Pharmacological Characterization of the Anandamide Cyclooxygenase Metabolite: Prostaglandin E$_2$ Ethanolamide

RUTH A. ROSS, SUSAN J. CRAIB, LESLEY A. STEVENSON, ROGER G. PERTWEE, ANDREA HENDERSON, JOHN TOOLE, and HEATHER C. ELLINGTON

Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland

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ABSTRACT

Anandamide can be metabolized by cyclooxygenase-2 to produce prostaglandin E$_2$ (PGE$_2$) ethanolamide. The purpose of this study was to investigate the pharmacology of this novel compound. Radioligand binding experiments in membranes from human embryonic kidney cells transfected with PGE$_2$ receptor subtypes EP$_1$, EP$_2$, EP$_3$, and EP$_4$ revealed that PGE$_2$ ethanolamide has pK$_i$ values of 5.61 ± 0.1, 6.33 ± 0.01, 6.70 ± 0.13, and 6.29 ± 0.06, respectively, compared with 8.31 ± 0.16, 9.03 ± 0.04, 9.34 ± 0.06, and 9.10 ± 0.04 for PGE$_2$. PGE$_2$ inhibits electrically evoked contractions of this tissue (EP2 receptor-mediated), with a pEC$_{50}$ value of 9.09 ± 0.09 compared with that of 7.38 ± 0.09 for PGE$_2$ ethanolamide. In the guinea pig trachea, 100 nM PGE$_2$ and 1 μM PGE$_2$ ethanolamide produced contractions of 51.8 ± 10.6 and 38.9 ± 5.6% (of the histamine E$_{max}$), respectively. The EP$_1$ receptor antagonist SC-51089 (10 μM) prevented the contractions induced by both compounds. In the presence of 10 μM 8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide, monohydrochloride (SC-51089), PGE$_2$ caused a concentration-related relaxation of histamine-induced contractions of this tissue (EP2 receptor-mediated), with a pEC$_{50}$ value being 8.29 ± 0.17 compared with that of 7.11 ± 0.18 for PGE$_2$ ethanolamide. In the rabbit jugular vein, PGE$_2$ induces relaxation (EP$_4$ receptor-mediated) with a pEC$_{50}$ of 9.35 ± 0.25, compared with 7.05 ± 0.4 for PGE$_2$ ethanolamide. In dorsal root ganglion neurons in culture, 3 μM PGE$_2$ ethanolamide evoked an increase in intracellular calcium concentration in 21% of small-diameter capsaicin-sensitive neurons. We conclude that this compound is pharmacologically active, however its physiological relevance has yet to be established.

There is evidence that the endogenous cannabinoid anandamide is effectively oxygenated by human cyclooxygenase (COX)-2 but not human COX-1 (Yu et al., 1997) and that the products are similar to those formed with arachidonic acid as the substrate. The major prostanoid product as determined by mass spectrometry was found to be prostaglandin E$_2$ ethanolamide. However, there are no studies reporting the presence of PGE$_2$ ethanolamide in intact cells or tissues. There is evidence that in RAW 264.7 macrophages PGE$_2$ ethanolamide may be synthesized from anandamide (PGE$_2$) ethanolamide. However, there are no studies reporting the presence of PGE$_2$ ethanolamide in intact cells or tissues. Anandamide can be metabolized by cyclooxygenase-2 to produce prostaglandin E$_2$ (PGE$_2$) ethanolamide. The purpose of this study was to investigate the pharmacology of this novel compound. Radioligand binding experiments in membranes from human embryonic kidney cells transfected with PGE$_2$ receptor subtypes EP$_1$, EP$_2$, EP$_3$, and EP$_4$ revealed that PGE$_2$ ethanolamide has pK$_i$ values of 5.61 ± 0.1, 6.33 ± 0.01, 6.70 ± 0.13, and 6.29 ± 0.06, respectively, compared with 8.31 ± 0.16, 9.03 ± 0.04, 9.34 ± 0.06, and 9.10 ± 0.04 for PGE$_2$. PGE$_2$ inhibits electrically evoked contractions of this tissue (EP2 receptor-mediated), with a pEC$_{50}$ value of 9.09 ± 0.09 compared with that of 7.38 ± 0.09 for PGE$_2$ ethanolamide. In the guinea pig trachea, 100 nM PGE$_2$ and 1 μM PGE$_2$ ethanolamide produced contractions of 51.8 ± 10.6 and 38.9 ± 5.6% (of the histamine E$_{max}$), respectively. The EP$_1$ receptor antagonist SC-51089 (10 μM) prevented the contractions induced by both compounds. In the presence of 10 μM 8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl]hydrazide, monohydrochloride (SC-51089), PGE$_2$ caused a concentration-related relaxation of histamine-induced contractions of this tissue (EP2 receptor-mediated), with a pEC$_{50}$ value being 8.29 ± 0.17 compared with that of 7.11 ± 0.18 for PGE$_2$ ethanolamide. In the rabbit jugular vein, PGE$_2$ induces relaxation (EP$_4$ receptor-mediated) with a pEC$_{50}$ of 9.35 ± 0.25, compared with 7.05 ± 0.4 for PGE$_2$ ethanolamide. In dorsal root ganglion neurons in culture, 3 μM PGE$_2$ ethanolamide evoked an increase in intracellular calcium concentration in 21% of small-diameter capsaicin-sensitive neurons. We conclude that this compound is pharmacologically active, however its physiological relevance has yet to be established.

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The physiological significance of this pathway has yet to be established. It may be that the prostaglandin ethanolamides are a new class of mediators and in some recent literature these novel prostaglandin products are referred to as “prostamides” (Chen et al., 2001). It has recently been demonstrated that mice lacking the fatty acid amide hydrolase (FAAH) enzyme have a 15-fold increase in endogenous brain levels of anandamide and display attenuated sensitivity to pain that is reversed by the CB$_1$ receptor antagonist SR141716A (Cravatt et al., 2001). Thus, FAAH may repre-
sent a key target for the treatment of pain. It is notable, however, that if anandamide levels increase in combination with the increased expression of COX-2 that is associated with inflammatory pain, there may be a significant increase in the production of prostaglandin ethanolamides. This scenario makes it particularly important to investigate the pharmacology of these novel metabolites.

Because of the structural similarity between this compound and PGE₂ we have hypothesized that it may interact with some of the PGE₂ receptor (EP) subtypes. PGE₂ exerts a range of actions, including contraction and relaxation of various types of smooth muscle, sensitizing sensory fibers to noxious stimulation and inflammation (Narumiya et al., 1999). The aim of this investigation was to characterize the interaction of PGE₂ ethanolamide with one or more of the four EP receptor subtypes: EP₁, EP₂, EP₃, and EP₄, all of which are activated by PGE₂. We have investigated the affinity of this compound for EP receptor subtypes in a radioligand binding assay using membranes from cells stably transfected with each receptor subtype. Functional assays have been performed in a range of isolated tissue preparations that have been established as assays for various EP receptor subtypes (Coleman et al., 1990). In the guinea pig trachea preparation, PGE₂ has an EP₁ receptor-mediated contractile action and a relaxant action, which is EP₂ receptor-mediated. Inhibition of electrically evoked contractions in the guinea pig vas deferens are EP₁ receptor-mediated (Lawrence et al., 1992). The rabbit jugular vein is thought to contain EP₂ receptors that mediate the relaxant actions of PGE₂ (Coleman et al., 1994; Milne et al., 1995). Recently, it has been shown that various arachidonoyl ethanolamides have affinity for the vanilloid VR₁ receptors (Ross et al., 2001). Thus, we have included an investigation of the activity of PGE₂ ethanolamide at VR₁ receptors. Prostaglandins are synthesized in sensory neurons and are important in the development and maintenance of hyperalgesia. A number of prostanoids both directly activate sensory neurons and sensitize these neurons to other potent nociceptive agents such as bradykinin and capsaicin. Thus, we have investigated the actions of PGE₂ ethanolamide in dorsal root ganglion (DRG) neurons in culture using Fura-2 calcium imaging.

It is possible that PGE₂ ethanolamide may interact with the enzyme responsible for the rapid hydrolysis of anandamide, FAAH; thus, its formation may lead to an increase in the intracellular concentration of anandamide. We have investigated whether this compound enhances the ability of anandamide to displace the CB₁ receptor agonist [³H]CP55940 from mouse brain membranes, comparing PGE₂ ethanolamide with the established FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) and the novel potent inhibitor OL-093 (compound 53 in Boger et al., 2000).

**Experimental Procedures**

**Materials**

Capsaicin, capsazepine, resiniferatoxin, and (+)-WIN55212 were obtained from Tocris Cookson (St. Louis, MO) and SR141716A from SANOFI Research Center (Montpellier, France). [³H]CP55940 and [³H]Resiniferatoxin were obtained from ABBIotech (Little Chalfont, UK). PGE₂ ethanolamide was obtained from Cayman Chemical (Ann Arbor, MI) and SC-51089 from BIOMOL Research Laboratories (Plymouth Meeting, PA). OL-093 is compound 53 in Boger et al. (2000) and was a gift from Dr. Boger (Scripps Research Institute, La Jolla, CA). Bovine serum albumin (BSA), cell culture medium, nonenzymatic cell dissociation solution, G418, t-glutamine, Krebs’ salts, penicillin with streptomycin, PMSF, and Triton X-100 were all obtained from Sigma-Aldrich (St. Louis, MO). Rat VR₁ transfected CHO cells were a gift from Novartis (London, UK). HEK cell membranes expressing hEP₁, hEP₂, and hEP₄ receptor subtypes were a gift from Merck Frosst (Montreal, QB, Canada) and hEP₅ was a gift from Allergan (Irvine, CA).

**Cell Culture**

**VR₁ Transfected CHO Cells.** rVR₁ transfected CHO cells were maintained in minimum essential medium-α minus medium containing 2 mM l-glutamine supplemented with 10% Hyclone fetal bovine serum, 350 µg ml⁻¹ G418, 100 units ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Cells were maintained in 5% CO₂ at 37°C. For the radioligand binding assay, cells were removed from flasks by scraping and then frozen as a pellet at −20°C for up to 1 month.

**Primary Cultured DRG Neurons.** Primary cultures of DRG neurons were prepared following enzymatic (0.125% collagenase, 0.25% trypsin, and 1.6 units ml⁻¹ DNase) and mechanical dissociation of dorsal root ganglia from decapitated 2-day-old Sprague–Dawley rats. The sensory neurons were plated on laminin-polyornithine-coated coverslips and bathed in Dulbecco’s modified Eagle’s medium/ F-12 Ham’s medium supplemented with 10% fetal bovine serum, 5000 IU ml⁻¹ penicillin, 5000 mg ml⁻¹ streptomycin, and 20 ng ml⁻¹ nerve growth factor. The cultures were maintained for up to 2 weeks at 37°C in humidified air with 5% CO₂ and refed with fresh culture medium every 5 to 7 days.

**Radioligand Binding Experiments**

**VR₁ Cell Membranes.** Assays were performed in Dulbecco’s modified Eagle’s medium containing 25 mM HEPES and 0.25 mg ml⁻¹ BSA. The total assay volume was 500 µl containing 20 µg of cell membranes. Binding was initiated by the addition of the VR₁ receptor agonist [³H]Resiniferatoxin (100 pM). Assays were carried out at 37°C for 1 h, before termination by addition of ice-cold wash buffer (50 mM Tris buffer and 1 mg ml⁻¹ BSA, pH 7.4) and vacuum filtration using a 12-well sampling manifold (cell harvester; Brandel, Inc., Gaithersburg, MD) and GF/B filters (Whatman, Maidstone, UK) that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction was washed nine times with a 1.5-mI aliquot of wash buffer. The filters were oven-dried for 40 min and then placed in 5 ml of scintillation fluid. Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was determined in the presence of 1 µM unlabeled resiniferatoxin. Protein assays were performed using a Bio-Rad DC kit (Bio-Rad, Hercules, CA).

**EP Receptor Membranes.** EP₁, EP₂, and EP₄ membranes were a gift from Merck Frosst, and EP₅ membranes were from Allergan. The transfection details can be found in Abramovitz et al. (2000). The assay conditions used in this study are identical to those used by Abramovitz et al. (2000) with the same membranes. Under these conditions it has been shown that the specific binding of PGE₂ 1) represents 75 to 95% of the total binding; 2) is linear with respect to the concentrations of radioligand and protein; and 3) has reached equilibrium within the stated incubation time and temperature. Assays were performed in 10 mM MES buffer containing 10 mM MgCl₂ and 1 mM EDTA, pH 6.0. The total assay volume was 200 µl containing 41, 40, 2.3, and 16.5 µg of cell membranes containing EP₁, EP₂, EP₃, and EP₄, respectively. Binding was initiated by the addition of 500 pM [³H]PGE₂. Assays were carried out at 30°C for 1 h, before termination by addition of ice-cold wash buffer (10 mM MES buffer, pH 6.0) and vacuum filtration using a 12-well sampling manifold (cell harvester; Brandel, Inc.) and GF/B filters (Whatman) that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction was washed six times with a 1.5-mI aliquot of wash buffer. The filters were oven-dried for 40 min and then placed in 5 ml of}
scintillation fluid. Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was determined in the presence of 1 μM unlabeled PGE₂. Protein assays were performed using a Bio-Rad DC kit.

**Drug Additions.** PGE₂ and PGE₂ ethanolamide were stored as 10 mM top stocks in dimethyl sulfoxide (DMSO); for the radioligand binding assay they were diluted in assay buffer and the concentration of DMSO kept constant at 0.1% throughout.

**Data Analysis.** The concentrations of competing ligands to produce 50% displacement of the radioligand (IC₅₀) from specific binding sites was calculated using GraphPad Prism (GraphPad Software, San Diego, CA). Kᵢ values for [³H]PGE₂ and [³H]Resiniferatoxin binding to EP receptor membranes and VR₁ receptor membranes were obtained from Abramovitz et al. (2000) and Ross et al. (2001), respectively. Dissociation constant (Kᵢ) values were calculated using the equation of Cheng and Prusoff (1972).

**Effect of FAAH Inhibitors and PGE₂ Ethanolamide on Binding of Anandamide to Mouse Brain Membranes.** Binding assays were performed with the CB₁ receptor agonist [³H]CP55940 (0.5 nM), 1 mM MgCl₂, 1 mM EDTA, 1 mg ml⁻¹ BSA, and 50 mM Tris buffer, with a total assay volume of 500 μL. Binding was initiated by the addition of mouse brain membranes (50–75 μg). Assays were carried out at 37°C for 60 min before termination by addition of ice-cold wash buffer (50 mM Tris buffer and 1 mg ml⁻¹ BSA) and vacuum filtration using a 12-well sampling manifold (cell harvester; Brandel, Inc.) and GF/B (Whatman) glass fiber filters that had been soaked in wash buffer at 4°C for 24 h. Each reaction tube was washed five times with a 4-mL aliquot of buffer. The filters were oven-dried for 60 min and then placed in 5 mL of scintillation fluid (Ultima Gold XR; Packard BioScience, Meriden, CT), and radioactivity quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabeled CP55940 and was 70 to 85% of the total binding. Membranes were preincubated with enzyme inhibitors (PMSF, OL-093, or PGE₂ ethanolamide) or vehicle (0.01% ethanol) for 30 min at 37°C before the addition of 100 nM anandamide or anandamide vehicle. The percentage of increase in the anandamide displacement of [³H]CP55940 due to the enzyme inhibitors was calculated as follows: [increase in displacement of [³H]CP55940 specific binding by 100 nM anandamide in the presence of enzyme inhibitor] / [increase in displacement of [³H]CP55940 specific binding by 100 nM anandamide in the presence of vehicle alone] × 100.

**Isolated Tissue Experiments**

**Guinea Pig Vas Deferens.** Vasa deferentia were obtained from Dunkin-Hartley guinea pigs weighing 300 to 800 g. Each tissue was mounted in a 4-mL organ bath at an initial tension of 0.5 g. The baths contained Mg²⁺-free Krebs' solution, which was kept at 37°C and bubbled with 95% O₂ and 5% CO₂. The composition of the Krebs’ solution was 118.2 mM NaCl, 4.75 mM KC1, 1.19 mM KH₂PO₄, 25.0 mM NaHCO₃, 11.0 mM glucose, and 2.54 mM CaCl₂ · 2H₂O. It also contained 10 μM indomethacin. At the end of each experiment the tissue was exposed to 100 μM histamine and the contractions for each compound expressed as a percentage of this response. Contractions were monitored by computer (Apple Macintosh LCIII and Performa 475) using a data recording and analysis system (MacLab) that was linked to either UF-1 transducers (Pioden Controls) or model 1030 transducers (UFI).

In experiments investigating the relaxation of the guinea pig trachea, the tissue was preincubated with the EP₁ receptor antagonists SC-51089 (10 μM). The tissues were then contracted with a concentration of 1 μM histamine and exposed to each concentration of agonist for 5 min. The relaxation of the tissue was expressed as a percentage of the inhibition of the histamine-induced contraction.

**Rabbit Jugular Vein.** Jugular veins were obtained from New Zealand White rabbits weighting 2 to 3 kg that had been anesthetized with 30 mg ml⁻¹ Sagatal injected into the marginal ear vein. The jugular veins were cut into rings of 3 to 5 mm wide. Each ring was mounted between two stainless steel hooks in a 4-mL organ bath at an initial tension of 0.75 g. The baths contained Krebs' solution (as for the trachea) containing 10 μM indomethacin, which was kept at 37°C and bubbled with 95% O₂ and 5% CO₂. Tissues were exposed to an initial concentration of 100 μM phenylephrine and allowed to recover before the addition of 1 μM phenylephrine. Prostanoids were diluted in ethanol from a top stock of 10 mM and were added cumulatively with no washout between additions. The vehicle did not significantly relax the tissue at the concentrations used. Contractions were monitored by computer (Apple Macintosh LCIII and Performa 475) using a data recording and analysis system (MacLab) that was linked to either UF1 transducers (Pioden Controls) or model 1030 transducers (UFI, California).

**Drug Additions.** All agonist additions were made cumulatively without washout in a volume of 10 μL. Top stocks of drugs were 10 mM in DMSO except indomethacin, which was a 20-mg ml⁻¹ stock in ethanol. For isolated tissue experiments, PGE₂ and PGE₂ ethanolamide were serially diluted in saline, SR141716A was diluted in a DMSO/saline (50:50) mixture, and capsaicin was diluted in neat DMSO. In control experiments, the appropriate vehicle was added instead of agonist or antagonist, and it had no effect when added alone in either the trachea (contraction or relaxation) or the vas deferens (n = 4, data not shown). Antagonists/vehicle were added 30 min before the addition of agonists.

**Analysis of Data.** Values have been expressed as means and variability as S.E.M. or as 95% confidence limits. The values for pEC₅₀ (log EC₅₀) are defined as the effective concentration producing 50% of the maximum response inducible by that compound. EC₅₀ and maximal effects (E₅₀max) and the S.E.M. or 95% confidence limits of these values have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism).

**Calcium Imaging Experiments**

**Calcium Imaging.** Cultured DRG neurons were incubated for 1 h in NaCl-based extracellular solution (130 mM NaCl, 3.0 mM KC1, 0.6 mM MgCl₂, 2.0 mM CaCl₂, 1.0 mM MgHCO₃, 10.0 mM HEPES, and 5.0 mM glucose; pH 7.4, 310–320 mOsM) containing 10 μM Fura-2 AM. The cells were constantly perfused with NaCl-based solution (1–2 mL/min) and viewed under an inverted BX50WI microscope with a KAI-1001 S/N 5B7890-4201 camera attached (Olympus, Tokyo, Japan). The fluorescence ratiometric images from data obtained at excitation wavelengths of 340 and 380 nm were viewed and analyzed using UltraView (Merlin morphometry). The DRG neurons were exposed to 3 μM PGE₂ ethanolamide for 3 min followed by 10 min of washout with NaCl solution. This was followed by exposure to 100 nM capsaicin for 30 s and washout for 5 min and finally exposure to...
30 mM KCl for 30 s. The Ca\(^{2+}\) transient (fluorescence ratio after background subtraction) generated by each compound was measured. All data are expressed as means ± S.E.M.

## Results

### Radioligand Binding Studies

**EP Receptor Membranes.** Figure 1 shows the displacement of 0.5 nM \[^{3}H\]PGE\(_2\) from membranes expressing EP1 to 4 receptors by PGE\(_2\) and PGE\(_2\) ethanolamide. PGE\(_2\) ethanolamide had significantly lower affinity than PGE\(_2\) for all the hEP receptor subtypes investigated in the radioligand binding assay (Fig. 1). At each receptor, the \(pK_i\) values for PGE\(_2\) ethanolamide (Table 1) are significantly higher than for PGE\(_2\) \((P < 0.01\), one-way ANOVA). The level of specific binding for \[^{3}H\]PGE\(_2\) observed in membranes was 0.9 ± 0.1 pmol mg\(^{-1}\) for EP\(_1\), 0.48 ± 0.05 pmol mg\(^{-1}\) for EP\(_2\), 17.3 ± 4.08 pmol mg\(^{-1}\) for EP\(_3\), and 2.45 ± 0.19 pmol mg\(^{-1}\) for EP\(_4\) (all \(n = 6\)). The specific binding represented 81.98 ± 3.87%, 66.55 ± 0.91%, 93.90 ± 2.39%, and 95.50 ± 0.29% (all \(n = 6\)) of the total binding for EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\), respectively. These data are in line with those published by Abramovitz et al. (2000) using the same membranes under identical conditions.

It is possible that, in the human embryonic kidney cell membranes, PGE\(_2\) ethanolamide is undergoing hydrolysis to be converted to PGE\(_2\). To further investigate this possibility experiments with the EP\(_3\) membranes were carried out in the presence of the nonselective amidase inhibitor PMSF. In the presence of 200 \(\mu M\) PMSF the \(pK_i\) values for PGE\(_2\) and PGE\(_2\) ethanolamide in the EP\(_3\) membranes were 9.52 ± 0.08 \((n = 3)\) and 6.64 ± 0.26 \((n = 3)\). These values were not significantly different from the values in the absence of PMSF \((P > 0.05\), unpaired \(t\) test).

**VR1 Receptor Membranes.** In cell membranes from rat VR1 transfected CHO cells, PGE\(_2\) ethanolamide produced only a modest displacement (23.58 ± 5.36% at 10 \(\mu M\), \(n = 4\)) of specifically bound \[^{3}H\]resiniferatoxin.

### Effect of FAAH Inhibitors and PGE\(_2\) Ethanolamide on Binding of Anandamide to Mouse Brain Membranes.

In mouse brain membranes the level of inhibition of specific binding of the CB\(_1\) receptor agonist \[^{3}H\]CP55940 by 100 nM anandamide were 535 nM (confidence limits, 170-1681) for PMSF and 0.22 nM (confidence limits, 0.065–0.71) for OL-093. There is evidence that the inhibition of FAAH is pH-sensitive and the inhibitory action

<table>
<thead>
<tr>
<th>Compound</th>
<th>hEP(_1)</th>
<th>hEP(_2)</th>
<th>hEP(_3)</th>
<th>hEP(_4)</th>
</tr>
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<tbody>
<tr>
<td>PGE(_2)</td>
<td>8.31 ± 0.16</td>
<td>9.03 ± 0.04</td>
<td>9.34 ± 0.06</td>
<td>9.10 ± 0.04</td>
</tr>
<tr>
<td>PGE(_2) ethanolamide</td>
<td>5.61 ± 0.10</td>
<td>6.33 ± 0.01</td>
<td>6.70 ± 0.13</td>
<td>6.29 ± 0.06</td>
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**Table 1**

\(pK_i\) values for displacement of \[^{3}H\]PGE\(_2\) by PGE\(_2\) and PGE\(_2\) ethanolamide from cell membranes transfected with EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\) receptors \((n = 3–8)\).

Fig. 1. Displacement of 0.5 nM \[^{3}H\]PGE\(_2\) by PGE\(_2\) and PGE\(_2\) ethanolamide (PGE\(_2\)Eth) from membranes of HEK cells transfected with human EP\(_1\) receptors, where the specific binding of PGE\(_2\) was 0.9 ± 0.1 pmol mg\(^{-1}\), representing 81.98 ± 3.87% of total binding (a); with EP\(_2\) receptors, where the specific binding of PGE\(_2\) was 0.48 ± 0.05 pmol mg\(^{-1}\), representing 66.55 ± 0.91% of the total binding (b); with EP\(_3\) receptors, where the specific binding of PGE\(_2\) was 17.3 ± 4.08 pmol mg\(^{-1}\), representing 93.90 ± 2.39% of the total binding (c); and with EP\(_4\) receptors, where the specific binding of PGE\(_2\) was 2.45 ± 0.19 pmol mg\(^{-1}\), representing 95.50 ± 0.29% of the total binding (d). Each symbol represents the mean percentage of displacement ± S.E.M. (\(n = 3–6\)).
of PMSF has been shown to be greater at low pH (Holt et al., 2001). However at pH 5, PGE\textsubscript{2} ethanolamide failed to alter the level of inhibition of specific binding of \[^{3}H\]CP55940 induced by 100 nM anandamide (8.27 ± 0.29 with vehicle and 7.80 ± 0.06 with capsazepine) were not significantly different ($P > 0.05$, unpaired $t$ test) (Fig. 3b). The inhibitory action of PGE\textsubscript{2} ethanolamide was not significantly affected by SR141716A ($P > 0.05$, unpaired $t$ test) (Fig. 3c). The $pEC_{50}$ values were 7.63 ± 0.12 ($n = 6$) in the presence of vehicle and 7.57 ± 0.11 ($n = 6$) in the presence of 100 nM SR141716A. The CB\textsubscript{1} receptor antagonist CP55940 also inhibited the electrically evoked contractions, and this effect was antagonized by the CB\textsubscript{1} receptor antagonist SR141716A (Fig. 3d). The $pEC_{50}$ value of CP55940 was 2.17 ± 0.71 nM ($n = 6$).

**Guinea Pig Trachea.** In the trachea, the behavior of PGE\textsubscript{2} ethanolamide was similar to that of PGE\textsubscript{2}, producing contractions at lower concentrations followed by relaxation at higher concentrations. Thus, the concentration-response curve for both compounds was bell-shaped (Fig. 4). Maximum contractions were produced by PGE\textsubscript{2} ethanolamide at 1 \mu M and by PGE\textsubscript{2} at 100 nM. These were 38.9 ± 5.6% and 51.8 ± 10.6% ($n = 6$), respectively. The EP\textsubscript{1} receptor antagonist SC-51089 (10 \mu M) abolished the contractions induced by both PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide (Fig. 5a). In the presence of 10 \mu M SC-51089, the relaxant effects of these compounds may be indicative of EP\textsubscript{2} receptor activation. Under these circumstances, both PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide caused a concentration-related relaxation of histamine-induced contractions of this tissue, the $pEC_{50}$ values being 8.29 ± 0.17 ($n = 6$) and 7.11 ± 0.18 ($n = 6$), respectively (Fig. 5b).

**Isolated Tissue Experiments**

**Guinea Pig Vas Deferens.** PGE\textsubscript{2} ethanolamide inhibited electrically evoked contractions of the vas deferens, with a $pEC_{10}$ of 7.38 ± 0.09 ($n = 8$) compared with that of 9.09 ± 0.06 ($n = 6$) for PGE\textsubscript{2} (Fig. 3a). In the presence of PMSF, the potency of both PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide was reduced by around 4-fold; however, the relative potency of the compounds was unchanged (data not shown). The VR1 receptor antagonist capsazepine (10 \mu M) caused a slight rightward shift in the log concentration-response curve for PGE\textsubscript{2} ethanolamide, but the $pEC_{50}$ values (8.27 ± 0.29 with vehicle and 7.80 ± 0.06 with capsazepine) were not significantly different ($P > 0.05$, unpaired $t$ test) (Fig. 3b). The inhibitory action of PGE\textsubscript{2} ethanolamide was not significantly affected by SR141716A ($P > 0.05$, unpaired $t$ test) (Fig. 3c). The $pEC_{50}$ values were 7.63 ± 0.12 ($n = 6$) in the presence of vehicle and 7.57 ± 0.11 ($n = 6$) in the presence of 100 nM SR141716A. Thus, the $K_B$ value for SR141716A was 2.17 ± 0.71 nM ($n = 6$).

![Fig. 2.](image-url) The percentage of increase in the displacement of 0.5 nM \[^{3}H\]CP55940 binding to mouse brain membranes by 100 nM anandamide in the presence of increasing concentrations of PMSF, OL-093, and PGE\textsubscript{2} ethanolamide (PGE\textsubscript{2}Eth). Each symbol represents the mean percentage of increase in displacement of \[^{3}H\]CP55940 by anandamide ± S.E.M. ($n = 6$).

![Fig. 3.](image-url) Inhibition of electrically evoked contractions of the guinea pig vas deferens by PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide (PGE\textsubscript{2}Eth) (a), PGE\textsubscript{2} ethanolamide in the presence of vehicle (vh) and 10 \mu M capsazepine (CPZ) (b), PGE\textsubscript{2} ethanolamide in the presence of vehicle and SR141716A (SR141) (c), and CP55940 in the presence of vehicle and 100 nM SR141716A (d). Each symbol represents the mean percentage of inhibition ± S.E.M. ($n = 5–6$).
Fig. 4. Histogram showing the bell-shaped concentration-response relationship for contraction of the guinea pig trachea by PGE, and PGE, ethanolamide (PGE,Eth). Each column represents the mean percentage of contraction (expressed as a percentage of the histamine amide (PGE2Eth). Each symbol represents the mean percentage of contraction (expressed as a percentage of the histamine E\textsubscript{max}) ± S.E.M. (n = 4–6).

Fig. 5. a, contraction of the guinea pig trachea by PGE, and PGE, ethanolamide (PGE,Eth) in the presence of vehicle and the EP\textsubscript{1}, receptor antagonist SC-51089 (SC) (10 \mu M). Each symbol represents the mean percentage of contraction (expressed as a percentage of the histamine maximum) ± S.E.M. (n = 4–6). b, relaxation by PGE, and PGE, ethanolamide of guinea pig trachea precontracted with 1 \mu M histamine in the presence of 10 \mu M SC-51089. Each symbol represents the mean percentage of inhibition ± S.E.M. (n = 4–6).

Rabbit Jugular Vein. The rabbit jugular vein is thought to contain EP\textsubscript{1} receptors that mediate relaxation (Coleman et al., 1994; Milne et al., 1995). In this tissue both PGE, and PGE, ethanolamide caused concentration-related relaxation of the rabbit jugular vein precontracted with phenylephrine (Fig. 6). However, PGE, ethanolamide was 200-fold less potent than PGE, The pEC\textsubscript{50} values were 9.35 ± 0.25 (n = 5) and 7.05 ± 0.4 (n = 6) for PGE, and PGE, ethanolamide, respectively.

Calcium Imaging. Of a total of 252 DRG neurons from 15 separate cultures there were three groups of responses observed (Fig. 7). In all the cells analyzed, high K\textsuperscript{+} (30 mM) evoked an increase in [Ca\textsuperscript{2+}], thus confirming the neuronal phenotype. One group (group 1) of neurons (160/252) responded only to 30 mM KCl; thus, this group constituted 63.5% of the total population. The second group (group 2) (92/252) responded to 100 nM capsaicin and constituted 36.5% of the total population. A third group (group 3) of cells responded to PGE, ethanolamide and this constituted 21% of the capsaicin-sensitive population (19/92). PGE, ethanolamide did not elicit a Ca\textsuperscript{2+} transient in any capsaicin-insensitive cells. The Ca\textsuperscript{2+} transient evoked by 3 \mu M PGE, ethanolamide was not significantly different (P > 0.05, one-way ANOVA) from that of evoked by 100 nM capsaicin in the same group of cells, the change in fluorescence being 0.55 ± 0.09 for PGE, ethanolamide and 0.75 ± 0.10 for capsaicin (Fig. 7c). In cells that did not respond to PGE, ethanolamide, the response to capsaicin (0.95 ± 0.09) was not significantly different (P > 0.05, one-way ANOVA) from that obtained in those that responded to PGE, ethanolamide (Fig. 7c). The response to 30 mM KCl was not significantly different in any of the three groups. The area (\mu m\textsuperscript{2}) of the DRG neurons that responded to KCl only (group 1) (334.64 ± 13.88 \mu m\textsuperscript{2}) was significantly greater than those that responded to capsaicin (group 2) (239.19 ± 10.77 \mu m\textsuperscript{2}) (P < 0.01, one-way ANOVA) and those that responded to PGE, ethanolamide and capsaicin (group 3) (212 ± 18.6 \mu m\textsuperscript{2}) (P < 0.001, one-way ANOVA) (Fig. 7d). The VR1 receptor antagonist capsazepine did not significantly attenuate the response to PGE, ethanolamide. Experiments were conducted in which the cells were exposed to 3 \mu M PGE, ethanolamide followed by 15 min washout and then treatment for 5 min with either capsazepine or vehicle (0.1% DMSO) before a second exposure to 3 \mu M PGE, ethanolamide. The change in fluorescence in response to PGE, ethanolamide was 0.240 ± 0.043 and 0.206 ± 0.052 (n = 3) (P > 0.05, one-way ANOVA) before and after pretreatment with 10 \mu M capsazepine, respectively. The change in fluorescence in response to PGE, ethanolamide was 0.277 ± 0.05 and 0.280 ± 0.033 (n = 3) (P > 0.05, one-way ANOVA) before and after pretreatment with the vehicle, respectively.

Discussion

It would seem that PGE, ethanolamide has a similar profile of action to that of PGE, in that it binds to EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} receptors. The affinity of PGE, ethanolamide for EP receptor subtypes is significantly lower than that of PGE, being 500-, 500-, 440-, and 651-fold lower than that of PGE, for hEP\textsubscript{1}, hEP\textsubscript{2}, hEP\textsubscript{3}, and hEP\textsubscript{4} receptors, respectively. The inclusion of the FAAH inhibitor PMSF did not alter the K\textsubscript{i} values for either PGE, or PGE, ethanolamide. This suggests that PGE, ethanolamide is acting on the receptor directly.
the first demonstration that OL-093 enhances the activity of anandamide with high potency, presumably by inhibition of FAAH. It has recently been demonstrated that the pharmacological properties of FAAH are highly pH-dependent (Holt et al., 2001) and PMSF has been shown to be almost 60-fold more potent as an inhibitor or FAAH at pH 5.28 than at pH 8.37. However at pH 5, PGE\textsubscript{2} ethanolamide did not enhance the activity of anandamide at concentrations up to 10 \textmu M.

In the guinea pig vas deferens preparation, which is reported to contain EP\textsubscript{3} receptors, PGE\textsubscript{2} ethanolamide was 45-fold less potent than PGE\textsubscript{2}, the EC\textsubscript{50} values being 37 and 0.82 nM, respectively. In the presence of PMSF the relative potency of these compounds was unaltered, indicating that PGE\textsubscript{2} ethanolamide is not inhibiting the twitch response via conversion to PGE\textsubscript{2}. The affinity of PGE\textsubscript{2} for hEP\textsubscript{3} receptors ($K_i = 0.48$ nM) is similar to its potency in the guinea pig vas deferens (EC\textsubscript{50} = 0.82 nM). In contrast, the potency of PGE\textsubscript{2} ethanolamide is higher than predicted from the low affinity of this compound for the hEP\textsubscript{3} receptor ($K_i = 250$ nM). At present, EP\textsubscript{3} receptor antagonists are not available but we have excluded the possibility that this compound is interacting with CB\textsubscript{1} or VR1 receptors in this tissue.

In the guinea pig trachea, PGE\textsubscript{2} ethanolamide exhibited a bell-shaped concentration-response relationship, as has previously been demonstrated for PGE\textsubscript{2} (Dong et al., 1986). PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide produced a maximal contraction of the preparation at 100 nM and 1 \textmu M, respectively, followed by a relaxation of the tissue at higher concentrations. The contractile action of both compounds was abolished by SC-51089, indicating that they are contracting the tissue via an interaction with the EP\textsubscript{1} receptor. When the tracheal preparations were precontracted with histamine, in the presence of SC-51089, both PGE\textsubscript{2} and PGE\textsubscript{2} ethanolamide produced concentration-related relaxation of the tissue. PGE\textsubscript{2} ethanolamide was only 15-fold less potent than PGE\textsubscript{2}, the EC\textsubscript{50} values being 76.9 and 5.19 nM, respectively. Previous pharmacological analysis indicates that this relaxant action of PGE\textsubscript{2} is mediated by the EP\textsubscript{2} receptor subtype (Coleman et al., 1990). The relatively high potency of PGE\textsubscript{2} ethanolamide at the EP\textsubscript{2} receptor in the trachea is not in line with the low affinity of the compound for the EP\textsubscript{2} receptor subtype. As with the EP\textsubscript{3} receptor, selective EP\textsubscript{2} receptor antagonists are not yet available. Thus, the potency of PGE\textsubscript{2} ethanolamide in both the vas deferens and the trachea (relaxation) is higher than predicted from the binding assays using human receptors. This may be accounted for by species differences. However, it is also possible that this compound may be interacting with other, yet uncharacterized receptors for prostaglandin ethanolamides. Interestingly, the pharmacology of PGF\textsubscript{2\alpha} ethanolamide (prostamide F\textsubscript{2\alpha}) suggests the existence of a novel prostamide receptor. Thus, PGE\textsubscript{2} ethanolamide contracts the cat iris sphincter with potent activity that is not exhibited in other preparations that respond to PGF\textsubscript{2\alpha} (Chen et al., 2001). Furthermore, this

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**Fig. 7.** Effect of PGE\textsubscript{2} ethanolamide (PGE\textsubscript{2} Eth) on [Ca\textsuperscript{2+}] in single DRG neurons. The traces (a and b) show examples of [Ca\textsuperscript{2+}] responses ($F_{i+}/F_{i-}$ ratio) evoked by 3 \textmu M PGE\textsubscript{2} ethanolamide, 100 nM capsaicin, and KCl in DRG neurons from two separate cultures. The agonists were applied for the periods shown by the horizontal lines. The dotted lines show a DRG neuron that only responds to KCl (group 1), the gray line shows a DRG neuron that responds to both capsaicin and KCl (group 2), and the black line shows a DRG neuron that responds to PGE\textsubscript{2} ethanolamide, capsaicin, and KCl (group 3). The histogram (c) shows the [Ca\textsuperscript{2+}] evoked in 252 DRG neurons obtained from 15 separate cultures. The data represent the mean change in fluorescence ratio ± S.E.M. The histogram (d) shows the area ($\mu m^2$) of the DRG neurons in each of the three groups ± S.E.M. The area of the DRG neurons that responded to KCl only (group 1) was significantly ($P < 0.01$, one-way ANOVA) greater than those that responded to capsaicin (group 2) and those that responded to PGE\textsubscript{2} ethanolamide and capsaicin (group 3) ($P < 0.001$, one-way ANOVA). The area of the DRG neurons that responded to PGE\textsubscript{2} ethanolamide (group 3) was not significantly different ($P > 0.05$, one-way ANOVA) from those that responded to capsaicin alone (group 2).

rather than being cleaved to form PGE\textsubscript{2}. It is notable that Kozak et al. (2001) have demonstrated that PGE\textsubscript{2} ethanolamide is subject to little or no hydrolysis in rat or human blood. It has been previously demonstrated that anandamide and other ethanolamides have significant affinity for the vanilloid VR1 receptor (Smart et al., 2000; Ross et al., 2001). However in this study, PGE\textsubscript{2} ethanolamide seemed to have limited affinity for the VR1 receptor with a $K_i$ value of $>10$ \textmu M, compared with a value of 1.66 \textmu M for anandamide in the same cell line (Ross et al., 2001).

PGE\textsubscript{2} ethanolamide does not seem to interact with FAAH. At pH 7.4, this compound did not enhance the ability of anandamide to displace $[^3H]$CP55940 from mouse brain membranes. Although this is indicative of a lack of modulation of FAAH by this compound, conclusive data can only be obtained with a more specific and direct measure of FAAH activity. In contrast to PGE\textsubscript{2} ethanolamide, the established FAAH inhibitors PMSF and OL-093 (compound 53 in Böger et al., 2000) produce a marked enhancement of the activity of anandamide in mouse brain membranes. This is the first demonstration that OL-093 enhances the activity of anandamide with high potency, presumably by inhibition of FAAH. It has recently been demonstrated that the pharmacological properties of FAAH are highly pH-dependent (Holt et al., 2001) and PMSF has been shown to be almost 60-fold more potent as an inhibitor or FAAH at pH 5.28 than at pH 8.37. However at pH 5, PGE\textsubscript{2} ethanolamide did not enhance the activity of anandamide at concentrations up to 10 \textmu M.

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compound has little affinity for the recombinant cat or human PGE_{2a} (FP) receptor. In contrast to PGE_{2a}, PGE_{2c} ethanola mide is not significantly hydrolyzed in plasma, is stable in cerebrospinal fluid, and is oxidized less efficiently than PGE_{2a} (Kozak et al., 2001). This raises the possibility that the higher relative potency of this compound in some tissues may be due to its resistance to metabolism by enzymes that are responsible for the rapid inactivation PGE_{2c}.

Prostanoids are known both to directly activate sensory neurons and to sensitize sensory neurons to other potent nociceptive agents such as bradykinin. PGE_{2c} has been shown to activate a subpopulation of small-diameter capsaicin-sensitive DRG neurons and to potentiate the bradykinin-evoked increases in [Ca^{2+}]_i. Both of these actions of PGE_{2c} seem to involve protein kinase A-dependent mechanisms (Smith et al., 2000). Smith et al. (2000) found that 1 μM PGE_{2c} evoked an increase in [Ca^{2+}]_i in 16% of capsaicin-sensitive DRG neurons. PGJ_{2} and PGF_{2α} (1 μM) also evoked calcium transients in 26 and 29% of DRG neurons, respectively. Similarly, in this study we found that 3 μM PGE_{2c} ethanamide evoked an increase in [Ca^{2+}]_i in 21% of small-diameter capsaicin-sensitive DRG neurons. The possibility that PGE_{2c} ethanamide shares the ability of PGE_{2a} to sensitize DRG neurons to capsaicin (Lopshire and Nicol, 1997) and bradykinin (Smith et al., 2000) is the subject of ongoing investigations. One would expect that activation of cyclic AMP-dependent kinase by PGE_{2c} ethanamide may enhance vanilloid receptor-mediated responses in DRG neurons (De Petrocellis et al., 2001). The receptor mechanisms underlying these actions of the prostanoids have not yet been investigated and it remains to be established whether the prostanoids are acting through the same or distinct sites of action to activate DRG neurons.

The physiological significance of the conversion of anandamide to PGE_{2c} ethanamide has yet to be established. It may be that the prostaglandin ethanamides are a new class of mediator; alternatively, it could be speculated that competing with arachidonic acid for COX-2, increasing levels of anandamide might modulate the local production of prostanoids by this enzyme. This, in turn, would result in less activation of EP receptors because the alternative product, PGF_{2α} ethanamide, has lower potency at these receptors than PGE_{2c}. There is strong evidence that anandamide is metabolized by COX-2 to produce PGE_{2c} ethanamide in physiologically relevant environments (Yu et al., 1997; Burton et al., 2000). However, it has yet to be established whether the levels of PGE_{2c} ethanamide synthesized by COX-2 metabolism of anandamide in vivo are sufficient to activate EP receptors. Potent FAAH inhibitors have recently been synthesized (Boger et al., 2000), which enhance the levels of anandamide significantly, and these compounds may be of considerable therapeutic benefit. Acute and chronic peripheral inflammation, interleukins, and spinal cord injury increase the expression of COX-2 (Vanegas and Schaible, 2001) in the spinal cord and DRG neurons. In the event of inhibition of FAAH metabolism of anandamide, increased levels of endogenous anandamide in combination with an up-regulation of COX-2 (inflammation, injury) may lead to the production of significant levels of the prostaglandin ethanamides. Recently, it has been shown that PGE_{2c} ethanamide is significantly more resistant to metabolism than PGE_{2a}, being detectable in rat plasma 2 h after administration (Kozak et al., 2001), thus raising the possibility that this compound may act systemically. In this study we have shown for the first time that PGE_{2c} ethanamide is indeed pharmacologically active in some tissues at relatively low concentrations. The physiological consequence and relevance of these actions remain to be established.

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References


Burstyn SH, Rossetti BG, Yagupsky P, and Nicol GD (2000) Prostaglandin E_{2c} ethanamide is not significantly hydrolyzed in plasma, is stable in cerebrospinal fluid, and is oxidized less efficiently than PGE_{2a}. In the rabbit jugular vein, which is thought to contain EP4 receptors, PGE_{2c} ethanamide is significantly more resistant to metabolism than PGE_{2a}, being detectable in rat plasma 2 h after administration (Kozak et al., 2001), thus raising the possibility that this compound may act systemically. In this study we have shown for the first time that PGE_{2c} ethanamide is indeed pharmacologically active in some tissues at relatively low concentrations. The physiological consequence and relevance of these actions remain to be established.


Dr. R. A. Ross, Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland, UK AB25 ZDD. E-mail: r.ross@abdn.ac.uk

**Address correspondence to:** Dr. R. A. Ross, Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland, UK AB25 ZDD. E-mail: r.ross@abdn.ac.uk