relaxations of phenylephrine precontracted preparations (Figure 2). Establishment of a rank order of potency using the EP₄>EP₂ agonist 11-deoxy PGE₁, and the EP₂>EP₄ agonist PGE₁-OH, suggested the presence of functional EP₄ receptors. The mean pEC₅₀ values for 11-deoxy PGE₁ and PGE₁-OH were 7.6±0.1 (*n*=6) and 6.4±0.1 (*n*=4), respectively. The selective EP₂ agonist butaprost was without effect at concentrations below 1 µM, suggestive of the absence of functional EP₂ receptors in human middle cerebral arteries (*n*=3).

In human middle cerebral arterial rings, the combined TP/EP₄ receptor antagonist AH23848 (10 µM) caused a surmountable, rightward shift in the PGE₂ concentration–response curve (pA₂ 5.7±0.1; *n*=3; Figure 3a). The high-affinity, selective EP₄ antagonist EP₄A caused rightward, surmountable displacement of PGE₂ concentration–effect curves (Figure 3b). Schild analysis generated a plot with slope not significantly different from unity (1.2±0.2) and a pK_B of 8.4 (*n*=3/4, at each concentration, Figure 3c).

Further characterisation of relaxatory prostanoid receptors demonstrated that the stable IP receptor agonists iloprost and cicaprost potently relaxed phenylephrine precontracted human middle cerebral arteries, exhibiting mean pEC₅₀ values of 8.3±0.1 (*n*=4) and 8.1±0.1 (*n*=9), respectively (Figure 2b). The DP receptor agonist PGD₂ produced only weak relaxations at high concentrations (*n*=5). PGF_{2α} was without appreciable effect at concentrations up to 100 µM (*n*=5). In the absence of phenylephrine precontraction and GR32191, the TP receptor agonist U-46619 caused concentration-dependent contractions, exhibiting a mean pEC₅₀ value of 7.5±0.1 (*n*=5, data not shown).

Discussion

In the present study, we have demonstrated that PGE₂ is a potent vasodilator of human precontracted middle cerebral artery, and have characterised the receptor mediating this response. The EP receptor family consists of four members that exhibit nanomolar affinity for the endogenous prostanoid PGE₂ (Coleman *et al.*, 1994a). Human middle cerebral arteries were found to express specific mRNA for each of the prostanoid receptors (unpublished observations, R. Davis). Pharmacological classification using endogenous and synthetic

Figure 1 Typical response to PGE₂ on phenylephrine precontracted human middle cerebral artery. The figure shows concentration-dependent relaxation in response to increasing concentrations of PGE₂ (0.1 nM–3 µM in half-log increments).

Figure 2 Characterisation of PGE₂ relaxatory response using a range of prostanoid ligands on human middle cerebral artery. (a) The EP₄>EP₂ agonist 11-deoxy PGE₁ and the EP₂>EP₄ agonist PGE₁-OH (1 nM–10 μ M), caused relaxation of cerebral artery rings, with a rank order suggestive of the presence of functional EP₄ receptors. Butaprost was without significant effect at concentrations below 1 μ M. (b) The IP receptor agonists iloprost and cicaprost (0.3 nM–10 μ M) equipotently caused concentration-dependent relaxations of phenylephrine precontracted cerebral rings. PGD₂ (1 nM–100 μ M) produced only weak relaxatory responses at high concentrations. Data are expressed as mean $\pm$ s.e.m. % of phenylephrine contraction.

ligands demonstrated that the EP₄>EP₂ agonist 11-deoxy PGE₁ and the EP₂>EP₄ agonist PGE₁-OH were both full agonists, exhibiting approximately two-fold and 40-fold lower potency than PGE₂, respectively. These data are consistent with the relative binding affinities reported for these agonists at cloned human, rat, rabbit and mouse EP₄ receptors (Breyer *et al.*, 1996; Boie *et al.*, 1997; Kiriya *et al.*, 1997; Davis & Sharif, 2000). Furthermore, butaprost, which exhibits nanomolar affinity for the EP₂ receptor (Gardiner, 1986) but micromolar affinity for the EP₄ receptor, was without effect at concentrations below 1 μ M, suggestive of the absence of functional EP₂ receptors. Characterisation of the presence of other prostanoid receptors functionally expressed in human middle cerebral arteries demonstrated contractile TP and relaxatory IP receptors, in good agreement with previous studies (Uski *et al.*, 1983), but the absence of EP₁, EP₂, EP₃, FP and DP receptors.

Although rank orders of agonist potency pointed to a role for EP₄ receptors in the cerebral vasodilator response to PGE₂, an evaluation of various EP₄ receptor antagonists was performed to consolidate this preliminary conclusion. The weak EP₄ antagonist activity of the TP antagonist AH23848 was first described on PGE₂-mediated relaxation of phenyl-

Figure 3 Effects of EP₄ receptor antagonists on PGE₂-mediated relaxation of phenylephrine pre-contracted human middle cerebral artery. Cerebral artery rings were preincubated for 60 min with (a) AH23848 (10 μ M) or (b) different concentrations of EP₄A (30, 300, 1000 and 3000 nM), and then cumulatively concentration-dependently relaxed with PGE₂ in the presence of GR32191 (1 μ M). Data are expressed as percentage of the phenylephrine contraction, and are given as mean $\pm$ s.e.m. for $n = 3/4$ donors. (c) Schild plot of the antagonist effect of EP₄A on responses to PGE₂ in human middle cerebral artery.

ephrine precontracted isolated smooth muscle rings of pig saphenous vein (Coleman *et al.*, 1994b). Although described as a low affinity, competitive antagonist at the EP₄ receptor in this tissue, there are reports suggesting that its functional antagonism is not truly surmountable (Blaschke *et al.*, 1996). In the present study, AH23848 (10 μ M) was found to cause a significant rightward shift in the PGE₂-mediated vasodilatation of human middle cerebral artery, exhibiting a pA₂ value (5.7) consistent with that previously reported (pA₂ 5.4; Coleman *et al.*, 1994b). Further support for the functional

expression of vasodilatory EP₄ receptors was demonstrated with the recently reported potent and selective EP₄ antagonist EP₄A (Machwate *et al.*, 2001; 2003). At human recombinant EP receptors, EP₄A exhibits high affinity for the EP₄ receptor ($pK_i = 7.6$), and approximately 80-, 800-, 280- and 30-fold selectivity over EP₃, EP₂/EP₁, IP and TP receptors, respectively. EP₄A caused concentration-related rightward, surmountable displacement of PGE₂ concentration–effect curves in the human middle cerebral artery, with a pK_B consistent with PGE₂ activity *via* EP₄ receptors. While the highest concentrations of EP₄A utilised may exhibit some weak antagonism at relaxatory IP receptors present in the human middle cerebral artery, it is unlikely that this contributes to its inhibition of PGE₂-mediated relaxation, given the low affinity of PGE₂ for IP receptors. The potency of EP₄A on IP receptor-mediated functions has not been reported.

The timing of the cerebral vascular component and augmentation of blood flow involved in the pathophysiology of migraine has been open to speculation. While the triptans (5-HT_{1B/1D} receptor agonists) can mediate vasoconstriction of the cerebral vasculature (Humphrey & Feniuk, 1991), their clinical efficacy may derive from inhibition of vasodilatation caused by neuropeptide or other inflammatory mediators released from trigeminal neurones (Goadsby & Edvinsson, 1993). Trigeminal sensory and visceral projections innervate the middle cerebral artery (Arbab *et al.*, 1988), and stimulation of these afferents in healthy volunteers with capsaicin can mediate pain and cranial vasodilatation (May *et al.*, 2001). Cortical spreading depression (CSD), a putative trigger for migraine with aura, has been shown experimentally to increase meningeal blood flow *via* vasodilatation associated with the stimulation of trigeminovascular afferents and trigeminal-dependent parasympathetic activation. However, the reverse is not true, such that sustained vasodilatation does not trigger trigeminal activation after CSD (Bolay *et al.*, 2002), and demonstrating that the vasodilatation component is secondary to afferent stimulation.

While much emphasis has focused on the neuropeptide CGRP as the agent mediating vasodilatation following

trigeminal activation, a role for prostaglandins in migraine has been implicated. Elevated levels of PGE₂ in the plasma, saliva and venous blood have been reported in migraineurs during an attack (Nattero *et al.*, 1989; Obach Tuca *et al.*, 1989; Sarchielli *et al.*, 2000), and inhibitors of prostaglandin synthesis have shown some clinical efficacy. Although CGRP and PGE₂ are not released in an *in vitro* model of CSD, (Ebersberger *et al.*, 2001), electrical or inflammatory mediated stimulation of rat trigeminal ganglia *in vitro* does cause a significant release of the CGRP from sensory nerve fibres, and a delayed synthesis and release of PGE₂ from dura mater (Rich *et al.*, 1996; Ebersberger *et al.*, 1999), an effect which can be inhibited by dihydroergotamide or sumatriptan (Buzzi *et al.*, 1991). Moreover, PGE₂-induced activation of EP₂ and possibly other EP receptors can stimulate the release of CGRP from rat cultured trigeminal neurones (Jenkins *et al.*, 2001) and sensory afferents *in vivo* following irritation-induced visceral pain (Friese *et al.*, 1997; Boku *et al.*, 2001). In rat dura (Zimmermann *et al.*, 2002) and rat tracheal afferent nerves (Hua *et al.*, 1994), PGE₂ released secondary to agonist stimulation can mediate CGRP release. Taken together, these data potentially suggest an early involvement of PGE₂ in the development and prolongation of migraine, potentially upstream of CGRP release.

In conclusion, accumulating evidence suggests that endogenously produced prostaglandins, most notably PGE₂, play a direct role in the pathophysiology of migraine by stimulating CGRP release from trigeminal afferents and thus mediating vasodilatation. In the present study, we have demonstrated for the first time that PGE₂ can also directly mediate vasodilatation of human cerebral arteries *via* EP₄ receptors. These data are hypothesised to be of clinical significance with reference to the aetiology of the vasodilator component of migraine. In addition, antagonism of PGE₂-induced cerebral vascular dilatation may be therapeutically beneficial. The characterisation of prostanoid receptors mediating CGRP release from human trigeminal afferents and vasodilatation in human meningeal and pial vessels is now the subject of on-going studies.

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