
The opposing role of two prostaglandin D₂ receptors, DP and CRTH2, in human eosinophil migration

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Background: Prostaglandin D₂ (PGD₂) regulates various immunological responses via two distinct PGD₂ receptors, prostaglandin D receptor (DP), and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). Recent studies have demonstrated that PGD₂ induces the migration of eosinophils through CRTH2. Although human eosinophils express both DP and CRTH2, it is unclear whether the function of DP is involved in eosinophil migration.

Objective: In this study, we investigated the roles of DP and CRTH2 in eosinophil migration by using selective agonists and antagonists.

Methods: Eosinophils were isolated from human subjects with mild eosinophilia by modified CD16-negative selection. After stimulation with or without DP receptor agonist, eosinophil migration was measured by Boyden chamber. The effect of DP agonists on CRTH2-induced eosinophil migration was studied in terms of CRTH2 expression, Ca²⁺ mobilization, ERK/MAPK phosphorylation, and cyclic AMP (cAMP) production.

Results: Treatment with DP agonists inhibited CRTH2-induced chemotaxis of eosinophils. Furthermore, we showed that DP agonists enhanced cAMP production in CRTH2 agonist stimulation without increasing CRTH2 expression. DP mediates eosinophils through the elevation of intracellular cAMP production but does not change CRTH2 expression.

Conclusion: Taken together, the balance between DP and CRTH2 could influence the degree of PGD₂-induced eosinophil migration and DP agonist might regulate eosinophil activation.

Ann Allergy Asthma Immunol. 2011;106:511–517.

INTRODUCTION

Allergic inflammation is characterized by a selective accumulation of inflammatory cells, such as activated Th2 lymphocytes, eosinophils, and mast cells. Eosinophils play an essential role in the pathogenesis of allergic disease such as atopic dermatitis. Atopic dermatitis patients exhibit increased numbers of peripheral eosinophils.

Prostaglandin D₂ (PGD₂), a biologic lipid swiftly synthesized by allergen-activated mast cells, is implicated in the pathogenesis of allergic diseases.¹ Indeed, elevation of PGD₂ levels was observed after allergen challenge, using the blister suction method in atopic dermatitis patients, and it acted directly on eosinophils to induce cell migration *in vitro*.^{2,3}

Two types of main G-protein–coupled receptors for PGD₂ have been cloned: D-prostanoid receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). D-prostanoid receptor is expressed on not only hematopoietic cells, eosinophils, basophils, and DCs but also on other types of cells,^{3–5} such as smooth muscle cells and nasal epithelial goblet cells.⁶ D-prostanoid receptor activation induces a Gs-mediated increase in intracellular cyclic adenosine monophosphate (cAMP) and consequently leads to inhibition of basophil and dendritic cell function, bronchodilation, and vasodilation.^{5,7–10} Conversely, CRTH2 expression has been specifically demonstrated on hematopoietic cells, eosinophils, basophils, Th2 cells, and normal human bronchial epithelial cells.^{8,11} Chemoattractant receptor-homologous molecule expressed on Th2 cells binds to a Gi-type G-protein, inducing the inhibition of cAMP production and increasing intracellular calcium.¹² As a result, CRTH2 agonists are believed to induce activation of eosinophils, basophils, and Th2 cells.

We and others have previously demonstrated that eosinophils express two PGD₂ receptors, CRTH2 and DP, and that PGD₂ induces eosinophil migration through CRTH2.^{2,3–5,13} Although CRTH2 was shown to be fully responsible for a PGD₂-induced migratory response in eosinophils, the direct effect of DP on eosinophil migration remains to be determined.

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Funding Sources: Supported in part by a grant from “the 21st Century Center of Excellence (COE) Program” and “the grants-in-aid for scientific research” supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosures: The authors have nothing to disclose.

Received for publication September 16, 2010; Received in revised form November 4, 2010; Accepted for publication January 21, 2011.

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doi:10.1016/j.anai.2011.01.027

The role of two PGD₂ receptors in the CRTH2-induced migration of human eosinophils was investigated. Our data show that the DP receptor regulates eosinophilic migration, by which it upregulates intracellular cAMP concentration. Thus, use of DP agonists might be a practical way to treat allergic diseases related to eosinophilic accumulation.

METHODS

Eosinophil Purification

Peripheral venous blood was obtained from subjects with mild to moderate eosinophilia. All subjects were either healthy or had mild allergic rhinitis, and blood was obtained at a time when they were relatively asymptomatic and off medication. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients as modified from the method of Hansel et al.^{14,15} The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils were then suspended in Hank's balanced salt solution (HBSS; Invitrogen Life Technologies, Grand Island, NY) with 1% fetal calf serum (FCS) in tubes coated with 3% human serum albumin. The resulting eosinophil purity was more than 99%. Oral informed consent was obtained from each participant, and the clinical research ethics committee of Akita University approved this study.

Migration Assay

Chemotaxis of eosinophils was conducted in duplicate using 5- μ m pore size, polycarbonate, polyvinylpyrrolidone-free membranes in Boyden chambers. The 13, 14-dihydro-15-keto-PGD₂ (DK-PGD₂), BW245C (Cayman Chemical, Michigan), or PGD₂ (Sigma, Poole, United Kingdom) was diluted in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen Life Technologies, Grand Island, New York) with 0.1% FCS and placed in the lower wells (100 μ L) at several concentrations. After incubation of the eosinophils with an agonist, antagonist, or medium at 37°C, aliquots of 100 μ L of the cell suspension at 2×10^6 cells/mL were suspended in the upper chambers. The loaded chambers were incubated at 37°C in humidified air with 5% CO₂ for 1 hour. Then, the membrane was removed, followed by fixation and staining for 3 minutes in May-Grünwald solution. The cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields by light microscopy. In this study, we used the following agonist or antagonist: PGD₂, DK-PGD₂, BW245C, and BWA868C.

Actin Polymerization Assay

The content of F-actin was analyzed by flow cytometry with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-phalloidin (Molecular Probes, Carlsbad, California) staining, as modified from the method described by Howard et al.^{16,17} Purified eosinophils were resuspended in phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺ and were left to stand for 5 minutes at room temperature. Aliquots of cells (1×10^6 cells)

were incubated with BW245C or medium in a 37°C shaking water bath for 10 minutes. The cells were added to DK-PGD₂ or medium in a final volume of 100 μ L. Twenty-five seconds later, they were fixed in 3.7% formaldehyde for 1 hour. Cells were washed; resuspended in PBS; mixed with a staining mixture containing 3.7% formaldehyde, 3.3×10^7 M NBD-phalloidin, and 100 μ g/mL of lysophosphatidylcholine (Sigma, Poole, United Kingdom; final concentrations); and incubated for 1 hour in the dark. The samples were then analyzed fluorospectrometrically. Data were acquired using the FL-1 fluorescence channel on a sensitive setting. The relative F-actin content was expressed as the ratio of the mean channel fluorescence between agonist and medium-stimulated cells.

CRTH2 Surface Expression

Purified eosinophils were incubated in RPMI 1640 (Gibco, Grand Island, New York) supplemented with 10% FCS with or without various concentrations of BW245C (Cayman Chemicals, Ann Arbor, Michigan) for 20 minutes at 37°C humidified air with 5% CO₂. The cells were washed twice with PBS and then stained with the isotype-matched control phycoerythrin-conjugated rat immunoglobulin G2a or phycoerythrin-conjugated rat anti-human CRTH2 antibody (Beckman Coulter, Inc, Fullerton, California). The percentage of eosinophils expressing CRTH2 was determined relative to the expression level on the isotype-negative control, and mean fluorescence intensities were measured as an index for the expression of CRTH2 using a flow cytometer (FACScan, Becton Dickinson).

Measurement of Intracellular Calcium

For intracellular calcium measurement, purified eosinophils (10×10^6 cells in HBSS/4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid without Ca²⁺) in suspension were loaded with 2 μ L of fura 2-AM (Dojindo, Kumamoto, Japan) for 40 minutes at room temperature in the dark. The cells were washed in HBSS/4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid with 4% FCS and then resuspended at a concentration of 5×10^6 cells/mL in HBSS with Ca²⁺. Aliquots of cells (50 μ L) were dispensed into cuvettes and equilibrated with 1 mM calcium at room temperature for 10 minutes. Changes in fluorescence were measured using a Fluorescence Drug Screening System 2000 (Hamamatsu Photoics K.K., Hamamatsu, Japan). Intracellular free calcium was calculated from fluorescence spectra (excitation wavelengths, 340 and 380 nm) in accordance with established methods.¹⁸ The eosinophils were incubated with 100 nM BW245C or medium for 20 minutes at 37°C, and then 10 nM DK-PGD₂ was added 90 seconds after commencing recording.

Gel Electrophoresis and Western Blotting

Purified eosinophils (1×10^6 cells) were treated with and without 100 nM BW245C for 20 minutes at 37°C followed by stimulation with and without 10 nM DK-PGD₂ for 3 minutes. The reaction was terminated by the addition of 15 volumes of ice-cold HBSS containing 1 mM Na₃VO₄. The cells were

pelleted by centrifugation and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM Na_3VO_4 , 1 mM NaF, 1 mM ethylenediaminetetra-acetic acid, 1 mM ethylene glycol tetra-acetic acid, 1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100, 10% glycerol, and 1 $\mu\text{g}/\text{mL}$ of aprotinin, leupeptin, and pepstatin). After 20 minutes on ice, detergent-insoluble materials were removed by centrifugation at 4°C at 15,000 g. The supernatants were mixed with sodium dodecyl sulfate (SDS) sample buffer and boiled for 4 minutes. The SDS-polyacrylamide (10%) gels (Ready Gel J; Bio-Rad, Hercules, California) were obtained from Bio-Rad (Tokyo, Japan). The electrophoresed gel was blotted onto Hybond ECL membranes (Amersham, Arlington Heights, Illinois). Blots were incubated in blocking buffer containing 5% bovine serum albumin in Tris-buffered saline-T buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween-20, pH 7.6) for 30 minutes followed by incubation in the primary 0.1- $\mu\text{g}/\text{mL}$ antibody (Ab; mouse monoclonal antibody against phospho-p38 from Cell Signaling Technology [Beverly, Massachusetts]; mouse monoclonal antibody against phospho-extracellular signal-regulated kinase [ERK] from Santa Cruz Biotechnology, Santa Cruz, California) for the phosphorylation state of signaling proteins with gentle agitation overnight at 4°C. After washing three times in Tris-buffered saline-T buffer, blots were incubated for 30 minutes with a horseradish peroxidase-conjugated secondary 0.04- $\mu\text{g}/\text{mL}$ antibody directed against the primary antibody. The blots were developed with the ECL substrate according to the manufacturer's instructions. Blots were subsequently re-probed with another antibody (rabbit polyclonal anti-p38 and anti-ERK2; Santa Cruz Biotechnology) for the nonphosphorylation state of signaling proteins after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS at 56°C for 30 minutes. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology.

Measurement of Intracellular cAMP Levels

Purified eosinophils (1×10^6 cells) in 100 μL of RPMI 1640 were preincubated with BW245C or medium for 10 minutes at 37°C and incubated with DK-PGD₂ or medium for a further 15 minutes. The intracellular cAMP was measured using the Correlate-EIA Direct cAMP Enzyme Immunoassay kit (Assay Designs, Ann Arbor, Michigan).

Statistical Analysis

Data are presented as mean $\pm$ standard deviation. A one-way analysis of variance with repeated measures was used for the comparison of more than two variables. When the initial *P* value was less than .05, Scheffe's test was used to determine the significance between groups. Significance was established at the *P* < .05 level.

RESULTS

The chemotactic effects of PGD₂ on eosinophils are mediated through CRTH2 activation. We initially investigated the effect of PGD₂, selective CRTH2 agonist DK-PGD₂, and se-

lective DP agonist BW245C on the migration of human purified eosinophils. In line with previous studies,¹⁹ PGD₂ and DK-PGD₂ induced eosinophil migration in a concentration-dependent manner. When stimulated at 1,000 nM, PGD₂ and DK-PGD₂ induced eosinophil migration that reached $310.2 \pm 105.1\%$ and $421.2 \pm 136.2\%$ vs vehicle control, respectively. In contrast, BW245C did not induce eosinophil migration (Fig 1). This result clearly indicates that PGD₂-induced eosinophil migration is mediated directly through CRTH2 activation.

The selective DP agonist BW245C inhibits CRTH2-mediated eosinophil migration and cytoskeletal rearrangement. To evaluate DP function in eosinophil migration, eosinophils were incubated with BW245C or vehicle for 10 minutes, and then migration assays were performed using Boyden chambers. Interestingly, BW245C significantly inhibited migration of eosinophils induced by PGD₂ or DK-PGD₂ in a concentration-dependent manner (Fig 2). Furthermore, to examine cytoskeletal rearrangement, we measured F-actin content using an actin polymerization assay. As shown in Figure 3, although DK-PGD₂ could enhance F-actin content, pretreatment of BW245C (100 nM) attenuated DK-PGD₂-induced actin polymerization. In addition, pretreatment of cells with the selective DP receptor antagonist BWA868C reversed the BW245C inhibition of eosinophil migration toward DK-PGD₂ (Fig 4). These data suggest that BW245C prevents CRTH2-mediated eosinophil migration and cytoskeletal rearrangement via DP activation.

BW245C does not affect expression of CRTH2 on eosinophils and intracellular Ca^{2+} levels or mitogen-activated protein kinase (MAPK)/ERK pathways. Next, to explore the mechanism of the inhibitory effect of DP on CRTH2-induced eosinophil migration, we investigated whether BW245C modulates CRTH2 surface expression on human eosinophils. Eosinophils were incubated for 20 minutes with BW245C. After that, we measured CRTH2 expression on the cells using a flow cytometer. However, BW245C did not reduce CRTH2 surface expression (Fig 5A). Because BW245C did not affect CRTH2 expression on eosinophils, to further investigate the

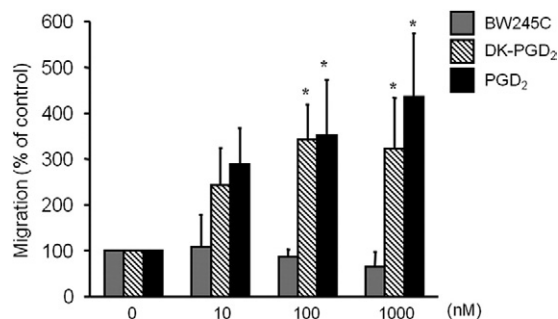


Figure 1. Effects of selective DP agonist BW245C, selective CRTH2 agonist DK-PGD₂, and PGD₂ on the migration of human purified eosinophils. Migration assays were measured using Boyden chambers. The chemotactic response to medium was considered as 100%. Data are expressed as means $\pm$ SD (*n* = 4). **P* < .05 versus control (medium alone).

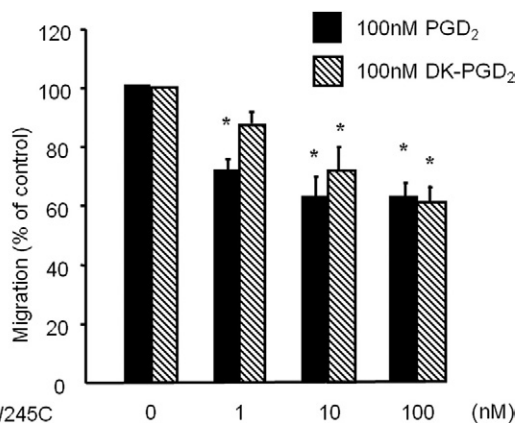


Figure 2. Inhibition of PGD₂- or DK-PGD₂-induced eosinophil migration by DP agonist (BW245C). Purified eosinophils were preincubated with increasing concentrations of BW245C at 37°C for 10 minutes. Migration assays were measured using Boyden chambers. The chemotactic response to 100 nM of PGD₂ or DK-PGD₂ was considered as 100% (**P* < .05 vs DK-PGD₂ alone, *n* = 4).

mechanism, we examined whether BW245C modulates intracellular Ca²⁺ levels and the ERK1/2 or p38 MAPK signal pathways. The Ca²⁺, ERK1/2, and p38 MAPK signals are partially responsible for eosinophils to allow CRTH2-mediated transmigration. However, neither Ca²⁺ mobilization (Fig 5B) nor the ERK1/2 and p38 MAPK signal pathways (Fig 5C) were affected by BW245C, suggesting that these pathways were not involved in the inhibitory effect of BW245C.

In eosinophils, BW245C regulates intracellular cAMP concentration when stimulated with CRTH2 agonist. A competitive protein-binding assay of intracellular cAMP was carried out because DP stimulates adenylyl cyclase through stimula-

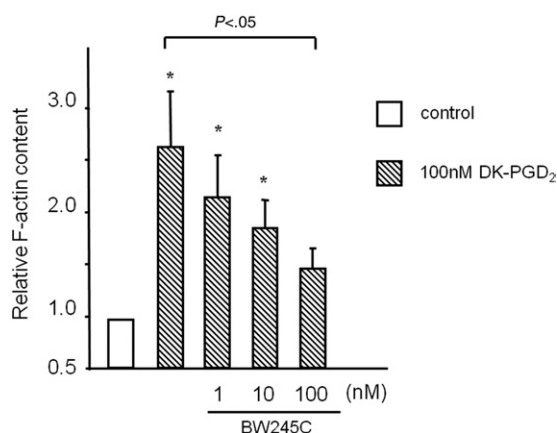


Figure 3. Inhibition of DK-PGD₂-induced cytoskeletal rearrangement by DP agonist (BW245C). Purified eosinophils were preincubated with increasing concentrations of BW245C for 10 minutes, followed by stimulation with 100 nM of DK-PGD₂ for 25 seconds, fixation with formaldehyde, and staining of F-actin with NBD-phalloidin. The F-actin content was measured by flow cytometry. (**P* < .05 vs DK-PGD₂ alone, *n* = 4).

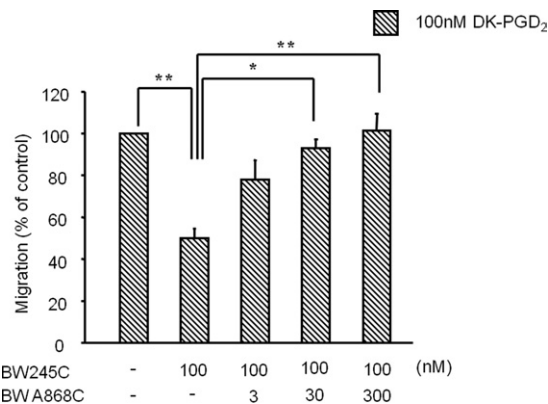


Figure 4. Effect of DP antagonist (BWA868C) on DP-mediated inhibition of chemotaxis. Purified eosinophils were further preincubated with BW245C and increasing concentrations of BWA868C at 37°C for 10 minutes. Then, migration assays were measured using Boyden chambers. The chemotactic response to 100 nM of DK-PGD₂ was considered as 100%. (**P* < .05, ***P* < .001, *n* = 4). Data are expressed as means ± SD.

tory G-protein-mediated increase, which results in an increase in intracellular cAMP concentration. In human eosinophils, BW245C and DK-PGD₂ did not influence intracellular cAMP concentration. In contrast, treatment of eosinophils with BW245C followed by DK-PGD₂ increased intracellular cAMP elevation (Fig 6).

DISCUSSION

Prostaglandin D₂ acts as both an activator and a regulator of allergic inflammation through DP and CRTH2. Studies using selective agonists and antagonists for each receptor have demonstrated that PGD₂ induces migration, actin polymerization, calcium mobilization, and adhesion molecule upregulation through CRTH2 on eosinophils.¹⁹⁻²³ In this study, to make progress in understanding the mechanisms of PGD₂ functions, we examined the role of DP in CRTH2-activated human eosinophil migration and the underlying signaling pathways. Our data showed that a DP-selective agonist, BW245C, enhanced intracellular cAMP production in CRTH2-selective agonist (DK-PGD₂) stimulation. We suggest that a DP agonist regulates intracellular cAMP concentration in CRTH2-activated eosinophil and concurrently controls eosinophil migration. Our study adds additional information about the role of DP in eosinophil functions.

We first confirmed the earlier findings of others that PGD₂ via CRTH2 induced human eosinophil migration.¹⁹ Our study showed that PGD₂ and DK-PGD₂ were potent stimulators of eosinophil migration and shape change with cytoskeletal rearrangement (Figs 1, 3), which was compatible with reports that CRTH2 stimulation induced eosinophil migration.^{20,22} In contrast, BW245C did not induce eosinophil migration. These results suggest that the effects of PGD₂ and DK-PGD₂ on eosinophil migration are mediated through CRTH2 but not DP receptors. In fact, the positive effect of CRTH2 on migration has been demonstrated in several kinds of human

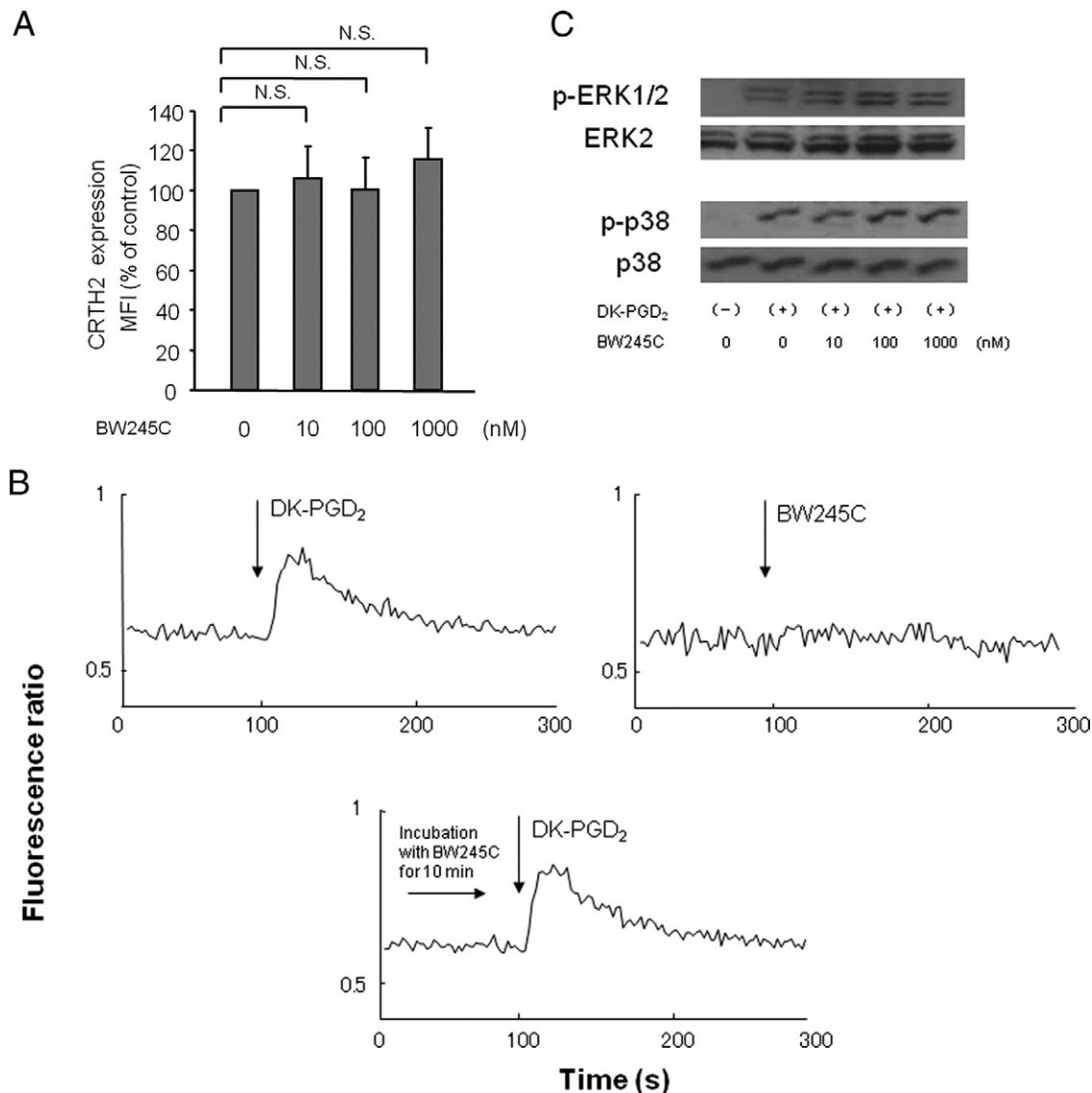


Figure 5. Examination of the mechanism of BW245-induced inhibitory effect. A, Effect of BW245C on eosinophil CRTH2 expression. Purified eosinophils were incubated with BW245C at 37°C for 20 minutes. The CRTH2 expression was measured by flow cytometry. The value is expressed as the percentage of the mean fluorescence intensity (MFI) on cells incubated in medium alone. Data are expressed as means $\pm$ SD. (* P < .05, n = 4). B, Effect of BW245C on DK-PGD₂-induced intracellular calcium influx. Purified eosinophils loaded with 2 μ M fura 2 were pre-equilibrated with Ca²⁺. Eosinophil preparations were then stimulated with medium or BW245C. After 20 minutes of incubation, DK-PGD₂ or BW245C was added. Changes in intracellular free calcium levels were detected as the increase in fluorescence intensity of the calcium-sensitive dye fura 2. The results are representative of experiments with similar results using eosinophils from 3 different donors. C, Effect of BW245C on DK-PGD₂-induced phosphorylation of ERK1/2 and p38 MAPK. Purified eosinophils were incubated with or without BW245C (100 nM) for 20 minutes. After incubation, eosinophils were stimulated with DK-PGD₂ for 1 minute (for ERK1/2) or 3 minutes (for p38). Phosphorylated ERK1/2 (p-ERK), total ERK2 (ERK2), phosphorylated p38 MAPK (p-p38), and total p38 MAPK (p38) were determined by Western blot analysis.

cells, including eosinophils.^{19,24,25} In the opposite direction, DP activation has been reported to inhibit cell migration in Langerhans cells, dendritic cells, monocytes, and fibroblasts.²⁶⁻²⁸ Before completion of the current study, Schratl et al.²³ reported that a DP-selective agonist, BW245C, attenuated PGD₂-induced eosinophil migration.²¹ Our findings also showed that DK-PGD₂-

induced migration and cytoskeletal rearrangement were inhibited by BW245C (Figs 2, 3). Blockade of DP with a selective DP antagonist, BWA868C, significantly removed this inhibitory effect in a concentration-dependent manner (Fig 4). We demonstrated the inhibition of eosinophil migration through DP in addition to cytoskeletal rearrangement.

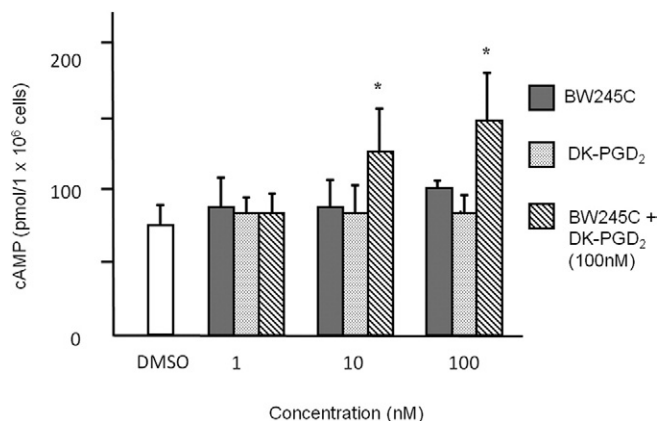


Figure 6. Effect of BW245C on cAMP levels in eosinophils. Purified eosinophils were preincubated with or without BW245C at 37°C for 10 minutes, followed by stimulation with DK-PGD₂ for 20 seconds. Then, cAMP levels were determined by using a competitive binding assay. Data are expressed as means $\pm$ SD ($n = 4$). * $P < .05$ vs control (dimethylsulfoxide alone).

Next, to determine the mechanism by which BW245C inhibits eosinophil migration, we conducted the following experiments. Because we previously found that PGD₂ reduced CRTH2 surface expression on human eosinophils,¹³ we investigated whether BW245C could reduce CRTH2 surface expression on human eosinophils. However, BW245C did not reduce CRTH2 expression on human eosinophils (Fig. 5A). The opposite effects on migration seen between DP and CRTH2 might be a result of a difference in the signaling pathway. Activation of CRTH2 causes a rise in intracellular Ca²⁺ levels and phosphorylation of MAPK signal,²⁸ which partly plays the important role of signaling pathway in eosinophil migration.^{29,30} Therefore, we checked whether BW245C suppressed these signal pathways. However, neither intracellular Ca²⁺ levels nor phosphorylation of MAPK were influenced by BW245C (Fig 5B, C).

Furthermore, we noted that both DP and CRTH2 belong to a G-protein-coupled receptor family. Activation of the DP receptor, a member of the prostanoid subfamily of GPCRs, leads to a G_s-mediated increase in intracellular cAMP.⁵ In contrast, CRTH2, very similarly to chemoattractant GPCRs, couples to a G_i-type G-protein, leading to inhibition of cAMP, and it increases intracellular Ca²⁺ in various types of cells.³¹ Beta₂ stimulants and phosphodiesterase inhibitors inhibit the activation of various leukocytes, including eosinophils.^{32,33} Contrary to a previous report,³¹ BW245C alone had limited effect in increasing cAMP concentration (Fig 6), which is probably because of the different experimental conditions. However, we demonstrated that pretreatment of BW245C followed by stimulation with DK-PGD₂ induced significant cAMP production in eosinophils. Thus, most likely DP and CRTH2 synergically control cAMP production, and increased intracellular cAMP concentration inhibits CRTH2-dependent eosinophil migration. Cyclic adenosine mono-

phosphate production inhibits CRTH2-dependent eosinophil migration.

In summary, we showed that eosinophil migration was induced by CRTH2 activation and was inhibited by DP activation. A negative regulatory effect of DP on migration may have induced an increase in intracellular cAMP concentration. In addition, a DP-induced inhibitory effect was demonstrated when activated by CRTH2. These findings suggest that a DP agonist might directly regulate human eosinophil migration in a specific condition, under the presence of PGD₂, for example. The control of DP receptor activation might be a novel therapeutic strategy for allergic diseases related to PGD₂.

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