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# Characterization of the prostanoid receptor types involved in mediating calcitonin gene-related peptide release from cultured rat trigeminal neurones

*,[1]David W. Jenkins, [1]Wasyl Feniuk & [1]Patrick P.A. Humphrey

[1]Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge, CB2 1QJ

**1** Prostaglandins and the vasodilator neuropeptide, calcitonin-gene related peptide (CGRP), have both been implicated in the pathogenesis of migraine headache. We have used primary cultures of adult rat trigeminal neurones to examine the effects of prostanoids on CGRP release *in vitro*.

**2** CGRP release was stimulated by prostaglandin $E_2$ ($PGE_2$) and the IP receptor agonist, carbaprostacyclin ($cPGI_2$). These responses were extracellular calcium-dependent, and the $PGE_2$-induced CGRP release was unaltered by inhibition of nitric oxide synthase (NOS), ATP receptor blockade, or the addition of adenosine deaminase.

**3** Increases in CGRP levels were also observed in response to prostaglandin $D_2$ ($PGD_2$), and the $EP_2$ receptor selective agonist, butaprost. No increases in CGRP release were observed in response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or the TP receptor selective agonist, U46619, or the $EP_3$ receptor selective agonist, GR63799X.

**4** The selective DP receptor antagonist, BWA868C, antagonized the $PGD_2$-, but not $PGE_2$- or $cPGI_2$-induced release. Furthermore, the $EP_1$ selective antagonist, ZM325802, failed to antagonize the $PGE_2$-induced CGRP release from these cells.

**5** These data indicate that activation of DP, EP and IP receptors can each cause CGRP release from trigeminal neurones, and suggest that the predominant EP receptor subtype involved may be the $EP_2$ receptor. Together with evidence that the cyclo-oxygenase inhibitor, aspirin, particularly when administered intravenously is effective in treating acute migraine, these findings further suggest a role for prostaglandins in migraine pathophysiology.
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**Keywords:** Prostanoids; prostaglandin $E_2$; prostacyclin; migraine; calcitonin gene-related peptide; trigeminal ganglion; primary culture

**Abbreviations:** CGRP, calcitonin gene-related peptide; $cPGI_2$, carbaprostacyclin; DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; L-NAME, $N^G$-nitro-L-arginine; NGF, nerve growth factor; NGS normal goat serum; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate buffered saline; $PGD_2$, prostaglandin $D_2$; $PGE_2$, prostaglandin $E_2$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; $PGI_2$, prostaglandin $I_2$; PPADS, pyridoxalphosphate-6-axophenyl-2′,4′-disulphonic acid; $TXA_2$, thromboxane $A_2$

## Introduction

Prostaglandins and thromboxanes are metabolites of arachidonic acid that act as local mediators in both the central nervous system and the periphery. There are five naturally occurring prostanoids, prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin ($PGI_2$) and thromboxane $A_2$ ($TXA_2$). They exert their physiological actions through binding to membrane-bound receptors that are coupled to heterotrimeric guanine nucleotide-binding (G) proteins and belong to the class of seven transmembrane domain receptors. The scheme of prostanoid receptor classification system identifies specific receptors for each of the natural prostanoids, termed DP, EP, FP, IP and TP receptors, respectively (Kennedy *et al*., 1982; Coleman *et al*., 1994). At each of these receptors one of the natural prostanoids is at least one order of magnitude more potent than the other four. The EP receptor has been further classified into four subtypes, termed $EP_1$, $EP_2$, $EP_3$ and $EP_4$ receptors, based on studies using synthetic agonists and antagonists (Coleman *et al*., 1994).

The role of prostaglandins, particularly $PGE_2$ and $PGI_2$, in inflammatory pain is well accepted, partly because of the anti-nociceptive and anti-inflammatory effects of non-steroidal anti-inflammatory drugs (NSAIDS), and also from observations in several animal models of nociception that exogenous prostaglandins can cause hyperalgesia and allodynia (Vane, 1971; Bley *et al*., 1998). Prostaglandins have also been implicated in migraine headache. The cyclo-oxygenase inhibitor, aspirin, is effective in treating acute migraine, particularly when administered intravenously (Diener, 1999) and the effectiveness of this class of drug in migraine as well as other headaches has been comprehensively reviewed (Limmroth *et al*., 1999). It has also been reported that prostaglandins can cause migraine-like symptoms when administered to volunteers (see Coleman *et al*., 1990) and that levels of $PGE_2$ are increased in the saliva of patients undergoing migraine attacks (Vardi *et al*., 1983; Tuca *et al*., 1989). Recently, Ebersberger *et al*.

*Author for correspondence; E-mail: dwj94941@gsk.com

(1999) have also shown that $PGE_2$ is released from rat dura mater encephali following electrical stimulation of the trigeminal ganglion and chemical stimulation with inflammatory mediators (5-hydroxytryptamine, histamine and bradykinin). This provides further evidence for a possible role for prostaglandins in facilitating meningeal nociceptor activation, and promoting inflammation and pain in headache.

Also implicated in migraine is the potent vasodilator neuropeptide, calcitonin gene-related peptide (CGRP), which is localized within trigeminal afferents that innervate intracranial, extracerebral blood vessels (Edvinsson *et al*., 1987). The cell bodies of these trigeminal neurones are located in the trigeminal ganglion (external to the brain at the level of the pons) and stimulation of the trigeminal ganglion in both cats and humans causes CGRP release that can be inhibited by the clinically effective anti-migraine agent, sumatriptan (Goadsby *et al*., 1988; Goadsby & Edvinsson, 1993). The levels of this peptide are also raised in the cephalic venous blood of migraineurs during the headache-phase, and are subsequently reduced by sumatriptan (Goadsby *et al*., 1990). In light of this evidence, suggesting roles for both prostaglandins and CGRP in migraine, we have investigated the receptors and mechanisms involved in prostanoid-induced CGRP release in cultured rat trigeminal neurones.

## Methods

### *Preparation of primary cultures of adult rat trigeminal neurones*

Cultures of adult rat trigeminal neurones were prepared as previously described (Carruthers *et al*., 2001). Briefly, adult Wistar rats (175–250 g; either sex) were killed by $CO_2$ inhalation in strict accordance with U.K. Home Office regulations. Trigeminal ganglia were dissected out and placed in ice-cold calcium-, magnesium- and bicarbonate-free Hanks' balanced salt solution (CMF-Hanks'), before being chopped and incubated for 17 min at 37°C in 3 ml CMF-Hank's containing 20 u $ml^{-1}$ papain. Cells were pelleted by centrifugation at $250 \times g$ for 3 min and the supernatant was replaced with 3 ml CMF-Hank's supplemented with 0.3% (w $v^{-1}$) collagenase and 0.4% (w $v^{-1}$) dispase II. After a further incubation at 37°C for 20 min, the cells were re-spun at $250 \times g$ and the pellet resuspended in 3 ml CMF-Hank's and 2 ml Liebowitz's L-15 medium supplemented with 5 mM $Na^+$-HEPES, 5 mM D-Glucose, 10% (v $v^{-1}$) heat-inactivated foetal bovine serum, 100 IU $ml^{-1}$ penicillin, 100 $\mu$g $ml^{-1}$ streptomycin and 0.15% (w $v^{-1}$) deoxyribonuclease I. Clumps of cells were dissociated *via* trituration through a graded series of fire-polished Pasteur pipettes. After centrifugation at $250 \times g$ for 3 min, the cell pellet was resuspended in culture medium [Ham's F-12 (GlutaMAX-I) containing 10% heat-inactivated foetal bovine serum, 100 IU $ml^{-1}$ penicillin, 100 $\mu$g $ml^{-1}$ streptomycin, and nerve growth factor (m2.5S NGF; 50 ng $ml^{-1}$)] before being plated down on Poly-D-lysine (150K+; 0.1 mg $ml^{-1}$) and laminin (20 $\mu$g $ml^{-1}$) pre-treated 12-well plates. Cells (200–500 per well) were incubated at 37°C in a 5% $CO_2$/humidified air atmosphere for 4–6 days. After 24 h and every other day thereafter, the culture medium was replaced with F-12 medium further supplemented with the mitotic inhibitor cytosine-$\beta$-D-arabinofuranoside (20 $\mu$M) to limit the growth of non-neuronal cells.

### *CGRP release from trigeminal neuronal cultures*

After 4–6 days in culture, the medium was gently aspirated and replaced with 1 ml CGRP release buffer (Vasko *et al*., 1994) (composition mM): $Na^+$-HEPES 22.5, NaCl 135, KCl 3.5, $MgCl_2$ 1, $CaCl_2$ 2.5, D-glucose 3.3, 0.1% (w $v^{-1}$) bovine serum albumin, 0.003% (w $v^{-1}$) bacitracin, 1 $\mu$M phosphoramidon and 10 $\mu$M indomethacin (to inhibit tonic prostanoid synthesis), pH 7.4 at 37°C). Cells were incubated for 30 min at 37°C in release buffer, before this was replaced with 1 ml test agonist or vehicle (dimethylsuphoxide (DMSO), maximal concentration 0.1%) for a further 30 min. In some experiments, cultures were incubated for the first 30 min period in either the nitric oxide synthase (NOS) inhibitor, $N^G$-nitro-L-arginine (L-NAME; 10 $\mu$M), the P2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 30 $\mu$M), or with adenosine deaminase (1 u $ml^{-1}$). To investigate whether the CGRP release observed was extracellular-calcium dependent, experiments were conducted in calcium-free release buffer (equimolar substitution of $Ca^{2+}$ with $Mg^{2+}$). Following each incubation, 0.8 ml samples were removed and assayed for CGRP content. A commercial enzyme immunometric assay (SPIbio, Massy, France) was used for quantitative analysis of the CGRP content of the eluates. CGRP levels were determined photometrically at 405 nm using a microplate reader (Packard SpectraCount™). The antibody used in this assay is 100% cross-reactive between rat CGRP$\alpha$ and $\beta$ and $<0.01$% cross-reactive with substance P (SPIbio, commercial information) and the minimum assay detection limit was approximately 10 pg $ml^{-1}$. None of the compounds used in this study were found to cross-react non-specifically with the assay at the concentrations indicated.

### *Data analysis*

The CGRP concentrations in the samples were quantified in pg $ml^{-1}$. In order to account for differences in neuronal numbers and baseline CGRP concentrations between preparations and between individual wells, each well acted as its own control. The increase in immunoreactive CGRP concentrations following 30 min drug incubation was calculated as a percentage increase over the baseline concentration obtained in the 30 min prior to drug administration ((CGRP conc. after drug treatment − basal)/basal × 100). Only one drug treatment was given to any single well. Unless otherwise stated, values from individual experiments were pooled and expressed as a mean ± s.e.mean.

Individual concentration-effect curves were fitted to a logistic equation using Prism 3 (Graphpad Software Inc., San Diego, C.A., U.S.A.), with the maximum response for each agonist constrained to 100%. The mean $pEC_{50}$ value was calculated by taking the average of the individual estimates. No estimate of $pEC_{50}$ was made when a clearly defined maximum was not achieved. Statistical analysis between treatments was by one-way analysis of variance followed by the Bonferroni *post hoc* test, and $P$ values of less than 0.05 were considered statistically significant.

### *Materials*

All cell culture media was purchased from Gibco BRL (Paisley, U.K.) and 12-well plates were from Corning Costar (High Wycombe, U.K.). Collagenase (Type 2) and papain were from Worthington (Reading, U.K.) and dispase II was purchased from Roche (Lewes, U.K.). Bovine serum albumin (fraction V, protease-free), murine Engelbroth swarm laminin, bovine pancreas crude DNase I, cytosine-$\beta$-D-arabinofuranoside, poly-D-lysine (m.w. 150 $K^+$) and L-NAME were obtained from Sigma (Poole, U.K.). Forskolin and adenosine deaminase (2326 u $ml^{-1}$) were purchased from Calbiochem (Nottingham, U.K.). Nerve growth factor was from Alomone Labs (Botolph Clayton, U.K.). $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, carbaprostacyclin ($cPGI_2$), (15**S**)-hydroxy-11$\alpha$,9$\alpha$-(epoxymethano)prosta-5Z, 13E-dienoic acid (U46619), misoprostol methyl ester, sulprostone and indomethacin were all purchased from the Cayman Chemical Company (Ann Arbor, M.I., U.S.A.). Iloprost was obtained from Amersham (Little Chalfont, U.K.). Butaprost free acid, [1**R**-[1$\alpha$(Z), 2$\beta$(**R***),3$\alpha$]]-4-(benzoylamino)phenyl-7-[3-hydroxy-2-(2-hydroxy-3-phenoxy-propoxy)-5-oxocyclopentyl]-4-heptenoate (GR63799X), 3-benzyl-5-(6-carbohexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin (BWA868C), ZM325802 (Shaw *et al*., 1999) were obtained from Glaxo SmithKline Research & Development (Stevenage, U.K.). PPADS was purchased from RBI (Poole, U.K.).

$PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $cPGI_2$, U46619, sulprostone, butaprost free acid, GR63799X, ZM325802, BWA868C, forskolin and indomethacin were dissolved with DMSO at 10 mM and immediately frozen at $-20^{\circ}C$. Iloprost was supplied at 1 mM in Tris buffer and was diluted directly to 1 $\mu$M in release buffer. PPADS and L-NAME was prepared at 1 $\mu$M in water and serially diluted in release buffer.

## Results

### *Effects of $PGE_2$ and carbaprostacyclin ($cPGI_2$) on CGRP release from trigeminal ganglion neuronal cultures*

To investigate the effects of prostanoids on CGRP release from trigeminal neurones, we established primary cultures of adult trigeminal ganglion neurones. Incubation of the cultures in release buffer, containing indomethacin (10 $\mu$M) and phosphoramidon (1 $\mu$M), for 30 min resulted in an average baseline CGRP concentration of $112\pm8$ pg $ml^{-1}$ (range 23–251 pg $ml^{-1}$; $n=29$ independent culture preparations). Incubation of cells with $PGE_2$ (1 $\mu$M) or a stable analogue of prostacyclin, $cPGI_2$ (1 $\mu$M) caused significant ($P<0.001$) increases in the concentration of immunoreactive CGRP of $199\pm25\%$ and $177\pm26\%$ respectively, compared with vehicle treated controls ($7\pm10\%$; $n=8$). When cells were exposed to calcium-free release buffer (equimolar substitution of $Ca^{2+}$ with $Mg^{2+}$), the basal CGRP levels after 30 min were lower than in control solutions ($36\pm2.3$ pg $ml^{-1}$ *vs* $67\pm12$ pg $ml^{-1}$), but the increases in CGRP release over baseline in response to $PGE_2$ and $cPGI_2$ were abolished (Figure 1).

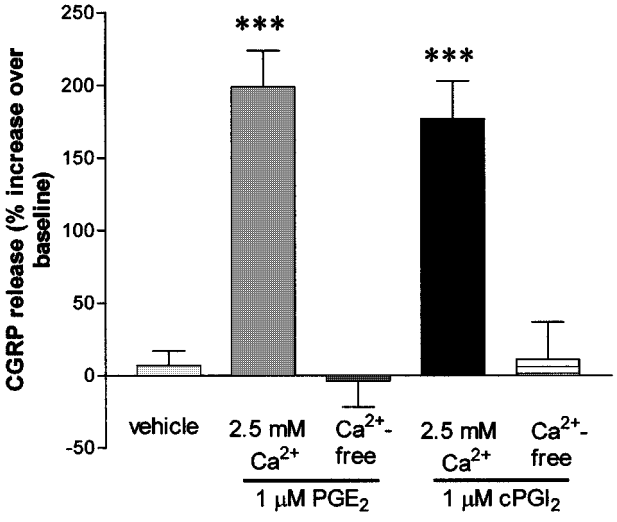


**Figure 1** Effects of extracellular calcium on $PGE_2$- and $cPGI_2$-induced CGRP release. Following 4–6 days in culture, adult trigeminal ganglion cells were exposed to either $PGE_2$ (1 $\mu$M) or $cPGI_2$ (1 $\mu$M) in the presence or absence of 2.5 mM extracellular calcium (equimolar substitution of $Ca^{2+}$ with $Mg^{2+}$ in the release buffer). Data are expressed as the percentage increase in CGRP release and are presented as the mean$\pm$s.e.mean from 3–8 independent experiments. ***$P<0.001$, significantly different from controls.

**Table 1** Effect of L-NAME, PPADS and adenosine deaminase on $PGE_2$-induced CGRP release

| *Treatment* | *30 min* | *60 min* | *Percentage increase over baseline* | n |
|---|---|---|---|---|
| Vehicle | $150\pm17$ | $113\pm15$ | $-23\pm8$ | 7 |
| $PGE_2$ (1 $\mu$M) | $155\pm15$ | $336\pm40$ | $116\pm27$ | 7 |
| $PGE_2$ (1 $\mu$M)+L-NAME (10 $\mu$M) | $170\pm16$ | $320\pm44$ | $86\pm23$ | 6 |
| $PGE_2$ (1 $\mu$M)+PPADS (30 $\mu$M) | $138\pm8$ | $337\pm38$ | $145\pm29$ | 6 |
| $PGE_2$ (1 $\mu$M)+ADA (10 u $ml^{-1}$) | $172\pm27$ | $338\pm44$ | $111\pm23$ | 5 |

The CGRP concentration in pg $ml^{-1}$ (mean$\pm$s.e.mean) in the medium of the cultured trigeminal neurones was determined after two successive 30 min periods in which the cells were first exposed to release buffer and then to $PGE_2$ (1 $\mu$M), with or without the following agents: L-NAME (10 $\mu$M), PPADS (30 $\mu$M) or ADA (10 u $ml^{-1}$). In addition, for the combinations, cells were pre-treated with the appropriate compound for the first 30 min incubation. None of these agents had any effect on baseline CGRP release (data not shown). The percentage increase was calculated for each individual experiment, and the data are presented as mean$\pm$s.e.mean from 5–7 independent experiments.

### *Effects of L-NAME, PPADS and adenosine deaminase on $PGE_2$-mediated CGRP release*

To further investigate the nature of the $PGE_2$-mediated CGRP release, the effects of the non-specific NOS inhibitor, L-NAME (10 $\mu$M), the P2 receptor antagonist, PPADS (30 $\mu$M), and the adenosine metabolizing enzyme, adenosine deaminase (1 u $ml^{-1}$), were tested. It was found that none of these agents modified baseline CGRP levels (data not shown), and, furthermore, that these agents did not modify CGRP release evoked by 1 $\mu$M $PGE_2$ (Table 1).

### *Effects of other prostanoid receptor agonists on CGRP release from cultured trigeminal neurones*

Stimulation of the cultures with $PGD_2$ (1 $\mu$M) significantly increased immunoreactive CGRP concentrations by $107 \pm 23\%$ ($n=5$; $P<0.05$ compared to vehicle controls). In addition, the effect of $cPGI_2$, indicative of IP receptor activation, was mimicked by another stable prostacyclin analogue, iloprost (1 $\mu$M) ($168 \pm 26\%$ increase in CGRP concentration; $n=3$). Neither $PGF_{2\alpha}$ (1 $\mu$M), nor the TP receptor agonist, U46619 (1 $\mu$M) had any significant effect on CGRP concentrations following a 30 min incubation period (Figure 2A).

To further characterize the receptor types involved in prostaglandin-induced increases in CGRP release, concentration-effect curves were generated to $PGE_2$, $cPGI_2$, the $EP_2$ receptor agonist butaprost (free acid), the $EP_3 > EP_2$ receptor agonist misoprostol, and the $EP_4 > EP_2$ receptor agonist, 11-deoxy-$PGE_1$ (Figure 2B; Table 2). $PGE_2$, 11-deoxy-$PGE_1$, $cPGI_2$ and butaprost all produced similar maximum increases in CGRP release, but the maximum response to misoprostol was lower. The overall rank order of agonist potency for these agonists was $PGE_2 = 11\text{-deoxy-}PGE_1 = cPGI_2 =$ butaprost > misoprostol. Neither the selective $EP_3$ receptor agonist, GR63799X (1 $\mu$M), nor the $EP_3 > EP_1$ receptor agonist, sulprostone (1 $\mu$M), had any significant effect on basal immunoreactive CGRP concentrations (both $n=5$). $PGD_2$ also caused concentration dependent increases in immunoreactive CGRP release (Figure 2B) but a clearly defined maximum response was not achieved even with a concentration as high as 10 $\mu$M.

### *Effects of prostanoid receptor antagonists on $PGD_2$ and $PGE_2$-evoked CGRP release from trigeminal neurones*

The specificity of the $PGD_2$ mediated peptide release was investigated using the DP receptor antagonist, BWA868C (Giles *et al*., 1989). When cells were pre-incubated for 30 min with BWA868C, the $PGD_2$-stimulated increases in CGRP release were abolished. In contrast, this antagonist had no effect on CGRP release stimulated by 1 $\mu$M $PGE_2$ or $cPGI_2$ (Figure 3A).

The $EP_1$ antagonist, ZM325802 (Figure 3B) and SC-19220 and SC-51322 (David W. Jenkins, unpublished observations) had no effect on $PGE_2$ (1 $\mu$M) stimulated CGRP release. We were unable to use the weak $EP_4$ receptor antagonist, AH23848B ($pA_2 = 5.4$, Coleman *et al*., 1994), to determine the possible involvement of $EP_4$ receptors in mediating $PGE_2$-induced CGRP release as this antagonist itself caused a marked increase in baseline CGRP secretion (446 and 391% at 10 $\mu$M, $n=2$).

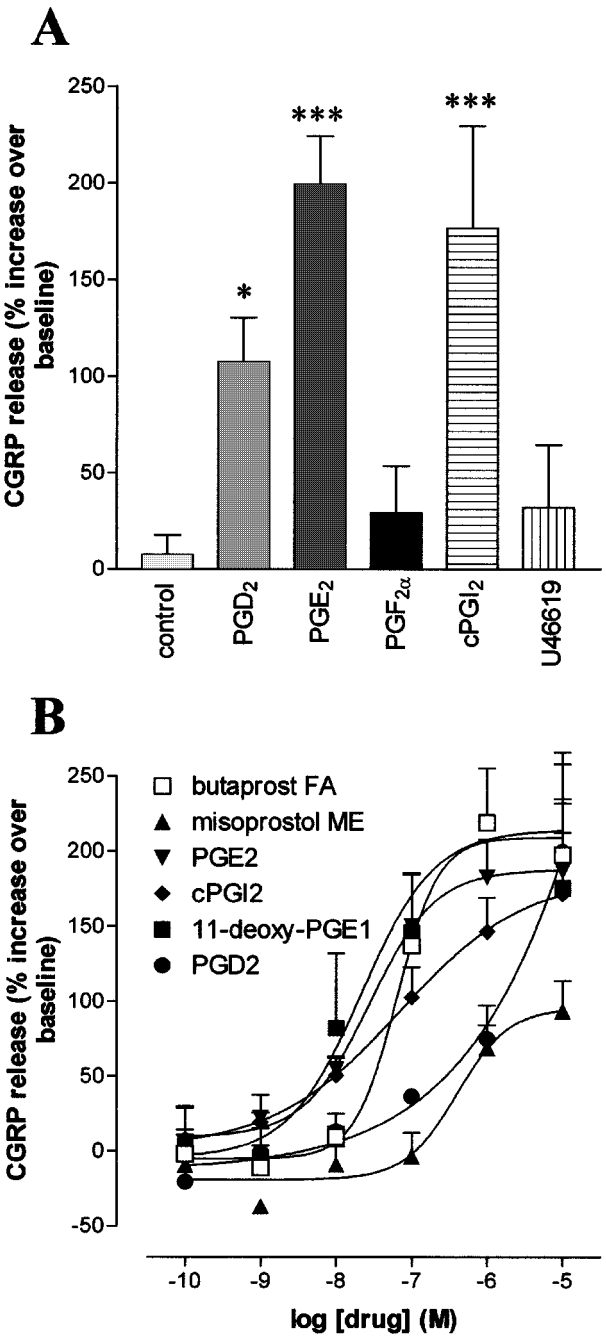


**Figure 2** Effects of prostanoid agonists on CGRP release from cultured adult rat trigeminal neurones. After 4–6 days in culture, cells were incubated in release buffer (Vasko *et al*., 1994), in the presence of 10 $\mu$M indomethacin for 30 min, before being challenged for a further 30 min with either vehicle (DMSO, 0.1%) or test agonist. Data are expressed as the percentage increase in CGRP release over baseline (A) Effects $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $cPGI_2$ and the TP receptor agonist, U46619 (each at 1 $\mu$M). $^*P<0.05$, $^{***}P<0.001$ over controls. (B) Effects of increasing concentrations of $PGD_2$, $PGE_2$, $cPGI_2$, the $EP_2$ receptor agonist, butaprost free acid, the $EP_3 > EP_2$ receptor agonist misoprostol and the $EP_4 > EP_2$ receptor agonist, 11-deoxy $PGE_1$. Data are presented as the percentage increase in CGRP secretion over baseline and represent the mean ± s.e.mean of 3–8 experiments.

**Table 2** Estimates of potency of natural and synthetic prostanoid agonists on CGRP release from primary cultures derived from adult rat trigeminal ganglia

| *Treatment* | *$pEC_{50}$* | *Slope* | *Max % increase over baseline* |
|---|---|---|---|
| $PGE_2$ | 7.44±0.07 | 0.90±0.40 | 187±70 |
| $cPGI_2$ | 7.34±0.35 | 0.63±0.07 | 171±40 |
| $PGD_2$ | N.D. | N.D. | 199±32 |
| Butaprost FA | 7.19±0.11 | 1.05±0.26 | 219±36 |
| Misoprostol ME | 6.61±0.14 | 0.90±0.23 | 93±20 |
| 11-deoxy-$PGE_1$ | 7.44±0.39 | 0.72±0.19 | 263±44 |

After 4–6 days in culture, cells were stimulated with varying concentrations of the agonists indicated. Concentration-effect curves were generated and estimates for the $pEC_{50}$, slope and maximum percentage increase over baseline were obtained. Values are represented as mean±s.e.mean for $pEC_{50}$, slope and maximum percentage increase from 3–4 experiments performed on independent culture preparations.

### *Effect of the $EP_3$ receptor agonist, GR63799X, on forskolin mediated CGRP release*

It has been reported that the $EP_3$ receptor exists in several splice variants that can couple differentially to several downstream signalling pathways, including $G_{i/o}$ to reduce cellular cyclic AMP levels and $G_s$ to induce the opposite effect (see Narimuya *et al*., 1999). Therefore, although we observed no CGRP release *per se* in response to the specific $EP_3$ receptor agonist, GR63799X (see above), we also tested the effects of this agonist against CGRP release stimulated by the adenylate cyclase activator forskolin. Incubation of the cultures in forskolin (1 $\mu$M) for 30 min increased basal immunoreactive CGRP concentrations by 102±9%. In the presence of GR63799X (1 $\mu$M), the forskolin-induced increase was 102±17%. Similar results were observed when the cultures were pre-incubated for 30 min in GR63799X (1 $\mu$M) before stimulation with forskolin (data not shown).

## Discussion

The trigeminal ganglion contains the cell bodies of the afferent neurones of the fifth cranial nerve, thought to be responsible for the pain associated with migraine. Although we have previously shown, using reverse transcription polymerase chain reaction (RT–PCR) that whole excised trigeminal ganglia express mRNA for all four EP receptors and also the IP receptor (Jenkins *et al*., 2000), the effects of prostanoids have remained poorly characterized with respect to both receptor localization and function. We have therefore used cultured trigeminal neurones to investigate the receptors involved in prostanoid-induced CGRP release, since this is one of the major neuropeptides thought to be implicated in the pathophysiology of migraine.

All experiments in the present study were carried out on cultures of adult trigeminal neurones and conducted in the presence of indomethacin to inhibit *de novo* prostaglandin synthesis, thus avoiding the confounding influences of endogenous prostanoid release. We have demonstrated that, in cultured trigeminal neurones, CGRP release can be stimulated by $PGD_2$, $PGE_2$ and $cPGI_2$, consistent with the activation of DP, EP, and IP receptors, respectively. The IP receptor agonist, iloprost, also caused CGRP release from these cells. In contrast, agonists at FP ($PGF_{2\alpha}$) and TP (U46619) receptors did not produce a significant effect. The release of CGRP by $PGE_2$ and $cPGI_2$ was not likely to have been compromised by a significant depletion of the releasable CGRP pool since release-induced by other agents, AH23848B (this study) and KCl (Carruthers *et al*., 2001) were substantially greater. In addition permeabilization of the trigeminal neurones with Triton X-100 (0.3%) causes an approximate 40 fold increase in the basal release of immunoreactive CGRP (unpublished observations).

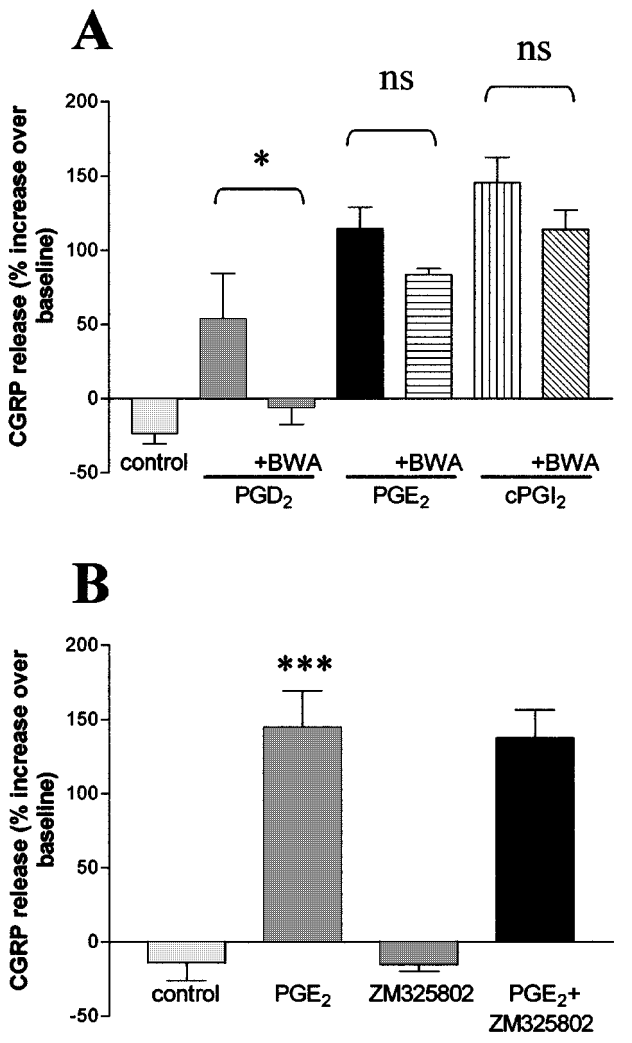


**Figure 3** Effects of prostaglandin receptor antagonists on $PGD_2$- and $PGE_2$-induced CGRP release. (A) Cultured trigeminal ganglion neurones were incubated with release buffer for 30 min and then subsequently challenged with 1 $\mu$M $PGD_2$, $PGE_2$ or $cPGI_2$ with or without the DP receptor antagonist, BWA 868C (1 $\mu$M). Co-incubation wells were pre-incubated with antagonist for 30 min before being incubated with the appropriate test agonist. *$P<0.05$ against $PGD_2$ alone. (B) Rat trigeminal ganglion neurones (4–6 days in culture) were incubated with either release buffer for 30 min and then $PGE_2$ alone or the $EP_1$ receptor antagonist, ZM325802, (both 1 $\mu$M) for another 30 min or ZM325802 (1 $\mu$M) for 30 min and then $PGE_2$ and ZM325802 (both 1 $\mu$M) for 30 min. Data are presented as the percentage increase in CGRP release over baseline and represent the mean±s.e.mean of 3–5 independent experiments. ***$P<0.001$ *vs* control.

Both the $PGE_2$- and $cPGI_2$-induced release of CGRP were highly dependent upon extracellular calcium entry, since removal of extracellular calcium effectively prevented agonist-induced release of CGRP. Furthermore, we also found that the non-specific NOS inhibitor, L-NAME, the non-selective ATP receptor antagonist, PPADS and the adenosine metabolizing enzyme, adenosine deaminase, did not modify the CGRP release induced by $PGE_2$. Taken together, these data suggest that the $PGE_2$ mediated effects are a direct consequence of EP receptor stimulation and not dependent on the secondary release of nitric oxide, ATP or adenosine. The lack of effect of L-NAME is particularly relevant since $PGE_2$ can cause nitric oxide release from rat spinal cord (Sakai *et al*., 1998).

The ability of both $cPGI_2$ and iloprost to release CGRP from cultured trigeminal neurones provides evidence for functional IP receptors in these neurones and is consistent with previous findings in DRGs (Hingtgen *et al*., 1995; Smith *et al*., 1998). Although several studies have demonstrated functional effects of $PGE_2$ in DRGs, this study is the first to show a direct $PGE_2$-induced release of CGRP from trigeminal neurones. This contrasts with findings in DRGs, which have shown $PGI_2$, but not $PGE_2$-induced CGRP release, although interestingly both agonists increased intracellular cyclic AMP (Vasko *et al*., 1994; Hingtgen *et al*., 1995; Hingtgen & Vasko, 1994). However, the present study was conducted in adult trigeminal neurones, rather than neonatal DRGs, and it would be necessary to carry out further studies to determine whether the differences in functional response observed were due to differences in source material (adult *vs* neonate) or tissue type (trigeminal ganglion *vs* DRG).

The response to $PGE_2$ may be mediated by several different receptors and the effects of several EP-receptor selective agonists and antagonists were evaluated to identify the EP receptor subtype involved. The endogenous prostanoids also show some degree of cross-reactivity between receptor types (Coleman *et al*., 1994), so it is possible that responses to $PGE_2$ may be mediated in part by the IP receptor. However, at present, no potent, selective IP antagonists are available to test this assertion. The $EP_1$ antagonist, ZM325802 (Shaw *et al*., 1999) had no effect on $PGE_2$-mediated CGRP release, suggesting that $EP_1$ receptors are not involved. In addition, no attenuation of peptide release was observed using the less potent putative $EP_1$ antagonists, SC-19220 and SC-51322 (D.W. Jenkins, unpublished observations). However, it has previously been reported that SC-19220 can block $PGE_2$-induced substance P release from cultured rat DRGs (White, 1996).

There is a considerable body of evidence to suggest that the effects of $PGE_2$ on sensory neurones are mediated by receptors that are coupled to increases in adenylate cyclase activity, implying the involvement of $EP_2$ and $EP_4$ receptors, and possibly an $EP_3$ receptor splice variant, all of which may couple to $G_s$ (Vasko *et al*., 1994; Cui & Nicol, 1995; Hingtgen *et al*., 1995; England *et al*., 1996; Smith *et al*., 1998; 2000). In this study, the $EP_2$ selective agonist, butaprost, stimulated CGRP release, and the estimated potency was similar to previously reported values measuring the ability of this agonist to stimulate cyclic AMP accumulation in Chinese Hamster Ovary (CHO) cells, stably transfected with the recombinant rat $EP_2$ receptor (Boie *et al*., 1997). Recently, the same agonist has also been reported to cause increases in cyclic AMP levels in neonatal DRG cultures that could be attenuated by an antisense oligonucletide directed at the $EP_2$ receptor (Southall & Vasko, 2001). In addition to butaprost, we showed that the mixed $EP_3 > EP_2$ receptor agonist, misoprostol, also caused a small, but significant increase in CGRP release, suggestive of activity at $EP_2$ receptors. The $EP_4 > EP_2$ receptor agonist, 11-deoxy-$PGE_1$, also caused CGRP release, with an estimated potency consistent with $EP_2$ receptor activation (Boie *et al*., 1997).

It seems unlikely that $EP_3$ receptors are involved in $PGE_2$-mediated CGRP release since the specific $EP_3$ receptor agonist, GR63799X, or the mixed $EP_3 > EP_1$ receptor agonist, sulprostone were without effect. In addition, GR63799X had no effect on forskolin-induced CGRP, an effect which we have previously shown to be inhibited by several agonists that couple to $G_{i/o}$ (Carruthers *et al*., 2001). This lack of functional activity associated with $EP_3$ receptors was somewhat surprising, since $EP_3$ receptors have been localized in the trigeminal ganglion at both the mRNA level in the mouse (Sugimoto *et al*., 1994) and, recently, the protein level in the rat (Nakamura *et al*., 2000). There are no potent, selective $EP_4$ receptor antagonists available at present, and the weak antagonist, AH23848B ($pA_2 \cong 5.4$; see Coleman *et al*., 1994) caused a large increase in CGRP release Since this release precluded further analysis, the potential significance of $EP_4$ receptor activation is unknown.

In conclusion, we have shown that stimulation of cultured rat trigeminal ganglion neurones with either D, E or I series prostaglandins causes a $Ca^{2+}$-dependent CGRP release. Since CGRP levels in jugular venous blood are known to be elevated during migraine (Goadsby *et al*., 1988) and electrical stimulation of the trigeminal ganglion is known to release $PGE_2$ (Ebersberger *et al*., 1999), $PGE_2$ may play an important role in the generation of migraine and in the peripheral sensitization thought to occur during migraine headache (see Burstein, 2001). Antagonists of prostanoid-induced CGRP release may provide novel therapeutic approaches to the treatment of migraine.

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