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The Role of the Prostaglandin D_2 Receptor, DP, in Eosinophil Trafficking[1]

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Prostaglandin (PG) D_2 is a major mast cell product that acts via two receptors, the D-type prostanoid (DP) and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptors. Whereas CRTH2 mediates the chemotaxis of eosinophils, basophils, and Th2 lymphocytes, the role of DP has remained unclear. We report in this study that, in addition to CRTH2, the DP receptor plays an important role in eosinophil trafficking. First, we investigated the release of eosinophils from bone marrow using the in situ perfused guinea pig hind limb preparation. PGD_2 induced the rapid release of eosinophils from bone marrow and this effect was inhibited by either the DP receptor antagonist BWA868c or the CRTH2 receptor antagonist ramatroban. In contrast, BWA868c did not inhibit the release of bone marrow eosinophils when this was induced by the CRTH2-selective agonist 13,14-dihydro-15-keto-PGD_2. In additional experiments, we isolated bone marrow eosinophils from the femoral cavity and found that these cells migrated toward PGD_2. We also observed that BWA868c inhibited this response to a similar extent as ramatroban. Finally, using immunohistochemistry we could demonstrate that eosinophils in human bone marrow specimens expressed DP and CRTH2 receptors at similar levels. Eosinophils isolated from human peripheral blood likewise expressed DP receptor protein but at lower levels than CRTH2. In agreement with this, the chemotaxis of human peripheral blood eosinophils was inhibited both by BWA868c and ramatroban. These findings suggest that DP receptors comediate with CRTH2 the mobilization of eosinophils from bone marrow and their chemotaxis, which might provide the rationale for DP antagonists in the treatment of allergic disease. *The Journal of Immunology,* **2007, 179: 4792–4799.**

Prostaglandin (PG)[3] D_2 is released by activated mast cells during the allergic response (1) and substantial evidence has accumulated that PGD_2 might be crucially involved in the initiation and perpetuation of allergic inflammation. A significant contribution of PGD_2 to the late phase allergic reaction is suggested by enhanced eosinophilic lung inflammation and cytokine release in transgenic mice over-expressing PGD_2 synthase (2). The biological effects of PGD_2 are principally mediated by two distinct receptors, the D-type prostanoid (DP) receptor and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (3). Moreover, at higher concentrations PGD_2 is a ligand for the thromboxane receptor (TP), which mediates the bronchoconstricting effect of PGD_2 (4).

CRTH2 is expressed on Th2-type T cells, eosinophils and basophils (5) and mediates their chemotaxis to PGD_2 (6). In addition, CRTH2 is activated by several PGD_2 metabolites, including 13,14-dihydro-15-keto-(DK)-PGD_2, PGJ_2, $\Delta^{12}PGJ_2$, and 15-deoxy-PGJ_2 (7, 8) and a thromboxane (TX) metabolite, 11-dehydro-TXB_2 (9). Moreover, CRTH2 mediates the respiratory burst and degranulation of eosinophils (8, 10), induces the production of proinflammatory cytokines in Th2 cells (11), and enhances the release of histamine from basophils (12). In humans, CRTH2 is the most reliable marker for Th2 cells (13), and in animal models, CRTH2 mediates eosinophil infiltration into the lungs and skin and aggravates the pathology of allergic responses (14–16). Therefore, CRTH2 antagonists are being considered as a potentially useful approach for the treatment of asthma and allergic disease. The cyclooxygenase inhibitor indomethacin was also found to be a potent CRTH2 agonist (17, 18) and has thus provided a useful pharmacophore for small molecule antagonists to CRTH2 (19).

The alternate PGD_2 receptor, DP, is expressed more widely, including platelets, several types of leukocytes, the vasculature, the CNS, retina, nasal mucosa, lungs, and intestine (6, 20–23). Functionally, DP-mediated responses include inhibition of platelet aggregation, induction of vasorelaxation, mucin secretion, and lowering intraocular pressure (23–25). DP agonists have been suggested to inhibit neutrophil, basophil, and dendritic cell function (26–29). Eosinophils have been found to express the mRNA for the DP receptor and to show delayed apoptosis after treatment with supramaximal concentrations (1 μM) of the selective DP agonist BW245c (10). In vivo, a DP antagonist blocks Ag-induced rhinitis, conjunctivitis, and pulmonary inflammation in the guinea

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[3] Abbreviations used in this paper: PG, prostaglandin; DP, D-type prostanoid receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DK-PGD_2, 13,14-dihydro-15-keto-PGD_2; 15-deoxy-PGJ_2, 15-deoxy-Δ(12, 14)-PGJ_2; LTB_4, leukotriene B_4; PMNL, polymorphonuclear leukocytes; Th2, Th 2; TP, thromboxane receptor; TXB_2, thromboxane B_2.

Table I. *Compounds used in this study and their receptor selectivity*

Name	Receptor of Selectivity and Rank Order of Affinity	Activity	Reference No.
PGD_2	DP = CRTH2 > TP	Agonist	(6, 48)
DK-PGD_2	CRTH2	Agonist	(6)
BW245c	DP	Agonist	(6)
ZK110841	DP	Agonist	(49)
BWA868c	DP	Antagonist	(50)
SQ29548	TP	Antagonist	(51)
Ramatroban	TP ≥ CRTH2	Antagonist	(52)

pig (30), and DP-deficient mice exhibit reduced pulmonary inflammation in response to allergen (31). These in vivo studies hence point to a proinflammatory role of DP but are difficult to explain based on the known functional responses of DP receptor activation.

We have previously shown that $\Delta^{12}PGJ_2$ and PGD_2 markedly prime eosinophils for chemotaxis toward other chemoattractants, such as the CCR3 ligand eotaxin, and that $\Delta^{12}PGJ_2$ is capable of mediating the rapid mobilization of eosinophils from the isolated hind limb of guinea pigs (8). These observations suggested novel mechanisms by which PGD_2 or its metabolites generated at sites of allergic inflammation could act as systemic signals for the supply of eosinophils from the bone marrow and enhance their extravasation to the tissue. Hence we set out to investigate the differential roles of CRTH2 and DP in the mobilization of eosinophils from the bone marrow and chemotaxis. Our data demonstrate that the DP receptor plays an important modulator role in the recruitment of eosinophils into the tissue. Thus, DP antagonists might be useful to treat conditions associated with eosinophilic inflammation such as asthma or other allergic diseases.

Materials and Methods

Animals

Adult Dunkin-Harley guinea pigs (either sex, 350–450 g body weight) were obtained from the Research Institute for Laboratory Animal Breeding (Medical University of Vienna, Himberg, Austria).

Chemicals

All laboratory reagents were obtained from Sigma-Aldrich, unless specified. Assay buffer as used in the shape change and chemotaxis experiments was made from Dulbecco's modified PBS (with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}; Invitrogen Life Technologies), 0.1% BSA, 10 mM HEPES, and 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM D-glucose, 3.33 mM $CaCl_2$, 0.49 mM $MgCl_2{\cdot}6H_2O$, 4.56 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO4, and 24 mM $NaHCO_3$, and supplemented with 0.1% BSA. Recombinant human eotaxin and synthetic guinea pig eotaxin were purchased from PeproTech and Cell Sciences, respectively. PGD_2, DK-PGD_2, BW245c, LTB_4, ramatroban, SQ29458, and BWA868c (for receptor selectivity and references, see Table I) were purchased from Cayman Chemical. Drugs were dissolved in ethanol or DMSO and further diluted in assay buffer to give a final concentration of the solvents <0.1%. $[^3H]PGD_2$ was obtained from PerkinElmer Life and Analytical Sciences.

Cell culture

The sequence of guinea pig DP (32) was amplified by PCR from guinea pig lung cDNA and inserted via 5′ *Hin*dIII and 3′ *Bam*HI into pcDNA3.1$^+$. The sequence identity of the inserts was verified by restriction endonuclease digestion and sequencing in both directions. HEK293 or 1321N1 cells expressing guinea pig DP were cultivated in DMEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 400 μg/ml G418.

Binding experiments

HEK293 cells expressing guinea pig DP receptors were seeded into 96-well plates at a density of 30,000 cells/well. Competition binding experiments on whole cells were then performed ~18–24 h later using $[^3H]PGD_2$ (PerkinElmer; 172 Ci/mmol) as a radiotracer in a binding buffer consisting of HBSS and 10 mM HEPES (pH 7.5); 0.6 nM $[^3H]PGD_2$ was used for guinea pig DP equilibrium competition binding. Competing ligands were diluted in DMSO that was kept constant at 1% (v/v) of the final incubation volume. Total and nonspecific binding were determined in the absence and presence of 10 μM PGD_2. Binding reactions were routinely conducted for 3 h at 4°C to exclude receptor internalization and terminated by two washes (100 μl each) with ice-cold binding buffer. Radioactivity was determined by liquid scintillation counting in a Topcount (Packard Instruments) following overnight incubation in Microscint 20.

cAMP accumulation assays

1321N1 cells expressing guinea pig DP were metabolically labeled with 2 μCi of $[^3H]$adenine (Amersham; TRK311) in 24-well plates for 18–24 h at 37°C. They were then washed twice with PBS and stimulated with increasing concentrations of PGD_2 in HEPES-buffered saline supplemented with 1 mM isobutylmethylxanthine for 30 min at 37°C. The reaction was stopped by adding 5% (w/v) ice-cold trichloroacetic acid supplemented with 0.1 mM cAMP and 0.1 mM ATP. $[^3H]$cAMP was separated from the remaining nucleotides using anion exchange chromatography, and radioactivity was counted after addition of HiSafe3 scintillation fluid (PerkinElmer Life and Analytical Sciences).

Leukocyte shape change assay

This study was approved by the Ethics Committee of the Medical University of Graz. Eosinophil shape change was recorded using human blood sampled from healthy volunteers or guinea pig blood obtained by cardiac puncture after an overdose of pentobarbital sodium (8). Ninety-microliter aliquots of citrated whole blood were stimulated with 10 μl of agonists for 4 min at 37°C. The samples were then transferred to ice and fixed with 250 μl of fixative solution followed by NH_4Cl-induced lysis of RBC (33). Cells were then washed and resuspended in 250 μl of fixative solution. Samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from other cells by means of their autofluorescence in the FL-1 and FL-2 fluorescence channels (8). Shape change was determined as the increase of forward scatter compared with vehicle stimulation. The forward scatter values are shown as dimensionless arbitrary units.

Chemotaxis of human eosinophils

Preparations of polymorphonuclear leukocytes (PMNL) were prepared by dextran sedimentation and Histopaque gradients, and eosinophils were further purified by negative magnetic selection using an Ab mixture directed against CD2, CD14, CD16, CD19, CD56, glycophorin A from StemCell Technologies. The purity of the eosinophil preparations was usually above 97%, the contaminating cells being mononuclear cells. The viability of the cells was >98%. Thirty microliters of assay buffer or agonists were placed into the bottom wells of a 48-well micro-Boyden chemotaxis chamber (NeuroProbe). Eosinophils were suspended in assay buffer at 2×10^6/ml, and 50 μl of the suspension were placed into the top wells of the plate, which was separated from the bottom wells by a 5-μm pore size polyvinylpyrrolidone-free polycarbonate filter. Baseline migration was determined in wells containing only assay buffer. The chamber was incubated at 37°C in a humidified CO_2 incubator for 1 h and the membrane was carefully removed. Cells that had migrated to the bottom wells were enumerated by flow cytometry (FACSCalibur, BD Biosciences), and contaminating cells were gated out by side scatter and autofluorescence as previously described (8).

Chemotaxis of guinea pig bone marrow eosinophils

The femoral bone cavity was flushed, erythrocytes were lysed by hypotonic shock, and PMNLs were prepared by Histopaque gradients. The cells were resuspended in RPMI 1640 (1% FCS) at 10×10^6/ml, and 100-μl aliquots of the suspension were placed into Transwell inserts with 5-μm pore size polyvinylpyrrolidone-free polycarbonate filters (Corning). The cells were allowed to migrate toward 600 μl of assay buffer or agonists in the bottom wells of 24-well tissue culture plates for 1 h at 37°C in a humidified CO_2 incubator. Baseline migration was determined in wells containing only assay buffer. Cells that had migrated to the bottom wells were enumerated by flow cytometry (FACSCalibur), and eosinophils were distinguished from neutrophils by side scatter and autofluorescence (34). To compare the migration of eosinophils and neutrophils, responses were expressed as the chemotactic index (i.e., relative to baseline migration).

In situ perfusion of the guinea pig hind limb

The guinea pig hind limb was perfused as previously described (8, 34). The external iliac artery and vein were exposed and the caudal abdominal artery, superficial iliac circumflex artery, and pudendoepigastric trunk along with their satellite veins were ligated. Polyethylene cannulas (0.8 mm outside diameter) were inserted into the external iliac artery and vein. Modified Krebs-Ringer bicarbonate buffer (gassed with 95% O_2 and 5% CO_2, 37°C) was infused at 4 ml/min via the arterial cannula and removed from the venous cannula using a peristaltic pump. After an equilibration period of 20 min, the perfusate fractions were collected every 10 min and centrifuged at 300 × *g* for 10 min. The cell pellet was resuspended in Kimura's stain and nucleated leukocytes and Kimura-positive eosinophils were counted in a Neubauer hemacytometer.

Immunofluorescence microscopy

The DP and CRTH2 receptors were visualized on human peripheral blood eosinophils by immunofluorescence following a double antibody staining protocol. For visualization of the CRTH2 receptor, a suspension of unfixed 1×10^7 PMNL in PBS was blocked with human IgG (Sigma-Aldrich) for 30 min at 4°C. Cells were washed once with PBS by centrifugation at 400 × *g* for 5 min and then incubated with rat mAb against CRTH2 (clone BM16; BD Biosciences) diluted 1/25, or the isotype control rat IgG2a (BD Biosciences) in PBS for 30 min at 4°C. Following a washing step, cells were incubated for 30 min in the dark at 4°C with the secondary Ab (Cy3-conjungated goat anti-rat secondary Ab; Chemicon; dilution 1/250). PMNL were washed twice with PBS, and cells were cyto-spinned on cover slips. Subsequently, PMNL were fixed with 3.7% formaldehyde in PBS at 4°C over night. After washing, the cells were blocked with powdered milk and human IgG (Sigma-Aldrich) in PBS for 60 min at room temperature. Cells were washed twice with PBS and then incubated with rabbit polyclonal Ab against the DP receptor (Sigma-Aldrich) diluted 1/125 or rabbit IgG (Sigma-Aldrich) as negative control in PBS with powdered milk. After incubation with primary Ab, PMNL were washed six times with PBS and then incubated for 30 min in the dark at room temperature with secondary Ab (AlexaFluor 488 goat anti-rabbit IgG; Invitrogen Life Technologies; dilution 1/500). Finally, after six washes with PBS, the nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen Life Technologies). The fluorescent signal was recorded with an Olympus IX70 fluorescence microscope and an Olympus UPlanApo - 60×/1.20 lens, using Olympus DP50-CU digital camera and Olympus CellP̂ software. Images were further processed also with CellP̂ for contrast and brightness adjustments.

Immunohistochemistry

The expression of DP and CRTH2 receptors was investigated in human bone marrow. Sections (3 μm) were prepared from EDTA decalcified material, deparaffinized by xylene, rehydrated in graded alcohols, and submitted to microwave epitope retrieval (10 min at 750 W using pH 9.0 Ag retrieval buffer S2367 obtained from Dako). After treatment in 3% H_2O_2 for 10 min, sections were incubated for 30 min using goat polyclonal Ab against eosinophil peroxidase (EPX; Santa Cruz Biotechnology) diluted 1/25, rabbit polyclonal Ab against the DP receptor (Sigma-Aldrich) diluted 1/200 and rat mAb against CRTH2 (clone BM16; BD Biosciences) diluted 1/10 in Dako Ab diluent. After enhancement using MultiLink (Dako E0453) diluted 1/100 and biotinylated StreptABComplex (Dako K5001) or alkaline phosphatase red (Dako K5005) diluted 1/100 for 30 min each, detection was performed using peroxidase substrate chromogen ready-to-use (Dako K5001) or alkaline phosphatase (fast red) under microscopic control. After each development procedure, blocking was performed for 5 min using 3% H_2O_2 with extensive rinsing using Tris-HCl buffer (pH 7.6) in between. Images were taken on a Nikon Eclipse E600 microscope with a Nikon Plan 40×/0.65 lens using Nikon DS-5M-U1 digital camera and Nikon Digital Sight DS-U1 software. Images were further processed with Adobe Photoshop CS, used for additional white balance, contrast, and brightness adjustments.

Calculations and data analysis

IC_{50} and EC_{50} values were determined by nonlinear regression analysis using the Prism 3.0 software (GraphPad Software). Antagonistic potencies in functional assays are given as pIC_{50} values that were obtained by competing with increasing concentrations of inhibitor compound for an agonist concentration required to elicit ~75–80% of the maximal agonist efficacy. IC_{50} values generated by this procedure are very closely matching the true affinity of antagonists determined by the analysis according to Arunlakshana and Schild (35).

Data are shown as the mean ± SEM except where otherwise stated. Statistical comparisons of groups were performed using two-way ANOVA for repeated measurements or the Mann-Whitney *U* test. Probability values of $p < 0.05$ were considered statistically significant.

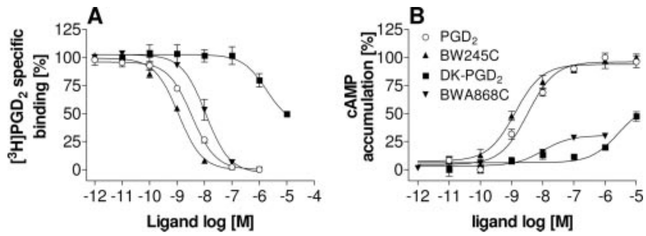

FIGURE 1. In vitro characterization of selective PGD_2 receptor ligands on recombinant guinea pig DP receptors. *A*, competition binding analysis in HEK293 cells expressing guinea pig DP. Values are the mean ± SD of three to six independent experiments performed in duplicate. Data are expressed as percentage of the maximal binding of $[^3H]PGD_2$ obtained in the absence of the indicated competitor compounds. *B*, cAMP production of 1321N1 cells transfected with guinea pig DP. Values are the mean ± SD of two independent experiments performed in duplicate and are normalized to the maximal cAMP production achieved by PGD_2.

Results

Pharmacological characterization of BWA868c

While BWA868c has been investigated before with respect to its affinity and selectivity toward human DP and CRTH2 receptors (36), guinea pig DP has been characterized so far only in functional assays. Thus we expressed the guinea pig DP receptor in HEK293 and 1321N1 cells for binding experiments and assay of cAMP accumulation, respectively. As expected, BWA868c and the DP-selective agonist BW245c exhibited high affinity for the guinea pig DP receptor, with log K_i values of −7.95 ± 0.08 and −8.95 ± 0.06, respectively, and displaced PGD_2 from the receptor (Fig. 1*A*). In assays of cAMP accumulation, BW245c was a full agonist of DP comparable with PGD_2, with pEC_{50} values of −8.89 ± 0.11 and −8.48 ± 0.09, respectively. In contrast, BWA868c caused a minute degree of cAMP accumulation at higher concentrations (Fig. 1*B*), which was compatible with partial agonism already reported for human DP (20).

The selectivity of BWA868c against guinea pig CRTH2 was investigated in eosinophils, which naturally express CRTH2, using the shape change assay. Stimulation with chemoattractants results in immediate responses of granulocytes including reorganization of the cytoskeleton and shape change, which can be detected by flow cytometry (8). PGD_2 was a potent and highly effective inducer of eosinophil shape change of guinea pig eosinophils, although with a 3- to 10-fold lower potency than in human whole blood (Fig. 2). Ramatroban inhibited the PGD_2-induced shape change of human and guinea pig eosinophils (Fig. 2), but had no effect on eotaxin-induced shape change (data not shown, $n = 4$). In contrast, BWA868c had no effect on shape change responses of guinea pig and human eosinophils, irrespectively of the stimulant used (PGD_2, Fig. 2; eotaxin, data not shown, $n = 4$). Since the DP agonist BW245c did not cause shape change (data not shown, $n = 4$), it seems that eosinophil shape change responses to PGD_2 are exclusively mediated by CRTH2.

Mobilization of eosinophils from the guinea pig femoral bone marrow

Using BWA868c and ramatroban as potent and selective antagonists for DP and CRTH2, respectively, we investigated which of the PGD_2 receptors was mediating the mobilization of mature eosinophils from bone marrow in the in situ perfused guinea pig hind limb preparation. PGD_2 caused an immediate increase of eosinophils in the perfusate when added during the period of 20–40 min

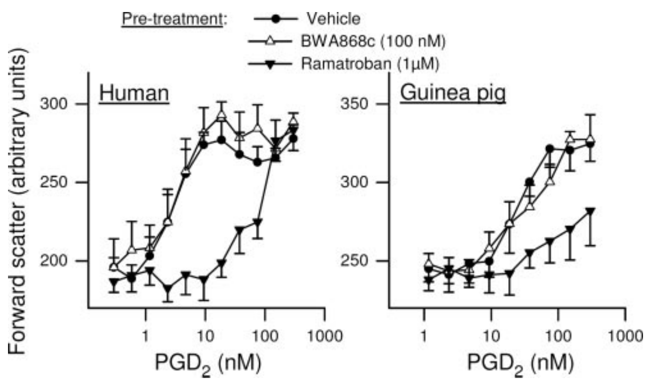

FIGURE 2. Selectivity of the DP antagonist BWA868C is demonstrated by the lack of effect on PGD_2-induced shape change of human and guinea pig eosinophils. Samples of human or guinea pig whole blood were pretreated with the CRTH2 antagonist ramatroban, BWA868c or vehicle at the concentrations indicated and stimulated with PGD_2. Shape change was measured by flow cytometry as increase in forward scatter. Data are shown as mean ± SEM, $n = 4$–10.

after the start of the experiments, whereas the vehicle of the prostanoid alone had no effect (Fig. 3*A*); 10 nM of PGD_2 caused a similar degree of eosinophil release as 1 nM eotaxin. The release of noneosinophilic cells from the bone marrow was not increased by PGD_2 or eotaxin, whereas LTB_4 nonselectively induced the release of both eosinophils and other nucleated cells (Fig. 3*A*).

To determine the role of CRTH2, DP, and TP receptors in eosinophil mobilization from bone marrow, PGD_2 antagonists or their vehicle were infused throughout the experiment, and PGD_2

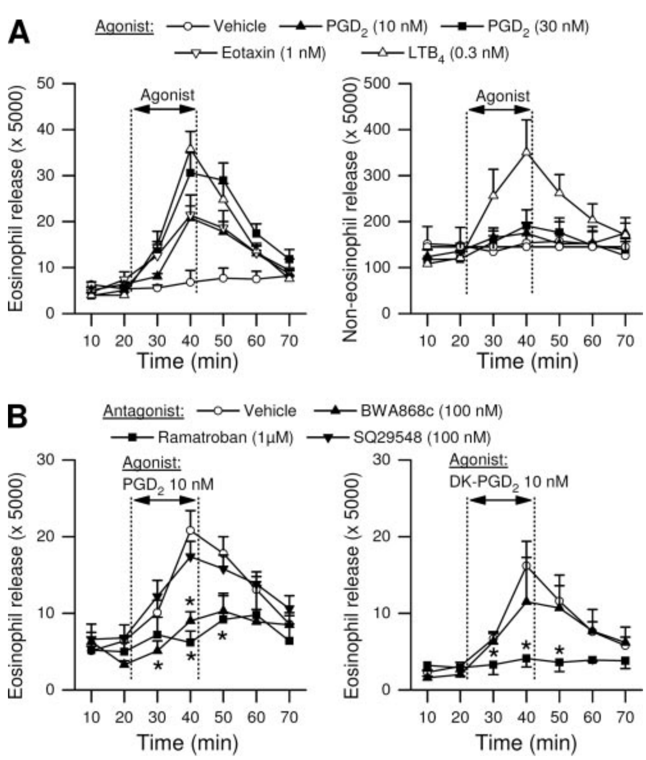

FIGURE 3. PGD_2 selectively induces the mobilization of eosinophils from guinea pig femoral bone marrow (*A*), and the DP antagonist BWA868c and the CRTH2 antagonist ramatroban, but not TP antagonist SQ29548, inhibit this response (*B*). The antagonists were infused into the in situ perfused hind limb preparation throughout the experiments, while the agonists were infused for 20 min as shown (↔). Data are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 5$–13.

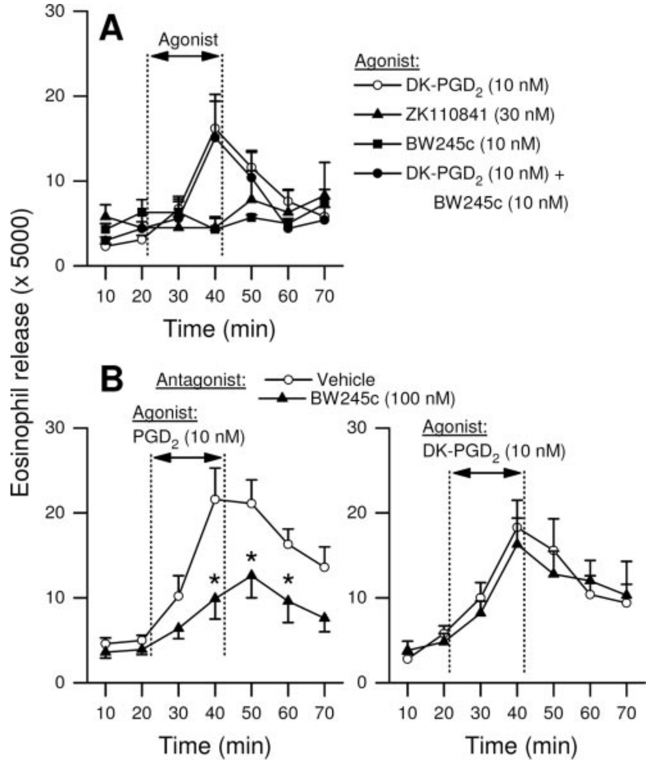

FIGURE 4. The DP agonists BW245c and ZK110841 do not induce eosinophil release from guinea pig femoral bone marrow (*A*), but BW245c inhibits the response to PGD_2 (*B*). The antagonists were infused into the in situ perfused hind limb preparation throughout the experiments, whereas the agonists were infused for 20 min as shown (↔). Data are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 6$–9.

was added to the perfusate for the period of 20–40 min after the start of the perfusion. The ability of PGD_2 to increase the release of eosinophils from bone marrow was not altered by the TP-selective antagonist SQ29548 (100 nM), but it was inhibited by the CRTH2 antagonist ramatroban (1 μM) by 70–80%, and also by the DP antagonist BWA868c (100 nM) to a similar extent (Fig. 3*B*). In contrast, the DP antagonist BWA868c did not inhibit the response to DK-PGD_2 (Fig. 3*B*). Therefore, these data show that PGD_2-induced release of eosinophils is mediated both by CRTH2 and DP receptors, while in agreement with its known selectivity for CRTH2, DK-PGD_2 mobilized eosinophils solely via CRTH2.

Unexpectedly, the DP agonists BW245c or ZK110841 did not induce eosinophil release by themselves and the addition of BW245c to the infusion of DK-PGD_2 did not enhance the response of bone marrow eosinophils when compared with DK-PGD_2 alone (Fig. 4*A*). Hence, these findings raised the question whether BW245c was inactive on DP receptors in this preparation or might even behave as a DP antagonist. When infused throughout the experiment, BW245c (100 nM) in fact reduced the ability of PGD_2 to release eosinophils from the bone marrow, whereas the response to DK-PGD_2 was not affected (Fig. 4*B*). From these observations it may be inferred that BW245c behaves as an antagonist of DP receptors in guinea pig bone marrow.

Chemotaxis of eosinophils

To further investigate the mechanisms by which CRTH2 and DP activation induce the release of eosinophils from bone marrow, we harvested the leukocytes from the cavity of the guinea pig femoral bone to determine their migration. Bone marrow eosinophils showed a chemotactic response to PGD_2 (3–300 nM) similar to

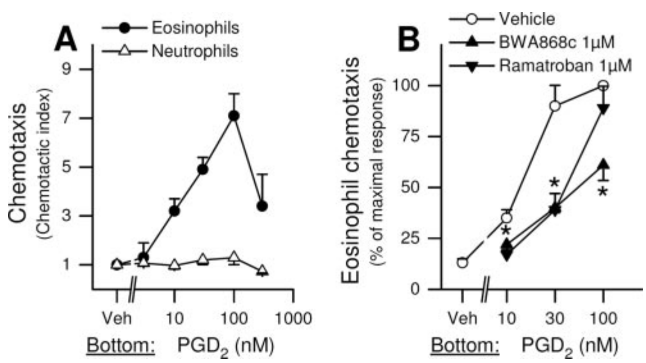

FIGURE 5. PGD_2 selectively induces the migration of guinea pig bone marrow eosinophils (A), and the DP antagonist BWA868c and the CRTH2 antagonist ramatroban inhibit this response (*B*). Eosinophils and neutrophils were isolated from the femoral bone cavity. Cells were placed on Transwell filter inserts and allowed to migrate toward PGD_2. In *A*, responses are expressed as the chemotactic index (i.e., relative to migration toward vehicle). In *B*, data are shown as percentage of the maximal control response (i.e., 100 nM PGD_2). Data are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 5–7$.

human peripheral blood eosinophils, whereas neutrophil chemotaxis was not stimulated by PGD_2 (Fig. 5*A*). Migration of bone marrow eosinophils was significantly inhibited by the CRTH2 antagonist ramatroban and, to a larger extent, also by the DP antagonist BWA868c (Fig. 5*B*). Human eosinophils purified from peripheral blood likewise migrated toward PGD_2, and this response was inhibited by ramatroban but not SQ29548 (Fig. 6*A*). BWA868c (100 nM) also attenuated the migration of human eosinophils by causing a 3- to 6-fold shift of the PGD_2 concentration-response curve to the right (Fig. 6*A*). The inhibitory effect of BWA868c was unrelated to nonspecific attenuation of eosinophil responsiveness, because BWA868c pretreatment did not alter the migration toward eotaxin (Fig. 6*A*) or LTB_4 ($n = 5$, data not shown). The DP-selective agonists BW245c (3–300 nM) or ZK110841 (10–30 nM) did not stimulate the chemotaxis of eosinophils, neither alone (Fig. 6*B*) nor in combination with DK-PGD_2 or eotaxin (data not shown). However, pretreatment of eosinophils with BW245c (100 nM) significantly attenuated the migratory response toward PGD_2 (Fig. 6*C*). This suggests again that, in contrast to PGD_2, conventional DP agonist do not activate chemotactic pathways in eosinophils, but might even act as antagonist at DP-mediated eosinophil responses.

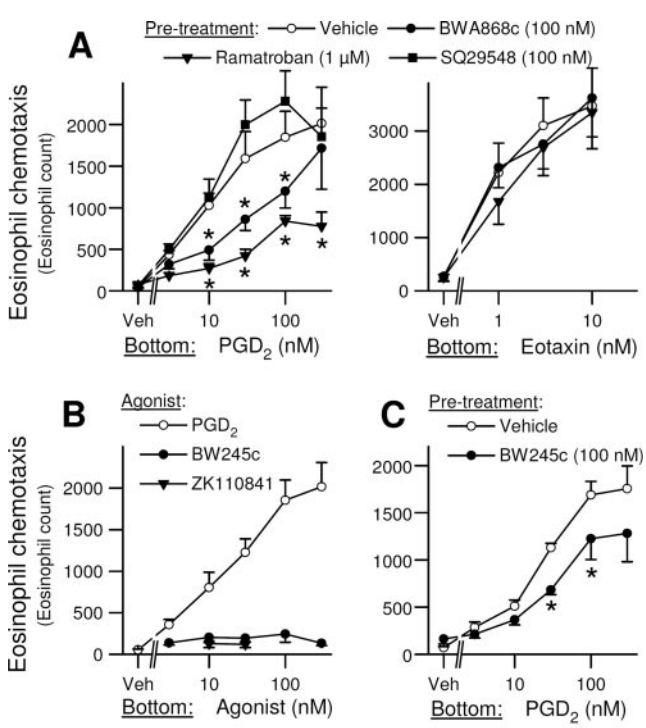

FIGURE 6. The PGD_2-induced migration of human peripheral blood eosinophils is inhibited by the DP agonist BWA868c and the CRTH2 antagonist ramatroban but not the TP antagonist SQ29548. In *A*, purified eosinophils were pretreated with the antagonists, were then loaded into the top wells of a microBoyden chamber and allowed to migrate toward PGD_2 or eotaxin. In *B*, the migration of eosinophils toward the DP agonists ZK110841 and BW245c was determined. In *C*, eosinophils were pretreated with BW245c and were then allowed to migrate toward PGD_2. Cells that had migrated into the bottom wells were enumerated by flow cytometry and the counts of eosinophil events gathered for 30 s are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 4–6$.

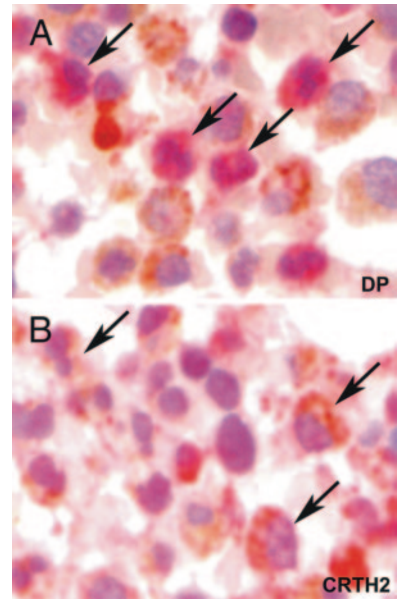

FIGURE 7. Human bone marrow eosinophils express DP and CRTH2 receptors. In *A*, eosinophil peroxidase-positive cells are stained brown by immunohistochemistry and DP-positive eosinophils (arrows) appear in red. In *B*, cells positive for eosinophil peroxidase are stained red, CRTH2-positive eosinophils and eosinophil precursors appear in brown (arrows).

Expression of CRTH2 and DP in human eosinophils

To further substantiate our findings of DP being involved in eosinophil release from bone marrow and eosinophil chemotaxis, we attempted to visualize the expression of DP and CRTH2 in human peripheral blood eosinophils and eosinophils in bone marrow biopsy specimens. Using immunohistochemistry, we identified DP and CRTH2 in normal bone marrow specimens of three different individuals (data not shown) and one patient suffering from hypereosinophilia of the bone marrow. In these samples, DP was located in mature eosinophils with typical bi-lobed nuclei that also stained positive for eosinophil peroxidase (Fig. 7*A*). CRTH2 could be observed in mature eosinophils and also in cells with larger, nonsegmented nuclei consistent with eosinophil precursors (Fig. 7*B*). Using double-staining immunofluorescence microscopy, we observed that eosinophils from peripheral blood expressed both DP and CRTH2, whereas neutrophils expressed DP only, although at higher levels than eosinophils (Fig. 8). In contrast, cells incubated with the respective isotype-matched primary control Abs followed by the secondary Abs did not exhibit positive staining.

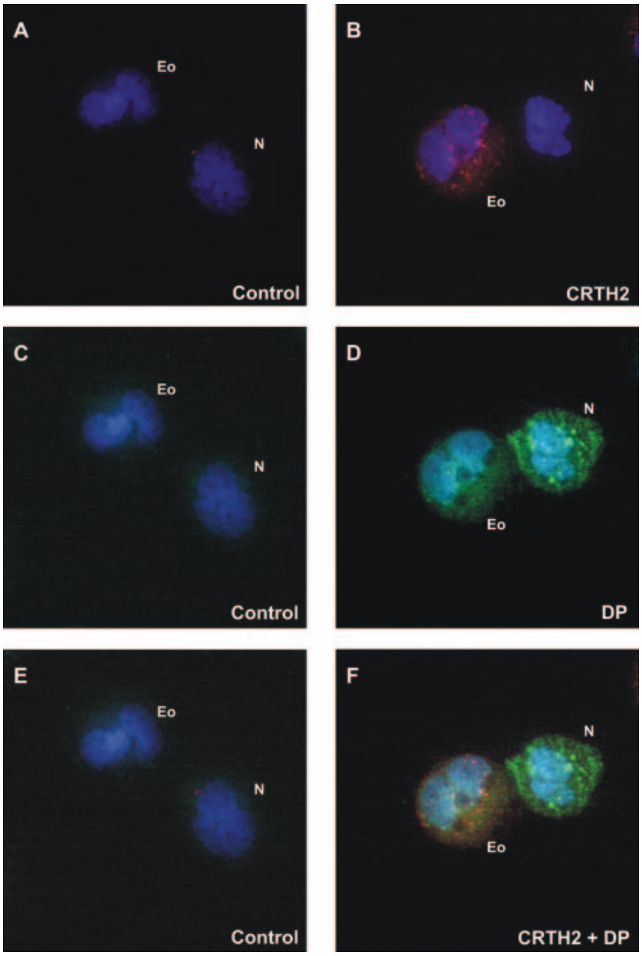

FIGURE 8. Human peripheral blood eosinophils express DP and CRTH2 receptors. In *D*, double-staining immunofluorescence shows an eosinophil (Eo) and a neutrophil (N) stained positive for DP (green), whereas *B* demonstrates that the same eosinophil also expresses CRTH2 (red). *A* and *C* are images taken with the respective control Abs. *E* and *F* show overlay-images of *A* and *C*, and *B* and *D*, respectively.

Discussion

In the current study, we show for the first time that the DP receptor plays an important role in eosinophil trafficking, i.e., chemotaxis and mobilization of eosinophils from the bone marrow. Hence, our data provide a rationale for the use of DP receptor antagonists or mixed CRTH2/DP antagonists in the treatment of allergic disease. In this study, we observed that PGD_2 induced the release of mature eosinophils from the bone marrow and that this effect was inhibited by either the CRTH2 antagonist ramatroban or the DP antagonist BWA868c, but not the TP antagonist SQ29548. The CRTH2-selective agonist DK-PGD_2 also mobilized eosinophils from the bone marrow, but BWA868c did not inhibit this response, which demonstrates the DP selectivity of this antagonist. The mobilization of eosinophils from the bone marrow was apparently linked with the chemotactic activity of PGD_2 as the prostanoid induced the in vitro migration of eosinophils isolated from the guinea pig femoral cavity. One possible mechanism underlying the inhibitory effect of BWA868c on eosinophil mobilization from bone marrow might be the blockade of DP receptors on endothelial cells, the activation of which by PGD_2 may support the release of bone marrow cells to the blood stream when the eosinophils are stimulated through CRTH2. However, both DP and CRTH2 seem to be involved in the migration of bone marrow eosinophils at the cellular level, because either BWA868c or ramatroban inhibited the in vitro chemotaxis of isolated bone marrow eosinophils. This was unexpected because the DP receptor has not been implicated in the chemotactic response of eosinophils to PGD_2 before. Therefore we further investigated the role of DP in the migration of human eosinophils purified from peripheral blood. As expected, the CRTH2 antagonist markedly attenuated the PGD_2-induced chemotaxis of human eosinophils purified from peripheral blood, whereas the TP-selective antagonist SQ29548 had no effect. However, the DP antagonist BWA868c also significantly reduced the migration toward PGD_2, by inducing a 6-fold rightward shift of the concentration response curve to PGD_2 without altering the maximal response.

Our data suggested for the first time that, in addition to CRTH2, the alternate PGD_2 receptor DP might also be involved in eosinophil chemotaxis and mobilization of eosinophils from the bone marrow. This conclusion was based on the observation that BWA868c inhibited the chemotaxis and mobilization from bone marrow of eosinophils that had been stimulated with PGD_2. BWA868c has previously been characterized as a highly selective antagonist at human DP receptors with virtually no affinity for human CRTH2 (36). The same seems to hold true for the guinea pig, since BWA868c potently inhibited the binding of PGD_2 to recombinant guinea pig DP, but it did not inhibit the PGD_2-induced shape change of guinea pig eosinophils, a response solely mediated by CRTH2. Moreover, BWA868c did not inhibit the chemotaxis of human eosinophils toward eotaxin and LTB_4. Finally, the selectivity of the DP antagonist was also demonstrated by its lack of effect when eosinophil mobilization from bone marrow was induced by the selective CRTH2 agonist, DK-PGD_2.

Whether eosinophils express the corresponding DP receptor protein has not been investigated before. Using immunohistochemistry we found that eosinophils in human bone marrow and in peripheral blood express both CRTH2 and DP receptors, which further emphasizes the importance of DP in eosinophil trafficking. BW245c and ZK110841 have previously been described as selective DP agonists, which can potently mimic those PGD_2 effects that are mediated by the DP receptor, such as inhibition of platelet aggregation, vasodilation and ocular hypotension (24, 37, 38). BW245c has been reported to delay eosinophil apoptosis at supramaximal (1 μM) concentration (10). In this study, we found that BW245c and ZK110841 were not chemotactic for eosinophils, and none of the DP agonists induced the mobilization of eosinophils from in the in situ perfused guinea pig hind limb preparation. On the contrary, BW245c actually inhibited the responses to PGD_2, i.e., the chemotaxis of peripheral blood eosinophils and eosinophil release from bone marrow. However, eosinophil mobilization induced by DK-PGD_2 was not attenuated by BW245c.

These observations suggest that the DP receptor, that is involved in eosinophil locomotion, behaves differently than the DP receptors in the vasculature or platelets: although BWA868c is a DP antagonist in all these cell types (38, 39), BW245c is a DP agonist in platelets and vascular smooth muscle (39, 40), but it does not activate chemotactic pathways in eosinophils and even might act as a DP antagonist in eosinophil responses. Similar disparities had been observed in the conjunctiva, where PGD_2 increased conjunctival microvascular permeability in a BWA868C-sensitive manner, despite the fact that BW245c failed to evoke a plasma exudation response (25). These findings might be explained by unique coupling of the DP receptor in eosinophils: previous studies by others and our own data suggest that activation of CRTH2 in eosinophils leads to shape change through G_q G-proteins, phospholipase C, Ca^{2+} mobilization from intracellular stores, MAP kinases, and actin polymerization (17, 41), while the CRTH2-mediated chemotaxis of eosinophils depends on $G_{i/o}$ (36). By contrast, DP is coupled to G_s and activation of adenylyl cyclase (3, 41). From our data it appears that the events leading to PGD_2-stimulated shape change are solely mediated by CRTH2 without an involvement of DP. Monneret et al. (41) have shown that both PGD_2 and BW245c stimulate cAMP formation in eosinophils in a BWA868c-sensitive manner. However, an increase of cAMP is likely to attenuate eosinophil chemotaxis (42) and blockade of DP with BWA868c amplifies the PGD_2-induced up-regulation of CD11b (41). Therefore, DP must be linked to additional yet unknown mechanisms that positively control eosinophil locomotion. Our data with chemotaxis of human eosinophils and release of bone marrow eosinophils suggest that this additional mechanism is not activated by BW245c. But since BW245c has the same binding site to the DP receptor as PGD_2 (Fig. 1), it acts as an antagonist with respect to this putative novel mechanism. This is reminiscent of a recent observation made by our group showing that some CRTH2 antagonists can selectively block PGD_2-induced arrestin translocation but not the activation of heterotrimeric G-proteins (43). Gervais et al. (10) have suggested that PGD_2 can delay the onset of apoptosis in cultured eosinophils through activation of DP. However, during the course of the chemotactic response (1 h of incubation at 37°C) we did not observe an anti-apoptotic effect of PGD_2 nor a proapoptotic or toxic effect of the DP antagonist BWA868c as investigated using annexin-V/propidium iodide staining ($n = 6$, data not shown). This demonstrated that the reduced migratory response toward PGD_2 after pretreatment with the DP antagonist cannot be attributed to reduced cell viability. Thus, further studies are needed to define the mechanisms by which DP activation positively modulates the PGD_2-induced locomotion of eosinophils.

Eosinophil influx to sites of allergic reactions is associated with tissue injury and airway hyperresponsiveness (44). In animal models of allergy, mice genetically deficient in eosinophils show reduced tissue damage and airway hyperresponsiveness (45, 46). Asthmatic patients that had been medicated with respect to eosinophil counts in sputum have significantly fewer severe asthma exacerbations than patients receiving conventional therapy (47). The magnitude of eosinophil infiltration at inflammatory sites is determined both by the supply of eosinophils from the bone marrow and the rate of cell extravasation into the tissue. Our current data suggest that CRTH2 is involved in the rapid mobilization of eosinophils from the bone marrow. In addition, previous in vivo studies have also suggested a proinflammatory role for the second PGD_2 receptor, DP; antagonists of DP were found to block Ag-induced inflammation in the guinea pig (30), and DP-deficient mice responded to allergen challenge with reduced pulmonary inflammation (31). However, the pharmacological mechanisms that underlie these observations have remained unclear. Here we have demonstrated for the first time that eosinophils in the bone marrow and in peripheral blood express the DP receptor and that DP is substantially involved in eosinophil recruitment. This notion applies both to eosinophil release from the bone marrow and chemotaxis. Our current data provide novel insights into the roles of PGD_2 in eosinophil function and hence add further evidence for the potential usefulness of DP antagonists in the treatment of allergic disease.

Disclosures

The authors have no financial conflict of interest.

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