D-type prostanoid receptor enhances the signaling of chemoattractant receptor–homologous molecule expressed on T_H2 cells

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Background: Prostaglandin (PG) D_2 is substantially involved in allergic responses and signals through the 7 transmembrane–spanning/G protein–coupled receptors, chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2), and D-type prostanoid (DP) receptor.

Objective: Although the proinflammatory function of CRTH2 is well recognized and CRTH2 is hence considered an important emerging pharmacotherapeutic target, the role of the DP receptor in mediating the biological effects of PGD_2 in patients with allergic inflammation has remained unclear.

Methods: The cross-talk of CRTH2 and DP receptors was investigated by using both a recombinant HEK293 cell model and human eosinophils in Ca^{2+} mobilization assays, coimmunoprecipitation, Western blotting, radioligand binding, and immunofluorescence.

Results: We show that CRTH2 and DP receptors modulate one another's signaling properties and form CRTH2/DP heteromers without altering their ligand-binding capacities. We find that the DP receptor amplifies the CRTH2-induced Ca^{2+} release from intracellular stores and coincidentally forfeits its own signaling potency. Moreover, desensitization or pharmacologic blockade of the DP receptor hinders CRTH2-mediated signal transduction. However, CRTH2 internalization occurs independently of the DP receptor. In cells that express both receptors, pharmacologic blockade of $G\alpha_{q/11}$ proteins abolishes the Ca^{2+} response to both CRTH2 and DP agonists, whereas inhibition of $G\alpha_i$ proteins selectively attenuates the CRTH2-mediated response but not the DP signal.

Conclusion: Our data demonstrate the capacity of DP receptors to amplify the biological response to CRTH2 activation. Therefore the CRTH2/DP heteromer might not only represent a functional signaling unit for PGD_2 but also a potential target for the development of heteromer-directed therapies to treat allergic diseases. (J Allergy Clin Immunol 2012;129:492-500.)

Key words: *Chemoattractant receptor–homologous molecule expressed on T_H2 cells, eosinophils, prostaglandin D_2, Ca^{2+} flux, G protein–coupled receptor, D-type prostanoid receptor*

Prostaglandin (PG) D_2 has been shown to be involved in allergic inflammation[1-3] and is emerging as a useful therapeutic target in patients with allergic diseases.[4] PGD_2 signaling is mediated by two 7 transmembrane–spanning (7TM)/G protein–coupled receptors (GPCRs) referred to as chemoattractant receptor–homologous molecule expressed on Th2 cells (CRTH2) and D-type prostanoid (DP) receptor.

On activation, CRTH2 mediates the release of Ca^{2+} from intracellular stores and inhibits cyclic AMP formation through $G\alpha_i$ proteins,[5] whereas the activation of DP has been suggested to result in a $G\alpha_s$ protein–induced increase in cyclic AMP levels.[6] However, previous studies also report a DP-mediated increase in intracellular Ca^{2+} levels in heterologous expression systems.[7] CRTH2 is found on T_H2 cells, eosinophils, basophils, dendritic cells, monocytes, and macrophages[8-10] and accounts for many of the proinflammatory effects of PGD_2.[5,11-15] In animal models CRTH2 propagates eosinophil mobilization from the bone marrow[13] and their infiltration into the lungs and skin[16-18] and consequently exacerbates the late phase of allergic inflammation.

The DP receptor is expressed more widely, including eosinophils, basophils, dendritic cells, T_H1 and T_H2 cells, platelets, and the vasculature, central nervous system, retina, nasal mucosa, lungs, and intestine.[5,7,19-23] Both proinflammatory and anti-inflammatory effects have been reported for the DP receptor. For instance, DP is involved in asthma suppression *in vivo*[24,25] and induces inhibitory responses in various immune cells *in vitro*.[10,15,23,26-30] In contrast, DP has also been reported to have proinflammatory effects in animal models of allergic inflammation, and DP-deficient mice were found to exhibit reduced pulmonary inflammation in response to allergens.[31,32] On the basis of these findings, the particular role of DP is controversial, and its effect on immune cell function, particularly when coexpressed with CRTH2, is unclear.

Many 7TM/GPCRs have been reported to form homomers or heteromers, which have been shown to be functionally relevant both *in vitro*[33] and *in vivo*.[34-37] Importantly, receptor heteromerization can alter (1) binding properties of ligands and (2) their efficacy and potency, as well as (3) their selectivity for G proteins or (4) receptor trafficking.[34] To date, it is unknown whether the PGD_2 receptors form functional heteromers and whether this

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Abbreviations used
CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 cells
DK-PGD_2: 13, 14-Dihydro-15-keto-PGD_2
DP: D-type prostanoid
GPCR: G protein–coupled receptor
HRP: Horseradish peroxidase
PG: Prostaglandin
PMNL: Polymorphonuclear leukocyte
PTX: Pertussis toxin
7TM: 7 Transmembrane spanning

results in altered signaling properties, trafficking properties, or both of CRTH2, DP, or both.

Here we report that CRTH2 and DP receptors form heteromers and modulate one another's signaling properties. Our data show that the responsiveness of CRTH2 is profoundly amplified in the presence of the DP receptor and might thus represent a crucial determinant for proper CRTH2 signaling in cells coexpressing these PGD_2 receptors.

METHODS

DNA constructs and generation of stable HEK293 cell lines

The cDNA coding for the human DP receptor was cloned into the modified eukaryotic expression vector SS-Flag pcDNA3.1 (+) zeo[35] by using the restriction sites *Not*I and *Xba*I and verified by means of sequencing. The following oligonucleotide primers (Operon, Ebersberg, Germany) were used: reverse, 5′-GCG CCC TCT AGA TCA CAG ACT GGA TTC CAT-3′; forward, 5′-ATG ACG CCG CGG CCG CCA AGT CGC CGT-3′. An N-terminal SS-Myc–tagged version of the human CRTH2 receptor was cloned into the eukaryotic expression vector pcDNA3.1 (+) neo. To generate stable HEK293 cell lines expressing Flag-DP (HEK-DP), Myc-CRTH2 (HEK-CRTH2), or both receptors (HEK-CRTH2+DP), cells were transfected with pcDNAs encoding SS-Flag-DP, SS-Myc-CRTH2, or both. Single colonies were propagated in selection media (0.2 mg/mL of Zeocin, 0.2 mg/mL G418, or both) in Dulbecco modified Eagle medium supplemented with 10% FBS at 37°C and 5% CO_2. For further details on reagents, see the Methods section in this article's Online Repository at www.jacionline.org.

Purification of human blood eosinophils

Blood was sampled from healthy volunteers after obtaining informed consent according to a local ethics committee–approved protocol. Polymorphonuclear leukocytes (PMNLs) were prepared by means of dextran sedimentation of erythrocytes and centrifugation on Histopaque gradients as described previously.[13] Eosinophils were further purified by means of negative magnetic selection, yielding a purity of greater than 97%.

Western blotting and coimmunoprecipitation

Sample preparation. Membranes of HEK293 cells were prepared as described previously.[36] Briefly, cells were harvested in HME buffer (25 mmol/L HEPES-NaOH [pH 7.5], 2 mmol/L $MgCl_2$, and 1 mmol/L EDTA). Cell membranes were disintegrated by using freeze-thaw cycles with liquid nitrogen and homogenized by means of sonication (15 × 1 second at 25% for 2 cycles). Lysates of isolated eosinophils were prepared in lysis buffer with 0.1% Triton X-100.

Western blotting. Samples were resolved on a 16% Tris-glycine gel and transferred to a polyvinylidene difluoride membrane. The membranes were probed with rabbit anti-DP receptor (2.5 μg/mL) or rabbit anti-CRTH2 (1 μg/mL) antibodies. β-Actin blots were probed with murine β-actin mAb (1 μg/mL). Proteins were visualized with horseradish peroxidase (HRP)–conjugated secondary antibodies (4 μg/mL), followed by enhanced chemiluminescence.

Coimmunoprecipitation. Receptors were immunoprecipitated from HEK293 cell membranes with anti–Flag M2 murine mAb or anti-c-Myc rabbit affinity matrix at 4°C overnight. Samples were treated with PNGase F (for 1 hour at 37°C) and incubated with a reducing sample buffer (100 mmol/L Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 0.2% bromphenol blue, and 200 mmol/L dithiothreitol) for 5 minutes at 95°C. Proteins were separated and immunoblotted as described above. Receptors were visualized with enhanced chemiluminescence after sequential incubation with anti-Myc IgG_1 or anti–Flag M2 IgG_{2b} antibodies (2 μg/mL) and the HRP-conjugated secondary antibodies (4 μg/mL).

Intracellular Ca^{2+} release assays

96-Well FlexStation II assay. Agonist-mediated intracellular Ca^{2+} release was measured in a 96-well plate format (FlexStation II; Molecular Devices, Sunnyvale, Calif), as previously described.[37] Briefly, cells were seeded at a density of 40,000 cells per well. For gene-dose experiments, cells were transfected with 50 to 200 ng per well of pcDNA by using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were loaded with the Ca^{2+} fluorophore for 60 minutes. Intracellular Ca^{2+} mobilization was measured after agonist stimulation and recorded in relative fluorescence units.

Ca^{2+} flux flow cytometric assay. PMNLs were treated with 2 μmol/L of the acetoxymethyl ester of Fluo-3 in the presence of 0.02% pluronic F-127 for 60 minutes at room temperature. Neutrophils were labeled with anti-CD16 (PE Cy5) antibody for 10 minutes. Changes in intracellular free Ca^{2+} levels were detected after addition of agonist as increases in fluorescence intensity in the FL-1 channel of a FACSCalibur flow cytometer (BD Biosciences, Mountainview, Calif).

Statistical analysis

Data are shown as means ± SEMs for n observations. Statistical analysis was performed with *t* tests, 1-way ANOVA followed by Bonferroni *post hoc* analysis, or 2-way ANOVA for repeated measurements with the Dunnett *post hoc* test, where appropriate. *P* values of less than .05 were considered statistically significant. Median effective concentration values were analyzed by means of nonlinear regression with Prism 4.02 software (GraphPad Software, Inc, San Diego, Calif).

RESULTS

DP receptor modulates CRTH2-induced intracellular Ca^{2+} release

In human peripheral blood eosinophils we observed intracellular Ca^{2+} release after stimulation with PGD_2 or the CRTH2-selective agonist 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2; Fig 1, *A*), as we have shown in earlier studies.[38] Notably, the DP-selective agonist BW245c failed to invoke Ca^{2+} mobilization (Fig 1, *A*). To further specify the respective roles of CRTH2 and DP in Ca^{2+} signaling, we tested these agonists in HEK-CRTH2+DP, HEK-CRTH2, and HEK-DP cell lines. Generation of HEK293 cell lines coexpressing CRTH2 and DP receptors is described in the Results section and Fig E1 in this article's Online Repository at www.jacionline.org. We found that PGD_2 failed to induce intracellular Ca^{2+} release in HEK-CRTH2 cells but evoked potent Ca^{2+} responses in HEK-DP and HEK-CRTH2+DP cells (Fig 1, *B*). Similarly, the CRTH2-selective agonist DK-PGD_2 failed to induce Ca^{2+} release in HEK-CRTH2 cells, as in HEK-DP cells (Fig 1, *C*). However, DK-PGD_2 evoked Ca^{2+} responses in HEK-CRTH2+DP cells (Fig 1, *C*). PGD_2 (Fig 1, *B*) and BW245c (Fig 1, *D*) strongly induced Ca^{2+} mobilization