15R-Methyl-Prostaglandin D$_2$ Is a Potent and Selective CRTH2/DP$_2$ Receptor Agonist in Human Eosinophils

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ABSTRACT

Prostaglandin D$_2$ (PGD$_2$) is a mast cell-derived mediator that seems to play a role in asthma and allergic diseases. It is the only primary prostanoid to activate human eosinophils, which it accomplishes through the DP$_2$ receptor/chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). In addition, PGD$_2$ has both pro- and anti-inflammatory effects via the adenylyl cyclase-coupled DP$_1$ receptor. To attempt to identify potent and selective DP$_2$ receptor agonists we compared the abilities of a series of PGD$_2$ analogs to activate eosinophils via the DP$_2$ receptor with their abilities to stimulate adenylyl cyclase in platelets via the DP$_1$ receptor. All of the PGD$_2$ analogs tested stimulated CD11b expression and actin polymerization with a rank order of potency of 15R-methyl-PGD$_2$ $>$ PGD$_2$ $>$ 17-phenyl-18,19,20-trinor-PGD$_2$ $>$ 15S-methyl-PGD$_2$ $>$ 16,16-dimethyl-PGD$_2$ $>$ 11-keto-fluprostain. Surprisingly, 15R-methyl-PGD$_2$, which has the unnatural R-configuration at carbon 15, was about 5 times more potent than PGD$_2$ and about 75 times more potent than 15S-methyl-PGD$_2$. 15R-methyl-PGD$_2$ (EC$_{50}$ value of 1.7 nM) was also much more potent as an eosinophil chemoattractant than PGD$_2$ (EC$_{50}$ value of 10 nM) and 15S-methyl-PGD$_2$ (EC$_{50}$ value of 128 nM). Cross-desensitization experiments indicated that 15R-methyl-PGD$_2$ acts through the DP$_2$ receptor. None of the PGD$_2$ analogs tested elevated platelet cAMP by more than 20% of the maximal level in response to PGD$_2$. However, in contrast to eosinophils, the most active was 15S-methyl-PGD$_2$. In conclusion, 15R-methyl-PGD$_2$ is the most potent known DP$_2$ receptor agonist, and because of its selectivity and resistance to metabolism, should be a useful tool in probing the physiological role of this receptor in inflammatory diseases.

Prostaglandin D$_2$ (PGD$_2$) is an arachidonic acid metabolite formed by hematopoietic-type PGD synthase in mast cells (Urade et al., 1990), dendritic cells (Urada et al., 1989), and Th2 cells (Tanaka et al., 2000), and by lipocalin-type PGD synthase in the central nervous system (Urada and Hayaishi, 2000). Large amounts of PGD$_2$ are released into the airways of asthmatics immediately after antigen challenge (Murray et al., 1986), suggesting that PGD$_2$ may be a mediator of asthma. Further evidence to support such a role for PGD$_2$ is the finding that mice lacking the DP receptor (referred to as the DP$_1$ receptor below) display reduced hyper-responsive-ness to acetylcholine, reduced pulmonary eosinophilia, and reduced Th2 cytokine levels after antigen challenge (Mat-suoka et al., 2000). Furthermore, pulmonary infiltration of inflammatory cells and Th2 cytokine production were elevated after antigen challenge of transgenic mice overexpressing lipocalin-type PGD synthase (Fujitani et al., 2002).

Until recently, PGD$_2$ was believed to act by raising intracellular cAMP levels through its action on a single G$_i$ protein-coupled receptor termed the DP receptor. However, Hirai et al. (2001) and our group (Monneret et al., 2001) independently discovered a second PGD$_2$ receptor that is coupled to a G$_i$ protein (Hirai et al., 2001). This receptor has been termed chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Hirai et al., 2001) or the DP$_2$ receptor (Monneret et al., 2001). Although there are some discrepancies between the binding affinities of certain PGD$_2$ degradation products (15-deoxy-$\Delta^{12,14}$-PGJ$_2$ and $\Delta^{12}$-PGJ$_2$) to K562 cells transfected with CRTH2 and the potencies of these compounds in activating eosinophils (Monneret et al., 2002), it would seem almost certain that CRTH2 and the DP$_2$ receptor are identical. These differences could possibly be explained by differences between the G protein complements of the two types of cell.

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ABBREVIATIONS: PG, prostaglandin; Th2, T helper cell type 2; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; PBS, phosphate-buffered saline; VLA-4, very late antigen 4; 17-Ph-PGD$_2$, 17-phenyl-18,19,20-trinor-PGD$_2$; LTB$_4$, leukotriene B$_4$; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid.
The DP₂ receptor/CRTH2 is found on eosinophils (Hirai et al., 2001; Monneret et al., 2001), Th2 cells (Hirai et al., 2001), and basophils (Hirai et al., 2001) and is responsible for the chemotactic effect of PGD₂ on each of these cell types. Activation of this receptor by PGD₂ also results in actin polymerization, CD11b expression, L-selectin shedding (Monneret et al., 2001), and calcium mobilization (Monneret et al., 2002) in eosinophils. Although the predominant response of eosinophils to PGD₂ is mediated by the DP₂ receptor, these cells also contain DP₁ receptors, which respond to PGD₂ and DP₁ receptor agonists such as BW245C with elevated levels of cAMP (Monneret et al., 2001). Activation of DP₁ receptors on eosinophils has also been reported to result in increased cell survival (Gervais et al., 2001).

Eludication of the precise roles of DP₁ and DP₂ receptors in asthma will require the availability of potent and selective agonists and antagonists for these receptors. Our previous work demonstrating that 13,14-dihydro-15-oxo-PGD₂ (Monneret et al., 2001) and 15-deoxy-Δ¹²,14-PGD₂ (Monneret et al., 2002) exhibit considerable activity at DP₂ receptors suggested that PGD₂ analogs with altered alkyl side chains may be good DP₂ receptor agonists. We therefore investigated the effects of a series of recently available PGD₂ analogs in which this side chain has been modified. We found that the DP₂ receptor strongly discriminates between the analogs tested, and that 15R-methyl-PGD₂₂₃, which has the unnatural R-configuration at carbon 15, is a potent and selective ligand for this receptor. In contrast, none of the compounds tested had substantial effects on DP₁ receptor-mediated responses.

**Materials and Methods**

**Materials.** All prostaglandins were purchased from Cayman Chemical (Ann Arbor, MI). 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) was synthesized chemically as described previously (Khanapure et al., 1998).

**Preparation of Leukocytes.** Unfractionated leukocytes were prepared as described previously by treatment of blood from healthy volunteers with Dextran T-500 (Amersham Biosciences, Piscataway, NJ) for 45 min at 4°C to remove red blood cells (Boiyum, 1968), followed by centrifugation of the supernatant at 200 g for 10 min. For experiments designed to measure CD11b expression, the cells were resuspended directly in phosphate-buffered saline (PBS). For other experiments, the cells were subjected to hypotonic lysis, followed by centrifugation and resuspension of the pellet in PBS.

Purified eosinophils, which were used for chemotaxis experiments, were prepared by centrifugation of unfractionated leukocytes over Ficoll-Paque, followed by immunomagnetic cell sorting using anti-CD16 coupled to paramagnetic microbeads to remove neutrophils from the granulocyte fraction (Hansel et al., 1991). The eosinophils obtained in the pass-through fraction were centrifuged and resuspended in RPMI 1640 medium containing ovalbumin (0.4%).

**Preparation of Platelets.** Whole blood (20 ml) was collected in medium (2.5 ml) containing citric acid (15.5 mM), sodium citrate (90 mM), Na₂HPO₄ (16 mM), dextrose (161 mM), and adenine (2 mM). After centrifugation at 200 g for 15 min, the supernatant was diluted with an equal volume of medium containing 94 mM citrate and 140 mM dextrose, pH 6.5. The mixture was centrifuged at 1000 g for 10 min, and the pellet was suspended in PBS to give a platelet concentration of 3 x 10⁹ cells/ml.

**Measurement of CD11b Expression.** Unfractionated leukocytes (5 x 10⁶ cells in 0.5 ml of PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂) were incubated with agonist for 10 min. The incubations were terminated by the addition of ice-cold FACSFLOW (BD Biosciences, San Jose, CA) and centrifugation. After staining with phycoerythrin-labeled anti-CD11b (30 min/4°C) as described previously (Powell et al., 2001), the cells were treated with OptiLyse C (0.25 ml; Beckman Coulter Inc., Fullerton, CA) for 15 min, centrifuged, and fixed in PBS (0.4 ml) containing 1% formaldehyde. The distribution of fluorescence intensities due to phycoerythrin isothiocyanate-anti-CD11b labeling was measured by flow cytometry (FACSCalibur; BD Biosciences) in eosinophils, which were gated out based on high side scatter and high VLA-4 expression (Powell et al., 2001). None of the prostanooids tested had an appreciable effect on VLA-4 expression or side scatter under the experimental conditions used.

**Analysis of Intracellular Calcium Levels by Flow Cytometry.** Calcium levels in eosinophils were measured by flow cytometry as described previously (Monneret et al., 2002). Unfractionated leukocytes (10⁵ cells/ml) were labeled with Fluo-3 acetoxy methyl ester (2 μM, 60 min at 23°C; Molecular Probes, Eugene, OR) in the presence of Phuronic F-127 (0.02%) and then stained with PCS-labeled mouse anti-human CD16 (3.3 μl/10⁶ cells; Beckman Coulter, Inc.) for 30 min at 0°C. PBS (25 ml) was then added, the mixture centrifuged as described above, and the pellet resuspended in PBS to give a concentration of 3 x 10⁶ leukocytes/ml. After incubation at 23°C for 30 min, an aliquot (0.95 ml) of the leukocyte suspension was removed and treated with PBS (50 μl) containing CaCl₂ (36 mM) and MgCl₂ (20 mM). After 5 min, Fluo-3 fluorescence was measured by flow cytometry in a total of approximately 10⁶ cells. Eosinophils and neutrophils were gated out on the basis of staining with anti-CD16 and side scatter.

**Measurement of Actin Polymerization.** Intracellular F-actin levels were measured in leukocytes prelabeled with PCS-labeled mouse anti-human CD16 as described previously (Monneret et al., 2002). Aliquots (90 μl) of the leukocyte suspension (5 x 10⁶ cells/ml) were preincubated for 5 min at 37°C before the addition of agonist or vehicle (10 μl of PBS containing 0.9 mM CaCl₂ 0.5 mM MgCl₂, and 0.1% bovine serum albumin). The incubations were terminated after 20 s by addition of formaldehyde (37%) to give a final concentration of 8.5%. After keeping the samples on ice for 30 min, a mixture of lysophosphatidylcholine (30 μg in 23.8 μl of PBS) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin (49 pmol in 6.2 μl of methanol; final concentration 0.3 μM; Molecular Probes) was added to each sample (Howard and Oresajo, 1985), followed by incubation overnight in the dark at 4°C. After washing by centrifugation, F-actin levels were measured by flow cytometry in eosinophils, which were gated out on the basis of high side scatter and low CD16 expression.

**Measurement of Eosinophil Migration.** Eosinophil migration was measured using 48-well microchemotaxis chambers (Neuro Probe Inc., Cabin John, MD) and Sartorius cellulose nitrate filters (8-μm pore size; 140 μm in thickness) (Neuro Probe, Inc.) (Powell et al., 1995). Agonists were added to the bottom well in a volume of 30 μl of PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂, and 0.3% bovine serum albumin, whereas eosinophils (150,000 cells in 55 μl of RPMI 1640 medium containing 0.4% ovalbumin) were added to each of the top wells. After incubation for 2 h at 37°C, the filters were fixed with mercuric chloride and stained with hematoxylin and chromotrope 2R (Kay, 1970). The numbers of cells on the bottom surfaces of the filters were counted in five different fields at a magnification of 400× for each incubation, each of which was performed in duplicate.

**Determination of cAMP Levels in Platelets.** Platelets (3 x 10⁹ cells in a final volume of 100 μl) were preincubated for 2 min at 37°C with isobutylmethylxanthine (1 mM) and then incubated for a further 2 min with prostanooids. The incubations were terminated by addition of ice-cold ethanol (300 μl), and the precipitated proteins were removed by centrifugation (600g for 10 min). cAMP in the supernatants was measured using a competitive protein-binding radiometric assay (Diagnostic Products, Los Angeles, CA) according to the manufacturer’s instructions.

**Statistical Analysis.** All EC₅₀ values are expressed as geometric means with 95% confidence limits. The EC₅₀ values of different
prostanoids were compared using one-way repeated measures analysis of variance followed by Student-Newman-Keuls test for multiple comparisons. Maximal responses are expressed as means ± S.E. Differences were considered to be statistically significant when P values were less than 0.05.

Results

Effects of PGD2 Analogs on CD11b Expression by Eosinophils. The effects of different PGD2 analogs on CD11b expression were determined by flow cytometric analysis of leukocytes labeled with anti-VLA-4 to distinguish eosinophils from neutrophils. All of the compounds tested stimulated CD11b expression but had markedly different potencies. 15R-Methyl-PGD2, in which the stereochemical configuration at carbon 15 is reversed compared with PGD2, was the most potent, having an EC50 value (1.4 nM) 5 times lower than that of PGD2 (7 nM; P < 0.001), whereas 17-phenyl-18,19,20-trinor-PGD2 (17-Ph-PGD2) was about one-half as potent as PGD2 (Fig. 1; Table 1). In contrast, 15S-methyl-PGD2, which has the natural S-configuration at carbon 15, is a much weaker stimulator of CD11b expression, with an EC50 value about 70 times higher than that of 15R-stereoisomer. 16,16-Dimethyl-PGD2 has an EC50 value similar to that of 15S-methyl-PGD2, whereas 11-keto-fluprostenol is about 4 times less potent.

Stimulation of Actin Polymerization in Eosinophils by PGD2 Analogs. Polymerized F-actin was measured in eosinophils treated with PGD2 and its synthetic analogs by labeling with fluorescently tagged phallacidin. The effects of these compounds on actin polymerization were similar to those on CD11b expression, except that the EC50 values were about 2 to 3 times higher (Fig. 2; Table 1). Of the compounds tested, 15R-methyl-PGD2 was the most potent stimulator of actin polymerization with an EC50 value of 3.8 nM compared with 13 nM for PGD2 and 333 nM for 15S-methyl-PGD2. 17-Ph-PGD2 was slightly less potent than PGD2, whereas 16,16-dimethyl-PGD2 was approximately equipotent with 15S-methyl-PGD2. Although the maximal response to 11-keto-fluprostenol did not seem to have been reached at the highest concentration tested (10 μM), it was calculated that it has an EC50 value of at least 1.9 μM.

15R-Methyl-PGD2 Is a Potent Stimulator of Eosinophil Migration. To determine whether 15R-methyl-PGD2 could induce functional changes in eosinophils we examined its chemoattractant effects on these cells using a modified Boyden chamber assay. 15R-Methyl-PGD2 was indeed a potent stimulator of eosinophil migration, with an EC50 value (1.7 nM) about 6 times lower than that of PGD2 (EC50 value of 10 nM; P < 0.001) (Fig. 3). In contrast, 15S-methyl-PGD2 was about 75 times less potent than the 15R-isomer and about 13 times less potent than PGD2 in stimulating eosinophil migration. The maximal responses to PGD2, 15R-methyl-PGD2, and 15S-methyl-PGD2 were 38 ± 4, 30 ± 2, and 30 ± 3 cells/high-power field, respectively, suggesting that 15R-methyl-PGD2 may not be a full agonist. However, when tested by analysis of variance, these differences were not significant.

Effects of PGD2 Analogs on cAMP Levels in Platelets. To determine the abilities of the ω-chain-modified PGD2 analogs to stimulate DP1 receptor-mediated responses, we examined their effects on cAMP levels in human platelets. Consistent with previous reports (Mills and Macfarlane, 1974), PGD2 was a strong stimulator of adenyl cyclase activity in platelets, increasing cAMP levels from a baseline of 0.21 ± 0.02 to 16.4 ± 1.8 pmol/10^7 platelets (Fig. 4A). The EC50 value for PGD2 is 109 nM (95% confidence limits 59–202 nM). None of the PGD2 analogs tested had substantial effects on platelet cAMP levels, even at concentrations up to 10 μM. The most potent analog was 15S-methyl-PGD2, which induced a response equivalent to 20 ± 1.6% of the maximal response to PGD2 at the highest concentration tested. The 15R-methyl isomer of PGD2 was considerably less potent, inducing a response only 5.9 ± 0.8% that of the maximal response to PGD2. As shown in Fig. 4B, the responses of platelets to the other analogs tested were also much lower than the maximal response to PGD2: 16,16-dimethyl-PGD2 (14 ± 2% of PGD2), 17-Ph-PGD2 (7.5 ± 1.6%), and 11-keto-fluprostenol (0.7 ± 0.2%). As an additional positive control we examined the effect of PGD3, which has been reported to be slightly more potent than PGD2 in inhibiting platelet aggregation (Whitaker et al., 1979). As expected, PGD3 strongly stimulated cAMP formation in platelets, inducing a similar maximal response to PGD2 with a slightly higher potency (EC50 value of 64 nM).

15R-Methyl-PGD2 is a Selective DP2 Receptor Agonist. We previously showed that PGD2 stimulates calcium mobilization in eosinophils but not neutrophils via the DP2 receptor (Monneret et al., 2002). To determine whether the effect of 15R-methyl-PGD2 is mediated by the DP2 receptor we investigated its effects on calcium transients elicited in eosinophils by PGD2 and other eicosanoids. Calcium levels were measured in anti-CD16-labeled unfractionated leukocytes by flow cytometry. Eosinophils and neutrophils were gated out on the basis of low and high CD16 expression, respectively. Like PGD2, 15R-methyl-PGD2 increased intracellular calcium levels in eosinophils but not neutrophils (Fig. 5). Furthermore, there was complete cross-desensitization between these two compounds, as shown in Fig. 5, top. In

**Fig. 1.** Effects of PGD2 analogs on CD11b expression by eosinophils. Unfractionated leukocytes were incubated for 10 min at 37°C with various concentrations of 15R-methyl-PGD2 (●, 15R; n = 6), PGD2 (○, D2; n = 7), 17-Ph-PGD2 (○, 17ph; n = 6), 15S-methyl-PGD2 (□, 15S; n = 7), 16,16-dimethyl-PGD2 (■, 16dm; n = 5), and 11-keto-fluprostenol (▲, 11kf; n = 4). Surface expression of CD11b was measured in eosinophils, which were gated out on the basis of high expression of VLA-4 and high side scatter, as described under Materials and Methods. The data are means ± S.E. of determinations on cells from the numbers of different donors indicated and are expressed as percentages of the maximal response to PGD2, which was 101 ± 14% above the basal level of expression of CD11b.
contrast, 15R-methyl-PGD$_2$ did not inhibit the effects of either 5-oxo-ETE or LTB$_4$ on calcium mobilization in either eosinophils or neutrophils (Fig. 5, bottom).

**Discussion**

The objective of the current study was to identify potent and selective DP$_2$ receptor agonists and to learn more about the structural selectivity of this receptor. Prompted by our earlier findings that the alkyl side chain of PGD$_2$ seems to be less critical for recognition by the DP$_2$ receptor than the DP$_1$ receptor, we investigated the biological effects of a series of PGD$_2$ analogs in which this side chain had been modified. Although relatively little was known about the effects of these compounds on PGD$_2$-mediated responses, they are structurally similar to analogs of other prostaglandins that were developed to increase metabolic stability by blocking metabolism by 15-hydroxy-prostaglandin dehydrogenase (e.g., 15S-methyl analogs and 16,16-dimethyl analogs) or by increasing receptor affinity (e.g., 17-phenyl analog). The potencies of these analogs in stimulating responses mediated by DP$_1$ and DP$_2$ receptors are summarized in Table 1. All of the PGD$_2$ analogs tested were capable of eliciting DP$_2$ receptor-mediated responses in eosinophils with efficacies close to that of PGD$_2$, on calcium mobilization in either eosinophils or neutrophils (Fig. 5, bottom).

**TABLE 1**

<table>
<thead>
<tr>
<th>Analogue</th>
<th>R</th>
<th>CD11b (DP$<em>2$) EC$</em>{50}$ (nM)</th>
<th>CD11b (DP$<em>2$) EC$</em>{50}$ (nM)</th>
<th>Chemotaxis (DP$<em>2$) EC$</em>{50}$ (nM)</th>
<th>cAMP (DP$<em>1$) EC$</em>{10}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD$_2$</td>
<td></td>
<td>7 (2, 18)</td>
<td>13 (6, 32)</td>
<td>10 (4, 27)</td>
<td>11 (7, 17)</td>
</tr>
<tr>
<td>PGD$_3$</td>
<td></td>
<td>8* (4, 16)</td>
<td></td>
<td></td>
<td>5 (1, 19)</td>
</tr>
<tr>
<td>15S-Me-PGD$_2$</td>
<td></td>
<td>99 (50, 202)</td>
<td>333 (251, 442)</td>
<td>128 (46, 361)</td>
<td>2,113 (1,577, 2,830)</td>
</tr>
<tr>
<td>15R-Me-PGD$_2$</td>
<td></td>
<td>1.4 (0.7, 2.6)</td>
<td>3.8 (2.5, 5.9)</td>
<td>1.7 (0.6, 5.3)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>16,16-diMe-PGD$_2$</td>
<td></td>
<td>101 (22, 461)</td>
<td>341 (217, 537)</td>
<td></td>
<td>5,481 (2,394, 12,548)</td>
</tr>
<tr>
<td>17-Ph-PGD$_2$</td>
<td></td>
<td>16 (6, 44)</td>
<td>34 (20, 57)</td>
<td></td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>11k-fluprostrenol</td>
<td></td>
<td>432 (158, 1,178)</td>
<td>1,937 (1,350, 2,779)</td>
<td></td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

* From Monneret et al., (2002)

The data for DP$_2$ receptor-mediated stimulation of eosinophils are geometric means of the EC$_{50}$ values with 95% confidence limits shown in brackets. The data for DP$_1$ receptor-mediated elevation of cAMP levels in platelets are geometric means of the EC$_{10}$ values with 95% confidence limits. EC$_{10}$ values are the concentrations of prostanoids required to elevate cAMP to a level equivalent to 10% of the maximal response to PGD$_2$, always determined in the same experiment. All values were calculated from the individual experiments used to calculate the data shown in Figs. 1–4. The numbers of experiments for each determination are given in the relevant figure legends.
unexpected because the former compound has the opposite configuration to PGD₂ at carbon 15. All naturally occurring prostanoids have the S-configuration at this carbon, and this is critical for recognition by their receptors. For example, 15S-methyl-PGF₂α has approximately the same affinity as PGF₂α, for FP receptors in the corpus luteum and is much more potent than PGF₂α in inducing luteal regression in vivo because of its resistance to metabolism by 15-hydroxy-prostaglandin dehydrogenase (Powell et al., 1975). In contrast, 15R-methyl-PGF₂α is about 150 times less potent in inducing this response (Miller and Sutton, 1976). Similarly, 15S-methyl-PGE₂ has a potency similar to that of PGE₂ on smooth muscle contraction in vitro and is much more potent than PGE₂ in stimulating intestinal muscle contraction and in inhibiting gastric acid secretion in vivo (Main and Whittle, 1975). In contrast, 15R-methyl-PGE₂ has very little biological activity itself, but undergoes isomerization to the biologically active 15S-isomer at acid pH (Main et al., 1975).

The cross-desensitization experiments shown in Fig. 5 provide strong evidence that 15R-methyl-PGD₂ stimulates eosinophils through the DP₂ receptor. Although we cannot completely rule out the possibility that this could be explained by heterologous desensitization between a selective 15R-methyl-PGD₂ receptor and the DP₂ receptor, this would seem unlikely in view of the fact that 15R-methyl-PGD₂ did not affect the responses of eosinophils to either 5-oxo-ETE or PGD₂. Furthermore, there are no prostanoid receptors other than the DP₂ receptor known to be associated with eosinophil activation (Monneret et al., 2001). Moreover, 15R-methyl-PGD₂ induces responses very similar to the DP₂ receptor-mediated effects of PGD₂ in that it is selective for eosinophils over neutrophils and elicits increased CD11b expression, actin polymerization, calcium mobilization, and chemotaxis in these cells. In all cases, 15R-methyl-PGD₂ is approximately 5 times more potent than PGD₂, making it the most potent agonist yet described for the DP₂ receptor.

The high degree of discrimination by the DP₂ receptor among different PGD₂ analogs with modified alkyl side chains was unanticipated because prior work suggested that this side chain may not be very important for recognition by this receptor. Oxidation of the 15-hydroxyl group coupled with reduction of the Δ¹⁵ double bond has only a small effect on DP₂ receptor-mediated responses (Gervais et al., 2001; Hirai et al., 2001; Monneret et al., 2001), whereas removal of this hydroxyl group and replacement of the Δ¹³ double bond by a Δ¹²,1⁴-conjugated system has virtually no effect (Monneret et al., 2002). However, the present results demonstrate that addition of two methyl groups to carbon 16 (16,16-dimethyl-PGD₂) lowers potency by about 20-fold compared with PGD₂ (Table 1). The maximal response to this analog also seems to be somewhat less than that to PGD₂ (Figs. 1 and 2). In contrast, the corresponding modification of prostaglandins F₂α and E₂ has little effect on the responses mediated by FP receptors (Powell et al., 1975) and some, but not all, EP receptor subtypes (Kiriyama et al., 1997), respectively. On the other hand, 17-Ph-PGD₂ was found to be a relatively potent DP₂ receptor ligand. Similarly, the 17-phenyl derivatives of prostaglandins F₂α and E₂ have been reported to be potent FP and EP receptor agonists (Powell et al., 1975; Kiriyama et al., 1997). On the other hand, 11-keto-fluprostenol is the least potent of the PGD₂ analogs tested, whereas the corresponding derivative of PGF₂α, is a potent FP receptor agonist (Dukès et al., 1974).

In contrast to their abilities to elicit DP₂ receptor-mediated responses, none of the PGD₂ analogs tested elicited a DP₁ receptor-mediated CAMP response in platelets exceeding 20% of the maximal response to PGD₂ at the highest concentration tested (10 μM). Because of the small response to these compounds, as an additional positive control we included PGD₃, which has a potency similar to or greater than that of PGD₂ in inhibiting platelet aggregation (Whitaker et al., 1979; Bundy et al., 1983). To permit an approximate comparison of the potencies of the PGD₂ analogs, the concentrations

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**Fig. 2.** Effects of PGD₂ analogs on actin polymerization in eosinophils. Unfractionated leukocytes were incubated for 20 s at 37°C with various concentrations of 15R-methyl-PGD₂ (▲, 15R), PGD₂ (●, D₁), 17-Ph-PGD₂ (○, 17ph), 15S-methyl-PGD₂ (□, 15S), 16,16-dimethyl-PGD₂ (■, 16dm), and 11-keto-fluprostenol (▼, 11kf). F-actin was measured in eosinophils, which were gated out on the basis of low expression of CD16 and high side scatter, as described under Materials and Methods. The results are means ± S.E. of determinations on cells from five different donors, except for 11-keto-fluprostenol (n = 4). They are expressed as percentages of the maximal response to PGD₂, which increased F-actin levels to 123 ± 13% above the level in control cells.

**Fig. 3.** Effects of 15-methyl analogs of PGD₂ on eosinophil migration. The migration of eosinophils, purified immunomagnetically using negative selection, through nitrocellulose filters was measured using microchemotaxis chambers, as described under Materials and Methods. The cells were placed above the filters, whereas 15R-methyl-PGD₂ (▲, 15R; n = 5), PGD₂ (●, n = 5), and 15S-methyl-PGD₂ (□, 15S; n = 4) were placed below. The results are means ± S.E. of determinations on eosinophils from the numbers of different donors indicated. The number of cells that migrated through the filter in controls was 3 ± 1 cells/high-power field (400× magnification), whereas the maximal number of cells induced to migrate through the filters by PGD₂ was 38 ± 4 cells/high-power field.
required to increase cAMP levels to 10% of the maximal level reached in the presence of PGD₂ were calculated (Table 1). The order of potencies for elevation of cAMP levels was PGD₃ > PGD₂ > 15S-methyl-PGD₂ > 16,16-dimethyl-PGD₂ > 17-Ph-PGD₂ ≈ 15R-methyl-PGD₂ > 11-keto-fluprostanol. This differs markedly from the rank order of potency for the DP₂ receptor. Among the PGD₂ analogs under study, 15S-methyl-PGD₂ was the most potent, and induced a substantially stronger response than 15R-methyl-PGD₂ (Fig. 4). Thus, unlike the DP₂ receptor, the DP₁ receptor is similar to other prostanoid receptors in its preference for the S-configuration at carbon 15. The only other PGD₂ analog that was capable of inducing a cAMP response greater than 10% of the maximal response to PGD₂ was 16,16-dimethyl-PGD₂. All of the other analogs had much smaller effects. These results are consistent with a previous report (Bundy et al., 1983) that 15S-methyl-PGD₂ is about 100 times less potent than PGD₂ in inhibiting platelet aggregation, whereas 17-Ph-PGD₂ is about 1000 times less potent. In contrast, 16,16-dimethyl-PGD₂ enhanced, rather than inhibited, platelet aggregation (Bundy et al., 1983). The effects of 11-keto-fluprostanol and 15R-methyl-PGD₂ on platelets have not previously been reported.

The basis for the enhanced potency at the DP₂ receptor due to the 15R-configuration is unclear. These results suggest that the 15-hydroxyl group of PGD₂ may play a role in its interaction with the receptor, and raise the possibility that affinity could be increased by the unnatural R-configuration at carbon 15. In this context, it would be very interesting to know whether inversion of the configuration at carbon 15 of PGD₂ itself (i.e., 15R-PGD₂) would result in enhanced DP₂ receptor activity. The answer to this question awaits the synthesis of this compound, which is currently unavailable.

The DP₂ receptor on eosinophils differs from other prostanoid receptors in several important aspects, in that it is activated by both metabolites (i.e., 13,14-dihydro-15-oxo-PGD₂) and degradation products (i.e., 15-deoxy-Δ12,14-prostaglandins D₂ and J₂) of PGD₂, and does not require the S-configuration at carbon 15. Activation of this receptor could thus be longer lasting than activation of other prostanoid receptors, which may be rapidly terminated due to the biological inactivation of their ligands. In addition, this receptor can be activated by the nonsteroidal anti-inflammatory drug indomethacin, which has a potency approximately 20 to 100 times less than that of PGD₂ in activating eosinophils, an effect not shared by other nonsteroidal anti-inflammatory drugs (Hirai et al., 2002; Stubbs et al., 2002). These differences between the classic prostanoid receptors and the DP₂ receptor may be due to the fact that the latter has evolved differently and has a higher degree of homology with chemotacticant and leukotriene receptors than with other prostanoid receptors (Hirai et al., 2001).
In conclusion, previous work has shown that PGD₂ can evoke proinflammatory responses through activation of both DP₁ and DP₂ receptors. The availability of a highly potent and selective DP₂ receptor/CRTH2 agonist will be of great utility in defining the physiological role of this receptor in asthma and other inflammatory diseases. 15R-Methyl-PGD₂ has considerable advantages over other known selective DP₂ receptor agonists. It is the most potent known ligand for this receptor, having an EC₅₀ value about 5 times lower than that of any other DP₂ agonist, and unlike PGD₂, is highly selective for DP₂ over DP₁ receptors. We have previously shown that both 15-deoxy-Δ¹²,1⁴-PGJ₂ and 15-deoxy-Δ¹₂,1⁴-PGJ₂ are also selective for DP₂ receptors (Monneret et al., 2002), but at higher concentrations these compounds also have peroxisome proliferator-activated receptor-γ-mediated anti-inflammatory effects, and can form covalent bonds with proteins (Jiang et al., 1998; Ricote et al., 1998; Rossi et al., 2000; Straus et al., 2000). Another selective DP₂ receptor agonist is the PGD₂ metabolite 13,14-dihydro-15-oxo-PGD₂ (Gervais et al., 2001; Hirai et al., 2001; Monneret et al., 2001), which is about one-half as potent as PGD₂ in activating eosinophils. However, as occurs for other 15-oxo-prostaglandins (Hamberg and Samuelsson, 1971), this substance could be reduced to 13,14-dihydro-PGD₂, which is likely to have DP₁ receptor activity. Thus, 15R-methyl-PGD₂ could be an important novel tool for defining the physiological role of the DP₂ receptor/CRTH2.

References


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