British Journal of Pharmacology (2004) 141, 580–585 © 2004 Nature Publishing Group All rights reserved 0007–1188/04 $25.00
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EP_4 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 E_2 (PGE_2) 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 μM) and the TP receptor antagonist GR32191 (1 μM), PGE_2 was found to relax phenylephrine precontracted cerebral arterial rings in a concentration-dependent manner (mean pEC_{50} 8.0 ± 0.1, $n = 5$).

3 Establishment of a rank order of potency using the $EP_4 > EP_2$ agonist 11-deoxy PGE_1, and the $EP_2 > EP_4$ agonist PGE_1-OH (mean pEC_{50} of 7.6 ± 0.1 ($n = 6$) and 6.4 ± 0.1 ($n = 4$), respectively), suggested the presence of functional EP_4 receptors. Furthermore, the selective EP_2 receptor agonist butaprost at concentrations < 1 μM failed to relax the tissues.

4 Blockade of EP_4 receptors with the EP_4 receptor antagonists AH23848 and EP_4A caused significant rightward displacements in PGE_2 concentration–response curves, exhibiting pA_2 and pK_B values of 5.7 ± 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 pEC_{50} values 8.3 ± 0.1 ($n = 4$) and 8.1 ± 0.1 ($n = 9$), respectively). In contrast, the DP and FP receptor agonists PGD_2 and $PGF_{2\alpha}$ failed to cause appreciable relaxation or contraction at concentrations of up to 30 μM. In the absence of phenylephrine contraction and GR32191, the TP receptor agonist U46619 caused concentration-dependent contraction of cerebral artery (mean pEC_{50} 7.4 ± 0.3, $n = 3$).

6 These data demonstrate the presence of prostanoid EP_4 receptors mediating PGE_2 vasodilatation of human middle cerebral artery. IP receptors mediating relaxation and TP receptors mediating contraction were also functionally demonstrated.
British Journal of Pharmacology (2004) **141,** 580–585. doi:10.1038/sj.bjp.0705645

Keywords: Prostaglandin E_2; EP_4 receptor; human middle cerebral artery; migraine

Abbreviations: AH23848, [1α(Z),2β,5α]-(+/−)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; GR32191, 1,11α,15*S*-trihydroxy-prost-13E-en-9-one;9-oxo-15*S*-hydroxy-prost-13E-en-1-oic acid; EP_4A, (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]; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PGE_1-OH, prostaglandin E_1 alcohol; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostaglandin I_2

Introduction

The actions of the five naturally occurring prostanoid metabolites of arachidonic acid (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2) 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 EP_1, EP_2, EP_3 and EP_4 subtypes, on the basis of their relative sensitivities to a range of naturally occurring and synthetic agonists and antagonists. While EP_1 and EP_3 receptors are coupled to Ca^{2+} mobilisation and the inhibition of cAMP *via* Gq/Gi G-proteins and mediate smooth muscle contraction, EP_2 and EP_4 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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Advance online publication: 26 January 2004

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 PGE_2 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 PGE_2-stimulated cAMP formation, stimulate prostaglandin biosynthesis and block prostaglandin metabolism (Parantainen, 1983). Furthermore, migraine-like symptoms can be induced in migraineurs 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 PGE_2 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 Ca^{2+}-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. PGE_2 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 PGE_2 in the pathophysiology of migraine, in the present study, we have pharmacologically classified prostanoid receptors mediating responses to PGE_2 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 ± 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% O_2/5% CO_2) Krebs' buffer containing (in mM); NaCl 118.2, KCl 4.69, $MgSO_4 \cdot 7H_2O$ 1.18, KH_2PO_4 1.19, glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2 \cdot 6H_2O$ 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 EC_{50} concentration of phenylephrine (1 μM) 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 μM) (Lumley *et al*., 1989); as in the absence of GR32191, PGE_2 caused vasodilatation at low concentrations, but vasoconstriction at concentrations greater than 0.1 μM ($n = 3$ data not shown). The maximal relaxatory responses of tissues were determined at the end of each experiment, by the addition of prostacyclin (1 μM).

Materials

The following compounds were used in this study: PGE_2, $PG1_2$, PGD_2, PGE_1-OH and 11-deoxy PGE_1, $PGF_{2\alpha}$ (Alexis Corp, U.K.), [1*R*-[1α(Z),2β,3β,5α]]-(+)-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, 1α(Z),2β,5α]-(+/−)-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 (EP_4A) (Machwate *et al*., 2001; 2003) were synthesised in-house.

Data analysis

To calculate a pK_B value for EP_4A, 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 pA_2 estimated using the Gaddum–Schild equation (where $pA_2 = \log[\text{concentration ratio} - 1] - \log[\text{antagonist}]$).

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 pEC_{50} of 5.8 ± 0.1 ($n = 7$). Contractions observed at all phenylephrine concentrations were well maintained. Application of increasing concentrations of PGE_2 to cerebral artery rings precontracted with an approximate pEC_{50} concentration of phenylephrine (1 μM) produced concentration-dependent relaxations to basal levels (Figure 1), exhibiting a mean pEC_{50} for PGE_2 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 PGE_2 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 EP_2 and EP_4 receptors potently mediate relaxatory responses of smooth muscle to PGE_2. PGE_2-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 EP_2 agonist, and finally with the use of two known EP_4 receptor antagonists.

A range of PGE_2-related analogues was shown to cause relaxations of phenylephrine precontracted preparations (Figure 2). Establishment of a rank order of potency using the $EP_4 > EP_2$ agonist 11-deoxy PGE_1, and the $EP_2 > EP_4$ agonist PGE_1-OH, suggested the presence of functional EP_4 receptors. The mean pEC_{50} values for 11-deoxy PGE_1 and PGE_1-OH were 7.6 ± 0.1 ($n = 6$) and 6.4 ± 0.1 ($n = 4$), respectively. The selective EP_2 agonist butaprost was without effect at concentrations below 1 μM, suggestive of the absence of functional EP_2 receptors in human middle cerebral arteries ($n = 3$).

In human middle cerebral arterial rings, the combined TP/EP_4 receptor antagonist AH23848 (10 μM) caused a surmountable, rightward shift in the PGE_2 concentration–response curve (pA_2 5.7 ± 0.1; $n = 3$; Figure 3a). The high-affinity, selective EP_4 antagonist EP_4A caused rightward, surmountable displacement of PGE_2 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 pEC_{50} values of 8.3 ± 0.1 ($n = 4$) and 8.1 ± 0.1 ($n = 9$), respectively (Figure 2b). The DP receptor agonist PGD_2 produced only weak relaxations at high concentrations ($n = 5$). $PGF_{2\alpha}$ 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 pEC_{50} value of 7.5 ± 0.1 ($n = 5$, data not shown).

Discussion

In the present study, we have demonstrated that PGE_2 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 PGE_2 (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

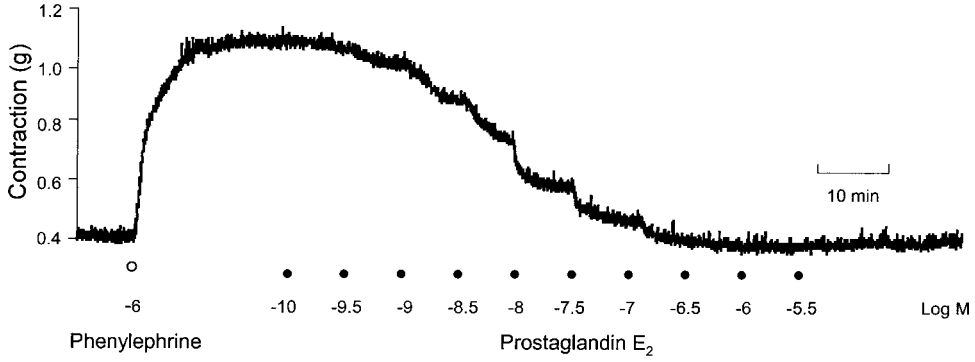

Figure 1 Typical response to PGE_2 on phenylephrine precontracted human middle cerebral artery. The figure shows concentration-dependent relaxation in response to increasing concentrations of PGE_2 (0.1 nM–3 μM in half-log increments).

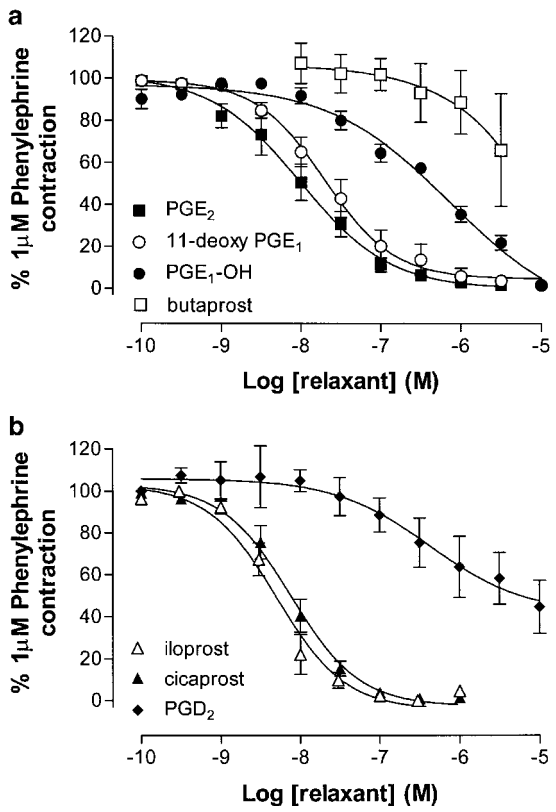

Figure 2 Characterisation of PGE_2 relaxatory response using a range of prostanoid ligands on human middle cerebral artery. (a) The $EP_4 > EP_2$ agonist 11-deoxy PGE_1 and the $EP_2 > EP_4$ agonist PGE_1-OH (1 nM–10 μM), caused relaxation of cerebral artery rings, with a rank order suggestive of the presence of functional EP_4 receptors. Butaprost was without significant effect at concentrations below 1 μM. (b) The IP receptor agonists iloprost and cicaprost (0.3 nM–10 μM) equipotently caused concentration-dependent relaxations of phenylephrine precontracted cerebral rings. PGD_2 (1 nM–100 μM) produced only weak relaxatory responses at high concentrations. Data are expressed as mean ± s.e.m.% of phenylephrine contraction.

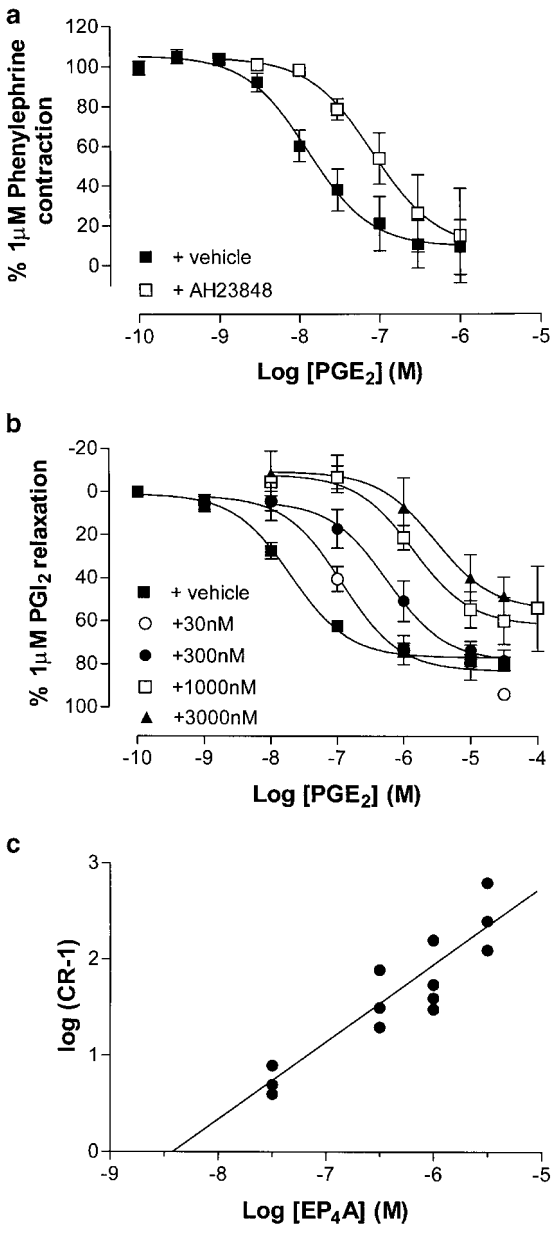

Figure 3 Effects of EP_4 receptor antagonists on PGE_2-mediated relaxation of phenylephrine pre-contracted human middle cerebral artery. Cerebral artery rings were preincubated for 60 min with (a) AH23848 (10 μM) or (b) different concentrations of EP_4A (30, 300, 1000 and 3000 nm), and then cumulatively concentration-dependently relaxed with PGE_2 in the presence of GR32191 (1 μM). Data are expressed as percentage of the phenylephrine contraction, and are given as mean ± s.e.m. for $n = 3/4$ donors. (c) Schild plot of the antagonist effect of EP_4A on responses to PGE_2 in human middle cerebral artery.

ligands demonstrated that the $EP_4 > EP_2$ agonist 11-deoxy PGE_1 and the $EP_2 > EP_4$ agonist PGE_1-OH were both full agonists, exhibiting approximately two-fold and 40-fold lower potency than PGE_2, respectively. These data are consistent with the relative binding affinities reported for these agonists at cloned human, rat, rabbit and mouse EP_4 receptors (Breyer *et al*., 1996; Boie *et al*., 1997; Kiriyama *et al*., 1997; Davis & Sharif, 2000). Furthermore, butaprost, which exhibits nanomolar affinity for the EP_2 receptor (Gardiner, 1986) but micromolar affinity for the EP_4 receptor, was without effect at concentrations below 1 μM, suggestive of the absence of functional EP_2 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 EP_1, EP_2, EP_3, FP and DP receptors.

Although rank orders of agonist potency pointed to a role for EP_4 receptors in the cerebral vasodilator response to PGE_2, an evaluation of various EP_4 receptor antagonists was performed to consolidate this preliminary conclusion. The weak EP_4 antagonist activity of the TP antagonist AH23848 was first described on PGE_2-mediated relaxation of phenylephrine precontracted isolated smooth muscle rings of pig saphenous vein (Coleman *et al*., 1994b). Although described as a low affinity, competitive antagonist at the EP_4 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 μM) was found to cause a significant rightward shift in the PGE_2-mediated vasodilatation of human middle cerebral artery, exhibiting a pA_2 value (5.7) consistent with that previously reported (pA_2 5.4; Coleman *et al*., 1994b). Further support for the functional

expression of vasodilatory EP_4 receptors was demonstrated with the recently reported potent and selective EP_4 antagonist EP_4A (Machwate *et al*., 2001; 2003). At human recombinant EP receptors, EP_4A exhibits high affinity for the EP_4 receptor ($pK_i = 7.6$), and approximately 80-, 800-, 280- and 30-fold selectivity over EP_3, EP_2/EP_1, IP and TP receptors, respectively. EP_4A caused concentration-related rightward, surmountable displacement of PGE_2 concentration–effect curves in the human middle cerebral artery, with a pK_B consistent with PGE_2 activity *via* EP_4 receptors. While the highest concentrations of EP_4A 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 PGE_2-mediated relaxation, given the low affinity of PGE_2 for IP receptors. The potency of EP_4A 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 ($5\text{-}HT_{1B/1D}$ 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 capsaisin 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 PGE_2 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 PGE_2 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 PGE_2 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, PGE_2-induced activation of EP_2 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), PGE_2 released secondary to agonist stimulation can mediate CGRP release. Taken together, these data potentially suggest an early involvement of PGE_2 in the development and prolongation of migraine, potentially upstream of CGRP release.

In conclusion, accumulating evidence suggests that endogenously produced prostaglandins, most notably PGE_2, 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 PGE_2 can also directly mediate vasodilatation of human cerebral arteries *via* EP_4 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 PGE_2-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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(Received November 11, 2003
Accepted November 24, 2003)