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RESEARCH PAPER

Co-operative signalling through DP_1 and DP_2 prostanoid receptors is required to enhance leukotriene C_4 synthesis induced by prostaglandin D_2 in eosinophils

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BACKGROUND AND PURPOSE
Prostaglandin (PG) D_2 has emerged as a key mediator of allergic inflammatory pathologies and, particularly, PGD_2 induces leukotriene (LT) C_4 secretion from eosinophils. Here, we have characterized how PGD_2 signals to induce LTC_4 synthesis in eosinophils.

EXPERIMENTAL APPROACH
Antagonists and agonists of DP_1 and DP_2 prostanoid receptors were used in a model of PGD_2-induced eosinophilic inflammation *in vivo* and with PGD_2-stimulated human eosinophils *in vitro*, to identify PGD_2 receptor(s) mediating LTC_4 secretion. The signalling pathways involved were also investigated.

KEY RESULTS
In vivo and *in vitro* assays with receptor antagonists showed that PGD_2-triggered cysteinyl-LT (cysLT) secretion depends on the activation of both DP_1 and DP_2 receptors. DP_1 and DP_2 receptor agonists elicited cysLTs production only after simultaneous activation of both receptors. In eosinophils, LTC_4 synthesis, but not LTC_4 transport/export, was activated by PGD_2 receptor stimulation, and lipid bodies (lipid droplets) were the intracellular compartments of DP_1/DP_2 receptor-driven LTC_4 synthesis. Although not sufficient to trigger LTC_4 synthesis by itself, DP_1 receptor activation, signalling through protein kinase A, did activate the biogenesis of eosinophil lipid bodies, a process crucial for PGD_2-induced LTC_4 synthesis. Similarly, concurrent DP_2 receptor activation used *Pertussis* toxin-sensitive and calcium-dependent signalling pathways to achieve effective PGD_2-induced LTC_4 synthesis.

CONCLUSIONS AND IMPLICATIONS
Based on pivotal roles of cysLTs in allergic inflammatory pathogenesis and the collaborative interaction between PGD_2 receptors described here, our data suggest that both DP_1 and DP_2 receptor antagonists might be attractive candidates for anti-allergic therapies.

LINKED ARTICLE
This article is commented on by Mackay and Stewart, pp. 1671–1673 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01236.x

Abbreviations

5-LO, 5-lipoxygenase; ADRP, adipose differentiation-related protein; CRTH2, chemoattractant receptor-homologous molecule expressed on T helper type 2 cell (Th2) cells; cysLTs, cysteinyl leukotrienes; DP_1, D prostanoid receptor 1; DP_2, D prostanoid receptor 2; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; LTC_4, leukotreine C_4; OVA, ovalbumin; PGD_2, prostaglandin D_2

Introduction

Eosinophil accumulation and subsequent activation at sites of allergic inflammation control the generation and release of diverse lipid and protein mediators critical to the development and perpetuation of allergic processes (Busse and Lemanske, 2001; Rothenberg and Hogan, 2006). Indeed, eosinophils represent a major source of leukotriene (LT) C_4, the intracellular parent of the cysteinyl LTs (cysLTs; $LTC_4/D_4/E_4$) (Weller *et al.*, 1983; Cowburn *et al.*, 1998; Bandeira-Melo and Weller, 2003). Central to the pathogenesis of allergic diseases, cysLTs cause bronchoconstriction, mucus hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, eosinophil infiltration and airway remodelling (Drazen and Austen, 1987). LTC_4, as the main eosinophil-derived product of the 5-lipoxygenase pathway of arachidonic acid metabolism in allergic diseases, is formed by the conjugation of LTA_4 with reduced glutathione by LTC_4 synthase. After active transport to the extracellular space, LTC_4 is converted to LTD_4 and LTE_4 through sequential enzymatic removal of glutamic acid and glycine. Even though the eosinophil LTC_4-synthesizing enzymatic pathways are well known (Bandeira-Melo *et al.*, 2002a), it remains of particular interest to fully characterize endogenous allergy-relevant stimuli, receptors and signalling pathways, as well as to understand the intracellular compartmentalization mechanisms that control allergen-induced, eosinophil-driven, LTC_4 synthesis.

Prostaglandin D_2 (PG) D_2 is another key lipid mediator of allergic airway inflammation that is produced following allergen exposure in patients with asthma, atopic dermatitis or allergic rhinitis. Similar to cysLTs, PGD_2 can mimic a number of important features of allergic processes (Pettipher, 2008). In regard to the sensitization phase of an allergic response, it has been postulated that PGD_2 modulates cytokine production by dendritic cells, leading to the polarization of T helper type 2 cell (Th2) cells (Theiner *et al.*, 2006; Hammad *et al.*, 2007). In addition to its immuno-regulatory role, PGD_2 is a highly effective trigger of blood flow changes, promoting oedema formation and therefore contributing to the nasal congestion symptom of allergic rhinitis (Doyle *et al.*, 1990; Widdicombe, 1990). PGD_2 also mediates the typical cell accumulation of late phase allergic responses by functioning as a selective chemoattractant for Th2 cells, basophils and eosinophils (Hirai *et al.*, 2001; Monneret *et al.*, 2001).

The immuno-modulatory and inflammatory functions of PGD_2 are mediated by high-affinity interaction with two receptors, the prostanoid DP_1 receptor and the chemoattractant receptor-homologous molecule expressed on Th2 cells receptor, now referred to as the DP_2 receptor (receptor nomenclature follows Alexander *et al.*, 2009). Although DP_1 and DP_2 receptors bind the same ligand, there is very little homology between the two receptors; DP_1 receptors being members of the prostanoid receptor family that includes $EP_{1\text{-}4}$, FP, IP and TP, whereas the DP_2 receptors are more closely related to other chemotactic receptors such as the LTB_4 receptors, BLT1 and BLT2, and the C5a receptor (Pettipher, 2008). Furthermore, while DP_1 receptors are coupled to $G\alpha_s$ proteins and signal through elevation of intracellular levels of cAMP, the DP_2 receptors are coupled to $G\alpha_i$ and their activation leads to the elevation of intracellular calcium, reduction in cAMP (Sawyer *et al.*, 2002) and downstream activation of phosphatidylinositol-3-kinase (PI3K) (Xue *et al.*, 2007).

Eosinophils co-express the classical DP_1 receptors coupled to adenylyl cyclase, as well as the *Pertussis* toxin (PTX)-sensitive DP_2 receptors (Monneret *et al.*, 2001). Although the ability of PGD_2 to activate eosinophils while concurrently elevating cAMP levels seems paradoxical, DP_1 receptors appear to be able to co-mediate, with DP_2 receptors, the mobilization of eosinophils from bone marrow as well as chemotaxis (Schratl *et al.*, 2007). Alternatively, it has also been shown that the final chemotaxis-related response of eosinophils to PGD_2 may be, ultimately, determined by a balance between two opposing downstream signalling pathways: the cAMP-dependent, inhibitory pathway activated via DP_1 receptors and the prevailing, stimulatory pathway activated via DP_2 receptors (Monneret *et al.*, 2001; Kostenis and Ulven, 2006; Sandig *et al.*, 2007).

Recently, we have shown that, in addition to its chemotactic activity towards eosinophils, PGD_2 controls allergy-relevant eosinophil activation, particularly the increased LTC_4 synthesizing capacity of these cells (Mesquita-Santos *et al.*, 2006). Indeed, other eosinophil chemoattractants, including eotaxin (CCL11), RANTES (CCL5) and platelet activating factor (PAF) are capable of triggering LTC_4 synthesis within eosinophils through the activation of their cognate $G\alpha_i$ -coupled chemotactic receptors, such as CCR3 (Bozza *et al.*, 1996; Bandeira-Melo *et al.*, 2001). Therefore, we had initially hypothesized that PGD_2-induced LTC_4 synthesis could be mediated by the stimulatory activation of DP_2 receptors while being counter-balanced by a parallel inhibitory cAMP-dependent DP_1 receptor activation. However, here by employing pharmacological strategies, we have uncovered a novel kind of interaction between the PGD_2 receptor types expressed on eosinophils. In contrast to the PGD_2-driven opposing signalling related to eosinophil chemotactic activities, eosinophil LTC_4 synthesis triggered by PGD_2 appeared to be controlled by the complementary stimulatory events between DP_1 receptor-activated, PKA-driven, lipid bodies and concurrent DP_2 receptor signalling. While PGD_2 emerges as a potent inflammatory mediator of allergic disorders and as an interesting therapeutic target, because of the mandatory dual activation of DP_1 and DP_2 receptors for increasing eosinophil LTC_4 synthesis, either DP_1 or DP_2 receptor antagonists might be highly effective candidates as anti-allergic tools to control cysLTs production regulated by the activation of eosinophils at sites of allergic reactions.

Methods

Animals

All animal care and experimental protocols were approved by Oswaldo Cruz Foundation Animal Welfare Committee. For *in vivo* experiments, male Swiss mice of 16–20 g were obtained from Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil).

PGD_2-induced and allergic pleurisy in sensitized mice

As previously described (Mesquita-Santos *et al.*, 2006), mice were sensitized with an s.c. injection (0.2 mL) of ovalbumin (OVA; 50 μg; Sigma, St. Louis, MO, USA) and $Al(OH)_3$ (5 mg) in 0.9% NaCl solution (saline) at days 1 and 7. Allergic challenge was performed at day 14 by means of an intrapleural injection of OVA (12 μg per cavity; 0.1 mL). Alternatively, sensitized mice were challenged with PGD_2 (35 pmol per cavity), BW245C (35 pmol per cavity), DK-PGD_2 (35 pmol per cavity) or a combination of the latter two agonists (both at 35 pmol per cavity) (all from Cayman Chemicals, Ann Arbor, MI, USA). All stimuli were diluted in sterile saline immediately before use. Control animals were injected with the same volume (0.1 mL) of vehicle. Mice were killed by CO_2 inhalation 24 h after challenge. Pleural fluid was obtained by rinsing cavities with 1 mL of phosphate-buffered saline containing BSA (0.1%). After samples were taken for lipid body analysis, pleural fluid was centrifuged and cell free supernatants were used for the quantification of cysLTs.

Isolation and in vitro *stimulation of human eosinophils*

Peripheral blood was obtained with informed consent from healthy donors under protocols approved by the ethical review boards of both the Federal University of Rio de Janeiro and the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Eosinophils were isolated by negative selection using EasySep™ system (StemCell Technologies Inc., Vancouver, Canada) (purity ~98%; viability ~95%) (Bezerra-Santos *et al.*, 2006). Purified eosinophils (2×10^6 cells·mL^{-1}) in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} ($HBSS^{-/-}$) were incubated for 1 h in a water bath (37°C) with PGD_2 (25 nM), BW245C (5–625 nM), DK-PGD_2 (5–625 nM), a combination of BW245C and DK-PGD_2 (both at 25 nM) or PAF (10 μM; from Cayman Chemicals). After samples were taken for lipid body analyses, eosinophils were resuspended in Hank's balanced salt solution with Ca^{2+} and Mg^{2+} ($HBSS^{+/+}$) and stimulated with 0.1 μM A23187 (Sigma) for another 15 min (37°C). Cell-free supernatants were then collected for cysLT quantification. Each *in vitro* experiment was repeated at least three times, with eosinophils purified from different donors.

Treatments

Using the pleurisy models, animals were pretreated with i.p. injections of the DP_1 receptor antagonist BWA868C (1 mg·kg^{-1}; Cayman Chemicals), the dual DP_2/TP receptor antagonist ramatroban (also known as Bay-u3405; 1 mg·kg^{-1}; Cayman Chemicals) or the selective DP_2 receptor antagonist Cay10471 (1 mg·kg^{-1}; Cayman Chemicals) 30 min before either PGD_2 or allergic challenges.

For *in vitro* studies, eosinophils in $HBSS^{-/-}$ were pretreated for 30 min with the DP_1 receptor antagonist BWA868C (200 nM), the dual DP_2/TP receptor antagonist ramatroban (200 nM), the selective DP2 antagonist Cay10471 (200 nM), two PKA inhibitors H-89 and PKI (both at 10 μM; Calbiochem, La Jolla, CA, USA); PTX (1 μg·mL^{-1}; Calbiochem) or cell-permeable calcium chelator BAPTA-AM (25 μg·mL^{-1}; Sigma). Of note, these pretreatments did not modify basal lipid body content found within cytoplasm of non-stimulated eosinophils or affected eosinophil viability (~90%) (data not shown).

Quantification of cysLTs

The amount of cysLTs found in cell-free pleural fluid and eosinophil supernatants was measured by the Cysteinyl Leukotriene EIA kit (catalog number 520501; from Cayman Chemicals), according to the manufacturer's instructions.

EicosaCell for intracellular LTC_4 immuno-detection

As previously described (Bandeira-Melo *et al.*, 2001) to localize LTC_4 at its sites of synthesis, *in vitro* stimulated eosinophils were mixed with a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC; 0.1% final concentration with cells in $HBSS^{-/-}$), used to crosslink eicosanoid carboxyl groups to amines in adjacent proteins. After 15 min incubation at room temperature with EDAC to promote both cell fixation and permeabilization, eosinophils were then washed with $HBSS^{-/-}$, cytospun onto glass slides and blocked with $HBSS^{-/-}$ containing 1% BSA for 30 min. Cells were incubated with rabbit anti-LTC_4 antibodies (Cayman Chemicals) or non-immune rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) overnight. Routinely, cells were co-incubated with guinea pig anti-adipose differentiation-related protein (ADRP) antibody (1:300 dilution; Fitzgerald Industries, North Acton, MA, USA) to distinguish cytoplasmic lipid bodies within eosinophils. The cells were washed 3× from 10 min with $HBSS^{-/-}$ containing 1% BSA and incubated with Alexa488-labelled anti-rabbit IgG and Alexa546-labelled anti-guinea pig secondary antibodies for 1 h.

The specificity of the LTC_4 immuno-labelling was confirmed by the: (i) lack of immunofluorescence within PGD_2-stimulated human eosinophils incubated with irrelevant IgG (data not shown); and (ii) lack of LTC_4 immuno-labelling within non-stimulated human eosinophils that were incubated with anti-LTC_4 antibody.

Images were obtained using an Olympus BX51 fluorescence microscope at 100× magnification and photographs were taken with the Olympus 72 digital camera (Olympus Optical Co., Tokyo, Japan) in conjunction with Cell^F Imaging Software for Life Science Microscopy (Olympus Life Science Europa GMBH, Hamburg, Germany). The images were edited using Adobe Photoshop 5.5 software (Adobe Systems, San Jose, CA, USA).

Lipid body staining and enumeration

To enumerate lipid bodies, cells recovered from pleural cavities or human eosinophils were cytocentrifuged (450 rpm, 5 min) onto glass slides. Cells, while still moist, were fixed in 3.7% formaldehyde (in $HBSS^{-/-}$; pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 (Sigma)

for 30 min, rinsed in distilled H_2O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO_4 for 3 min, rinsed in distilled water, and then air dried and analysed. Cell morphology was observed and lipid bodies were enumerated by light microscopy. Fifty consecutively scanned eosinophils were evaluated by more than one individual and results were expressed as the number of lipid bodies *per* eosinophil.

Statistical analysis

Results are expressed as mean ± SEM and were analysed statistically by means of analysis of variance followed by the Newman–Keuls Student test, with the level of significance set at $P < 0.05$.

Results

Both DP_1 and DP_2 receptors contribute to PGD_2-induced enhanced LTC_4 secretion from eosinophils

We have reported that PGD_2, formerly recognized only as an eosinophil chemoattractant, is also able to trigger eosinophil activation, characterized by enhanced LTC_4 synthesis (Mesquita-Santos *et al.*, 2006). Here, by employing a mouse model of PGD_2-induced eosinophil activation *in vivo* (Mesquita-Santos *et al.*, 2006), as well as human-purified eosinophils stimulated *in vitro* with PGD_2, we investigated the molecular and cellular mechanisms involved in PGD_2-induced LTC_4 synthesis within eosinophils. Our initial goal was to identify the specific PGD_2 receptor, either DP_1 or DP_2, the two well recognized, cloned and eosinophil-expressed receptors for PGD_2 that was involved in this response. *In vivo,* as illustrated in Figure 1, both PGD_2 receptors appeared to contribute to the LTC_4 secretion from eosinophils, inasmuch as pretreatment with either the DP_1 receptor antagonist BWA868C or the dual DP_2/TP receptor antagonist ramatroban displayed high levels of inhibition. BWA868C and ramatroban significantly reduced LTC_4 secretion levels found increased in sites of PGD_2-triggered eosinophilic inflammation by 63 and 81%, respectively (Figure 1A). Similarly, Figure 1B shows that increased amounts of LTC_4 detected in the supernatants of *in vitro* PGD_2-stimulated human eosinophils were also inhibited by either the DP_1 receptor antagonist BWA868C, the dual DP_2/TP receptor antagonist ramatroban or by the selective DP_2 antagonist Cay10471 (83, 96 and 89% inhibition, respectively), indicating essential roles for each receptor type, which in turn, perhaps by acting synergistically, evoke PGD_2-driven secretion of LTC_4 from eosinophils. Of note, by showing that (i) pretreatments with BWA868C and Cay10471 did not interfere with PAF-induced LTC_4 synthesis by human eosinophils (Table 1), or (ii) the pretreatment with the selective TP antagonist SQ29548 failed to alter LTC_4 synthesis triggered by *in vitro* stimulation of eosinophils (Figures 1B; 10% inhibition), respectively, we excluded potential non-specific effects of PGD_2 receptor antagonists and demonstrated that the inhibitory effects of ramatroban were dependent on DP_2, rather than TP receptor antagonism.

DP_1 and DP_2 receptors cooperate to trigger PGD_2-driven enhanced LTC_4 secretion from eosinophils

To test the hypothesis that a synergistic mechanism of action between the PGD_2 receptors, DP_1 and DP_2, controls PGD_2-driven induction of LTC_4 secretion from eosinophils, selective agonists of DP_1 receptors (BW245C) or of DP_2 receptors (DK-PGD_2) were applied to *in vivo* and *in vitro* systems of eosinophil activation. Figure 2 shows that, alone, neither BW245C nor DK-PGD_2 was able to elicit LTC_4 release from eosinophils at concentrations that PGD_2 by itself is able to trigger LTC_4 secretion both *in vivo* (35 pmol per cavity; Figure 2A) and *in vitro* (25 nM; Figure 2B). In contrast, by co-stimulating

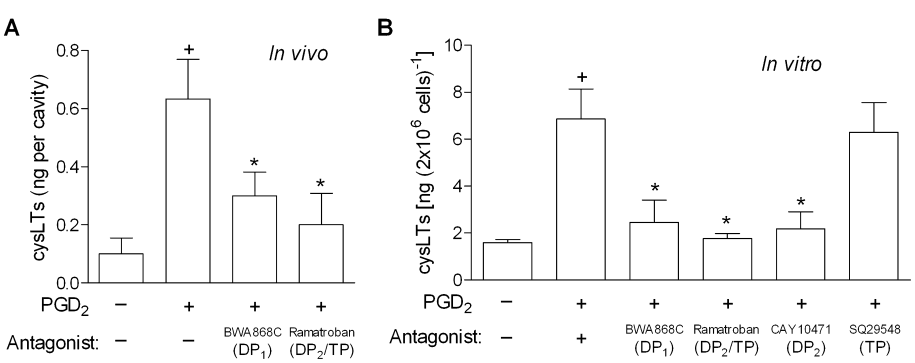

Figure 1

Both DP_1 and DP_2 receptors control cysLTs production triggered by PGD_2. In A, sensitized mice were pretreated with BWA868C (1 mg·kg^{-1}) or ramatroban (1 mg·kg^{-1}) and then stimulated with an i.pl. injection of PGD_2 (35 pmol per cavity). Analysis of cysLTs synthesis was performed 24 h after PGD_2 administration. Results are expressed as the means ± SEM from at least six animals. $^{\dagger}P \leq 0.05$ compared with control animals and $^{*}P \leq 0.05$ compared with PGD_2-injected mice. In B, for *in vitro* analysis of LTC_4 synthesis, human eosinophils were pretreated for 30 min with BWA868C (200 nM), ramatroban (200 nM), Cay10471 (200 nM) or SQ29548 (200 nM), and then stimulated for 1 h with PGD_2 (25 nM). *In vitro* results are expressed as the means ± SEM from at least three independent experiments with eosinophils purified from different donors. $^{\dagger}P \leq 0.05$ compared with control. $^{*}P \leq 0.05$ compared with PGD_2-stimulated eosinophils. cysLT, cysteinyl leukotriene; DP_1, D prostanoid receptor 1; DP_2, D prostanoid receptor 2; i.pl., intrapleural; PGD_2, prostaglandin D_2.

Table 1

PAF-induced lipid body biogenesis and LTC_4 synthesis by human eosinophils are dependent on PTX-sensitive protein $G\alpha_i$ and on intracellular calcium mobilization, but not on activation of DP_2 receptors or PKA activity

Conditions	Treatments	Lipid bodies/ eosinophil	cysLTs (ng/2 × 10^6 cells)
Control		7.7 ± 0.4	1.1 ± 0.6
PAF		16.4 ± 1.4†	4.4 ± 1.4†
	+BWA868C	16.9 ± 1.7	4.2 ± 1.3
	+CAY10471	16.7 ± 1.6	4.5 ± 1.2
	+H-89	13.6 ± 2.3	4.5 ± 1.6
	+PTX	10.5 ± 1.4*	1.6 ± 0.8*
	+BAPTA-AM	8.6 ± 0.5*	1.5 ± 0.8*

†$P \leq 0.05$ compared with control group. *$P \leq 0.05$ compared with PAF-stimulated eosinophils.
Human eosinophils were pretreated for 30 min with BWA868C (200 nM), CAY10471 (200 nM), H-89 (10 μM), *Pertussis* toxin (PTX; 1 μg·mL^{-1}) or BAPTA-AM (25 μM), and stimulated with PAF (10 μM). Analysis of lipid body biogenesis and LTC_4 synthesis were performed 1 h after PAF stimulation. Results were expressed as the means ± SEM from at least three different experiments.
cysLT, cysteinyl leukotriene; DP_2, D prostanoid receptor 2; PAF, platelet activating factor.

eosinophils with a mixture of DP_1 and DP_2 agonists, increased cysLTs levels were detected at eosinophilic inflammatory sites (Figure 2A) and human eosinophil-free supernatants (Figure 2B), with comparable magnitude to those found *in vivo* (Figure 2A) or *in vitro* (Figure 2B) stimulation with PGD2, reinforcing the possibility of a synergism between DP_1 and DP_2 receptors.

Cooperation between DP_1 and DP_2 receptors controls LTC_4 synthesis within eosinophil cytoplasmic lipid bodies

Stimulus-dependent extracellular detection of cysLTs depends on sequential intracellular events, including: (i) an initial step of activation and proper compartmentalization of both substrate and enzymatic machinery, culminating in intracellular LTC_4 synthesis; (ii) intracellular transport to direct newly synthesized LTC_4 to secretory pathways; that is followed by (iii) an active carrier-dependent LTC_4 release through the plasma membrane (see Bandeira-Melo *et al.*, 2002a). Therefore, as virtually no cysLTs can be detected in cell-free supernatants of BW245C- or DK-PGD_2-stimulated eosinophils, one can argue that, under these stimulatory conditions, the intracellular step of LTC_4 synthesis actually occurs, without the subsequent LTC4 transport/release events, which would only follow after the co-stimulation of both PGD_2 receptors. By employing EicosaCell technology, a microscopic method that immobilizes and immuno-detects newly synthesized lipid mediators at their sites of synthesis, we have excluded this hypothesis by showing that LTC_4 synthesis itself is elicited

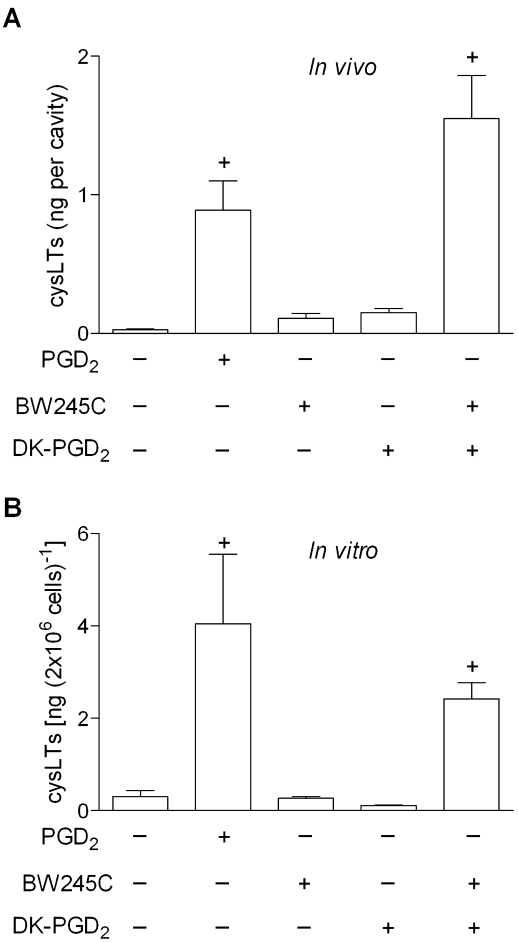

Figure 2

DP_1 and DP_2 receptors cooperate to trigger cysLTs production. In A, sensitized mice received an i.pl. injection of PGD_2 (35 pmol per cavity), BW245C (35 pmol per cavity), DK-PGD_2 (35 pmol per cavity) or BW245C plus DK-PGD_2 (both at 35 pmol per cavity). Analysis of cysLTs production within pleural fluids was performed 24 h after i.pl. administration. Results are expressed as the means ± SEM from at least six animals. †$P \leq 0.05$ compared with control animals. In B, for *in vitro* analyses of LTC_4 production in cell-free supernatants, human eosinophils were stimulated for 1 h with PGD_2 (25 nM), BW245C (25 nM), DK-PGD_2 (25 nM) or with a combination of BW245C plus DK-PGD_2 (both at 25 nM). *In vitro* results are expressed as the means ± SEM from at least three independent experiments with eosinophils purified from different donors. †$P \leq 0.05$ compared with control. $P \leq 0.05$ compared with PGD_2-stimulated eosinophils. cysLT, cysteinyl leukotriene; DP_1, D prostanoid receptor 1; DP_2, D prostanoid receptor 2; i.pl., intrapleural; PGD_2, prostaglandin D_2.

in vitro within human eosinophils only if both DP_1 and DP_2 receptors are simultaneously engaged by either PGD_2 itself (Figure 3; top panel) or by the combination of both PGD_2 receptor agonists BW245C and DK-PGD_2 (Figure 3; botton panel). Eosinophils activated only with DP1 or DP2 agonists show no immuno-fluorescent LTC_4 (Figure 3; middle panels) in EicosaCell preparations, indicating that the LTC_4 synthesizing machinery was indeed not activated under these conditions of stimulation of a single PGD_2 receptor.

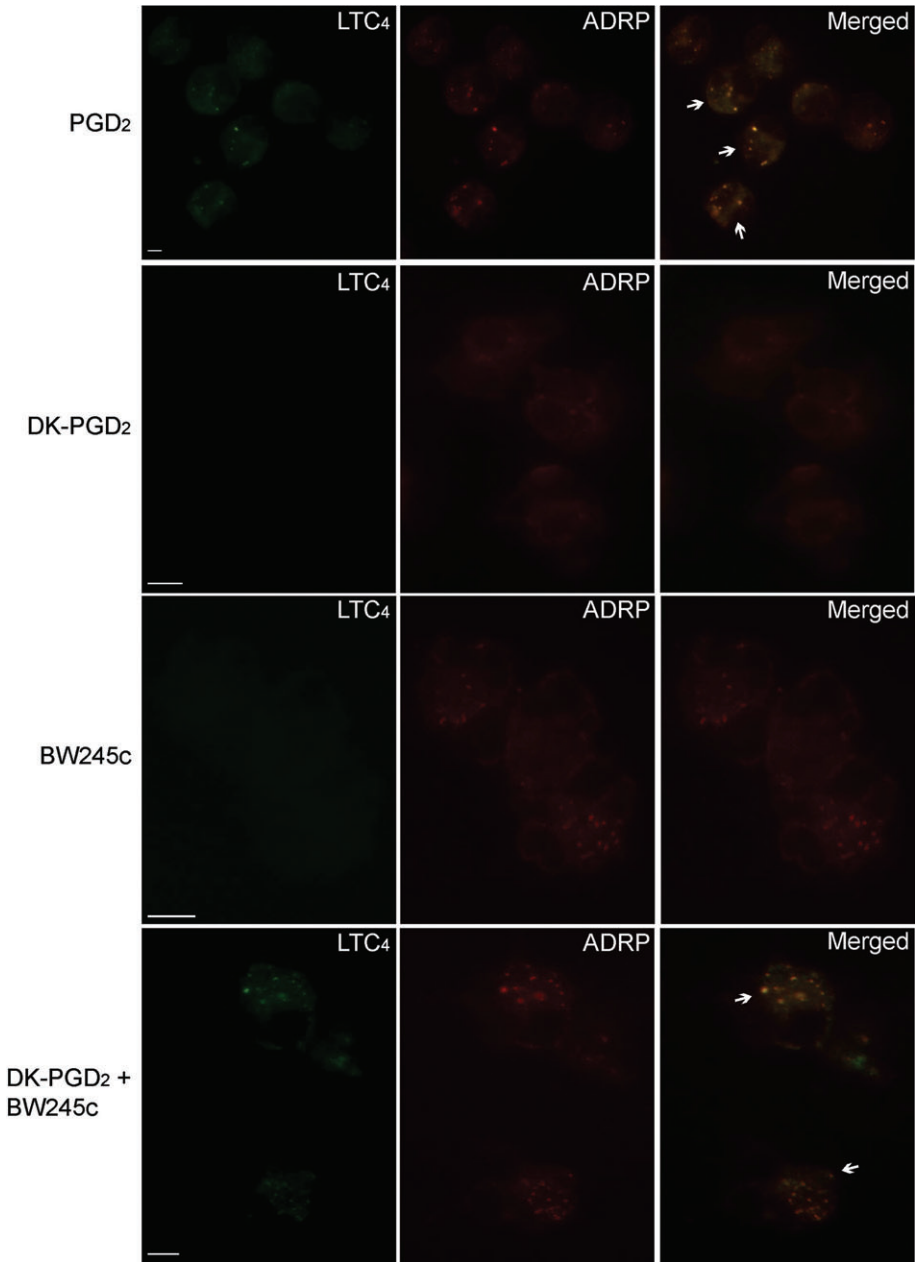

Figure 3

LTC_4 synthesis is triggered within eosinophil cytoplasmic lipid bodies by simultaneous activation of DP_1 and DP_2 receptors by either PGD_2 or the combination of BW245C and DK-PGD_2 stimulation of human eosinophils *in vitro*. EicosaCell images illustrate intracellular immuno-detection of newly formed LTC_4 (green) and of ADRP (red) in PGD_2-stimulated, BW245-stimulated, DK-PGD_2-stimulated or BW245C/DK-PGD_2 co-stimulated human eosinophils (as indicated). Overlay images of identical fields are shown in the right column. Arrows indicate co-localization of immuno-labelled synthesized LTC_4 with ADRP-bearing lipid bodies. For EicosaCell analyses, cells were fixed and permeabilized with EDAC and sequentially incubated with anti-LTC_4 and anti-ADRP antibodies and Alexa488-labelled anti-rabbit IgG plus Alexa546-labelled anti-guinea pig secondary antibodies. Images are representative of three independent experiments. ADRP, adipose-differentiation-related protein; DP_1, D prostanoid receptor 1; DP_2, D prostanoid receptor 2; PGD_2, prostaglandin D_2.

A more detailed analysis of these preparations revealed that the intracellular LTC_4-synthesizing compartment within either PGD_2- or BW245C/DK-PGD_2-stimulated eosinophils was in a punctate cytoplasmic pattern, proximate to, but separate from the nucleus, and fully consistent in size and form with eosinophil lipid bodies. In fact, the compartmentalization of newly formed LTC_4 to eosinophil lipid bodies was confirmed by their co-localization with ADRP (Figure 3; top and bottom panels), a lipid body marker protein. Virtually no LTC_4 was immuno-localized within non-stimulated eosinophils (not shown), thus showing that the newly formed lipid bodies of *in vitro* DP_1/DP_2-engaged eosinophils

are the enzymatically fully equipped organelles responsible for the effective LTC_4 synthesis. Of note, eosinophils stimulated solely with BW245C, but not with DK-PGD_2, display a lipid body-enriched cytoplasm as detected by the punctate cytoplasmic ADRP immuno-labelling (Figure 3; middle panel as indicated), suggesting that the initially hypothesized synergistic effect at receptor level between DP_1 and DP_2 does not take place. Instead, PGD_2-induced LTC_4 synthesis by eosinophils appears to be due to distinct mechanistic roles of DP_1 and DP_2 receptors with complementary functional features.

DP_1 activation, but not that of DP_2, evokes biogenesis of eosinophil lipid bodies

To study whether the induction of lipid body assembly driven by DP_1-receptor pathways contributed to PGD_2-induced LTC_4 synthesis, we employed an osmium-based staining methodology that allows the enumeration of these organelles to check for lipid body biogenesis, under either single or combined receptor stimulation. In agreement with EicosaCell images shown previously (Figure 3), Figure 4 shows that selective activation of DP_1 receptors by BW245C triggers rapid (within 1 h) assembly of new lipid bodies within human eosinophils in a dose-dependent manner. DP_2 receptor activation by DK-PGD_2, on the other hand, even when used in high concentrations (Figure 4B) failed to induce eosinophil lipid body biogenesis. No alteration of DP_1 receptor-induced lipid body biogenesis was observed when eosinophils were co-stimulated with both DP_1 and DP_2 receptor agonists (Figure 4A), suggesting that only the DP_1 receptor controls the formation of these LTC_4-synthesizing organelles.

To further investigate the role of the DP_1 receptor on lipid body biogenesis, we evaluated the participation of each receptor on PGD_2-induced lipid body formation (osmium-stained cells) by pretreating PGD_2-challenged sensitized mice (Figure 5A) and PGD_2-stimulated human eosinophils (Figure 5B) with either DP_1 or DP_2 receptor antagonists. *In vivo*, while the pretreatment with the DP_2 receptor antagonist ramatroban did not affect the number of cytoplasmic lipid bodies found within infiltrating eosinophils of PGD_2-elicited inflammatory reaction, pretreatment with the DP_1 antagonist BWA868C nearly abolished this *in vivo* biogenic process. Similarly, *in vitro* pre-treatment of human eosinophils with the DP_1 receptor antagonist BWA868C significantly inhibited PGD_2-induced eosinophil lipid body biogenesis, while two different DP_2 receptor antagonists, ramatroban and Cay10471, failed to modify the lipid body assembly triggered by PGD_2. Thus, it appears reasonable to postulate that the main role of DP_1 receptors in PGD_2-induced LTC_4 synthesis is to initiate the intracellular signalling pathway that leads to the biogenesis of LTC_4 synthesizing organelles in eosinophils.

DP_1 receptors signal via PKA activation to trigger eosinophil lipid body biogenesis: a requirement for PGD_2-induced LTC_4 synthesis

To further evaluate the role of DP_1 receptors in PGD_2-induced lipid body-driven LTC_4 synthesis, we have studied the contribution of DP_1 receptor-related cAMP-dependent signalling on PGD_2-induced assembly of new lipid bodies within eosinophils. As shown in Figure 6, *in vitro* lipid body assembly

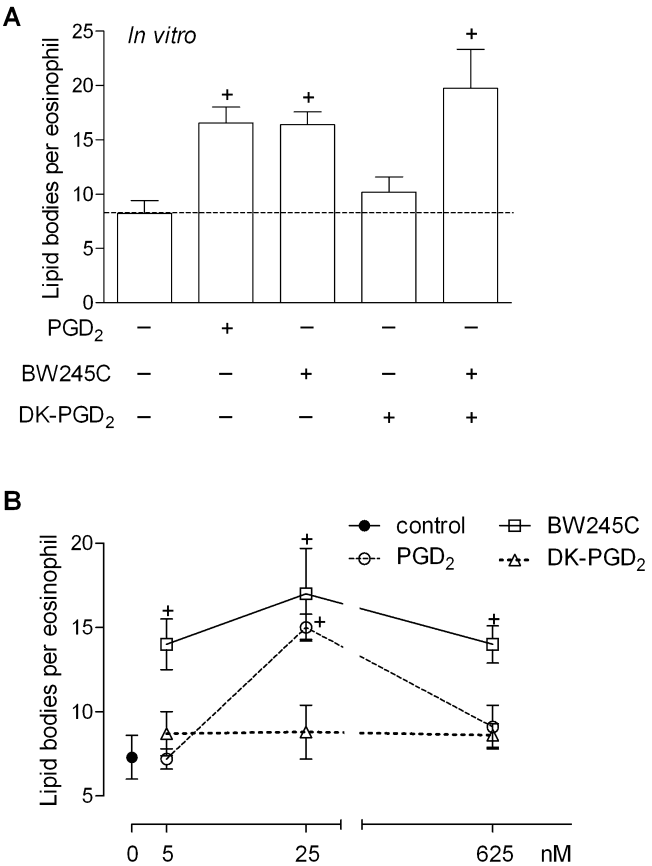

Figure 4

DP_1, but not DP_2, activation triggers lipid body biogenesis within human eosinophils *in vitro*. In A, human eosinophils were stimulated with PGD_2 (25 nM), BW245C (25 nM), DK-PGD_2 (25 nM) or with a combination of BW245C plus DK-PGD_2 (both at 25 nM). B shows a dose-response effect of PGD_2 (25 nM), BW245C (25 nM) or DK-PGD_2 (25 nM) on lipid body biogenesis after stimulation of human eosinophils. Analysis of lipid body biogenesis was performed 1 h after stimulation in osmium-stained cells. Results are expressed as means ± SEM from at least three different experiments with eosinophils purified from distinct donors. $^{\dagger}P \leq 0.05$ compared with control. DP_1, D prostanoid receptor 1; DP_2, D prostanoid receptor 2; PGD_2, prostaglandin D_2.

triggered by stimulation with either PGD_2 (Figure 6A) or BW245C (Figure 7) was consistent with the central role of DP_1 receptors, as pretreatment with H-89 or PKI (10 μM), two non-structurally related inhibitors of PKA activation, decreased the numbers of cytoplasmic lipid bodies found within PGD_2- (Figure 6A; bottom panel) and BW245C-stimulated human eosinophils (Figure 7). While the specificity of PKA involvement in DP_1 receptor-driven effect was strengthened by the lack of effect of H-89 on PAF-induced eosinophil lipid body biogenesis (Table 1), the ability of forskolin, a well-known activator of adenylate cyclase, to trigger rapid formation of lipid bodies within human eosinophils substantiates the role of cAMP/PKA signalling pathway on the regulation of lipid body biogenic process (C. Bandeira-Melo, unpubl. data).

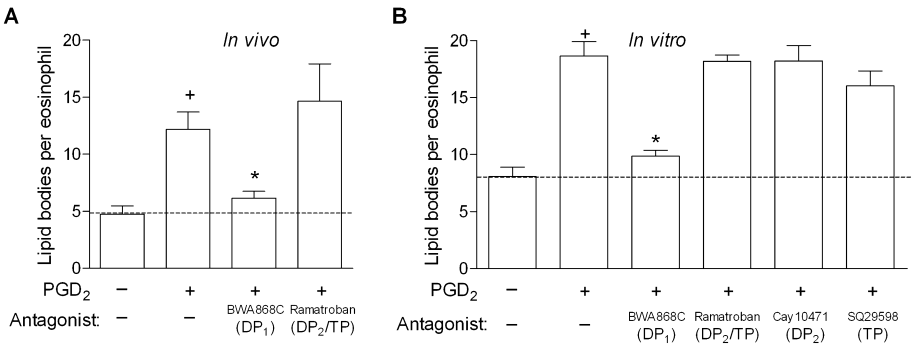

Figure 5

DP$_1$, but not DP$_2$ receptors, control eosinophil lipid body biogenesis triggered by PGD$_2$ either *in vivo* or *in vitro*. In A, sensitized mice were pretreated with BW868c (1 mg·kg^{-1}) or ramatroban (1 mg·kg^{-1}), and then stimulated with an i.pl. injection of PGD$_2$ (35 pmol/cavity). Analysis of lipid body biogenesis was performed 24 h after PGD$_2$ administration in osmium-stained cells. Results are expressed as means ± SEM from at least six animals. $^{\dagger}P \leq 0.05$ compared with control animals and $*P \leq 0.05$ compared with PGD$_2$-injected mice. In B, for *in vitro* analysis of lipid body biogenesis, human eosinophils were pretreated for 30 min with BW868c (200 nM), ramatroban (200 nM), Cay10471 (200 nM) or SQ29548 (200 nM), stimulated for 1 h with PGD$_2$ (25 nM) and subsequently stained with osmium. *In vitro* results are expressed as the means ± SEM from at least three different experiments with eosinophils purified from distinct donors. $^{\dagger}P \leq 0.05$ compared with control. $*P \leq 0.05$ compared with PGD$_2$-stimulated eosinophils. DP$_1$, D prostanoid receptor 1; DP$_2$, D prostanoid receptor 2; i.pl., intrapleural; PGD$_2$, prostaglandin D$_2$.

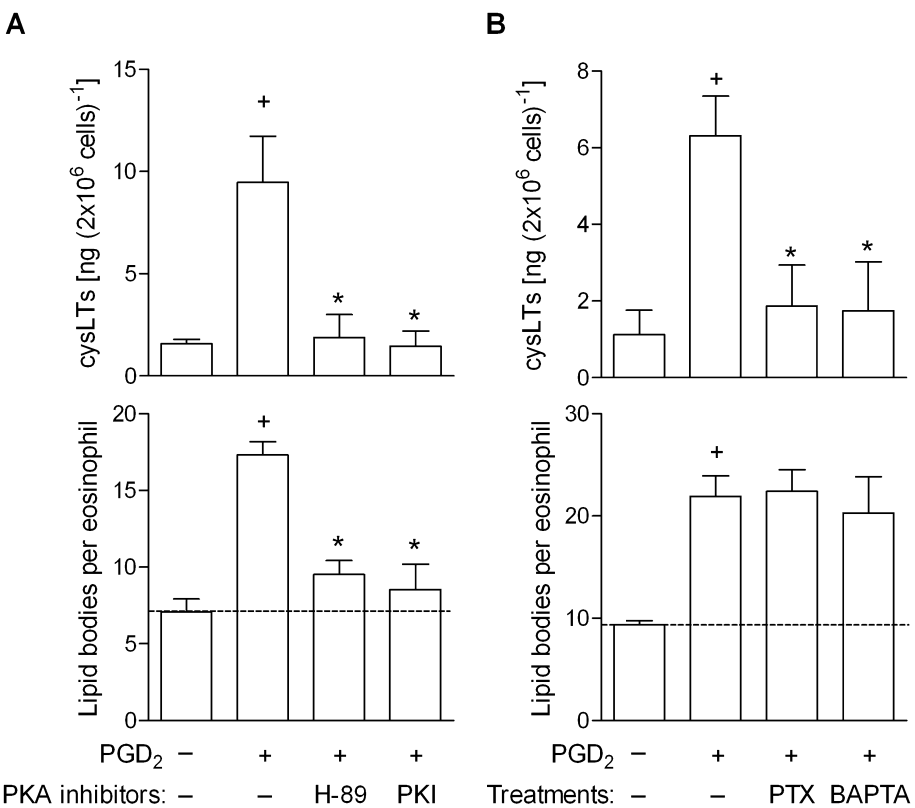

Figure 6

DP$_1$ receptor-driven PKA activation cooperates with DP$_2$-driven Gα_i protein activation and calcium influx to mediate lipid body-driven LTC$_4$ sytnhesis within human eosinophils triggered by *in vitro* PGD$_2$. Human eosinophils were pretreated for 30 min with H-89 (10 μM) and PKI (10 μM) in A, or with PTX (1 μg·mL^{-1}) or BAPTA-AM (25 μg·mL^{-1}) in B and then stimulated with PGD$_2$ (25 nM). *In vitro* analysis of LTC$_4$ production in cell-free supernatants and lipid body biogenesis were analysed 1 h after PGD$_2$. Results are expressed as the means ± SEM from at least three different experiments with eosinophils purified from different donors. $^{\dagger}P \leq 0.05$ compared with control group. $*P \leq 0.05$ compared with PGD$_2$-stimulated eosinophils. DP$_1$, D prostanoid receptor 1; DP$_2$, D prostanoid receptor 2; PGD$_2$, prostaglandin D$_2$; PTX, *Pertussis* toxin.

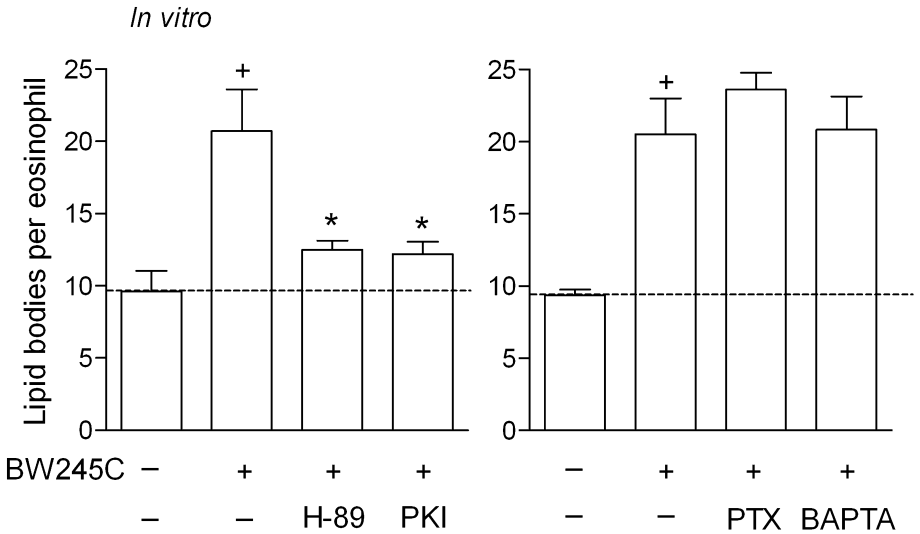

Figure 7

PKA activation, but not $G\alpha_i$ protein and calcium influx, mediates lipid body biogenesis within human eosinophils triggered by BW245C *in vitro*. Human eosinophils were pretreated for 30 min with PTX (1 $\mu g \cdot mL^{-1}$), BAPTA-AM (25 $\mu g \cdot mL^{-1}$), H-89 (10 μM) or PKI (10 μM), and then stimulated with BW245C (25 nM). Lipid body biogenesis was analysed 1 h after BW245C stimulation. Results are expressed as the means ± SEM from at least three different experiments with eosinophils purified from different donors. $^{\dagger}P \leq 0.05$ compared with control group. $^{*}P \leq 0.05$ compared with PGD_2-stimulated eosinophils. PTX, *Pertussis* toxin.

In agreement with DP_1-driven induction of new lipid bodies as a prerequisite to concurrent DP_2-elicited signalling required for successful LTC_4 synthesis, PKA inhibition by either H-89 or PKI pretreatment also consequently reduced LTC_4 synthesis triggered *in vitro* by the PGD_2 stimulation of human eosinophils (Figure 6A; upper panel).

DP_2 receptor activation signals via PTX-sensitive $G\alpha_i$ protein and calcium mobilization to prompt DP_1 receptor-driven, newly formed lipid bodies to synthesize LTC_4

To establish how the concurrent activation of DP_2 receptors contributes to PGD_2-induced lipid body-driven LTC_4 synthesis, we have studied the potential role of $G\alpha_i$ activation and cytoplasmic calcium mobilization elicited by PGD_2. Lipid body assembly triggered *in vitro* by stimulation with either PGD_2 (Figure 6B) or BW245C (Figure 7) was not modified by pretreatments with inhibitors of either $G\alpha_i$ activation or cytoplasmic calcium influx, PTX (1 $\mu g \cdot mL^{-1}$) and BAPTA-AM (25 $\mu g \cdot mL^{-1}$), respectively, ruling out once more the involvement of DP_2 receptors in lipid body formation triggered by PGD_2. As shown in Table 1, PTX and BAPTA-AM were also able to reduce lipid body biogenesis induced by PAF within human eosinophils.

Although playing no role in PGD_2-induced lipid body assembly, the inhibition of $G\alpha_i$ protein and calcium mobilization by, respectively, PTX and BAPTA-AM pretreatment, reduced LTC_4 synthesis triggered *in vitro* by PGD_2 stimulation of human eosinophils (Figure 6B; upper panel), indicating that DP_2 receptors, by activating calcium-dependent signalling, converted DP_1 receptor-induced lipid bodies into enzymatically active organelles capable of LTC_4 synthesis.

Eosinophil lipid body-driven LTC_4 synthesis elicited in vivo *by allergic inflammation is also mediated by a complementary signalling between DP_1 and DP_2 receptors*

To verify whether the cooperative signalling between DP_1 and DP_2 receptors also operates under allergic inflammatory conditions *in vivo*, we employed a mouse model of allergic inflammation characterized by eosinophil accumulation and activation. As shown in Figure 8, while the DP_1 antagonist BWA868C reduced both eosinophil lipid body biogenesis and increased levels of cysLTs without significantly affecting eosinophil infiltration found in allergic reaction sites, the DP_2 receptor antagonists ramatroban and Cay10471 decreased the numbers of recruited eosinophils and inhibited allergic cysLT production but failed to alter eosinophil lipid body biogenesis; thus, yet again, illustrating the mandatory cooperation between the two PGD_2 receptors to evoke LTC_4 synthesis, as well establishing the relevance of such cooperation to the molecular mechanisms underlying allergy

Discussion

PGD_2 is now emerging as a potential mediator of allergic inflammatory pathologies, because it modulates the polarization of Th2 cells, oedema formation and eosinophil recruitment (Pettipher, 2008). In addition, PGD_2 is able to directly activate recruited eosinophils, particularly by eliciting the capacity of eosinophils to synthesize LTC_4 at sites of allergic inflammation. While the enzymic pathway by which eosinophils synthesize LTC_4 is well characterized, the pathophysiological stimuli and intracellular signalling cascades that

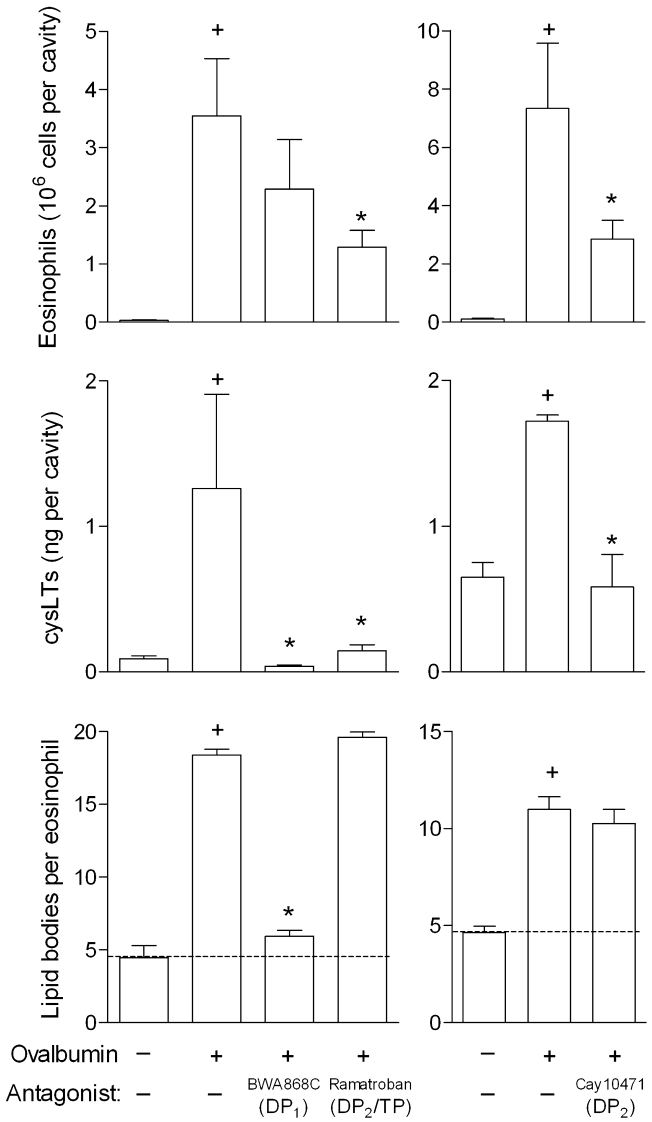

Figure 8

Cooperation between DP$_1$ and DP$_2$ receptors to trigger lipid body-driven LTC$_4$ synthesis within human eosinophils also takes place in allergic inflammatory response *in vivo*. Sensitized mice were pre-treated with BWA868C (1 mg·kg^{-1}), ramatroban (1 mg·kg^{-1}) or Cay10471 (1 mg·kg^{-1}), and then challenged with an i.pl. injection of ovalbumin (12 µg per cavity). Analyses of lipid body biogenesis and cysLTs production were performed 24 h after allergic challenge. Results are expressed as means ± SEM from at least six animals. $^{\dagger}P \leq 0.05$ compared with saline-challenged mice and $^{*}P \leq 0.05$ compared with ovalbumin-challenged mice. cysLT, cysteinyl leukotriene; DP$_1$, D prostanoid receptor 1; DP$_2$, D prostanoid receptor 2; i.pl., intrapleural.

govern such activity remain to be fully elucidated. Seeking such characterization, it is important to consider the evolving understanding of the potential roles that some eosinophil chemoattractants, which participate in the recruitment of eosinophils to sites of allergic inflammation, have as priming stimuli on eosinophil LTC$_4$ synthesis.

Our previous studies have demonstrated that, besides PGD$_2$, other eosinophil chemoattractants, such as PAF, CCL11 and CCL5, are secreted in response to allergic challenge and, by acting on their specific receptors expressed on eosinophil membranes, initiate an intracellular cascade leading to enhanced LTC$_4$ synthesis (Bozza *et al.*, 1996; Bandeira-Melo *et al.*, 2001). Our attempts to characterize the intracellular signalling pathways committed to chemoattractant-induced enhanced LTC$_4$ synthesis revealed that diverse stimuli-specific intracellular signalling events control LTC$_4$ synthesis within eosinophils. For instance, while PAF, acting via its PTX-sensitive Gα_i-protein-linked receptor appears to induce LTC$_4$ synthesis via a downstream signalling involving PKC and phospholipase C (PLC) activation (Bozza *et al.*, 1996; 1997; 1998), CCL11 and CCL5, acting via Gα_i protein-linked CCR3 receptors, signal via the activation of mitogen-activated proteins kinases and phosphatidylinositide 3-kinase, but not PKC or PLC (Bandeira-Melo *et al.*, 2001). Even though diverse downstream cascades for LTC$_4$ synthesis can be engaged, common upstream steps triggered by the activation of CCR3 and PAF chemotactic receptors share Gα_i protein- and calcium influx-regulated cellular activities that, besides culminating in cell polarization/migration, also leads to LTC$_4$ synthesis. Moreover, the well-documented role of PGD$_2$ in eosinophil trafficking as well as a variety of migration-related cellular responses, including actin polymerization and increased expression of adhesion molecules, depends on the activation of Gα_i proteins and calcium influx (Monneret *et al.*, 2001; Sawyer *et al.*, 2002). Accordingly, we initially hypothesized that the PGD$_2$ chemotactic DP$_2$ receptor expressed on eosinophils, signalling via the activation of Gα_i proteins and calcium influx, would be responsible for PGD$_2$-induced LTC$_4$ synthesis. However, our data showed that specific DP$_2$ receptor stimulation, by itself, was not sufficient to trigger LTC$_4$ synthesis.

Another shared intracellular event triggered by eosinophil chemoattractants that is essential for the successful LTC$_4$ synthesis is the rapid assembly of new cytoplasmic lipid bodies – a biological process recognized as an acute, highly regulated cellular event that is stimulus- and cell-specific (Bozza *et al.*, 2007). As multifunctional organelles, lipid bodies are a hallmark of leukocyte activation and, together with perinuclear envelope (Bandeira-Melo *et al.*, 2001; Tedla *et al.*, 2003) and phagosomes (Balestrieri *et al.*, 2006), represent a potential intracellular domain for LTC$_4$ synthesis. Within eosinophils, compartmentalized LTC$_4$ synthesis triggered by eosinophil chemotactic agents, including PGD$_2$, has been located specifically within lipid bodies, thereby explaining why the biogenesis of lipid bodies critically affects the biosynthesis of LTC$_4$ (Bandeira-Melo *et al.*, 2001; 2002b; Mesquita-Santos *et al.*, 2006). Again, different from the other eosinophil chemotactic receptors, DP$_2$ receptor stimulation alone did not promote PGD$_2$-induced lipid body biogenesis, which was also not dependent on DP$_2$ receptor-related Gα_i and calcium signalling. Inasmuch as the lipid body biogenic process is mandatory for LTC$_4$ synthesis, the inability of DP$_2$ receptors to trigger lipid body biogenesis in part explains its inability to promote enhanced LTC$_4$ synthesis within eosinophils.

We found that the discrepancy between DP$_2$ receptors and the other eosinophil chemotactic receptors, in terms of eliciting LTC$_4$ synthesis, relies on the more complex PGD$_2$ receptor system expressed on eosinophils. Besides DP$_2$ receptors,

eosinophils also express the non-chemotactic DP_1 receptor. By dissecting the specific contributions that each receptor makes to PGD_2-induced LTC_4 synthesis, we demonstrated that the PGD_2-elicited rapid *de novo* assembly of lipid bodies was insensitive to PTX or a calcium chelator, but was largely dependent on the activation of DP_1 receptor-elicited PKA signalling. Of note, the DP_1 receptor activation by PGD_2 signalling through $G\alpha_s$ proteins leads to increased cAMP and PKA activity – an intracellular signal transducing cascade that is classically related to the inhibition of chemoattractant-induced eosinophil motility (Hirai *et al.*, 2001; Monneret *et al.*, 2001), and consistent with the idea of cAMP elevating agents as powerful anti-inflammatory (Teixeira *et al.*, 1995; Diaz *et al.*, 1996) or pro-resolution (Sousa *et al.*, 2009) agents for the treatment of diseases in which eosinophil accumulation is thought to play a relevant role (Sousa *et al.*, 2009). In this context, it was noticeable that DP_1 receptor-driven, PKA-dependent, newly formed lipid bodies were not able to synthesize LTC_4, as shown in the EicosaCell preparations. In contrast to PAF- or CCL11-induced LTC_4-synthesizing lipid bodies, we demonstrated that under PGD_2 stimulation, compartmentalized LTC_4 synthesis within DP_1 receptor-driven eosinophil lipid bodies, which were assembled under cAMP/PKA regulation, demands concurrent DP_2 receptor stimulation, inasmuch as: (i) antagonists for either receptor were equally able to reduce PGD_2-induced LTC_4 synthesis; (ii) DP_1, but not DP_2 receptor antagonists, inhibited PGD_2-induced lipid body biogenesis; (iii) only by co-stimulating eosinophils with both DP_1 and DP_2 receptor agonists, was PGD_2-induced LTC_4 synthesis mimicked; (iv) inhibition of PKA inhibited PGD_2-induced DP_1 receptor-driven lipid body biogenesis and subsequent lipid body-compartmentalized LTC_4 synthesis; and (v) PTX and BAPTA-AM, while failing to interfere with DP_1 receptor-dependent lipid body biogenesis, inhibited PGD_2-induced lipid body-driven LTC_4 synthesis. Therefore, the molecular mechanisms orchestrating how DP_2 receptor activation converts DP_1 receptor-driven lipid bodies into enzymatically active organelles capable of effective LTC_4 synthesis depend on the coordinated $G\alpha_i$ activation and calcium mobilization.

Recently, focusing on eosinophil-driven allergic pathologies, we have found that, alongside CCL11, CCL5, PAF and macrophage migration inhibitory factor (Bandeira-Melo *et al.*, 2001; Vieira-de-Abreu *et al.*, 2005; 2010), PGD_2 is as an endogenous and potent biogenic stimulus of enzymatically active lipid bodies, organelles involved in LTC_4 synthesis by eosinophils (Mesquita-Santos *et al.*, 2006). Here, despite the evidence showing that PGD_2-driven eosinophil migration-related activities are mediated by a balance of opposing intracellular signalling cascades downstream of DP_1 and DP_2 receptor activation within eosinophils (Monneret *et al.*, 2001), we uncovered that the intracellular mechanisms of receptor-mediated PGD_2-induced LTC_4 synthesis rely on the collaborative signalling between both PGD_2 receptors. The PGD_2-elicited LTC_4 synthesis is dependent on the activation of DP_1 receptor-elicited PKA-regulated lipid bodies, in addition to an equally important and concomitant DP_2 receptor-elicited $G\alpha_i$/calcium-regulated signaling pathway, which prompts DP_1 receptor-driven, newly formed lipid bodies to synthesize LTC_4.

Collectively, our findings indicate that PGD_2 binding to DP_1 receptors triggers PKA-driven biogenesis of cytoplasmic lipid bodies, but is incapable of activating the LTC_4-synthesizing machinery, which is switched on by concurrent DP_2 receptor activation. Furthermore, by using PGD_2 receptor antagonists, we also demonstrated that during allergen-elicited eosinophilic inflammatory reactions, cysLTs production is also regulated by DP_1/DP_2-orchestrated eosinophil activation, thus indicating that either DP_1 or DP_2 antagonists might be highly effective at controlling eosinophil activation-regulated LTC_4 synthesis at sites of allergic reactions. However, considering the disappointing clinical trial results of the DP_1 receptor antagonist laropiprant in asthmatics and allergic rhinitis patients (Philip *et al.*, 2009), therapies based on dual blockade of DP_1 and DP_2 receptors or PGD_2 synthesis inhibition may display increased beneficial outcome.

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Conflict of interest

The authors have declared that no competing interests exist.

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