Brief Definitive Report

Prostaglandin D2 Selectively Induces Chemotaxis in T Helper Type 2 Cells, Eosinophils, and Basophils via Seven-Transmembrane Receptor CRTH2

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Abstract

Prostaglandin (PG)D$_2$, which has long been implicated in allergic diseases, is currently considered to elicit its biological actions through the DP receptor (DP). Involvement of DP in the formation of allergic asthma was recently demonstrated with DP-deficient mice. However, proinflammatory functions of PGD$_2$ cannot be explained by DP alone. We show here that a seven-transmembrane receptor, CRTH2, which is preferentially expressed in T helper type 2 (Th2) cells, eosinophils, and basophils in humans, serves as the novel receptor for PGD$_2$. In response to PGD$_2$, CRTH2 induces intracellular Ca$^{2+}$ mobilization and chemotaxis in Th2 cells in a G$_{ai}$-dependent manner. In addition, CRTH2, but not DP, mediates PGD$_2$-dependent cell migration of blood eosinophils and basophils. Thus, PGD$_2$ is likely involved in multiple aspects of allergic inflammation through its dual receptor systems, DP and CRTH2.

Key words: prostanoid receptor • G$_{ai}$-type G protein • T cells • cell migration • allergic inflammation

Introduction

Leukocytes migrate from the peripheral blood to sites of inflammation through interaction with various cellular and humoral components such as selectins, integrins, and locally produced chemoattractants (1, 2). Allergic inflammation is characterized by selective accumulation of Th2 cells, eosinophils, and basophils, and is often triggered by antigen-stimulated mast cells (3, 4), although the mechanisms supporting these phenomena are largely unclear.

Recently, we cloned a novel putative chemoattractant receptor named CRTH2, which is a seven-transmembrane G protein–coupled receptor structurally related to members of the N-formyl peptide receptor (FPR) subfamily (5). CRTH2 is intriguing in that it is selectively expressed in Th2 cells, T cytotoxic type 2 cells, eosinophils, and basophils (5–7). Furthermore, CRTH2 can mediate intracellular Ca$^{2+}$ mobilization in response to a factor(s) released from activated mast cells, suggesting that CRTH2 may be closely involved in mast cell–mediated allergic inflammation (6).

In this study, we show that the mast cell–derived CRTH2 agonist is prostaglandin (PG)D$_2$. This result was somewhat surprising because PGD$_2$ is generally considered to exert its action via the DP receptor (DP; references 8–10) and because CRTH2 has no significant homology in amino acid sequence with DP or all other known prostanoid receptors (10). However, in view of the structure, it may be notable that, like CRTH2, some other lipid mediator receptors such as lipoxin (LX)$A_4$ receptor FPRL1 and the new leukotriene (LT)$B_4$ receptor BLT2 are also more closely akin to FPR than the prostanoid receptors (11, 12). PGD$_2$ is the major prostanoid that is produced by activated mast cells and thereby has long been implicated in allergic diseases (10, 13). Most recently, involvement of the DP-mediated activities of PGD$_2$ in the formation of allergic asthma has been demonstrated with DP-deficient mice.
However, contribution of DP to the direct effects of PGD₂ on leukocytes, such as induction of eosinophil migration (15), has not yet been established, raising new questions such as how these receptors are being used.

Our results show that although CRTH2 functions as a selective PGD₂ receptor as well as DP, it sharply differs from DP in signaling pathways. Furthermore, CRTH2 but not DP induces migration of Th2 cells, eosinophils, and basophils in response to PGD₂. Our findings elucidated a novel function of PGD₂ on T cell responses, and CRTH2 is the first receptor that directly and selectively connects a major lipid mediator of activated mast cells with Th2 cells, eosinophils, and basophils.

Materials and Methods

DP Cloning. Human DP cDNA was generated by reverse-transcription (RT)-PCR using a small intestine poly(A)+ RNA (CLONTECH Laboratories, Inc.) as a template and the following primers: 5'-CTTCCGAGCCCTCCTACGCCCTCTGGTCCCG (sense) and 5'-GTTCTTTCTCATAGAAAATGTCAGCAGGCTACCT (antisense). The DP cDNA was cloned into the HindIII-XbaI sites of pRc/CMV (Invitrogen), yielding an expression plasmid pRc/DP.

Abs. CRTH2-specific rat mAbs BM16 and BM7 were described previously (5, 6). Both mAbs were generated by immunizing Wister rats with CRTH2-transfected rat T cell line TART-1, and their epitopes are still unclear.

Cells. Mast cell supernatants were prepared as reported previously (6). K562 and Jurkat lines were transduced with pRc/B19 (a CRTH2 expression vector; references 5, 6), pRc/DP, or pRc/CMV and cloned as described (5). Human Th1 and Th2 lines were generated from the PBMCs of healthy adults by stimulation with a purified protein derivative of Mycobacterium tuberculosis in the presence of IL-12 and IFN-γ (Th1 condition) or with an extract of Dermatophagoides in the presence of IL-4 and neutralizing anti-IFN-γ and anti-IL-12 mAbs (Th2 condition), then expanded by IL-2 and used at days 7–14 for examinations as described previously (5). In some chemotaxis assays, Th2 cells highly expressing CRTH2 were enriched by sorting with anti-CRTH2 mAb BM16 (5) a few days before use. PBMCs and granulocytes were isolated as described (6).

Ca²⁺ Mobilization. Ca²⁺ mobilization assays were performed as described (6). LTC₄, LTD₄, LXA₄, prostanooids, and their synthetic analogues were purchased from Cayman Chemical except for fluprostenol (Biomol) and PGD₂ methyl ester (Sigma-Aldrich). Thrombin and monocyte chemotactic protein 1 (MCP-1) were obtained from Sigma-Aldrich and PeproTech, respectively. For inactivation of G proteins, cells (2.5 × 10⁶ cells/ml) were incubated with pertussis toxin (PTX; Sigma-Aldrich) or cholera toxin (CTX; Sigma-Aldrich) at 1 µg/ml for 24 h at 37°C.

3H-PGD₂ Binding Analysis. Cells washed and suspended in HBSS containing 10 mM Hepes, pH 7.3 (5 × 10⁶ cells/100 µl) were incubated with 1 nM 3H-PGD₂ (Amersham Pharmacia Biotech) for 60 min on ice. Cells were then separated by centrifugation on RPMI 1640 containing 1 M sucrose, and radioactivity bound to the cells was measured on a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 µM cold PGD₂.

RT-PCR Analysis. Basophils and eosinophils were purified as described (6). Neutrophils (99% pure as determined by May-
Grünewald Giemsa staining) were purified from granulocytes with anti-CD16-coated magnetic beads (Miltenyi Biotec). Monocytes (98% pure), T cells (92% pure), and B cells (91% pure) were isolated from PBMCs with anti-CD14–, anti-CD3–, and anti-CD19–coupled magnetic beads (Miltenyi Biotec), respectively. RT-PCR was performed as described previously (5, 6). Primers for DP were 5′-GCAAACCTCTATGCCATGCA (sense) and 5′-CAAGGCTGAGGCTTCTTCT (antisense). PCR conditions were 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Chemotaxis Assay. This was performed on a 96-well microchemotaxis chamber with a 5-μm-pore filter (Neuroprobe) according to the manufacturer’s instructions. In brief, cells (1–2 × 10⁵ cells/50 μl) and test samples (10 μl) were applied to top and bottom wells, respectively, of the chamber. After incubation at 37°C for 2 h, cells in the bottom wells were counted by flow cytometry. In the case of blood leukocytes, cell types were determined by Wright’s staining.

Results

**CRTH2 Is a Novel Receptor for PGD₂.** We previously found that mast cells produce a CRTH2-specific agonist(s) that can induce Ca²⁺ mobilization in CRTH2-transfected K562 (K562/CRTH2) but not mock-transfected K562 (K562/neo) (Fig. 1 A; reference 6). In a screening of known mast cell products (13, 16), we found that only PGD₂ can induce Ca²⁺ mobilization in K562/CRTH2 but not K562/neo at nanomolar concentrations (Fig. 1 B). Other mast cell–related and –unrelated inflammatory mediators such as histamine, serotonin, platelet-activating factor, LTB₄, LTC₄, regulated upon activation normal T cell expressed and secreted (RANTES), MCP-1, eotaxin, IL-8, LXA₄, fMLP, C5a, and angiotensin II did not induce any significant CRTH2-mediated Ca²⁺ mobilization (5; and data not shown). Treatment of mast cells with aspirin, a PG synthesis inhibitor, completely impaired production of the active component. The active component of the mast cell supernatants showed an elution profile identical to that of PGD₂ on a reverse phase chromatography using a μRPC C2/C18 SC 2.1/10 column (Amersham Pharmacia Biotech) (data not shown). Having once responded to PGD₂, K562/CRTH2 did not show any appreciable Ca²⁺ mobilization by the mast cell active fraction, and vice versa (cross-desensitization; Fig. 1 B). These observations demonstrate that PGD₂ is the CRTH2 agonist that is produced by activated mast cells.

**Table 1. Kᵢ Values of Various Compounds on the Specific Binding of [³H]-PGD₂ to CRTH2 and DP**

| Ligand       | K562/CRTH2 (nM) | K562/DP (nM) |
|--------------|-----------------|--------------|
| PGD₂         | 61 ± 23         | 45 ± 17      |
| DK-PGD₂      | 160 ± 35        | >30,000      |
| PGJ₂         | 460 ± 160       | 64 ± 13      |
| 11β-PGF₂α     | 20,000 ± 160    | 1,200 ± 470  |
| Δ¹²-PGJ₂      | 7,100 ± 1,300   | 5,200 ± 1,700|
| 15-deoxy-Δ¹²,¹⁴-PGJ₂ | 2,300 ± 400    | >30,000      |
| PGE₂         | 3,100 ± 1,100   | 2,300 ± 1,100|
| PGE₂α        | 2,000 ± 780     | >30,000      |
| PGA₄         | >30,000         | >30,000      |
| U46619       | >30,000         | >30,000      |
| BW245C       | >100,000        | 50 ± 20      |
| PGD₂ methyl ester | 460 ± 120     | 270 ± 46     |
| LTB₄         | >30,000         | >30,000      |
| LXA₄         | >30,000         | >30,000      |

Inhibitor constant (Kᵢ) values (nM ± SD, n ≥ 3) are shown. ICₕ₀ values were determined by the binding of [³H]-PGD₂ to the indicated cells in the presence of various concentrations of competing compounds and converted to Kᵢ values according to the equation Kᵢ = ICₕ₀/[1 + [L]/Kᵢₐ], where [L] is the concentration of [³H]-PGD₂ (1 nM) and Kᵢₐ is its dissociation constant (taken as 31.3 nM for CRTH2 and 37.7 nM for DP) (reference 17).

![Figure 2. Functional discrimination between CRTH2 and DP.](image-url)

(A) DP but not CRTH2 mediates PGD₂-induced cAMP generation. Intracellular cAMP levels were determined by enzyme-linked immunosassay (mean, n = 2; Amersham Pharmacia Biotech). (B) CTX and PTX differentially affect PGD₂-induced (25 nM) Ca²⁺ mobilization in K562/CRTH2 and K562/DP. (C) Chemotactic responses to agonists (PGD₂, DK-PGD₂, and BW245C) are opposite in Jurkat/CRTH2 and Jurkat/DP (mean ± SD, n = 3). Numbers of migrated cells in controls were 727 ± 134 (Jurkat/CRTH2), 439 ± 80 (Jurkat/DP), and 661 ± 152 (Jurkat/neo).
The above finding was supported by the experiments using K562/CRTH2 and DP-transfected K562 (K562/DP; Fig. 1A). Both lines exhibited specific binding of $^3$H-PGD$_2$ with similar numbers of binding sites per cell and $K_d$ values of 31.3 and 37.7 nM, respectively, whereas $^3$H-PGD$_2$ did not bind to K562/neo (Fig. 1C and D). A neutralizing CRTH2-specific mAb BM7 selectively inhibited the binding of $^3$H-PGD$_2$ to K562/CRTH2 but not K562/DP, whereas control rat IgG had no effect on this assay (Fig. 1D). To establish ligand specificity, binding affinities to the CRTH2 and DP of PGD$_2$ analogues, major prostanoids, a thromboxane (TX)A$_2$ receptor agonist U46619 (8), a synthetic DP agonist BW245C (8), LTB$_4$, and LXA$_4$ were determined by competitive binding assay (Table I). The results demonstrate that PGD$_2$ binds to CRTH2 as well as DP with the highest affinity among various prostanoids. Interestingly, one of the PGD$_2$ metabolites, 13,14-dihydro-15-keto-PGD$_2$ (DK-PGD$_2$), showed an affinity to CRTH2 as high as PGD$_2$ to CRTH2, whereas DP showed at least 600-fold lower affinity to DK-PGD$_2$ than PGD$_2$ as estimated by the competition assay. In contrast, BW245C was highly selective for DP.

Consistently, Ca$^{2+}$ mobilization was induced in both K562/CRTH2 and K562/DP by PGD$_2$, whereas DK-PGD$_2$ and BW245C were selectively effective in K562/CRTH2 and K562/DP, respectively, in Ca$^{2+}$ mobilization assay (Fig. 1E). No significant Ca$^{2+}$ mobilization was induced in K562/CRTH2 by up to 250 nM PGA$_2$, PGI$_2$, and TXB$_2$ (data not shown). From these findings, we have concluded that CRTH2 serves as the novel selective receptor for PGD$_2$. Furthermore, DK-PGD$_2$ and BW245C are useful reagents that specifically induce CRTH2- and DP-mediated responses, respectively.

**Differences in Signaling Pathways Associated with CRTH2 and DP.** To gain insight into functional differences between CRTH2 and DP, signaling pathways associated with CRTH2 expression in representative Th1 and Th2 lines. Levels of CRTH2 expression are presented as described in the legend to Fig. 1A. (B) PGD$_2$ and DK-PGD$_2$ selectively induce Ca$^{2+}$ mobilization in Th2 cells via CRTH2. Arrows indicate the time of addition of the test samples (black arrows) and 62.5 nM MCP-1 (white arrows). MCP-1 was used as an irrelevant stimulant. BM7 and rat IgG (each 600 μg/ml) were added 20 min before the stimulant addition. (C) Chemotaxis to PGD$_2$ and DK-PGD$_2$ is selectively induced in Th2 but not Th1 lines (mean ± SD, n = 3). The numbers of migrated cells in controls were 4,015 ± 627 (Th1) and 1,374 ± 225 (Th2). (D) Chemotaxis to PGD$_2$ and DK-PGD$_2$ of Th2 cells is inhibited by BM7 (mean ± SD, n = 3). Th2 cells were treated with PBS, BM7, or normal rat IgG as described in B before being subjected to chemotaxis assay. Numbers of migrated cells in controls were 4,146 ± 433 (PBS), 3,283 ± 349 (BM7), and 3,246 ± 65 (normal rat IgG). (E) mRNA levels for CRTH2, DP, and β-actin (internal control) in peripheral blood leukocytes and Th lines as determined by RT-PCR. (F) Migration of basophils and eosinophils is induced by PGD$_2$ (mean ± SD, n = 3). (G) Migration of eosinophils and basophils to PGD$_2$ and DK-PGD$_2$ is dose dependent (mean ± SD, n = 3). In this experiment, neutrophil (CD16$^+$ cell)-depleted leukocyte samples were used. (H) PGD$_2$-induced migration of basophils and eosinophils is inhibited by BM7 (mean ± SD, n = 3). Neutrophil-depleted leukocytes were treated as described in D. Asterisks indicate statistical significance with a probability of <0.01 in Student’s t-test.
the two receptors were examined. DP induced cAMP generation in response to PGD₂ as reported previously (Fig. 2 A; reference 9), and DP-mediated Ca²⁺ mobilization was blocked by CTX but not by PTX as expected (Fig. 2 B). In contrast, PTX but not CTX inhibited CRTH₂-mediated Ca²⁺ mobilization (Fig. 2 B), and no production of cAMP was seen by interaction of PGD₂ with CRTH₂, as expected (Fig. 2 A). These results indicate that CRTH₂ is coupled with Gα₁₆-type G protein and DP with Gαi₁-dependent protein.

Since many seven-transmembrane Gαi₁-coupled receptors are involved in chemotaxis of leukocytes (1), we then addressed the issue of whether CRTH₂ was responsible for chemotaxis. CRTH₂-transfected Jurkat (Jurkat/CRTH₂) was indeed attracted by PGD₂ and DK-PGD₂ but not by chemotaxis. CRTH₂-transfected Jurkat (Jurkat/CRTH₂) was succeeded by interaction of PGD₂ with CRTH₂, as expected (Fig. 2 A). These results indicate that CRTH₂ mediates chemotactic/chemokinetic signal of PGD₂ in a Gα₁₆-dependent manner in Th₂ cells, eosinophils, and basophils.

Discussion

Prostanoid receptors have been classified into TX receptor (TP), PGE receptor (EP₁, EP₂, EP₃, and EP₄), PGI receptor (FP), PGI receptor (IP), and PGD receptor (DP; references 8, 10). In view of the structure, CRTH₂ appears to be unique among members of prostanoid receptors in that it lacks any consensus amino acid sequence motifs that are shared by all other known prostanoid receptors (10). Indeed, a phylogenetic analysis shows that CRTH₂ is more akin in the overall structure to the FPR subfamily members and receptors for LTs than known prostanoid receptors (Fig. 4). Furthermore, the chromosomal location of on Th₂ cells was mostly chemotactic, whereas on basophils and eosinophils there was a partly chemokinetic effect as reported on eosinophils (15). PGD₂ is also known to weakly cross-react with receptors for PGE₂, PGF₂α, and TXA₂ (18). However, involvement of these receptors in chemotaxis was ruled out because PGE₂, fluprostenol (a PGF₂α receptor-selective agonist; reference 8), and U46619 (a TXA₂ receptor-selective agonist; reference 8) had no significant chemotactic effect on these three cell types at nanomolar ranges. Taken together, these results indicate that CRTH₂ mediates chemotactic/chemokinetic signal of PGD₂ in a Gα₁₆-dependent manner in Th₂ cells, eosinophils, and basophils.

![Figure 4](image-url)

**Figure 4.** A phylogenetic tree for human receptors to classical chemoattractants and major prostanoids. The tree was constructed by the N-J method using CLUSTAL X software. The sequence of the human α²B adrenergic receptor was used as an outer group to obtain a root. Chromosomal locations are shown in parentheses. Accession nos. for the receptors are (from top to bottom) GenBank/EMBL/DDBJ AF005900, D38081, L24470, L22647, L27490, L28175, D25418, U19487, Q13258 (GenPept accession no.), AF119711, AF254664, AB008193, AB008193, AB008535, M62505, L14061, L18020, and M85462. Cys LT, cysteinyl LT receptor; FPRL₁, formyl peptide receptor-like receptor.
CRTH2 (11q12.2) (sequence data available from GenBank/EMBL/DDBJ under accession no. AC004126) differs from those of other receptors listed in Fig. 4, suggesting that CRTH2 might form a novel receptor gene subfamily. The mechanisms by which CRTH2 binds PGD2 with an affinity as high as DP remain to be clarified.

PGD2 is currently considered to elicit its biological actions through DP (8, 10). In fact, well-established PGD2-induced activities such as vasodilation, relaxation of other various smooth muscles, and inhibition of platelet aggregation are apparently mediated by DP because they are also induced by a DP-specific agonist BW245C (18, 19). However, several in vivo and ex vivo effects of PGD2 such as an increase in microvascular permeability, eosinophil infiltration, and goblet cell depletion are not mimicked by BW245C (20). Thus, questions on the existence of a BW245C-insensitive PGD2 receptor subtype(s) has been repeatedly proposed (20–22). In addition, although DK-PGD2 is generally thought to be biologically inactive in many systems, several investigators actually observed its effects on some PGD2-sensitive tissues (23). Our finding that CRTH2 is the novel PGD2 receptor functioning differently from DP may lead to the resolution of such long-standing questions.

A notable difference between CRTH2 and DP is the signaling molecules: Gαi is used for CRTH2 whereas Gαs is for DP. As shown by many cell types with Gαi-coupled receptors, CRTH2 leads Th2 cell, eosinophil, and basophil induction of chemotaxis/chemokinesis in response to PGD2. Consequently, these types of cells may accumulate at the sites of allergic inflammation (3, 4). Indeed, it was demonstrated that topical application of PGD2 causes significant accumulation of eosinophils in the guinea pig conjunctiva and the dog trachea (20, 24). In contrast, DP-mediated PGD2 signals caused reduction in spontaneous cell migration in DP-transfected Jurkat cells and had no effect on the migration of DP-expressing basophils in our study. These results suggest that DP-mediated signals may not, in nature, lead to chemotactic or chemokinin behavior in leukocytes. Although PGD2 has been reported to modulate various activities of neutrophils, we could not detect any substantial expression of CRTH2 or DP in blood neutrophils (25). However, further studies are required to finally establish the actual involvement of these receptors in the PGD2-induced modulation of neutrophils.

Although the mechanisms by which DP contributes to the formation of allergic inflammation remain to be clarified, our results suggest that DP and CRTH2 may be cooperatively involved in allergic inflammation through different processes. A plausible scenario could be that PGD2 is largely produced by mast cells upon antigen stimulation, inducing local vasodilation via DP, which enhances extravasation of blood leukocytes (26), followed by chemotactic migration of Th2 cells, eosinophils, and basophils via CRTH2 in cooperation with other chemotactic mediators such as CC chemokines TARC and eotaxin (27, 28). Thus, CRTH2 may be a new favorable target for allergic disease therapies. Generation of selective inhibitors for CRTH2 or CRTH2-deficient mice should help to elucidate the physiological and pathophysiological roles of the CRTH2/PGD2 system and its relative importance in the host defense mechanisms.

We thank Drs. K. Katayama and F.B. Hoshino (BML) for drawing the phylogenetic tree.

Submitted: 31 August 2000
Revised: 15 November 2000
Accepted: 20 November 2000

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