Dissertation

Signaling and trafficking properties of the PGD_2 receptors CRTH2 and DP in a recombinant HEK293 cell model and in human peripheral blood eosinophils

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Date, *Signature*

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Abbreviations

AC	Adenylyl cyclase
ADP	Adenosine diphosphate
Ag	Agonist
AKT	V-akt murine thymoma viral oncogene homolog 1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3', 5'-monophosphate
CD	Cluster of differentiation
CREB	cAMP response element binding
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
Da	Dalton
DK-PGD_2	13,14 dihydro-15-keto prostaglandin D2
DMEM	Dulbecco's modified Eagle's Medium
DNA	Desoxyribonucleic acid
DOP	Delta opioid receptor
EC_{50}	Half maximal effective concentration
ECL	Enhanced chemoluminescence
e.g.	Exempli gratia
ELISA	Enzyme-linked immunosorbent assay
E_{max}	Maximal possible effect
EPAC	Exchange protein activated by cAMP
Ex	Excitation
FACS	Fluorescence assisted cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLEX	Fluid-Excitation
GABA	Gamma-butyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIRK	G protein-coupled inwardly-rectifying potassium channels
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein coupled receptor
GRK	G protein-coupled receptor kinases

GTP	Guanosine triphosphatase
GTPase	Guanine-nucleotide triphosphate
HEK	Human embryonic kidney
HMVEC-L	Human microvascular endothelial cells-lung
IL	Interleukin
IP_3	Inositol triphosphate
KOP	Kappa opioid receptor
MAPK	Mitogen.activated protein kinase
MOP	Mu opioid receptor
mRNA	Messenger ribonucleic acid
NSF	*N*-methylmaleimide-sensitive factor
PBS	Phosphate-buffered saline
PCAM	Platelet-endothelial cell adhesion molecule
pH	Potential of hydrogen (lat. Potential Hydrogenii)
PI3K	Phosphatidyl inositol-3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PK	Protein kinase
PL	Phospholipase
PTX	Pertussis toxin
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
RFU	Relative Fluorescence Units
RLU	Relative Light Units
S.E.M.	Standard error of means
TGF	Tumor growth factor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule

Summary

Prostaglandin (PG) D_2 is a cardinal mediator released from activated immune cells during the allergic response and thus, is fundamentally involved in the initiation and perpetuation of allergic inflammation. The effects of PGD_2 are mediated by the seven transmembrane spanning (7TM)/ G-protein coupled receptors (GPCRs) D-type prostanoid receptor (DP) and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). Both receptors are co-expressed in eosinophils, among other immune cells. PGD_2 and its two receptors have raised considerable attention in the field of allergy research during the past years and have become attractive therapeutic targets. CRTH2 is well recognized to mediate proinflammatory effects, *in vitro* as well as *in vivo*, and a variety of CRTH2 antagonists are being developed. However, the particular role of the DP receptor in allergic inflammation is controversial and many of the observed DP-mediated effects both *in vitro* and *in vivo* are difficult to connect with the signaling pathways so far described for this receptor. During the last decades, the importance of 7TM/GPCR heteromerization has been recognized. A plethora of studies showed, that heteromerization of 7TM/GPCR can profoundly alter the pharmacological profile of a receptor and consequently the cellular response.

In my PhD thesis I aimed to investigate individual and combinatorial signaling and trafficking properties of CRTH2 and DP receptors. I hypothesized that the divisive signaling properties of the DP receptor are based on a cross-talk with CRTH2. I studied both receptors in a HEK293 cell model and in human peripheral blood eosinophils and could thereby demonstrate that the cell model recombinantly expressing the PGD_2 receptors resembles eosinophils endogenously expressing CRTH2 and DP. I show that CRTH2 and DP receptors essentially modulate the signaling properties of each other and form CRTH2/DP heteromers without altering their ligand binding capacities. The DP receptor amplifies the CRTH2-induced Ca^{2+} release from intracellular stores and, coincidentally, forfeits its own signaling potency. Moreover, desensitization or pharmacological blockade of the DP receptor hinders CRTH2-mediated signal transduction. In contrast, CRTH2 internalization occurs independently of the DP receptor. In cells that express both receptors pharmacological blockade of $G\alpha_{q/11}$-proteins abolishes the Ca^{2+} response to both CRTH2 and DP agonists, while inhibition of $G\alpha_i$-proteins selectively attenuates the CRTH2-mediated response but not the DP signal. Further, I could show that CRTH2 and DP receptors synergistically mediate actin polymerization and eosinophil adhesion to activated endothelial cells. In doing so, CRTH2 is the dominant receptor as revealed by application of selective antagonists. I also demonstrate the interactive involvement of the PGD_2 receptors in activating transcription

factors, which play essential roles in allergic inflammation: NFAT activation induced by CRTH2 relies on the presence of the DP receptor and can be modulated by both, CRTH2 and DP antagonists. SRE can be solely activated by DP, but underlies an inhibitory regulation by CRTH2. CREB is exclusively activated by the DP receptor. NF-κB is not regulated by PGD_2 receptors.

In summary, my PhD thesis demonstrates outstanding cooperativity between the PGD_2 receptors, CRTH2 and DP. They exist as a dynamic signaling unit, whose functionality might be altered by the G-protein pathway activated. Thus, the PGD_2 receptor signaling unit represents a potential target for development of heteromer-directed therapies to treat allergic diseases.

Zusammenfassung

Prostaglandin (PG) D_2 wird von aktivierten Immunzellen während der allergischen Reaktion ausgeschüttet und ist somit ein zentraler Mediator im Verlauf des allergischen Entzündungsprozesses. Die biologischen Effekte von PGD_2 werden von zwei G-Protein-gekoppelten Rezeptoren vermittelt, D-type prostanoid (DP) Rezeptor und Chemoattractant Receptor-Homologous Molecule Expressed on Th2 Cells (CRTH2). Diese Rezeptoren werden endogen in Eosinophilen und anderen Immunzellen exprimiert. Während der letzten Jahre hat das Forschungsinteresse an PGD_2 und seinen Rezeptoren als potentielle therapeutische Angriffspunkte stark zugenommen. Sowohl *in vitro* als auch *in vivo* wird CRTH2 eine proinflammatorische Rolle zugeschrieben und mehrere selektive CRTH2 Antagonisten befinden sich in verschiedenen Phasen von klinischen Studien. Im Gegensatz dazu wird die inflammatorische Rolle des DP Rezeptors kontrovers diskutiert. Viele der beschriebenen DP-induzierten Effekte sind den bisher bekannten Signalwegen des Rezeptors nur schwer zuzuordnen. Gleichzeitig wurde im Feld der G-Protein-gekoppelten Rezeptoren die Bedeutung der Heteromerisierung intensiv untersucht. Eine Vielzahl von Studien hat gezeigt, dass Heteromerisierung von G-Protein-gekoppelten Rezeptoren das pharmakologische Profil eines Rezeptors und somit die damit verbundene zelluläre Antwort nachhaltig verändern kann.

Das Hauptziel meiner Dissertation bestand in der Charakterisierung von individuellen sowie kombinatorischen Eigenschaften der Signalwege dieser PGD_2 Rezeptoren. Ich prüfte die Hypothese, dass die zwiespältige Signaltransduktion des DP Rezeptors durch eine direkte Interaktion mit CRTH2 bedingt ist. Zu diesem Zweck wurden folgende Zellsysteme verwendet: i) HEK293 Zellen mit rekombinanter Expression von CRTH2 und DP Rezeptoren und ii) humane Eosinophile mit endogener PGD_2 Rezeptorexpression. Prinzipiell konnte ich dabei zeigen, dass die untersuchten Effekte des rekombinanten Zellmodells denen der Primärzellen ähneln. Es bestätigte sich die Annahme, dass die Signaltransduktion von CRTH2 und DP einer engen Kooperation zwischen beiden Rezeptoren unterliegt. Ich konnte zeigen, dass CRTH2 und DP Rezeptoren Heteromere bilden, ohne die jeweiligen Liganden-Bindungseigenschaften zu verändern. Der DP Rezeptor verstärkt das CRTH2-vermittelte Ca^{2+} Signal mit einhergehender Verminderung der eigenen Signalkapazität. Darüber hinaus wird die CRTH2-induzierte Signaltransduktion durch pharmakologische Inhibierung sowie durch Agonisten induzierte Desensibilisierung des DP Rezeptors vehindert. Dennoch ist CRTH2 in der Lage, unabhängig vom DP Rezeptor – dessen Desensibilisierung an der Zelloberfäche stattfindet - zu internalisieren. In Zellen, die beide PGD_2 Rezeptoren koexprimieren, führt

eine Inhibierung der $G\alpha_{q/11}$-Proteine zu einer kompletten Eliminierung der CRTH2- und DP-vermittelten Ca^{2+} Antwort, während Hemmung der $G\alpha_i$-Proteine selektiv das CRTH2-generierte Signal blockiert. Desweiteren ermittelte ich die Rolle von CRTH2 und DP Rezeptoren in der Reorganisation des Zytoskelets, sowie Adhäsion von Eosinophilen an aktivierten Lungenendothelzellen. Die PGD_2 Rezeptoren fungieren synergistisch bei der Aktin-Polymerisierung, als auch bei der Adhäsion von Eosinophilen an Endothelzellen, wobei in beiden Prozessen CRTH2 eine dominate Rolle übernimmt. Mit Hilfe des HEK293 Zellmodells untersuchte ich die Beteiligung von CRTH2 und DP Rezeptoren bei der Aktivierung verschiedener Transkriptionsfaktoren, die mit allergischer Entzündung in Verbindung stehen. NFAT-Aktivierung durch CRTH2 ist abhängig vom DP Rezeptor und kann sowohl von CRTH2 als auch von DP Antagonisten moduliert werden. SRE wird ausschließlich durch DP aktiviert, unterliegt aber einer inhibitorischen Regulierung durch CRTH2. CREB-Aktivierung ist DP-abhängig und CRTH2-unabhängig, wohingegen NF-κB Aktivität weder von CRTH2 noch von DP induziert wird.

Meine Arbeit veranschaulicht eine enge Interaktion beider PGD_2 Rezeptoren. Sie scheinen als dynamische Signaleinheit zu existieren, dessen Funktionalität von den jeweilig aktivierten G-Protein Signalwegen bedingt und reguliert wird, und welche demzufolge ein potentielles Ziel für Heteromer-spezifische Therapien in der Allergie bildet.

Preface

Atopy belongs to the most common chronic diseases in the industrialized world with increasing prevalence, health-care costs and the urgent need for a causal therapy. Atopy is a multi-causal disorder - often with a genetic background - leading to a typical inflammatory response to antigens. Common features of atopic diseases are a reduction in quality of life and – in severe cases - in life expectancy. A portion of patients with allergic diseases do not respond well to the current treatment and remain poorly controlled[1;2], although some progress in efficient medication has been achieved over the last decades. In particular, children are in need of an alternative treatment to corticosteroids. An exclusive use of bronchodilators, however, increases the risk of pulmonary inflammation with subsequent airway remodelling[3].

The molecular mechanisms underlying the pathology of allergy are still poorly understood, but detailed knowledge thereof is essential for the development of effective therapeutics. The major intention is to develop drugs that target important mechanisms of the initiation and progression of the disease on the one hand, but to minimize their side effects on the other hand. Recent reports emphasize the importance of prostaglandin (PG) D_2 as a therapeutic target[4], since it might essentially link the early and late phases of allergic inflammation by modulating immune cell responses. The deorphanization of the G-protein coupled receptor GPR44 as a novel receptor for PGD_2, now termed chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2)[5;6], has raised further attention to the role of PGD_2 in allergic inflammation. To date, it is well-known that the biological effects of PGD_2 are mediated by two seven transmembrane spanning receptors (7TM)/G-protein coupled receptors (GPCR), the D type prostanoid (DP) receptor and CRTH2. It has been estimated that about 30 % of the clinically prescribed drugs on the current market[7;8] target GPCR and modulate their activity. The majority of these small-molecule medicines are agonists and antagonists directed against the ligand-binding site of the receptor.

1. Introduction

1.1. G-protein coupled receptors (GPCRs)

GPCRs, also referred to as seven transmembrane (7TM) spanning receptors or heptahelical receptors, embrace a large and diverse family of receptors. 7TM/GPCRs sense molecules outside the cell and translate extracellular information to intracellular signaling pathways which, consequently, elicit cellular responses. In doing so, the remarkable diversity of 7TM/GPCRs results from both, the high range of stimuli which they respond to and the number of signal transduction pathways they initiate. These include endogenous ligands like biogenic amines, lipids, proteins, amino acids, hormones, nucleotides, chemokines, ions, proteases, neurotransmitters, as well as exogenous stimuli like light, odorants and flavors[9;10].

A considerable number of diseases and malfunctions have been associated with genetic polymorphisms in 7TM/GPCRs[11;12].

1.2. Function of 7TM/GPCRs

Ligand-binding to the receptor induces a conformation of the transmembrane helices which enables a direct interaction with heterotrimeric guanine nucleotide binding proteins (G-proteins)[13-15]. The type of coupled G-protein further transduces the activation of downstream signaling pathways as reviewed by Marinissen and Gutkind[16]. 7TM/GPCR activity can then shut down[17] – i.e. desensitized by arrestin-mediated or arrestin-independent mechanisms - to protect the cell against overstimulation.

1.3. Heterotrimeric G-proteins

G-proteins consist of three subunits, α, β and γ[18;19], forming a heterotrimer. In its inactive state, guanine-nucleotide diphosphate (GDP) is bound to the alpha-subunit. Upon activation by a receptor, GDP is released from the Gα-subunit followed by binding of guanine-nucleotide triphosphate (GTP)[20]. Subsequently, the GTP-bound form of the Gα-subunit dissociates from the receptor as well as from the stable Gβγ-dimer. Both, Gα-GTP and the free Gβγ-dimer can modulate several cellular downstream effectors, like adenylyl cyclases (AC), phospholipases (PL), protein kinases (PK) and potassium and calcium channel activity (as reviewed by Hamm et al., 1998)[21]. The intrinsic GTPase activity of the Gα-subunit terminates the G-protein activity by hydrolysis of GTP and initiates the reassociation of Gα-GDP with the beta-gamma subunit[22]. The Gα-protein family is divided into four groups: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ [23].

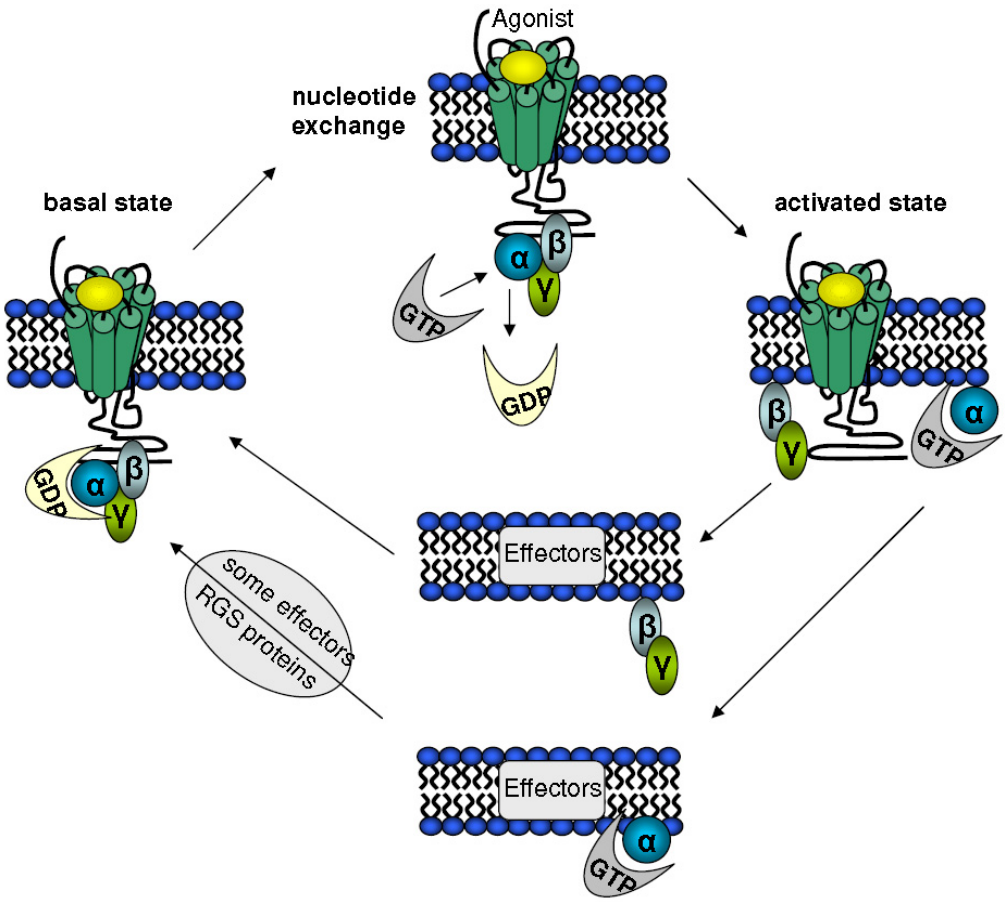


Figure 1. ***Cycle of G-protein activity*** **(modified from Wettschureck and Offermanns, 2005[24])**. Agonist-binding to a 7TM/GPCR initiates replacement of GDP with GTP at the Gα-subunit. GTP-Gα and Gβγ then dissociate and modulate downstream effector functions. The spontaneous hydrolysis of GTP can be accelerated by different cellular effectors or by regulators of G-protein signaling (RGS) proteins leading to the recombining of the Gα- and Gβγ-subunits.

1.3.1. The Gα-subunits

The $G\alpha_s$-protein stimulates the adenylyl cyclase leading to an elevation of intracellular cyclic adenosine 3', 5'-monophosphate (cAMP) levels, which subsequently convert the enzyme PKA into its active form[18;25]. Activated PKA phosphorylates a wide range of target proteins in a cell context-dependent manner to modulate their function. Alternatively, PKA can translocate into the nucleus to phosphorylate gene regulatory proteins, like the 43 kDa cAMP response element-binding (CREB) protein[26]. Moreover, cAMP can act in a PKA-independent manner via the exchange protein activated by cAMP 1 (EPAC1), which serves as a guanine-nucleotide exchange factor for the small guanosine triphosphatase (GTPase) Rap[27], thereby linking the $G\alpha_s$-protein pathway to the mitogen-activated protein kinase (MAPK) signaling pathway.

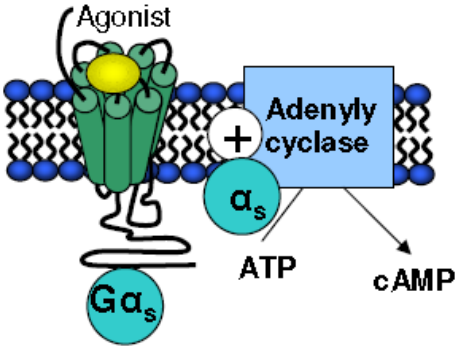


Figure 2. *Gα$_s$-protein coupling* (modified from Wettschureck and Offermanns, 2005[24]). The GTP-Gα$_s$-subunit activates the adenylyl cyclase which in turn catalyses the conversion of adenosine triphosphate to cAMP.

The Gα$_{i/o}$-proteins inhibit adenylyl cyclase activity and cAMP production. This pathway was originally identified by the ability of *Bordetella pertussis* or Pertussis toxin (PTX) to ribosylate the Gα$_{i/o}$-protein subunit with adenosine diphosphate (ADP), thus preventing it from interacting with the receptor[28;29].

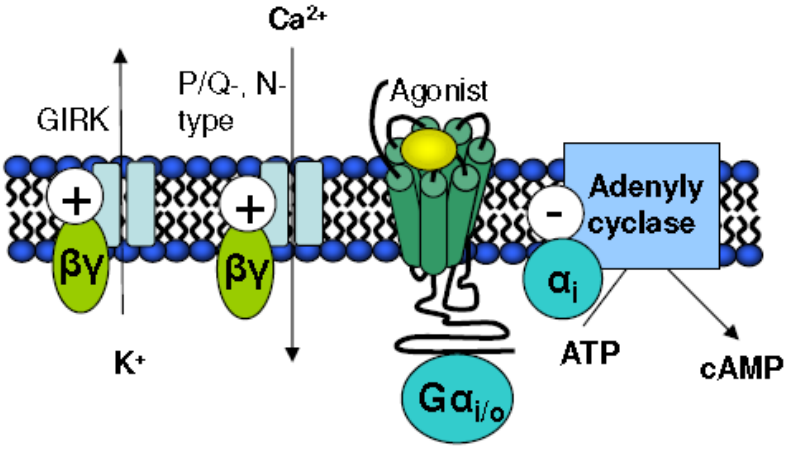


Figure 3. *Gα$_{i/o}$-protein coupling* (modified from Wettschureck and Offermanns, 2005[24]). The GTP-Gα$_{i/o}$-subunit inhibits the adenylyl cyclase and thus the conversion of adenosine triphosphate to cAMP. The Gβγ-subunit stimulates PLC-β, phosphatidylinositol-3-kinases (PI-3-K) β/γ and the G-protein-coupled inwardly-rectifying potassium channels (GIRK). P/Q- and N-type calcium channels are inhibited by the the Gβγ-subunit.

The Gα$_{q/11}$-protein was first identified by Taylor *et al*. in 1990[30]. It stimulates PLC-*β* to produce the intracellular messengers inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol (DAG)[18;31]. IP_3 binds to IP_3 receptors (calcium channels) and triggers the release of calcium from intracellular stores, while DAG recruits PKC to the membrane and activates it (reviewed

by Neves, 2002)[32]. $G\alpha_q$-proteins are also reported to activate the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB)[33].

The $G\alpha_{12/13}$-proteins are involved in signaling pathways connected to cellular shape and morphology (actin cytoskeleton) as well as proliferation[34]. $G\alpha_{12}$-proteins activate Ras, Bryton's tyrosine kinase, PLD, c-Srk and PKC[35]. $G\alpha_{13}$-proteins activate the small GTP-binding proteins of the Rho family[36] and are entailed in the PI3K pathway stimulating the protein kinase Akt and NFκB[33].

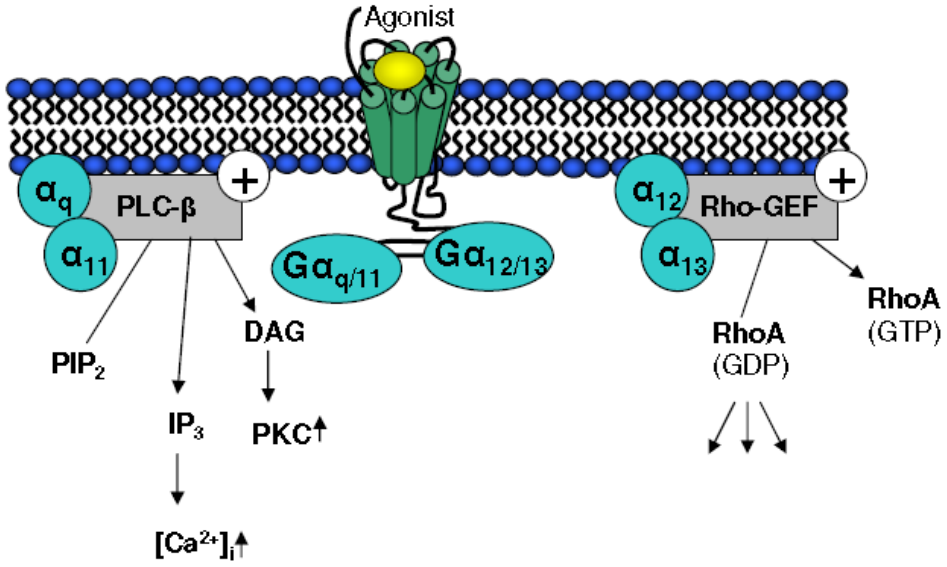


Figure 4. *$G\alpha_{q/11}$- and $G\alpha_{12/13}$-protein coupling* (modified from Wettschureck and Offermanns, 2005[24]). The $G\alpha_{q/11}$-subunit stimulates PLC-β and generation of IP_3 and DAG through cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2). In turn, IP_3 increases the intracellular calcium concentration ($[Ca^{2+}]_i$) and DAG activates PKC. The $G\alpha_{12/13}$-subunit stimulates the Rho-guanine nucleotide exchange factor (GEF) to activate the small GTPase RhoA by substitution of GDP with GTP.

1.3.2. The Gβγ-subunit

The Gβγ-subunit is a functional dimer whose importance in transducing signals has long been underestimated. In 1987, it was first shown that the Gβγ-subunit modulates an effector, e.g potassium (GIRK) channels[37]. Later, it was shown that Gβγ-subunit stimulates phospholipase C activity[38-41], phospholipase A_2[42], adenylyl cyclase[43] and calcium channels[44;45]. The number of activated effectors underlines the relevance of Gβγ-signaling regarding diseases such as hypertension, metabolic syndrome and artherosclerosis[46].

1.3.3. Role of G-proteins in allergic inflammation

Allergic inflammation as it occurs in asthma bronchiale and other allergic diseases is hallmarked by profound lung-infiltration with inflammatory cells including lymphocytes, granulocytes and mast cells. Release of mediators such as histamine, leukotriens, prostanoids, cytokines and chemokines by these cells activate and modulate immune cell function[47-49] and accumulation at the site of inflammation via GPCRs and G-proteins[50]. In general, immune cells express a variety of 7TM/GPCRs, coupling to all classes of G-proteins. Smooth muscle contraction, one characteristic feature of asthma, depends on an increase in intracellular Ca^{2+} which is mediated by $G\alpha_q$- [51] or $G\alpha_{12/13}$-proteins[52;53]. In contrast, smooth muscle relaxation is mediated by $G\alpha_s$-proteins which increase cAMP levels, and via PKA, which in turn blocks the actin/myosin machinery[54].

1.4. 7TM/GPCR trafficking

7TM/GPCR trafficking comprises the delivery of the receptor to the plasma membrane following synthesis, folding in the endoplasmatic reticulum and modification in the Golgi apparatus as well as the preceding retrieval from the cell surface upon activation. Receptor function underlies the equilibrium of receptor signaling, desensitization and resensitization (see Fig. 5). Prolonged or repeated activation by ligands regularly leads to a rapid attenuation of receptor responsiveness[55]. Receptor desensitization is an important physiological feedback mechanism that protects the cell against acute and chronic overstimulation. Prominent examples are the β1–receptor downregulation in the heart[56] and β2 adrenoceptor desensitization in bronchial smooth muscle in asthma[57]. The extent of receptor desensitization varies from complete termination of signaling - e.g. as observed in the visual and olfactory systems - to the attenuation of agonist-potency and maximal responsiveness as observed e.g. for the β2 adrenoceptor[58]. The receptor is desensitized by uncoupling from the responsive G-protein[59] and then phosphorylated by intracellular kinases[17;60-62]. Second messenger-dependent PKA and PKC[63] as well as G-protein-coupled receptor kinases (GRK) phosphorylate serine and threonine residues within the intracellular loop and carboxy-terminal tail domains[59;64;65]. Notably, the GRK-mediated receptor phosphorylation requires two conditions: First, the presence of an agonist-bound receptor [64;66] and second, the binding of cytosolic regulatory proteins, the arrestins[67]. Besides preventing the G-protein to couple to the receptor, arrestins further initiate a process called “receptor internalization or endocytosis”, which can be mediated by clathrin-coated pits, caveolae or uncoated pits[68;69]. In most cases, the receptor undergoes clathrin-mediated endocytosis. The GTPase dynamin pinches the pits

off the cell surface[70] and the receptor is either dephosphorylated and recycled back to the plasma membrane or targeted to the lysosomes for degradation[61].

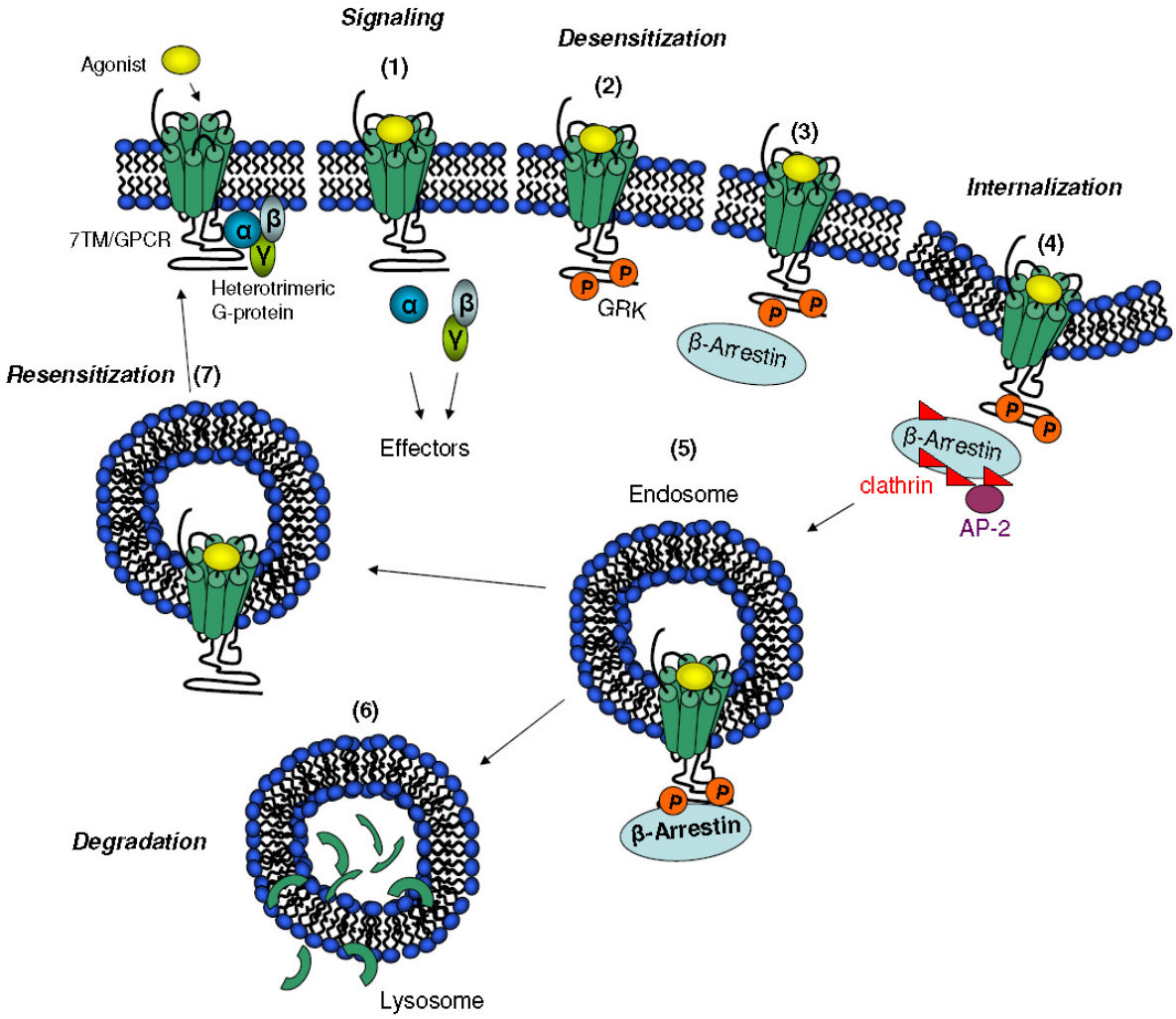


Figure 5. 7TM/GPCR trafficking cycle (modified from Bockaert *et al.*, 2004[71] and Moser *et al.*, 2010[72]). Agonist-binding to 7TM/GPCRs activates the heterotrimeric G-proteins. The Gα- and Gβγ-subunits dissociate and induce signal transduction (1). The receptor is desensitized through phosphorylation by G-protein-coupled receptor kinases (GRK) (2) followed by β-arrestin-binding (3). Clathrin and the adapter protein 2 (AP-2) facilitate the internalization of the receptor-arrestin-complex (4). The internalized receptor is localized in the endosome (5) and is either targeted to the lysosomal pathway for degradation (6) or recycled back to the cell surface for resensitization (7).

1.5. Heteromerization of 7TM/GPCRs

Cross-talk between receptors and the signaling pathways to which they are connected can significantly affect the cellular responses[73]. Recently, the meaning of physical interactions between 7TM/GPCRs and the formation of homomultimers and particularly heteromultimers has been recognized[74]. The importance of heteromerization has first been demonstrated for the metabotropic γ-butyric acid $(GABA)_B$ receptor[75]. Agonist-binding and signal transduction

requires co-expression of both $GABA_B$ receptor subtypes forming a functional heteromer. Ever since, a plethora of studies followed to show the existence of 7TM/GPCR heteromers and their functional significance *in vitro* [76-83]. As a proof-of-principle, the clinical relevance of 7TM/GPCR heteromers was highlighted in a number of *in vivo* studies. To name a few, in the opioid receptor research field it was shown that delta and mu opioid receptor (DOP/MOP) heteromers might improve morphine-based analgesia by limiting tolerance and dependence[78;84;85]. The *in vivo* relevance of delta and kappa opioid receptor (DOP/KOP) heteromers was shown by a heteromer-selective agonist[86]. Further on, Kostenis *et al*. showed a control of the angiotensin II AT_1 receptor by heteromerformation with the Mas oncogene, also a GPCR[81].

7TM/GPCR heteromers represent a functional unit with a unique pharmacological profile and can affect receptor properties such as cellular trafficking[87;88], cell-surface delivery[89;90], ligand-selectivity, -binding[76;91] and –potency[92;93] and G-protein-coupling[94;95]. This heteromer-directed signal specificity magnifies the repertoire of receptor signaling and consequently cellular responses. In conclusion, the existence of the numerous 7TM/GPCR heteromers in tissues and primary cells highlights their relevance as novel therapeutic targets for the pharmaceutical industry.

1.6. Prostaglandin (PG) D_2 as a novel therapeutic target in allergic disease

PGD_2 is the most important lipid mediator released from Immunoglobulin (Ig) E-activated mast cells[96;97], dendritic cells, macrophages, eosinophils, Th2 cells and endothelial cells[98-104]. Thus, PGD_2 is regarded as a key mediator in the course of inflammatory diseases. Besides allergens, other stimuli such as cytokines and oxidative or mechanical stress can induce PGD_2 synthesis via the phospholipase A_2 and cyclooxygenase pathway[105;106]. Local allergen challenge in patients with allergic rhinitis[107], bronchial asthma[108], allergic conjunctivitis[109], and atopic dermatitis[110] has been shown to result in instant increase of local PGD_2 levels. Moreover, PGD_2 accounts for multiple inflammatory effects including an increase in vascular permeability[111;112], airway narrowing and bronchoconstriction[113], and eosinophil infiltration in the airways[114]. PGD_2 induces chemotaxis of Th2 cells, eosinophils and basophils *in vitro* and *in vivo*[6;115-119] and propagates release of leukotrienes and cytokines from eosinophils and Th2 cells[120;121]. In conclusion, PGD_2 significantly contributes to the late phase of the allergic response and has thus become a relevant target for the pharmaceutical industry.

1.7. PGD_2 signaling via G protein-coupled receptors

1.7.1. DP receptor

The DP receptor was first cloned in mice[122], and subsequently in human[123]. DP, also referred to as DP1, is widely expressed, including eosinophils, basophils, dendritic cells, Th1 and Th2 cells, platelets, vasculature, central nervous system, retina, nasal mucosa, lungs and intestine[6;115;123-127]. Activation of the DP receptor stimulates the adenylyl cyclase through $G\alpha_s$-proteins[123] leading to an increase in cAMP. Previous studies report a DP-mediated elevation of intracellular Ca^{2+} levels via $G\alpha_{q/11}$-proteins in heterologous expression systems[123].

Both pro- and antiinflammatory effects have been reported for the DP receptor. For instance, DP-mediated responses include inhibition of platelet aggregation, induction of vasorelaxation and bronchodilatation in humans, mucin secretion, and lowering of intraocular pressure[112;126;128;129]. In T cells and other immune cells, accumulation of cAMP is generally associated with inhibition of effector cell function[129]. Indeed, DP agonists have been shown to inhibit neutrophil, basophil and dendritic cell function[130-132]. However, *in vivo* studies have demonstrated a pro-inflammatory role of DP, since DP antagonists prevent antigen-induced rhinitis, conjunctivitis, and pulmonary inflammation in the guinea pig[133]. Furthermore, DP-deficient mice exhibit a reduced pulmonary inflammation in response to allergen[134]. Recently, Schratl *et al.*[135] demonstrated that the DP receptor plays an important modulatory role in stimulating the mobilization of eosinophils from the bone marrow and their chemotaxis.

1.7.2. CRTH2

CRTH2, also referred to as DP2 receptor, was deorphanized upon the observation that not all of PGD_2-induced effects on immune cells were depending on the DP receptor[116]. CRTH2 is found on Th2 cells, eosinophils, basophils, dendritic cells, monocytes and macrophages[5;98;132] and couples to $G\alpha_i$-proteins, thereby leading to the inhibition of cAMP formation and an increase in intracellular calcium[6;136]. However, some PGD_2-induced effects, like e.g. shape change, activation of PI3K, PLC and p38 MAPK in eosinophils were found to be pertussis toxin-insensitive and thus ascribed to $G\alpha_{q/11}$-protein coupling[137].

CRTH2 accounts for many proinflammatory effects of PGD_2. These include i) the chemotaxis of immune cells such as eosinophils, Th2 cells, basophils, monocytes and macrophages[6;116;138], ii) respiratory burst of eosinophils[139;140], iii) histamine release from basophils[141], and iv) cytokine release and enhanced survival of Th2 cells[119]. In animal models, CRTH2 propagates eosinophil mobilization from the bone marrow[139] and their infiltration into the lungs and skin[117;118;142] and – as a consequence - exacerbates the late phase of allergic

inflammation. The severity of asthma has even been linked with single nucleotide polymorphisms in the CRTH2 gene[143].

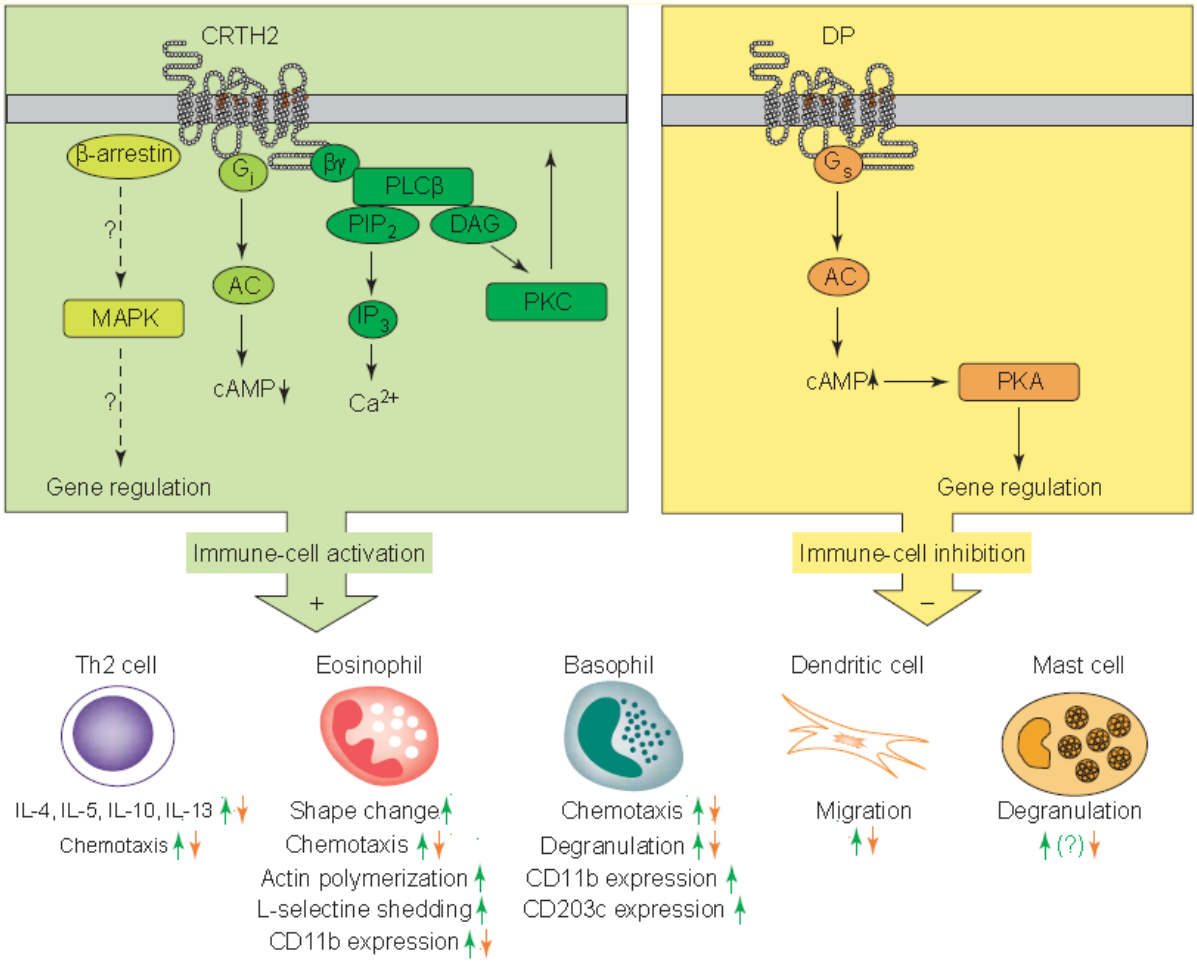


Figure 6. Effects of CRTH2 and DP on immune cell function (adapted from Kostenis and Ulven, 2006[105]). CRTH2 couples to $G\alpha_i$-proteins and inhibits adenylyl cyclase (AC) leading to a decrease in cAMP levels. The $G\beta\gamma$-subunit activates PLC-β which in turn generates diacylglycerol (DAG) and inositol triphosphate (IP_3) by cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 induces release of Ca^{2+} from intracellular stores. DAG activates PKC. CRTH2-mediated arrestin recruitment might activate MAP kinases and regulate genes in a G-protein-independent manner. Collectively, CRTH2 signaling induces immune cell activation as listed below the cells in the figure. The DP receptor couples to $G\alpha_s$-proteins and stimulates adenylyl cyclase leading to an increase in cAMP levels which in turn activate PKA. Elevation of the cAMP level is generally associated with immune cells inhibition. The functional consequences are indicated below.

1.8. Eosinophils in allergic diseases

Eosinophils are a hallmark of allergic inflammation and are involved in the initiation and propagation of the inflammatory response[47;144]. Eosinophils, which are end stage cells derived from myeloid precursor cells in the bone marrow, comprise about 2-5 % of leukocytes. They mature under the influence of the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and the late differentiation factor IL-5[145]. Typically, only a small portion of eosinophils circulates in the blood stream. The majority migrates to tissues and eosinophils are most abundant in the connective tissue underneath respiratory, gastrointestinal and urogenital epithelium[146;147].

The pathology of eosinophils is determined by secretion of free radicals and cytotoxic basic proteins, namely major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil derived neurotoxin, which can induce tissue damage and dysfunction, particularly of the bronchial epithelium[148]. This ultimately leads to the phenotype of asthma bronchiale, characterized by airway hyperresponsiveness, increased mucus production, smooth muscle hyperplasia and deposition of extracellular matrix components[149]. In addition, activated eosinophils release a number of chemical mediators, such as cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, TGF-α/β), chemokines (regulated upon activation, normal T cell expressed and secreted (RANTES), eostaxin) and lipid mediators (sulphidopeptide leukotriens, prostaglandins and platelet activating factor)[150]. These molecules amplify the inflammatory response by upregulating adhesion molecules, modulating cellular trafficking and regulating vascular permeability, mucus secretion and smooth muscle constriction.

The activation of eosinophils is mediated at different levels. The synthesis of eosinophils in the bone marrow is influenced by IL-5. In the absence of any immune stimulation few cells are produced, whereas the activation of Th2 cells and thus cytokine production increases the release of eosinophils into the circulation[151]. However, CC chemokines such as RANTES and eotaxins[152] as well as adhesion molecules (e.g. P-selectin, vascular cell adhesion molecule (VCAM) -1)[153] are coordinative key factors for chemotaxis and initiate the migration of eosinophils from the blood into inflammatory tissue. In allergic diseases, eosinophil infiltration into the tissue is a characteristic feature. Eosinophils can be enriched in the tissue up to 100-fold over neutrophil granulocytes[151].

1.9. Roles of CRTH2 and DP receptors on eosinophil function *in vitro*

CRTH2 and DP are co-expressed in eosinophils. Due to the fact, that both receptors are coupled to different – opposing - signaling pathways, but PGD_2 binds with equal affinity to CRTH2 and DP[136;154], the underlying mechanism(s) of PGD_2 action has remained unclear. Nevertheless, PGD_2-induced responses have been shown to be often dominated by CRTH2 over DP-signaling[115;141;155], although PGD_2 binds with equal affinity to both receptors[136;142]. For example, PGD_2-induced Ca^{2+} mobilization and chemotaxis of Th2 cells as well as eosinophil shape change, chemokinesis and degranulation was mediated through CRTH2, but not through DP receptors[6;115]. It was speculated that a reason for this might be the overall lower expression level of the DP receptor as compared to CRTH2 in various cell types[115;119;120;127;141;156]. Notably, the expression levels of both receptors underlie stimulus-dependent dynamic up- or downregulation[134;157], so that the net effect of PGD_2 might be differential. As mentioned above, CRTH2 activation induces eosinophil shape change, chemotaxis, enhancement of chemotactic responsiveness to other chemoattractants and degranulation[115;116;139;158]. DP activation, on the other hand, has been reported to enhance eosinophil survival[115].

1.10. Roles of CRTH2 and DP receptors on eosinophils function *in vivo*

The complexity of the inflammatory role of PGD_2 becomes evident when data from *in vivo* studies are considered. The antiinflammatory effects of DP observed *in vitro* are deemphasized by a proinflammatory action *in vivo* as reviewed by Kostenis and Ulven[105] and summarized in Table 1. The proinflammatory action of CRTH2 is depicted in Table 2.

Table 1. *In vivo* role of the DP receptor in inflammation

Proinflammatory effects	**Species/ Model**
No allergen-induced airway inflammation[134]	DP-/- mouse
Inflammation-induced upregulation of DP expression[120;134]	Mouse
SNP-associated lower risk of asthma[159]	Human
DP-receptor antagonists prevent allergic rhinitis, conjunctivitis and asthma[133;160;161]	Guinea-pig, sheep
DP agonist-induced conjunctivitis[162]	Human, guinea-pig
Antiinflammatory effects	
DP-activation mediates reduction of atopic dermatitis[163;164]	Mouse
DP-activation reduces tissue inflammation and eosinophilia[142]	Mouse
DP agonist inhibits dendritic cell migration in the airway and consequently prevents T cell activation[165]	Mouse

Table 2. *In vivo* role of CRTH2 in inflammation

Proinflammatory effects effects	**Species/ Model**
CRTH2 activation enhances eosinophilia and pathology of allergic asthma and atopic dermatitis[142]	Mouse
Intratracheal PGD_2 induces eosinophil trafficking into the airways via CRTH2[117;118]	Rat
Inhibition of CRTH2 prevents contact hypersensitivity[166]	Mouse
Increased number of CRTH2-positive $CD4^+$ T cells in atopic dermatitis[6;165;167;168]	Human
Genetic CRTH2 variants with higher mRNA stability are associated with higher degree of bronchial hyper-responsiveness and near-fatal asthma[143]	Human
Antiinflammatory effects	
CRTH2-mediated limitation of IL-5 production and eosinophil recruitment[169]	Mouse

2. Specific aims of the thesis project

AIM 1:

To characterize the functional consequences of CRTH2 and DP receptor co-expression in a heterologous cell model

I will investigate expression, signaling, ligand binding, heteromer formation and internalization of the respective receptors alone and in combination.

AIM 2:

To test the functional relevance of CRTH2 and DP receptors in human peripheral blood eosinophils

I will compare the results from freshly isolated eosinophils to the data obtained from AIM 1.

3. Material and Methods

3.1. Material

3.1.1. Chemicals

3, 3', 5, 5'-Tetramethylbenzidine	Sigma-Aldrich, St. Louis, USA
Aqua bidestillata	Mayrhofer Pharmazeutika, Linz, Austria
BD Cell FIX	BD Biosciences, San Jose, CA, USA
BD FACS Flow	BD Biosciences, San Jose, CA, USA
BD FACS Lysing Solution	BD Biosciences, San Jose, CA, USA
Bovine serum albumine	Sigma-Aldrich, St. Louis, USA
Bromphenol blue	Sigma-Aldrich, St. Louis, USA
$CaCl_2$	Sigma-Aldrich, St. Louis, USA
Dextran	Sigma-Aldrich, St. Louis, USA
Dulbecco's Modified Eagle's Medium	Invitrogen, Lofer, Austria
Dry milk	Fixmilch Instant, Wien, Austria
DTT	Sigma-Aldrich, St. Louis, USA
Dulbecco´s PBS 1x liquid - $CaCl_2$, Mg_2	Invitrogen, Lofer, Austria
Dulbecco´s PBS 1x liquid +$CaCl_2$, Mg_2	Invitrogen, Lofer, Austria
ECL	GE Healthcare, Vienna, Austria
EDTA Pulver cell culture tested	Sigma-Aldrich, St. Louis, USA
EDTA solution	Sigma-Aldrich, St. Louis, USA
EGM-2 MV Bullet medium	Lonza, Basel, Switzerland
FBS	Hyclone Distributor VWR, Logan, USA
Fibronectin	Sigma-Aldrich, St. Louis, USA
Fluo-3	Sigma-Aldrich, St. Louis, USA
Formaldehyde, 37 %	Carl Roth GMBH & Co KG, Karlsruhe, Germany
Gelatine Type B, cell culture tested	Sigma-Aldrich, St. Louis, USA
Geneticin Selective Antibiotic	Invitrogen, Karlsruhe, Germany
Glucose	Sigma-Aldrich, St. Louis, USA
Glycerol	Sigma-Aldrich, St. Louis, USA
HBSS	Invitrogen, Lofer, Austria
HCl	Merck, Darmstadt, Germany
HEPES cell culture tested	Sigma-Aldrich, St. Louis, USA
Histopaque	Sigma-Aldrich, St. Louis, USA
Immersion Oil for Microscopy, Type A	Nikon Instruments, Badhoevedorp, NL

KCl	Sigma-Aldrich, St. Louis, USA
$KHCO_3$	Sigma-Aldrich, St. Louis, USA
Lipofectamin2000	Invitrogen, Lofer, Austria
Mautner-Markhof Ethanol absolut	Lactan GMBH &Co.KG
MgCl	Sigma-Aldrich, St. Louis, USA
Na_2HPO_4	Merck, Darmstadt, Germany
NaCl	Carl Roth GMBH&Co.KG
NH_4Cl	Sigma-Aldrich, St. Louis, USA
OPTIMEM	Invitrogen, Lofer, Austria
PageRuler Prestained Protein Ladder	Fermentas, St.Leon-Rot, Germany
PBS	Invitrogen, Lofer, Austria
Phalloidin-Texas Red	Invitrogen, Lofer, Austria
Pluronic F-127	Sigma-Aldrich, St. Louis, USA
Poly-D-Lysine	Sigma-Aldrich, St. Louis, USA
SDS	Sigma-Aldrich, St. Louis, USA
Sulfuric acid	Carl Roth GMBH&Co.KG
Texas-Red Phalloidin	Invitrogen, Lofer, Austria
TRIS Base	Carl Roth GMBH&Co.KG
Tris-Glycin gel, 16%	Invitrogen, Lofer, Austria
Tris-HCl	Carl Roth GMBH&Co.KG
Triton-X 100	Sigma-Aldrich, St. Louis, USA
Tween	Sigma-Aldrich, St. Louis, USA
Ultra-V Block	Labvision, Westinghouse, CA, USA
Vectashield Mounting Medium	Vector Laboratories, Burlingam, CA, USA
Zeocin	Invitrogen, Lofer, Austria

3.1.2. Kits and Enzymes

Complete Protease Inhibitor Cocktail (Roche Applied Science, USA)

Eosinophil Isolation Kit (StemCell Technologies, Vancouver, Canada)

Fast SYBR Green PCR Master Mix (Applied Biosystems, Vienna, Austria)

FLIPR® Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA, USA)

Hight Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vienna, Austria)

HiSpeed Plasmid Maxi Kit (QIAGEN, Hilden, Germany)

PNGaseF (Fermentas, St.Leon-Rot, Germany)

RNeasy Mini Kit (QIAGEN, Hilden, Germany)

Steadylite Plus Kit (Packard Instrument Company, Meriden, CT, USA)

3.1.3. Plasmids

The plasmids pCREB-luc, pNFAT-luc and pNFκB-luc for luciferase reporter gene assays were purchased from Stratagene, La Jolla, CA, USA. pSRE-luc was a kind gift from Silvio J. Gutkind form the National Institutes of Health (NIH), Bethesda, MA, USA.

3.1.4. Cells

HEK293 cells were purchased from American Type Culture Collection, Wesel, Germany.

HMVEC-L were purchased from Lonza, Basel, Switzerland.

3.1.5. Pharmacological substances

PGD_2; 13,14-di-hydro, 15- keto prostaglandin D_2 (DK-PGD_2); (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidin heptanoic acid (BW245c); 3-[(2-cyclohexyl-2-hydroxyethyl)amino]-2,5-dioxo-1-(phenylmethyl)-4-imidazolidine-heptanoic acid (BWA868c), (+)-3-[[(fluorophenyl)sulfonyl] methyl amino]-1,2,3,4-tetrahydro-9H-carbazole-9-acetic acid (CAY10471) and MK0524 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). MH-362-63-8 was a kind gift from Ed Cannon, Unigen Inc. (Lacey, Washington, USA). Pertussis toxin was from Sigma-Aldrich (St. Louis, USA).

3.1.6. Antibodies

All antibodies were purchased from Invitrogen, Karlsruhe, Germany[1], BD Biosciences, San Jose, CA, USA[2], Abcam, Camebridge, UK[3], Sigma-Aldrich[4], or Santa Cruz[5].

Alexa Fluor 488 IgG_1[1]

Alexa Fluor 488 IgG_{2B} [1]

Alexa Fluor 594 IgG_1 [1]

Alexa Fluor 594 IgG_{2B} [1]

Alexa Fluor 647 mouse anti-human CD3 [2]

Alexa Fluor 647 rat anti human-CRTH2 [1]

Alexa Fluor 647 rabbit anti-goat [1]

Anti-c-Myc rabbit affinity matrix [4]

FITC anti-human CD16 [2]

HRP-conjugated secondary antibodies

Mouse monoclonal (9E10) ab32 anti-Myc IgG_1 [3]

Mouse monoclonal M1 anti-Flag IgG_{2b} [3]

Mouse monoclonal anti-β-actin

Mouse monoclonal anti-Flag M2 affinity gel [4]

PE Cy5-anti- human CD16 [2]

PE-anti-human CD16 [2]

Polyclonal goat anti-human DP [5]

Rabbit anti-CRTH2 [4]

Rabbit anti-DP receptor [4]

3.2. Methods

3.2.1. DNA constructs

The cDNA coding for the human DP receptor was cloned into the modified eukaryotic expression vector SS-Flag pcDNA3.1 (+) zeo[170] by using the restriction sites NotI and XbaI and verified by 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.

3.2.2. Cells and cell culture

3.2.2.1. Generation of stable HEK293 cell lines

Human embryonic (HEK) 293 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) at 37 ºC in a 5 % CO_2, humidified atmosphere. Stable cell lines expressing recombinant receptors were generated as described before (Schuligoi et al., 2009; Sedej et al., 2011). HEK293 cells stably expressing

Myc-CRTH2 (HEK-CRTH2), Flag-DP (HEK-DP), or both receptors (HEK-CRTH2+DP) were transfected with pcDNAs encoding SS-Flag-DP and/ or the SS-Myc-CRTH2. Single colonies were propagated in selection media (0.2 mg/mL Zeocin and/or 0.2 mg/mL Geneticin) in DMEM supplemented with 10 % FBS.

3.2.2.2 . Preparation and isolation of human peripheral blood eosinophils

Blood was taken from healthy volunteer blood donors and sampled according to a local ethics committee-approved protocol. The blood donors signed an informed consent form prior to blood sampling. Polymorphonuclear leukocytes (PMNL) containing neutrophils and eosionophils were prepared by dextran sedimentation of whole blood and centrifugation on Histopaque gradients as described before[139]. Briefly, whole blood supplemented with 3.8 % sodium citrate was centrifuged (20 min, 400 x g) and platelet-rich plasma was removed. Blood cells were then subjected to 6 % of dextran and erythrocytes were allowed to sediment (30 min, room temperature). The upper phase (leukocytes) was placed onto the polysucrose solution Histopaque with a defined density of 1.077 g/mL and centrifuged (20 min at 400 x g) to split up peripheral blood mononuclear cells (PBMC), PMNL and erythrocytes. PBMC, saline and Histopaque were removed and the pellet consisting of PMNL was washed with PBS without Ca^{2+} and Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM glucose pH 7.4. Remaining erythrocytes were lysed with 0.2 % saline. Eosinophils were further purified by negative magnetic selection using an antibody mixture directed against CD2, CD14, CD16, CD19, CD59 and glycophorin A and colloidal magnetic beads. The purity and viability of isolated eosinophils was > 97 %.

3.2.2.3. Cultivation of human lung microvascular endothelial (HMVEC-L) cells

HMVEC-L cells were maintained in EGM-2 MV Bullet medium supplemented with 5 % FCS as previously described[171]. Cells were grown to about 90 % confluence and used for experiments from passage six to nine.

3.2.3. Flow cytometry

3.2.3.1. Analysis of receptor expression in HEK293 cells

Expression levels of Myc-CRTH2 and Flag-DP in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells were determined by flow cytometry. Cells were blocked in 1 % BSA in PBS (30 min, 4 °C) and sequentially stained with anti-Myc IgG_1 or anti-Flag M1 IgG_{2b} antibodies (1 µg/mL, 1 hour, 4 °C) and Alexa Fluor 488 goat anti-mouse IgG_1 or IgG_{2b}

antibodies (2 µg/mL, 45 min, 4 °C). Cells were washed with PBS and fixed with Cell FIX and analyzed in a FACSCalibur flow cytometer (BD Biosciences).

3.2.3.2. CRTH2 and DP receptor internalization assay in HEK293 cells

To determine CRTH2 and DP receptor internalization in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells, cells were incubated with the agonist for 60 min following optional treatment with antagonist or vehicle for 15 min at 37 °C. Cells were transferred to ice, washed with PBS and fixed with Cell FIX. Then, cells were stained as described above (3.2.3.1). Samples were analyzed in a FACSCalibur flow cytometer.

3.2.3.3 Ca^{2+} flux assay in eosinophils

Intracellular Ca^{2+} flux in eosinophils was measured as previously described[139;172]. PMNL were treated with 2 µM of the acetoxymethyl ester of Fluo-3 in the presence of 0.02 % pluronic F-127 (60 min, room temperature). Neutrophils were labeled with PE Cy5-conjugated anti-CD16 antibody (10 min, room temperature). Cells were pretreated with antagonist or vehicle for 10 min at 37 °C and 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). Between the treatments cells were washed with assay buffer made from Dulbesso's modified phosphate-buffered saline without Ca^{2+}/ Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM Glucose, pH 7.4.

3.2.3.4. Leukocyte shape change assay

The leukocyte shape change assay was performed essentially as described before[106]. In brief, citrated whole blood was stimulated with the agonists (4 min, 37 °C). The blood was then transferred to ice and fixed with Cell FIX followed by NH_4Cl-induced lysis of red blood cells[173]. Cells were then washed in assay buffer made from Dulbesso's modified phosphate-buffered saline without Ca^{2+}/ Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM Glucose, pH 7.4 and resuspended in 250 µL of fixative solution. To record shape change responses samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). Eosinophils were identified according to their autofluorescence in the FL-1 and FL-2 fluorescence channels[139].

3.2.3.5. CRTH2 and DP receptor internalization assay in eosinophils

Whole blood was stimulated with the agonist for 1 hour at 37 °C. Cells were fixed on ice with Cell FIX. Erythrocytes were lysed with BD FACS Lysing solution and cells were

blocked with Ultra V Block (30 min, 4 ºC). Cells were stained with Alexa Fluor 647 rat anti-human CRTH2 (10 µg/mL) or polyclonal goat anti-human DP (20 µg/mL) antibodies, respectively, for 30 min at 4 ºC. The goat anti-human DP antibody was detected with the Alexa Fluor 647 rabbit anti-goat antibody (2 µg/mL, 30 min at 4 ºC). Respective control antibodies were used to determine non-specific binding. Between the treatments cells were washed with PBS without Ca^{2+} and Mg^{2+}. Neutrophils were distinguished from eosinophils in PMNL preparations by labeling with PE-conjugated anti-CD16 antibody. Cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences).

3.2.4. Immunofluorescence microscopy

3.2.4.1. Antibody feeding experiments

Antibody feeding experiments were conducted as previously described[86;170]. Briefly, living cells were loaded with anti-Flag M1 or anti-Myc antibodies (1 µg/mL, 30 minutes, 37 ºC). For receptor internalization studies, cells were then stimulated with the agonist (1 µM, 1 hour, 37 ºC). Cells were fixed with 3.7 % paraformaldehyde, blocked and permeabilized with blotto (50 mM Tris HCl pH 7.5, 1 mM $CaCl_2$, 3 % dry milk, 0.1 % TritonX-100) and then stained with Alexa Fluor 488 or 594-conjugated isotype-specific secondary antibodies (2 µg/mL, 20 min). Between the treatments cells were washed with TBS (25 Mm Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, pH 7.4). Cells were mounted with Vectashield mounting medium and analyzed with a Zeiss LSM 510 Meta Axioplan confocal microscope.

3.2.4.2. F-actin (Phalloidin) staining in HEK293 cells

F-actin staining in HEK293 cells was performed essentially as described before[174]. Briefly, HEK293 cells were grown on glass coverslips for 48 hours and starved in Optimem for 16 hours prior to the experiment. Cells were stimulated with the agonist (10 min) at 37 °C following antagonist or vehicle treatment (10 min). Cells were fixed in 3.7 % paraformaldehyde (10 min, room temperature), permeabilized with 0.1 % TritonX-100 in PBS (5 min, room temperature) and blocked with 1 % BSA in PBS (25 min, room temperature). To visualize actin polymerization cells were stained with 1 U of methanolic Texas-Red Phalloidin (20 min, room temperature). Between the treatments cells were washed with PBS. Cells were mounted with Vecatshield-DAPI mounting medium on glass slides and analysed with an Olympus IX70 fluorescence microscope and an Olympus UPlanApo-60x/1.20 lens using an Olympus DP50-CU digital camera and Olympus CellˆP software (Olympus, Lake Success, NY, USA).

3.2.4.3. F-actin (Phalloidin) staining in eosinophils

Isolated eosinophils in were seeded in Dulbesso's modified phosphate-buffered saline without Ca^{2+}/ Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM Glucose, pH 7.4 on glass coverslips coated with fibronectin (25 µg/mL). Cells were treated as described above (3.2.4.2). To visualize actin polymerization cells were stained with 0.4 U of methanolic Texas-Red Phalloidin (20 min, room temperature) and analysed using an Olympus DP50-CU digital camera and Olympus CellˆP software (Olympus, Lake Success, NY, USA).

3.2.5. Enzyme-linked immunosorbent assay (ELISA)

As previously described[175], cells were fixed with 3.7 % paraformaldehyde (20 min, room temperature) and then blocked and permeabilized with blotto (50 mM Tris HCl pH 7.5, 1 mM $CaCl_2$, 3 % dry milk, 0.1 % TritonX-100). Cells were incubated with anti-Flag M1 antibody (1 µg/mL, over night, 4 °C) and HRP-conjugated secondary antibody (4 µg/mL, 2 hours, room temperature). Between the treatments cells were washed with TBS (25 Mm Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, pH 7.4). Cell numbers were determined by optical density in a FlexStation II device (Molecular Devices). TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate was added, neutralized by 0.5 M sulfuric acid and color intensity was measured at the optical density of 450 nm in a BioRad xMark microplate spectrophotometer (Vienna, Austria).

3.2.6. Western blot and co-immunoprecipitation

Sample preparation - Cell membranes of HEK293 cells were prepared as previously described (Waldheor et al., 1998, Mol Pharmacol). Briefly, cells were grown to 100 % confluency in 10 cm dishes, washed in ice-cold PBS and harvested in HME buffer (25 mM HEPES·NaOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA) supplemented with 1 % Triton X-100. Cell membranes were disintegrated by two freeze/thaw cycles with liquid nitrogen, and homogenized by sonication (15 x 1sec at 25 %, two cycles). Cell membranes were resuspended in HME buffer and stored at -70 °C. Whole cell lysates of isolated eosinophils were prepared in lysis buffer (150 mM NaCl, 25 mM KCl, 10 mM Tris pH 7.4, 1 mM $CaCl_2$) with 0.1 % Triton X-100. Protein concentrations were determined using the colorimetric BioRad protein assay which is based on the Bradford method.

Western blot – Samples were resolved on a 16 % Tris-glycine gel, transferred to a polyvinylidene difluoride membrane and blocked with TBST buffer (135 mM NaCl, 25 mM Tris base pH 7.4, 2.5 mM KCl, 1 mM $CaCl_2$, 1 % Tween) supplemented with 10 % dry milk. The membranes were probed with rabbit anti-DP receptor (2.5 µg/mL) or rabbit anti-CRTH2

(1 µg/mL) antibodies overnight at 4 °C. β-actin blots were probed with mouse monoclonal β-actin antibody (1 µg/mL) overnight at 4 °C. Proteins were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (4 µg/mL) for 2 hours at room temperature followed by enhanced chemiluminescence (ECL).

Co-immunoprecipitation – CRTH2 and DP receptors were immunoprecipitated from HEK293 cell membranes with 50 µL of anti-c-Myc rabbit affinity matrix or 20 µL of anti-Flag M2 murine monoclonal affinity gel at 4 °C overnight. Beads were extensively washed with cold HME buffer followed by a washing step with 10 mM Tris HCl pH 7.4. Samples were treated with PNGase F in 10 mM Tris HCl pH 7.4 (1 hour, 37 °C) and incubated with a reducing sample buffer (100 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 0.2 % bromphenol blue, 200 mM DTT) for 5 minutes at 95 °C. Proteins were separated and immunoblotted as described above. Receptors were visualized with ECL 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).

3.2.7. Calcium (Ca^{2+}) signaling assay (96-well FlexStation II assay)

Agonist-mediated intracellular Ca^{2+} release in HEK293 cells was measured in a 96-well plate format with a FlexStation II (Molecular Devices, Sunnyvale, CA, USA) as previously described[86]. Briefly, cells were seeded in black 96 well assay plates with clear bottom (Corning Inc.) at a density of 40,000 cells/ well and grown to approximately 90 % confluency. For gene dose experiments cells were transiently transfected with 50 to 200 ng/well of pcDNA using 0.6 µL/well of Lipofectamine2000 according to the manufacturer's instruction. Twenty-four hours post-transfection medium was exchanged to Optimem and cells were loaded with the Ca^{2+} fluorophore (FLIPR Calcium 4 Assay Kit) for 60 minutes at 37 °C. Intracellular Ca^{2+} mobilization was measured following agonist stimulation and recorded as relative fluorescence units (RFU) for 2 minutes in a FlexStation II.

3.2.8. Luciferase reporter gene assays

Agonist-induced activation of the transcription factors nuclear factor of activated T-cells (NFAT), cAMP response element-binding protein (CREB), serum response element (SRE) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) was measured in HEK293 cells using the Steadylite Plus Kit essentially as described before[176]. Cells were seeded in white 96 well assay plates with clear bottom (Perkin Elmer) at a density of 60,000 cells/ well and grown to approximately 90 % confluence. Cells were transiently transfected with the following luciferase reporter plamids using Lipofectamine2000 according to the

manufacturer's instruction: pNFAT-luc (200 ng/ well), pCREB-luc (200 ng/well), pSRE-luc (100 ng/well) or pNFκB-luc (50 ng/well). Twenty-four hours post transfection cells were stimulated with agonist or vehicle in serum-free medium (Optimem) for 5 hours at 37 °C. Cells were washed with PBS and cell numbers were determined by optical density in a FlexStation II device (Molecular Devices). Luciferase activity was determined by measuring luminescence in a TopCounter device (TopCount NXT), recorded as relative light units (RLU).

3.2.9. Quantitative real-time PCR

Total RNA from isolated eosinophils was prepared using the RNeasy Mini Kit, followed by reverse transcription of 1 µg total RNA with the Hight Capacity cDNA Reverse Transcription Kit. The amplification was performed with primers for the human GAPDH (forward 5'-ATGGGGAAGGTGAAGGTCG-3'; reverse 5'-GGGGTCATTGATGGCAACAATA-3'), CRTH2 (forward 5'-TCTGCACTTCCAACTTAGGCG-3'; reverse 5'-GCTCTGGAGACGGCTCATC-3') and DP (forward 5'-GCTGCCTACGCTCAGAACC-3'; reverse 5'-TGTCGAGGAGAGCCCAAAGA-3') using the Fast SYBR Green PCR Master Mix in a total volume of 10 µL in a Mastercycler (Eppendorf). Data analysis of relative expression levels was based on the $2^{-\Delta Ct}$ calculation[177].

3.2.10. Eosinophil adhesion to endothelial cells under flow conditions

Eosinophil adhesion to HMVEC-L cells was investigated under flow conditions as described before[178]. Briefly, endothelial cells were seeded on 1 % gelatine-coated substrates at a density of 400,000 cells. Confluent monolayers of endothelial cells were stimulated with 10 pM of TNF-α or vehicle for 4 h. Substrates containing the cells were then assembled to the flow chamber (*VenaEC*^{TM} Biochips, Cellix) and freshly isolated eosinophils (3 x 10^5 cells) were perfused across the endothelial monolayer. Eosinophils were pre-treated with antagonist for 5 min followed by stimulation with agonist for further 5 min. Adhesion of eosinophils was recorded with a CCD camera using 10 x magnification for 2.5 min at 2 dyn/ cm^2.

4. Results

Part I: Signaling and trafficking properties of CRTH2 and DP receptors in a recombinant HEK293 cell model

4.1. Generation of HEK293 cell lines co-expressing CRTH2 and DP receptors

In order to examine the individual roles of CRTH2 and DP receptors in agonist-mediated signaling and trafficking patterns, I generated HEK293 cells stably expressing a tagged version of CRTH2 (HEK-CRTH2), DP (HEK-DP) or a combination of both receptors (HEK-CRTH2+DP). Immunofluorescence microscopy (Fig. 7A) using anti-Flag (DP) and anti-Myc (CRTH2) antibodies revealed that both receptors were expressed on the cell surface (Fig.7A, left and middle panel) and co-localized in HEK-CRTH2+DP cells (Fig 7A, right panel). Expression of DP (dark blue line) and CRTH2 (red line) in HEK-CRTH2+DP cells was also confirmed by flow cytometry (Fig. 7B, right panel). I found that expression levels of DP receptors on one hand, and CRTH2 on the other hand were similar in HEK-CRTH2+DP cells as in HEK-DP and HEK-CRTH2 cells, respectively (Fig.7B, left and middle panel). Next, I assessed the expression of both, CRTH2 and DP receptor protein in membrane preparations of HEK-DP and HEK-CRTH2 cells (Fig. 7C). Using antibodies specifically targeting the respective receptors, I found that DP (upper panel) and CRTH2 (lower panel) proteins were expressed at the appropriate protein sizes, i.e. receptor monomers at ~37 kDa and higher order homomers at ~ 70 kDa. Untransfected HEK293 cells served as negative control (con).

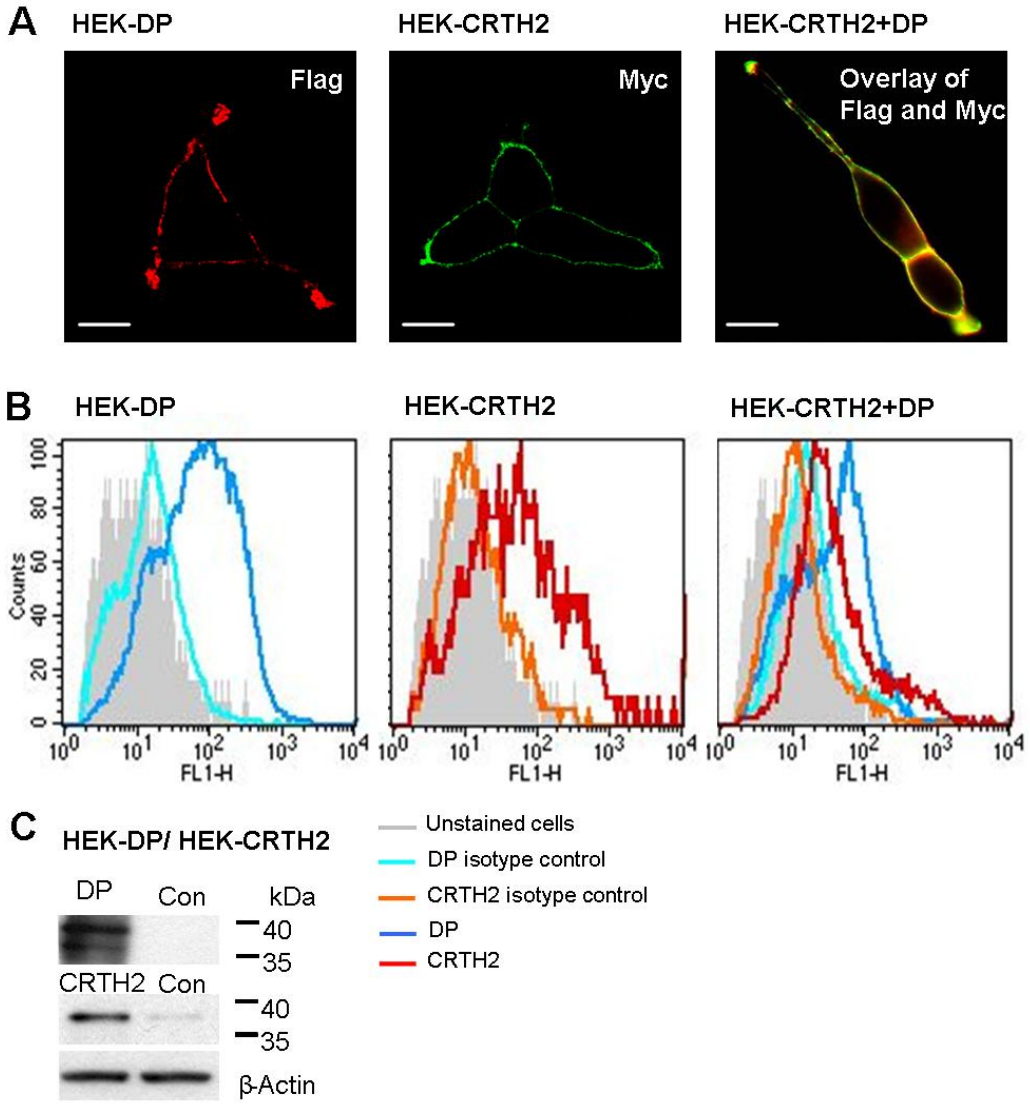


Figure 7. Expression patterns of CRTH2 and DP receptors in HEK-DP, HEK-CRTH2 and HEK-CRTH2+DP cells. HEK293 cells were stably transfected with Myc-CRTH2 (CRTH2) and Flag-DP (DP) and expression levels were determined by (**A**) immunofluorescence microscopy, (**B**) flow cytometry and (**D**) Western blot. (**A**) The DP (red) and CRTH2 (green) receptors were targeted with anti-Flag or anti-Myc antibodies and stained with Alexa Fluor 488 IgG_{2B} or Alexa Fluor 594 IgG_1, overlay (yellow), scale bar = 10 µm. (**B**) DP (dark blue line, anti-Flag) and CRTH2 (red line, anti-Myc) receptors were stained with Alexa Fluor 488 IgG_{2B} or IgG_1, respectively. **(C)** DP and CRTH2 protein expression was determined in membrane preparations from HEK-DP (upper panel) or HEK-CRTH2 (middle panel) cells using polyclonal rabbit anti-PGD_2 receptor and polyclonal rabbit anti-human CRTH2 antibodies, respectively. Plain HEK293 cells (Con) are shown for comparison. Lysate samples immunoblotted for β-actin served as loading controls.

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

Previously, I observed an agonist-mediated intracellular Ca^{2+} release in human peripheral blood eosinophils after stimulation with PGD_2 or the CRTH2-selective agonist 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) (see Schuligoi *et al*., 2009[179] and results part II, Fig. 27). Notably, the DP-selective agonist BW245c failed to invoke Ca^{2+} mobilization. To further specify the respective roles of CRTH2 and DP in Ca^{2+} signaling, I tested these agonists in HEK-CRTH2+DP (■), HEK-CRTH2 (▲), HEK-DP (▼) and HEK (○) cell lines (Fig. 8). I found that PGD_2 failed to induce intracellular Ca^{2+} release in cells expressing CRTH2 alone, but evoked potent Ca^{2+} responses in HEK-DP cells or HEK-CRTH2+DP cells (Fig. 8A). Similarly, the CRTH2-selective agonist DK-PGD_2 failed to induce Ca^{2+} release in HEK-CRTH2 cells and was without effect in HEK-DP cells (Fig. 8B). However, DK-PGD_2 evoked potent Ca^{2+} responses in cells expressing both receptors (Fig. 8B). In agreement with the potent PGD_2 responses in DP-expressing cells (see Fig. 8A), the DP-selective agonist BW245c strongly induced intracellular Ca^{2+} mobilization in HEK-DP cells (Fig. 8C). In HEK-CRTH2+DP cells, this effect was significantly diminished, which was also reflected by a nine-fold increase in EC_{50} values for BW245c in HEK-CRTH2+DP cells versus HEK-DP cells (Table 3). As expected, HEK-CRTH2 cells did not respond to BW245c (Fig. 8C), similar to untransfected HEK293 cells which showed no Ca^{2+} response to any of the agonists (Figs. 8A-C). In contrast, all cell lines responded in a similar manner to the Ca^{2+} ionophor A23187 (Fig. 8D).

These data clearly suggest that the DP receptor is critically involved in mediating Ca^{2+} flux in response to CRTH2 activation and *vice versa*.

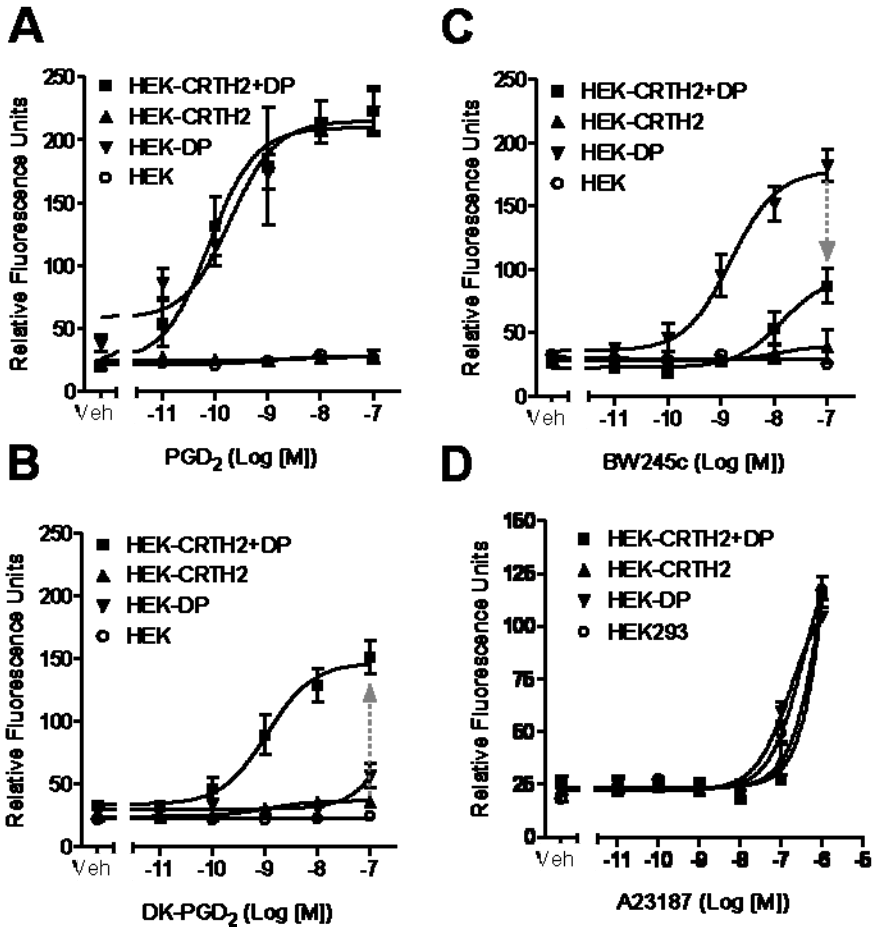


Figure 8. The DP receptor modulates CRTH2-induced intracellular Ca^{2+} release in HEK-CRTH2+DP cells. Agonist-induced intracellular Ca^{2+} release was measured in a FLEXStation II in HEK-CRTH2+DP (■), HEK-CRTH2 (▲), HEK-DP (▼) or untransfected HEK (○) cells. Cells were stimulated with (**A**) PGD_2, (**B**) DK-PGD_2, (**C**) BW245c or (**D**) the Ca^{2+} ionophor A23187. Data were normalized to cell numbers and are shown as relative fluorescence units (means of 3-5 experiments ± S.E.M.) carried out in duplicates.

Table 3. EC_{50} and E_{max} values of ligand-induced Ca^{2+} release in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells.

	Ca^{2+} release EC_{50} (nM)			Ca^{2+} release E_{max} (RFU)		
	CRTH2	DP	CRTH2+DP	CRTH2	DP	CRTH2+DP
PGD_2	-	0.19 ± 0.27	0.08 ± 0.16	-	157 ± 1.82	186 ± 3.40
+Cay10471	-	0.51 ± 0.13	1.02 ± 0.28	-	183 ± 0.70	108 ± 2.80
+BWA 868c	-	-	-	-	-	-
DK-PGD_2	-	-	1.09 ± 0.10	-	-	113 ± 1.20
+Cay10471	-	-	-	-	-	-
+BWA 868c	-	-	-	-	-	-
BW245c	-	1.51 ± 0.09	13.7± 0.12	-	143 ± 1.70	73.2 ± 3.40
+Cay10471	-	6.07 ± 0.08	12.9 ± 0.19	-	133 ± 3.10	51.7 ± 3.90
+BWA 868c	-	-	-	-	-	-

Data are means ± S.E.M. of three to five independent experiments performed in duplicates. RFU, relative fluorescence units; EC_{50}, half maximal effective concentration; E_{max}, maximal possible effect-, no response

4.3. Increased DP receptor levels enhance CRTH2-mediated intracellular Ca^{2+} release

To further substantiate the observation that CRTH2-mediated Ca^{2+} signaling was regulated by the DP receptor I performed gene dose experiments (Fig. 9). Here, HEK-CRTH2 cells were transiently transfected with increasing doses of DP receptor DNA and stimulated with 100 nM of DK-PGD_2. I found that an increase in DP receptor expression in the presence of CRTH2 resulted in a concordant dose-dependent increase in DK-PGD_2-mediated intracellular Ca^{2+} release (Fig. 9A). To rule out the possibility that CRTH2-mediated responses might be regulated non-specifically by any 7TM/GPCR, I repeated these experiments with the DNA of the CB2 cannabinoid receptor, which is also expressed in human eosinophils[180]. As Fig. 9B shows, no change in DK-PGD_2-mediated Ca^{2+} mobilization was observed in HEK-CRTH2 cells co-transfected with increasing amounts of CB2 receptor DNA. The expression levels of DP and CB2 receptors were confirmed by ELISA (Figs. 9C and D).

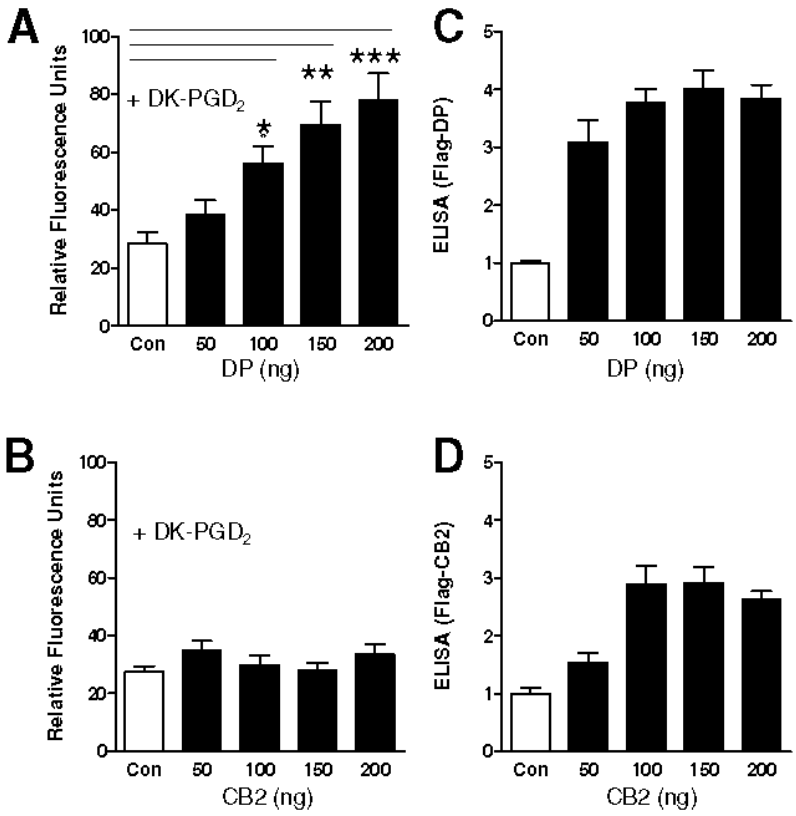


Figure 9. Increasing levels of DP receptor enhance the CRTH2-mediated Ca^{2+} response. (A, B) Agonist-induced intracellular Ca^{2+} release was measured in a FLEXStation II. HEK-CRTH2 cells were transiently co-transfected with DP or CB2 pcDNA and stimulated with 100 nM of DK-PGD_2. Data were normalized to cell numbers (triplicates of *n*=3-5) and are shown as relative fluorescence units (means ± S.E.M.) * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. (**C, D**) Receptor expression levels were determined by ELISA against the Flag-epitopes of DP and CB2, respectively. Data are shown as fold increase of the control.

4.4. Desensitization of the DP receptor prevents CRTH2-induced intracellular Ca^{2+} release

Rapid desensitization of 7TM/GPCR signaling is an important regulatory mechanism to maintain cellular homeostasis in response to a given agonist. If the DP receptor was required for a proper Ca^{2+} response via CRTH2, then the desensitization of the DP receptor should prevent agonist-mediated Ca^{2+} release via CRTH2. Hence, HEK-DP and HEK-CRTH2+DP cells were pre-treated with 10 nM of the DP agonist BW245c or its vehicle at 37 °C for 10 min to desensitize the DP response followed by stimulation with 100 nM of agonist (Fig. 10). HEK-CRTH2+DP (Fig. 10A) and HEK-DP (Fig. 10B) cells pre-treated with vehicle showed effective Ca^{2+} responses to DK-PGD_2 and PGD_2, respectively, while cells pre-incubated with BW245c were unresponsive.

This implicates that the agonist-induced desensitization of DP receptors caused blockade of the CRTH2 signaling capacity.

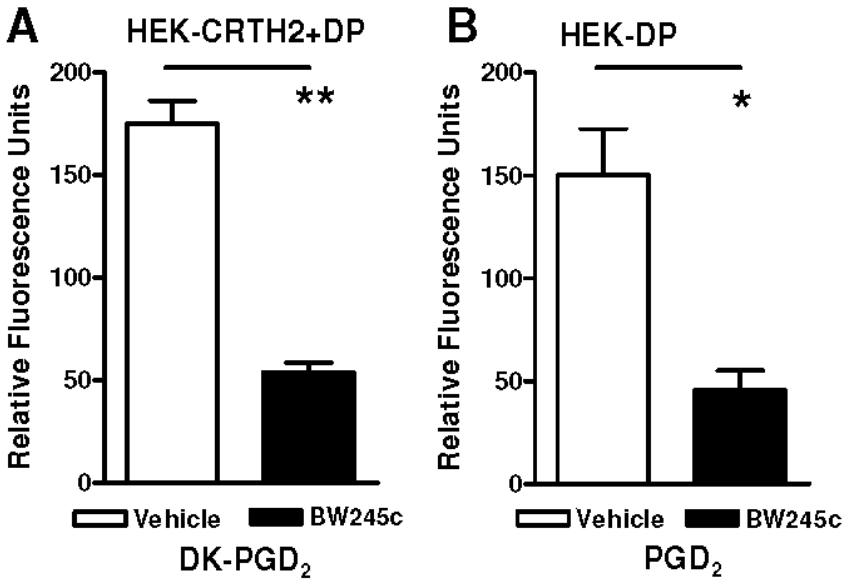


Figure 10. DP desensitization prevents CRTH2-mediated Ca^{2+} mobilization in HEK-CRTH2+DP and HEK-DP cells. Agonist-induced intracellular Ca^{2+} release was measured in a FLEXStation II. **(A)** HEK-CRTH2+DP and **(B)** HEK-DP cells were pretreated with BW245c (10 nM) or vehicle for 10 min and were then stimulated with 100 nM (**A**) DK-PGD_2 or (**B**) PGD_2. Data are shown as relative fluorescence units (means of 4 experiments ± S.E.M.) carried out in duplicates. * $P < 0.001$, ** $P < 0.0001$.

4.5. Selective CRTH2 or DP receptor antagonists modulate the Ca^{2+} response in HEK-CRTH2+DP cells

In further experiments, I explored how CRTH2- and DP-selective antagonists were able to modulate this putative cross-regulation of CRTH2 and DP receptors (Figs. 11 and 12). I found that 1 µM of the CRTH2-selective antagonist Cay10471 (Fig. 11A) only partially prevented the PGD_2-induced Ca^{2+} response in HEK-CRTH2+DP cells. In contrast, Cay10471 completely prevented the Ca^{2+} response to DK-PGD_2 in HEK-CRTH2+DP cells (Fig. 11B) suggesting that the binding of this CRTH2-selective agonist to its receptor is a prerequisite for Ca^{2+} signaling. Cay10471 did not significantly alter the responses to BW245c in HEK-CRTH2+DP cells (Fig. 11C). Likewise, the CRTH2-antagonist Cay10471 had no effect on PGD_2- (Fig. 11D), or BW245c- (Fig. 11E) induced Ca^{2+} release in HEK-DP cells.

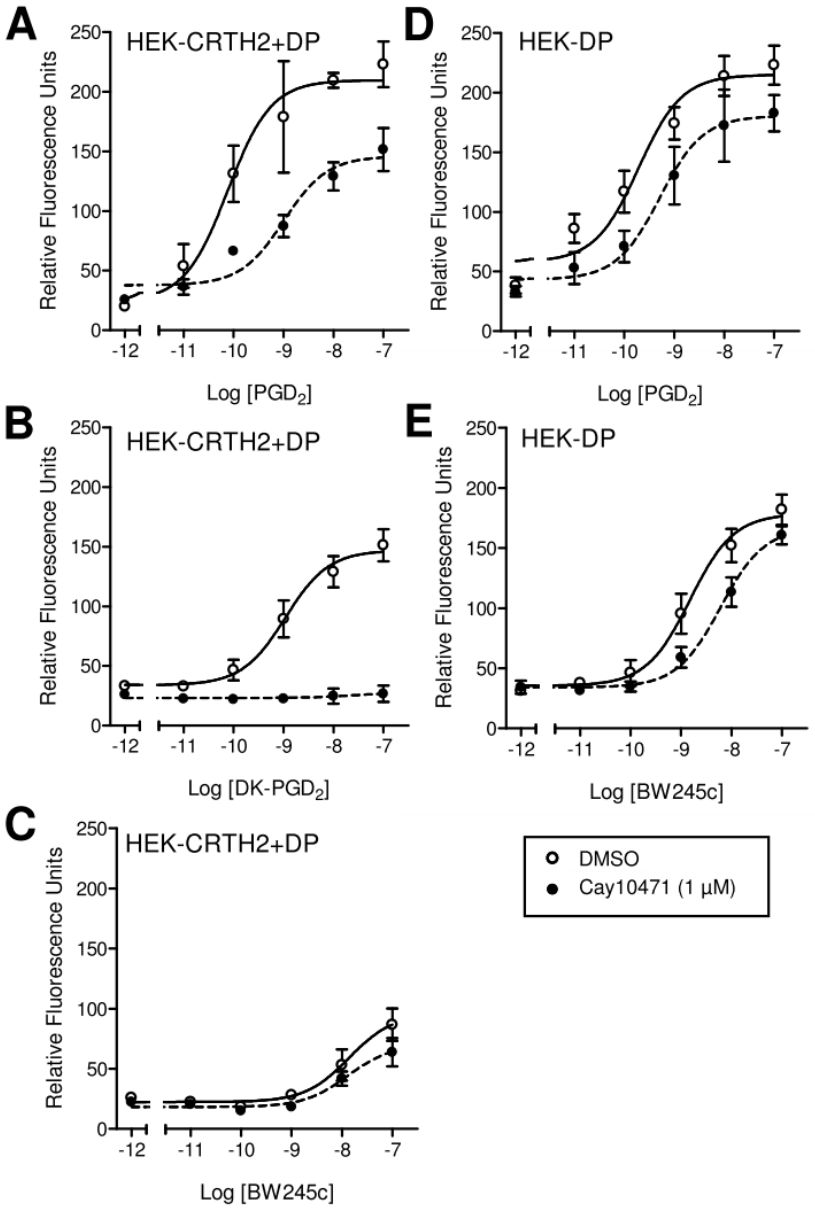


Figure 11. Effect of the CRTH2 antagonist Cay10471 on agonist-induced intracellular Ca^{2+} release in HEK-CRTH2+DP and HEK-DP cells. Agonist-induced intracellular Ca^{2+} release was measured in a FLEXStation II. Cells were treated with Cay10471 (1 µM) or vehicle 15 minutes before agonist application. Data were normalized to cell numbers and are shown as relative fluorescence units (means ± S.E.M.) carried out in duplicates (*n*=3-5).

Next, I tested whether 1 µM of the selective DP antagonist BWA868c would alter the signaling properties of HEK-CRTH2+DP cells. BWA868c was able to completely abolish both the PGD_2- (Fig. 12A) and DK-PGD_2- (Fig. 12B) mediated Ca^{2+} release in HEK-CRTH2+DP cells. Moreover, BWA868c blocked the Ca^{2+}-release in HEK-CRTH2+DP cells after stimulation with the DP-selective agonist BW245c (Fig. 12C), as well as the PGD_2- (Fig. 12D) or BW245c- (Fig. 12E) mediated Ca^{2+} release in HEK-DP cells.

Together with the impact of DP desensitization on CRTH2 signaling, these data indicate a prominent role for the DP receptor in mediating Ca^{2+} release when co-expressed with CRTH2.

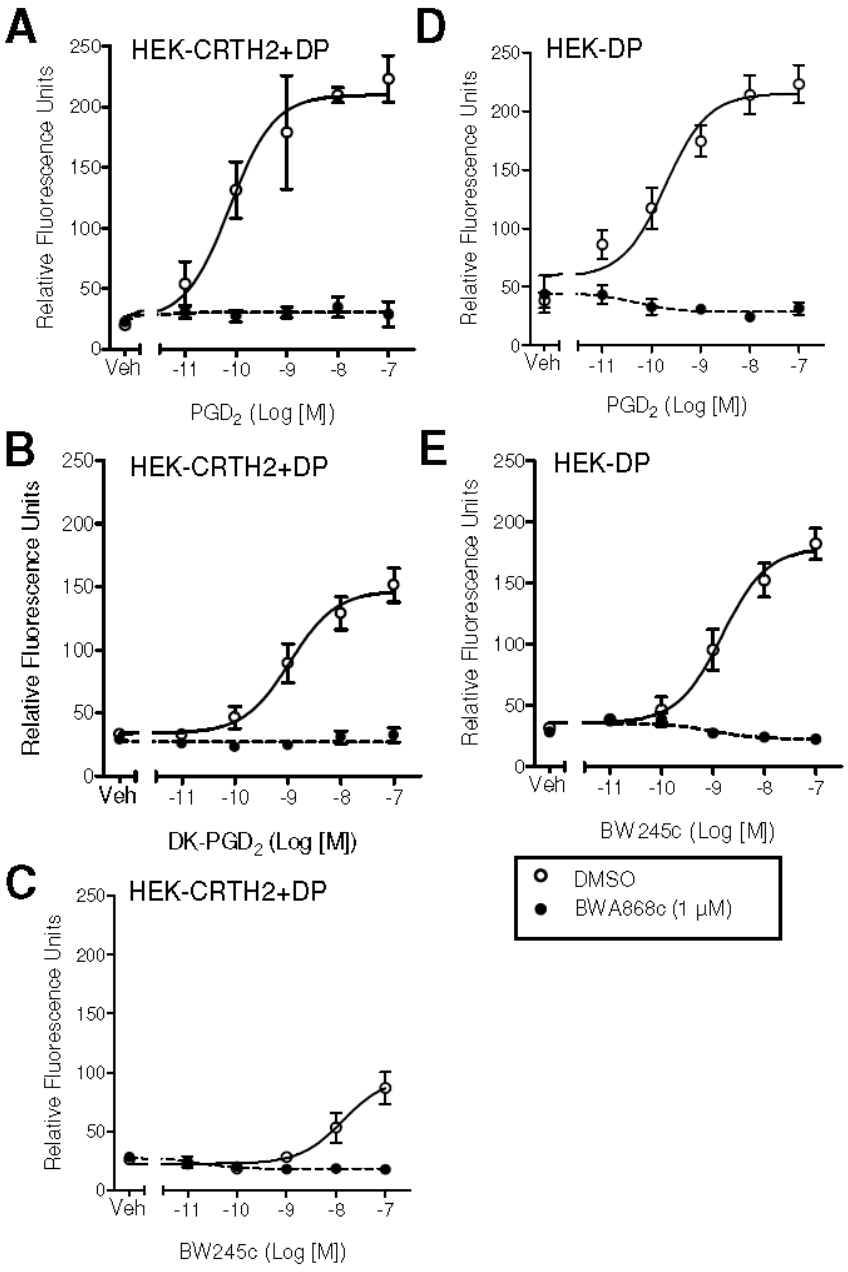


Figure 12. The DP antagonist BWA686c prevents agonist-induced intracellular Ca^{2+} release in HEK-CRTH2+DP and HEK-DP cells. Agonist-induced intracellular Ca^{2+} release was measured in a FLEXStation II. Cells were treated with BWA868c (1 µM) or vehicle 15 minutes before agonist application. Data were normalized to cell numbers and are shown as relative fluorescence units (means ± S.E.M.) carried out in duplicates (*n*=3-5).

4.6. DP and CRTH2 receptors form heteromers

Based on the co-localization (Fig. 7) and the altered calcium signaling properties (Figs. 8 and 9) of the CRTH2 and DP receptors in cells co-expressing both receptors, I wondered whether a heteromerization between CRTH2 and DP receptors might explain my observations. To this end, I co-immunoprecipitated CRTH2 with the DP receptor and *vice versa* from membrane preparations of HEK-CRTH2+DP cells using an anti-Flag or anti-c-Myc antibody affinity matrix (Fig. 13). Proteins were separated and immunoblotted with anti-c-Myc or anti-Flag antibodies, yielding a band that corresponded to the molecular size (37 kDa) of the CRTH2 protein (Fig. 13A, top panel, CRTH2+DP lane) and the DP receptor protein (Fig. 13A, second panel, CRTH2+DP lane). Plain HEK293 cells served as a negative and HEK-CRTH2 (Fig. 13A, third panel, CRTH2 lane) or HEK-DP (Fig. 13A, bottom panel, DP lane) cells as positive controls. Collectively, these data suggested that DP and CRTH2 receptors physically interact with each other, thus implicating the formation of CRTH2/DP heteromers. Since I found that the DP antagonist BWA868c completely abolished PGD_2- and DK-PGD_2-induced Ca^{2+} release in HEK-CRTH2+DP cells (Fig. 12A and B), I tested whether BWA868c could also prevent CRTH2/DP heteromer formation. Cells were incubated with 1 μM of BWA868c for 15 min before isolating the membranes. Subsequent co-immunoprecipitation of DP receptors with CRTH2 revealed that CRTH2/DP heteromer formation was not affected by the presence of the DP antagonist (Fig. 13B, top panel, CRTH2+DP lanes BWA868c-treated/ vehicle-treated). Expression of CRTH2 and DP receptors in HEK-CRTH2, HEK-DP, HEK-CRTH2+DP and plain HEK293 cells is shown in the middle and the bottom panel of Fig. 13B, respectively.

Conclusively, these findings suggest that the presence of CRTH2/DP heteromers accounts for the altered calcium signaling in HEK-CRTH2+DP cells.

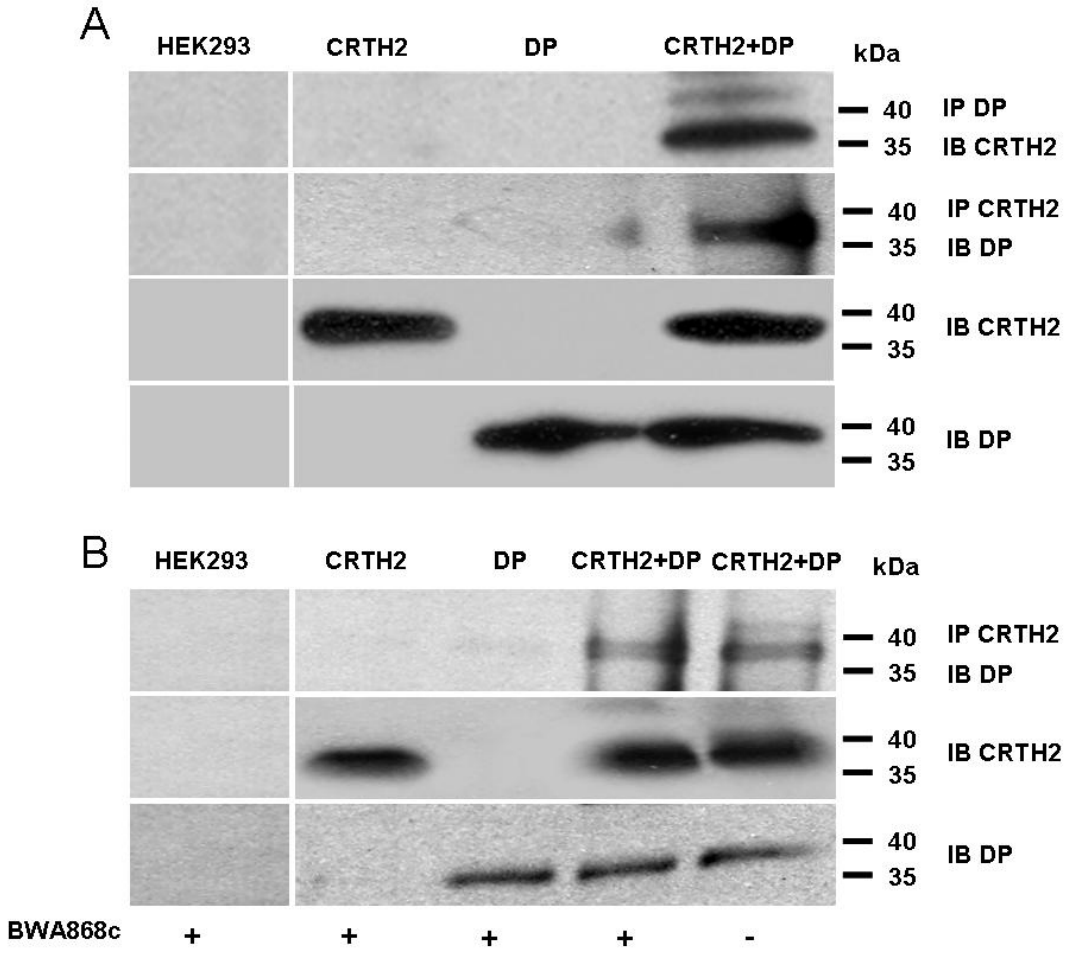


Figure 13. CRTH2 and DP receptors form heteromers in HEK-CRTH2+DP cells. **(A)** CRTH2 or DP receptors were co-immunoprecipitated from HEK-CRTH2+DP membranes with either Flag (first panel, IP DP) or Myc (second panel, IP CRTH2) antibodies. Myc-CRTH2 and Flag-DP receptors were Western-blotted using anti-c-Myc (third panel, IB CRTH2,) or anti-Flag (last panel, IB DP) antibodies. **(B)** Cells were treated with 1 μM of BWA868c for 15 minutes before membrane isolation (first panel). CRTH2 and DP expression was visualized as described above and is shown in the middle and the last panel, respectively. Shown are representative blots out of three independent experiments.

4.7. Ligand-binding properties are not altered in CRTH2/DP heteromers

Formation of 7TM/GPCR heteromers is known to potentially affect their ligand-binding properties[181]. I found that the Ca^{2+} signaling properties of CRTH2 and DP co-expressed in HEK293 cells are profoundly altered (see Fig. 8 and 9). Accordingly, in collaboration with Evi Kostenis' team at the Institute of Pharmaceutical Biology in Bonn, Germany we set out to determine whether ligand-binding is affected in HEK-CRTH2+DP cells. Ralf Schröder and Kathrin Bell performed competition binding experiments on HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells, which had been generated in our laboratory, with [^{3}H]PGD_2 as the

radioligand (Fig. 14). Even though the CRTH2 agonists failed to induce a Ca^{2+} response in HEK-CRTH2 cells - while HEK-CRTH2+DP cells efficiently responded (see Fig. 8A and B) - they found that the binding of PGD_2 and DK-PGD_2 to CRTH2 was not compromised in HEK-CRTH2 cells, as reflected by the pIC_{50} values of 7.51 $\pm$ 0.05 and 7.56 $\pm$ 0.07 (Fig. 14A) compared to 7.79 $\pm$ 0.11 and 7.97 $\pm$ 0.14 in HEK-CRTH2+DP cells (Fig. 14C). Likewise, PGD_2 and BW245c had equal affinities of pIC_{50} = 8.05 $\pm$ 0.09 and 7.99 $\pm$ 0.10 to DP in HEK-DP cells (Fig. 14B) and pIC_{50} = 7.79 $\pm$ 0.11 and 8.33 $\pm$ 0.28 in HEK-CRTH2+DP cells (Fig. 14C). In addition, binding of the CRTH2 and DP antagonists was not influenced by the presence of receptor heteromers. Antagonist competition curves in HEK-CRTH2+DP cells were best fitted using a two-site model. The affinity values for Cay10471 were pIC_{50} = 8.63 $\pm$ 0.07 (HEK-CRTH2, Fig. 14A) and pIC_{50high} = 8.72 $\pm$ 0.23, pIC_{50low} = 5.17 $\pm$ 0.95 (HEK-CRTH2+DP, Fig. 14C). BWA868c binding was estimated to be pIC_{50} = 8.17 $\pm$ 0.07 (HEK-DP, Fig. 14B) and pIC_{50high} = 8.74 $\pm$ 0.24, pIC_{50low} = 5.20 $\pm$ 0.39 (HEK-CRTH2+DP, Fig. 14C). Non-specific binding of Cay10471 and BWA868c to DP and CRTH2, respectively, occurred at high concentrations (>1 μM). A summary of pIC_{50} values is shown in Table 4.

In conclusion, these data implicate that i) receptor-binding of CRTH2 and DP ligands to the respective receptors is similar in all three cell lines and ii) the altered signaling properties of CRTH2/DP heteromers are not caused by changes in the respective ligand-binding sites.

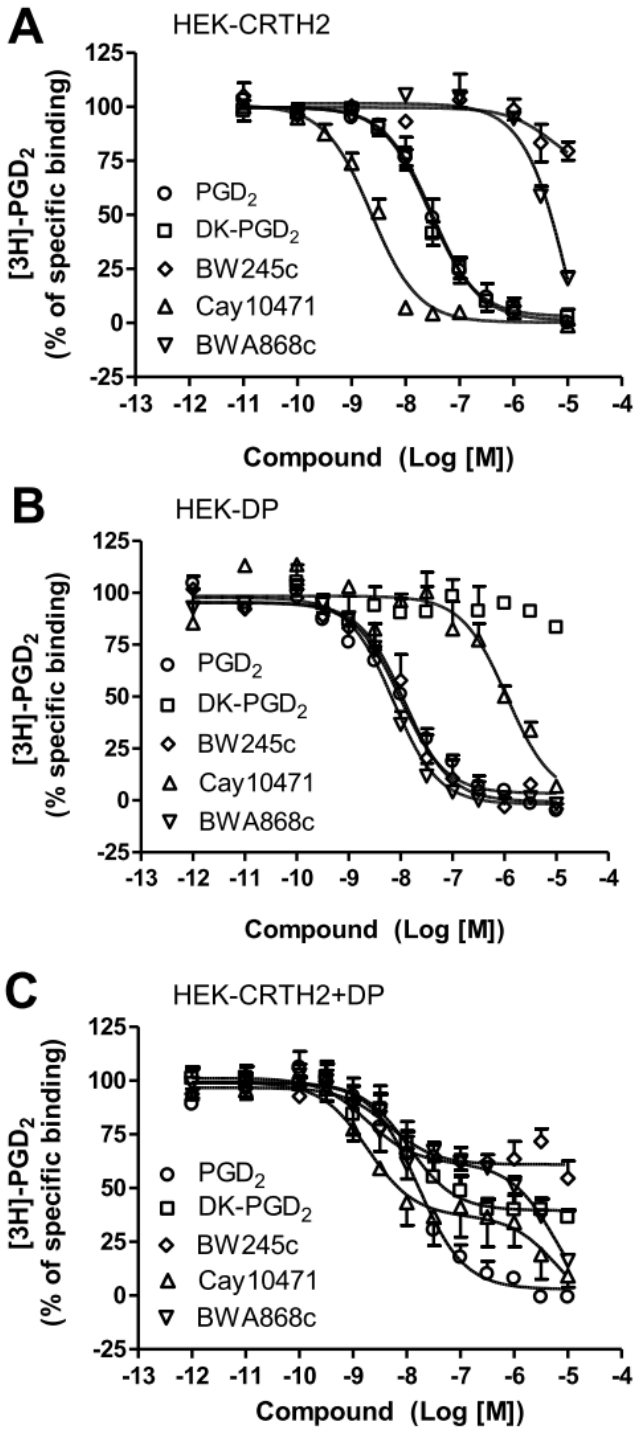


Figure 14. Ligand-binding properties of CRTH2 and DP receptor mono- and heteromers. Competition-binding experiments were performed using 2 nM of [^{3}H]PGD_2 on whole **(A)** HEK-CRTH2, **(B)** HEK-DP and **(C)** HEK-CRTH2+DP cells. Data points represent the mean values ± S.E.M of three to five independent experiments, each performed in triplicate. ***Performed by Ralf Schröder and Kathrin Bell, Institute of Pharmaceutical Biology, Bonn, Germany***

Table 4. pIC_{50} values of ligand competition-binding experiments on HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells.

	Affinity (pIC_{50})		
	CRTH2	DP	CRTH2+DP
PGD_2	7.51 ± 0.05	8.05 ± 0.09	7.79 ± 0.11
DK-PGD_2	7.56 ± 0.07	-	7.97 ± 0.14
BW245c	-	7.99 ± 0.10	8.33 ± 0.28
Cay10471	8.63 ± 0.07	6.02 ± 0.22	pIC_{50high} = 8.72 ± 0.23 pIC_{50low} = 5.17 ± 0.95
BWA868c	4.90 ± 0.33	8.17 ± 0.07	pIC_{50high} = 8.74 ± 0.24 pIC_{50low} = 5.20 ± 0.39

Data are means ± S.E.M. of three to five independent experiments performed in triplicates. -, not calculated

4.8. The Ca^{2+} signals in HEK-CRTH2+DP are mediated by both $G\alpha_i$-and $G\alpha_{q/11}$-proteins

Based on our finding that ligand-binding in the CRTH2/DP heteromer is not altered, I hypothesized that the modulation of CRTH2 and DP calcium signaling might occur on the level of Gα-protein coupling and/or further downstream in the signaling cascade. Therefore, I retraced the origin of the CRTH2- and DP-mediated Ca^{2+} signals in HEK-CRTH2+DP cells to decipher the involvement of $G\alpha_i$- and $G\alpha_{q/11}$-proteins (Fig.15). Thus, cells were pre-incubated with 100 ng/mL of the $G\alpha_i$-protein inhibitor pertussis toxin (PTX) for 4 h, with 0.1 nM - 1 µM of the $G\alpha_{q/11}$-protein inhibitor MH-362-63-8[182] for one hour, or their respective vehicles prior to CRTH2 or DP stimulation. While PTX partially inhibited the PGD_2-mediated Ca^{2+} release (Fig. 15A) and completely prevented the DK-PGD_2-mediated Ca^{2+} signal (Fig. 15B), it had no effect on BW245c-induced Ca^{2+} release in HEK-CRTH2+DP cells (Fig. 15C). In contrast, PTX caused no inhibition of PGD_2-mediated Ca^{2+} release in HEK-DP cells (Fig. 15G). MH-362-63-8 completely eliminated the PGD_2- and DK-PGD_2-mediated Ca^{2+} release in HEK-CRTH2+DP cells (Fig. 15D and 15E). The $G\alpha_{q/11}$-protein inhibitor also blocked BW245c-induced Ca^{2+} release in HEK-CRTH2+DP (Fig. 15F) and HEK-DP cells (Fig. 15H). HEK293 cells endogenously expressing the *bona fide* $G\alpha_q$ protein-linked muscarinic M3 receptor served as positive control. The carbachol-induced Ca^{2+} response was completely inhibited by MH-362-63-8 (Fig. 15I).

In summary, CRTH2-elicited Ca^{2+} release - in the presence of the DP receptor - is dependent on $G\alpha_{q/11}$- *and* $G\alpha_i$-proteins while DP-mediated Ca^{2+} responses are solely derived

from $G\alpha_{q/11}$-proteins. The proposed mechanism of calcium signaling in HEK293 cells expressing CRTH2 and DP receptors and the consequences of co-expression of both receptors are illustrated in Fig. 16.

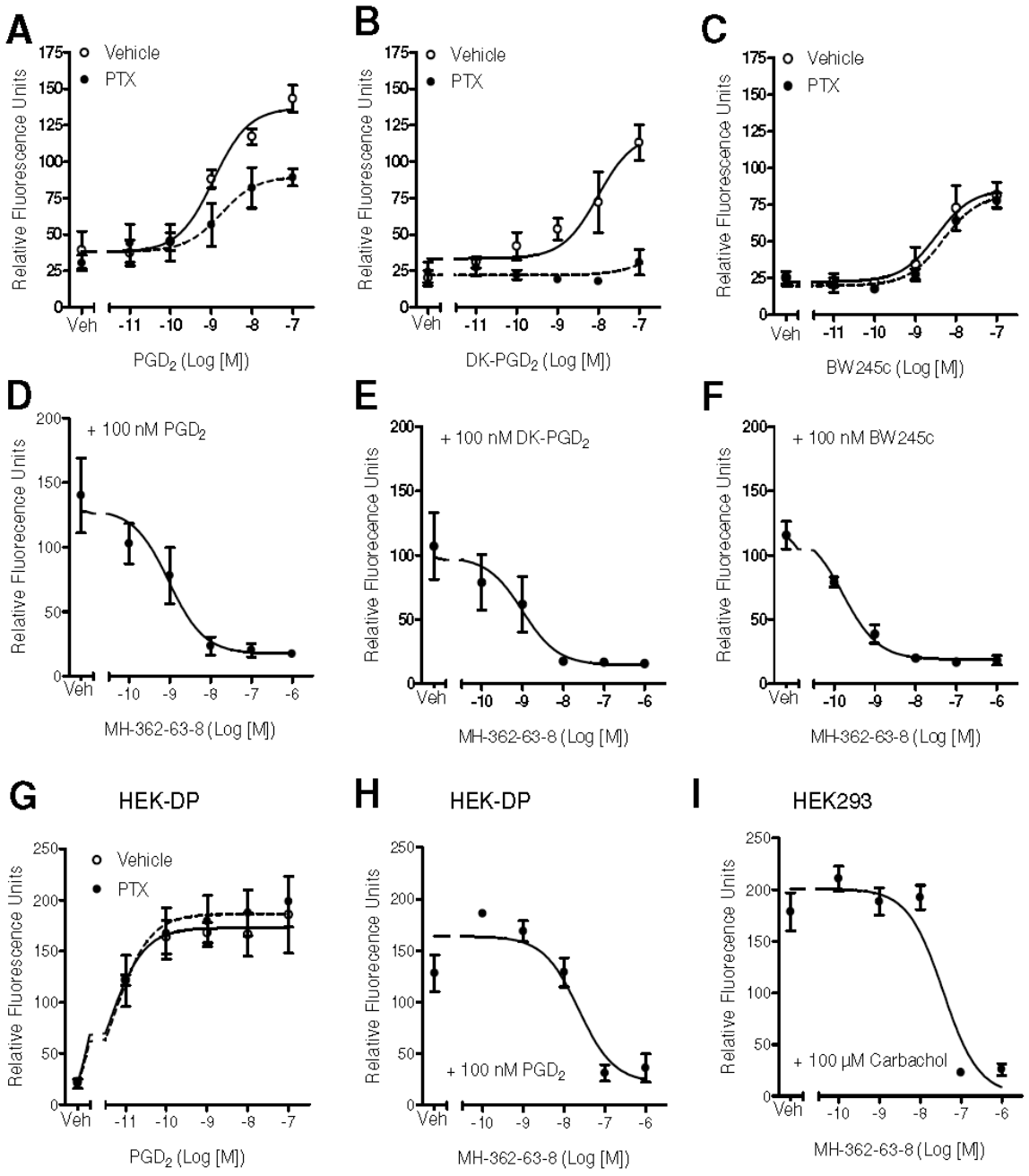


Figure 15. The Ca^{2+} signals in HEK-CRTH2+DP are mediated by both $G\alpha_i$-and $G\alpha_{q/11}$-proteins. Agonist-induced intracellular Ca^{2+} release was measured in HEK-CRTH2+DP cells treated with **(A-C, G)** 100 ng/mL PTX for 4 h or **(D-F, H-I)** MH-362-63-8 for 1 h. Data were normalized to cell numbers and are shown as relative fluorescence units (means ± S.E.M.) carried out in duplicates (*n*=3-5).

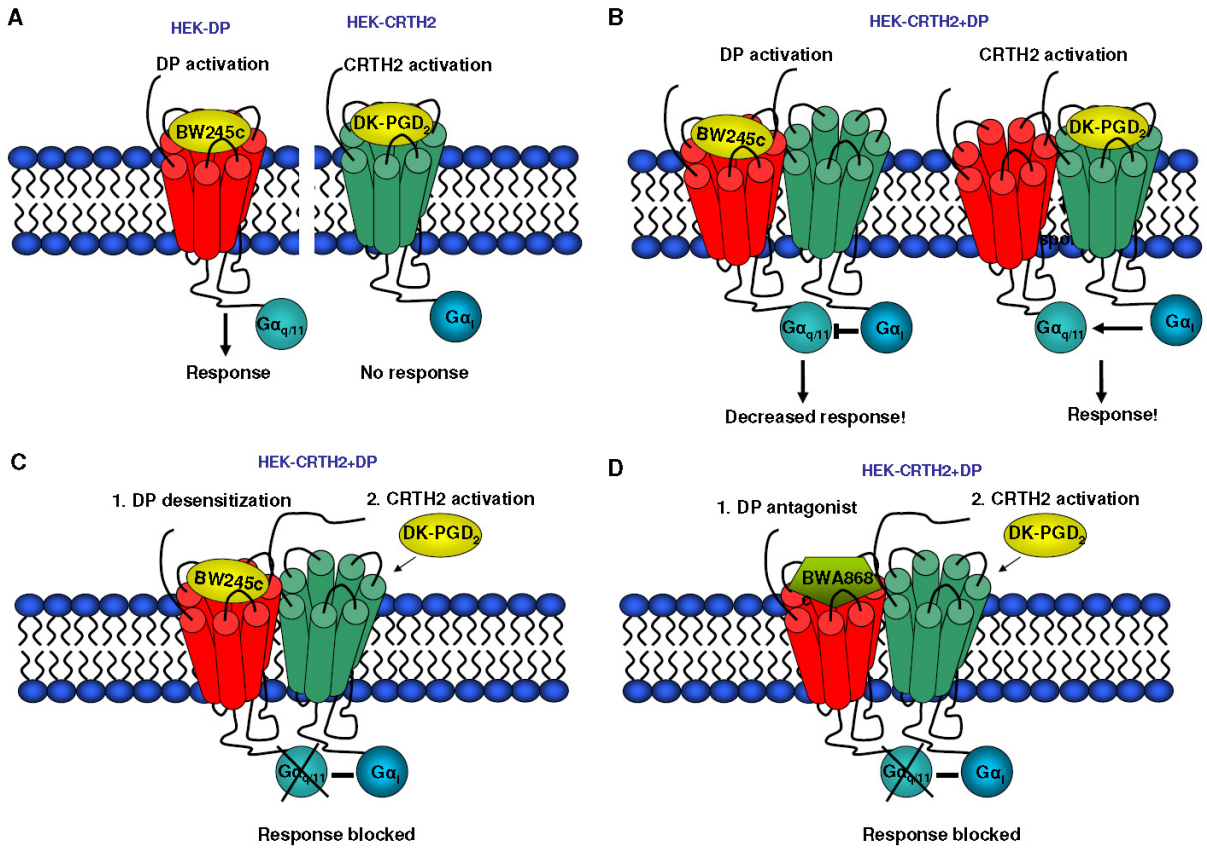


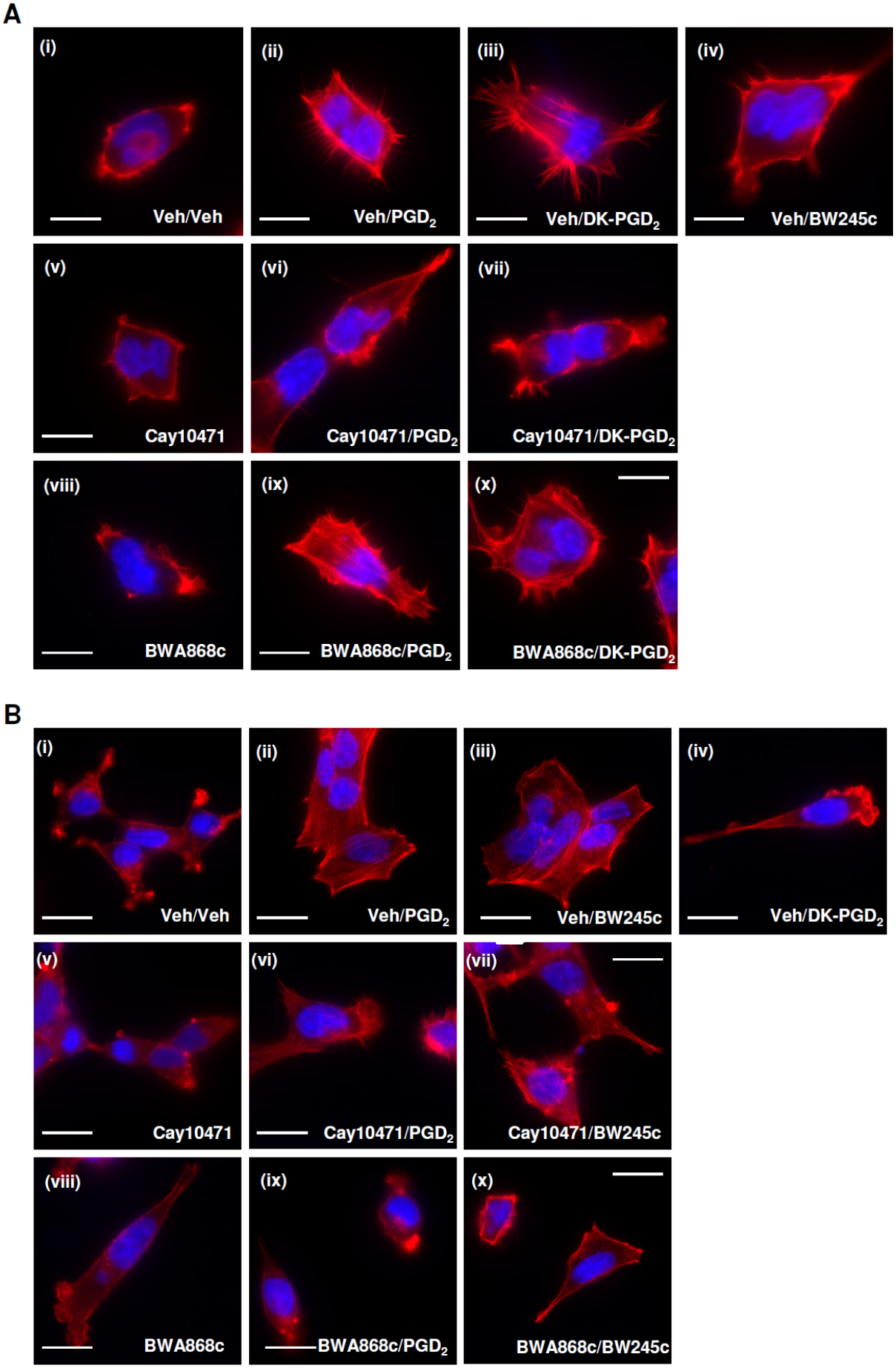
Figure 16. Proposed mechanism of CRTH2 and DP signaling in HEK-DP, HEK-CRTH2 and HEK-CRTH2+DP cells. (A) In HEK-DP cells, activation of the DP receptor with the DP agonist BW245c induces a $G\alpha_{q/11}$-protein mediated Ca^{2+} response. In contrast, stimulation of HEK-CRTH2 cells with the CRTH2 agonist DK-PGD_2 fails to activate intracellular Ca^{2+} release. **(B)** Upon CRTH2/DP heteromerization in HEK-CRTH2+DP cells, the BW245c-induced DP response is decreased in the presence of CRTH2. DK-PGD_2 induces intracellular Ca^{2+} release in HEK-CRTH2+DP by transactivating the DP receptor in a $G\alpha_i$-protein-dependent manner. **(C)** Desensitization of the DP receptor by BW245c in HEK-CRTH2+DP cells prevents the CRTH2-induced Ca^{2+} response. **(D)** The DP antagonist BWA868c abolishes the CRTH2-induced Ca^{2+} response in HEK-CRTH2+Dp cells.

4.9. CRTH2 and DP receptors mediate F-actin formation in HEK293 cells

Intracellular Ca^{2+} levels closely correlate with the migratory capacity of a given cell. It is known that PGD_2-induced chemotaxis of eosinophils does not only engage CRTH2[115], but also that the DP receptor plays a role in eosinophil migration[135]. Cellular movement requires rearrangement of the actin cytoskeleton involving actin monomers (G-actin) forming polymers (F-actin). Hence, I set out to decipher the impact of both PGD_2 receptors on cytoskeletal rearrangement and investigated actin polymerization in HEK293 cells (Fig. 17). For visualization of polymerized actin I used Phalloidin, which is a phallotoxin isolated from the *Amanita phalloides* mushroom and selectively attaches to F-actin[183]. HEK-CRTH2, HEK-

DP and HEK-CRTH2+DP cells were stained for F-actin following stimulation with 100 nM of agonist or vehicle for 10 min. Pre-treatment with 1 µM of antagonists or vehicle were performed for 10 min at 37 °C. Unstimulated HEK293 cells showed regular polygonal shapes with dense cortical F-actin fibers but without protrusions and stress fibers in the cytoplasm (i). In HEK-CRTH2 cells (Fig. 17A), PGD_2 (ii) as well as DK-PGD_2 (iii) induced characteristic formation of fuzzy protrusions and stress fibers all over the cell, while BW245c (iv) treatment caused no cytoskeletal rearrangement. This phenotype was completely prevented by the CRTH2 antagonist Cay10471 (v-vii), but was not affected by the DP antagonist BWA868c (viii-x). In HEK-DP cells (Fig. 17B) treatment with PGD_2 (ii) or BW245c (iii) resulted in prominent stress fiber formation, while DK-PGD_2 (iv) had not effect in HEK-DP cells. PGD_2- and BW245c-induced changes were not prevented by Cay10471 (v-vii), but were blocked upon pre-treatment with BWA868c (viii-x). In HEK-CRTH2+DP cells (Fig. 17C) the PGD_2-induced F-actin formation was partially reduced by Cay10471 (ii and vi), but was not inhibited by BWA868c (x). DK-PGD_2- and BW245c-mediated stress fiber formation was selectively blocked by Cay10471 (iii and vii) and BWA868c (iv and xii), respectively. Both, vehicle and antagonists had no effect *per se* in any cell line (Fig. 17A-C, (i), (v) and (ix)).

In conclusion, this set of data indicates that both, CRTH2 and DP receptors can induce actin polymerization, whereby CRTH2 predominatly activates spreading of protrusions from the membrane, while the DP receptor strongly induces intracellular stress fiber formation. When both receptors are co-expressed and stimulated simultaneously with PGD_2, CRTH2 seems to be the predominant receptor to induce changes in cell morphology and the cytoskeleton.


A
(i)
Veh/Veh
(ii)
Veh/PGD2
(iii)
Veh/DK-PGD2
(iv)
Veh/BW245c
(v)
Cay10471
(vi)
Cay10471/PGD2
(vii)
Cay10471/DK-PGD2
(viii)
BWA868c
(ix)
BWA868c/PGD2
(x)
BWA868c/DK-PGD2
B
(i)
Veh/Veh
(ii)
Veh/PGD2
(iii)
Veh/BW245c
(iv)
Veh/DK-PGD2
(v)
Cay10471
(vi)
Cay10471/PGD2
(vii)
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(x)
BWA868c/BW245c

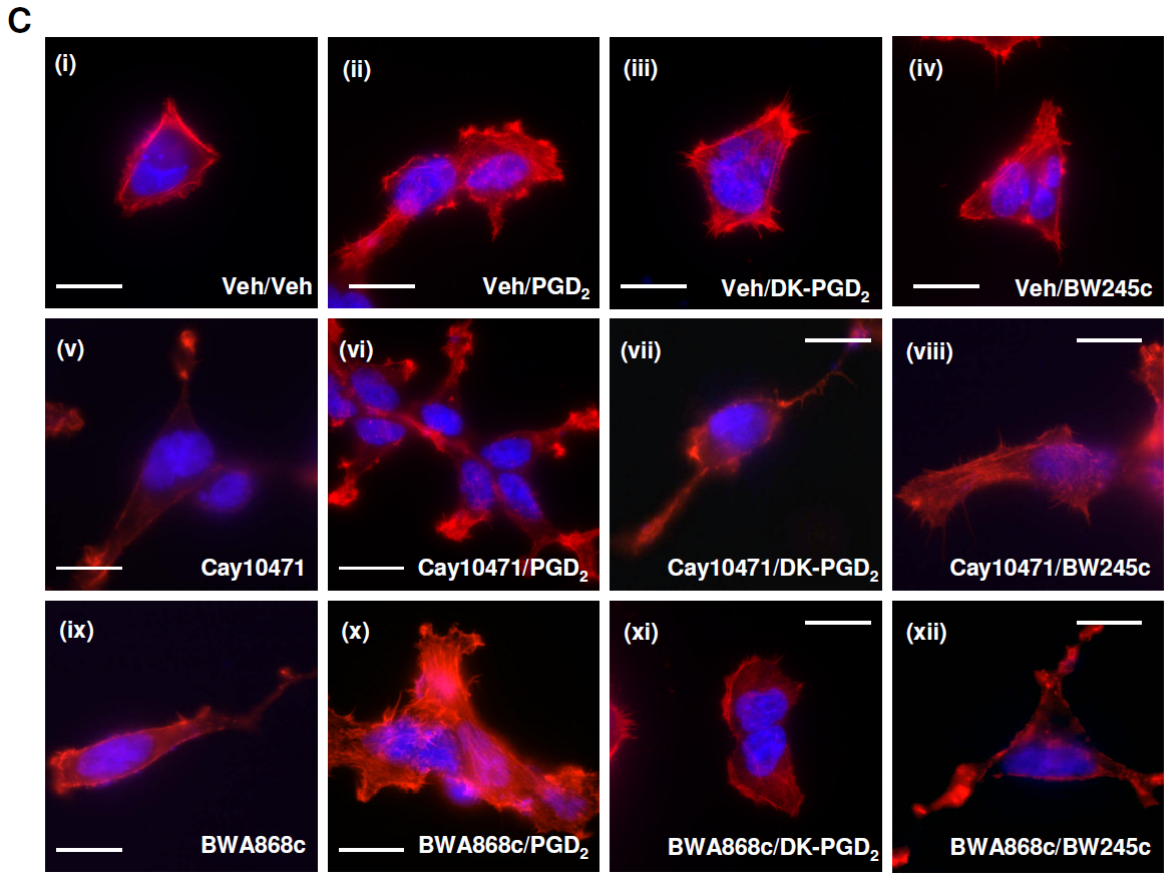

Figure 17. Agonist-induced actin polymerization in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells. (A) HEK-CRTH2, **(B)** HEK-DP and **(C)** HEK-CRTH2+DP cells were stimulated with 100 nM of PGD_2, DK-PGD_2, BW245c or vehicle for 10 min at 37 °C following treatment with 1 µM of Cay10471, BWA868c or vehicle for 10 min at 37 °C. F-actin was visualized with 1 U/ mL Texas Red Phalloidin. Cells were analysed with an Olympus IX70 fluorescence microscope and an Olympus DP50-CU digital camera. Shown are representative images of 3 independent experiments.

4.10. CRTH2 and DP receptors do not internalize as a heteromer

Many 7TM/GPCRs have been found to undergo agonist-induced internalization. Here I tested whether i) CRTH2 and DP receptors could internalize as heteromers, and/or ii) whether the ability of CRTH2 to internalize is altered in the presence of the DP receptor. I performed antibody feeding experiments and flow cytometric measurement of cell surface receptor expression in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells (Fig. 18). I observed by immunofluorescence microscopy (Fig. 18A) that in HEK-CRTH2 cells CRTH2 receptors readily internalized in course of the 60 min treatment with 1 µM of PGD_2 (ii) or the CRTH2 agonist DK-PGD_2 (iii) with respect to cells incubated in the presence of vehicle (i). As expected, no internalization was observed following stimulation with the DP agonist BW245c (iv). In contrast to previously published data[184], no agonist induced internalization could be

observed in HEK-DP cells, when they were stimulated with either PGD_2 (vi) or the DP-selective agonist BW245c (viii). HEK-DP cells were also unresponsive to DK-PGD_2 (vii). Likewise, I could observe no agonist-induced internalization of DP receptors in HEK-CRTH2+DP cells (x-xii, red), whereas CRTH2 receptors readily underwent internalization in the presence of DP receptors when treated with PGD_2 (x, green) or DK-PGD_2 (xi, green). Analogous to fluorescence microscopic visualization of receptor distribution, flow cytometric analysis of HEK293 cells revealed that CRTH2 (Fig. 18B) - but not DP (Fig. 18C) - underwent agonist-induced internalization after stimulation with PGD_2 over a time course of 60 min. Loss of CRTH2 surface expression increased with time. To further substantiate that CRTH2 internalization occurs independently from DP receptors, I pre-treated cells with a CRTH2 or DP antagonist. Both, in HEK-CRTH2 (Fig. 18D) and HEK-CRTH2+DP (Fig. 18E) cells reduction of CRTH2 surface expression was blocked by the CRTH2 antagonist Cay10471, but was not modulated by the DP antagonist BWA868c. Hence, in cells co-expressing CRTH2 and DP, CRTH2 internalized independently from the DP receptor.

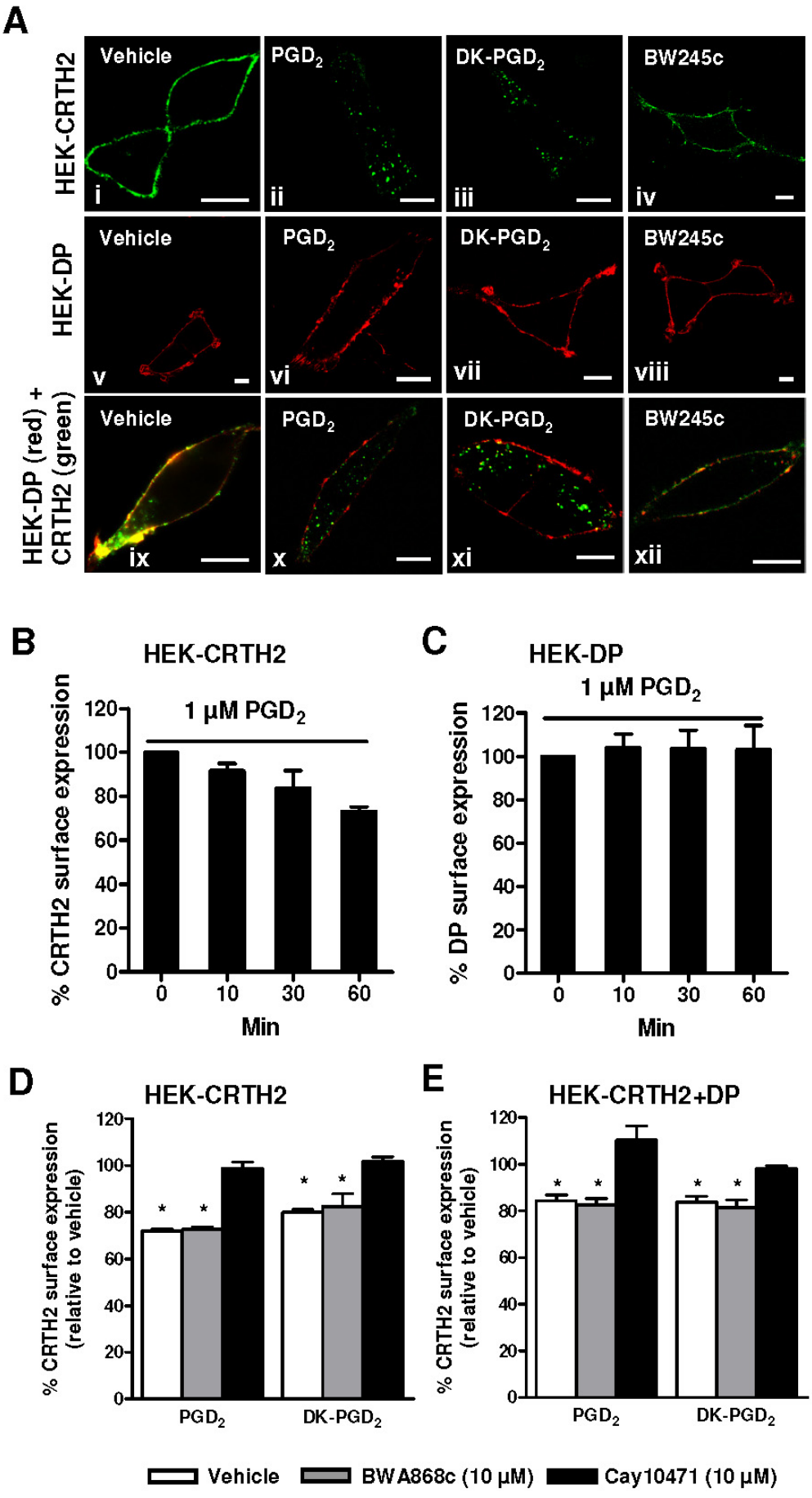
A
HEK-CRTH2
HEK-DP
HEK-DP (red) + CRTH2 (green)
Vehicle
PGD2
DK-PGD2
BW245c
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
B
HEK-CRTH2
1 µM PGD2
% CRTH2 surface expression
Min
C
HEK-DP
1 µM PGD2
% DP surface expression
Min
D
HEK-CRTH2
% CRTH2 surface expression (relative to vehicle)
PGD2
DK-PGD2
E
HEK-CRTH2+DP
% CRTH2 surface expression (relative to vehicle)
PGD2
DK-PGD2
Vehicle
BWA868c (10 µM)
Cay10471 (10 µM)

Figure 18. CRTH2 and DP receptor internalization in HEK293 cells. CRTH2 and DP receptor internalization in HEK293 cells was investigated by **(A)** immunofluorescence microscopy (antibody feeding experiments**)** and **(B-E)** flow cytometry. **(A)** Cells were stimulated with 1 µM of PGD_2, DK-PGD_2, BW245c or vehicle for 60 min following antibody feeding with anti-Myc (CRTH2-Myc) and/or anti-Flag M1 (DP-Flag) antibodies for 30 min. Cells were stained with Alexa Fluor 488 IgG_1 (CRTH2-Myc, green) and/or Alexa Fluor 594 IgG_{2B} (DP-Flag, red). Shown are representative images of three independent experiments. Scale bar = 10 µM. **(B-C)** Cells were stimulated with 1 µM of PGD_2 for up to 60 min. **(D-E)** Cells were pre-treated with 10 µM of Cay10471, BWA868c or vehicle followed by stimulation with 1 µM PGD_2 or DK-PGD_2. Cells were labeled with anti-Myc and/or anti-Flag M1 antibodies and stained with Alexa Fluor 488 IgG_1 and/or Alexa Fluor 488 IgG_{2B}. Data are means + S.E.M. of n = 3-5 * $P < 0.05$.

4.11. Pharmacological characterization of CRTH2- and DP-induced transcription factor activation

Little is known about the activation of transcription factors downstream of the signal transduction pathways of the PGD_2 receptors. In this part of my thesis, I want to provide an overview of the involvement of the following transcription factors in CRTH2 and DP receptor signaling and their possible consequences on cellular function by means of luciferase reporter gene assays:

1. Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB)
2. cAMP response element-binding protein (CREB)
3. Nuclear factor of activated T-cells (NFAT)
4. Serum response element (SRE)

4.11.1. CRTH2 and DP receptors do not activate the transcription factor NF-κB

NF-κB is recognized as a crucial regulator of inflammation[185]. To determine whether PGD_2 has any impact on NF-κB activation, plain HEK293 cells, HEK-DP, HEK-CRTH2 and HEK-CRTH2+DP cells were transiently transfected with 50 ng of the plasmid pNF-κB-Luc (Fig. 19). 24 hours post transfection cells were stimulated with the agonists for five hours at 37 °C before quantifying the cells and measuring luciferase activity. As shown in Fig. 19, upon stimulation with 1 µM of PGD_2 (black bars) both, CRTH2 and DP receptors did not significantly induce NF-κB luciferase activity when compared to vehicle treatment (white bars) in any of the cell lines tested.

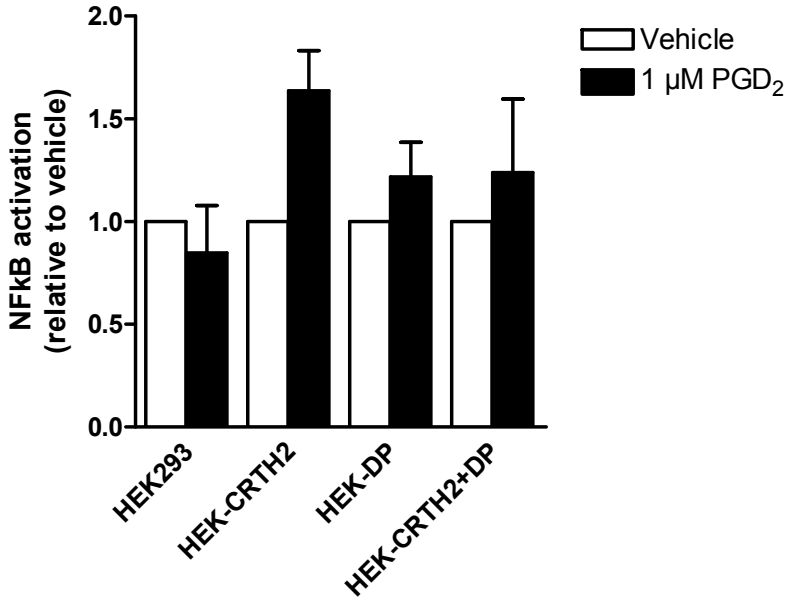

Figure 19. PGD_2-stimulated CRTH2 and DP receptors do not activate NF-κB in HEK293 cells. Agonist-induced NF-κB activation was measured in plain HEK293, HEK-CRTH2, HEK-DP or HEK-CRTH2+DP cells in luciferase reporter gene assays. Cells were transiently transfected with 50 ng of the plasmid pNF-κB-Luc and luciferase activity was measured in relative light units after five hours of agonist (black bars) or vehicle (white bars) stimulation. Data were normalized to vehicle and are shown as means + S.E.M. of *n* = 2-4 independent experiments.

4.11.2. The DP receptor, but not CRTH2, activates CREB.

CREB can be activated by several signaling pathways, such as PKA and MAPK pathways[186]. Involvement of CRTH2 and DP receptors in CREB activation was determined as described above (4.10.1) and shown in Figs. 20 and 21. While PGD_2-induced CRTH2 activation in HEK-CRTH2 (▲) cells had no impact on CREB activation, the same treatment resulted in three to four fold increase in CREB activity in HEK-DP (▼) cells when compared to vehicle (Fig. 20A). A remarkable increase in CREB activation – up to ten-fold as compared to vehicle and about three-fold as compared to HEK-DP cells - could be detected in HEK-CRTH2+DP (■) cells. This observation might indicate an indirect involvement of CRTH2, possibly functioning as an amplifier of the DP receptor in cAMP/CREB signal transduction. A similar effect could be observed in cells stimulated with the DP agonist BW245c (Fig. 20C). CREB activation in HEK-DP (▼) cells was increased about four-fold over control levels while in HEK-CRTH2+DP (■) cells CREB activity reached about nine-fold when compared to vehicle. BW245c stimulation of HEK-CRTH2 cells was not determined in this and all following experiments and non-specific binding of the DP agonist to CRTH2 has not been described in the literature[115;118;187] and in my experiments (see Ca^{2+} signaling assays above, Fig. 8). The slight DK-PGD_2-mediated CREB activation in HEK-CRTH2+DP cells

was neglectable due to the high EC_{50} value in the micromolar range which is 10^{4}-fold higher as compared to BW245c and PGD_2. A summary of all EC_{50} values is shown in Table 5.

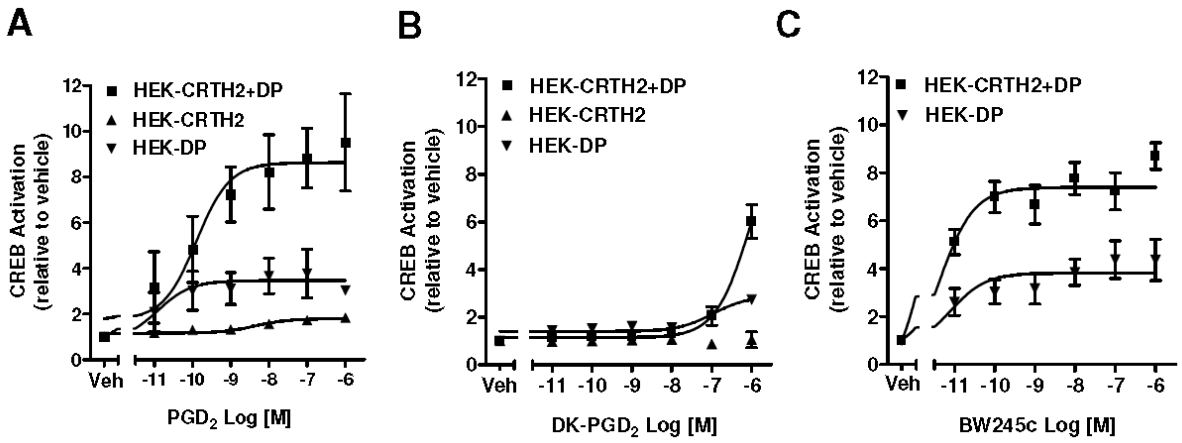

Figure 20. The DP receptor, but not CRTH2, activates CREB in HEK293 cells. Agonist-induced CREB activation was measured in HEK-CRTH2 (▲), HEK-DP (▼) and HEK-CRTH2+DP (■) cells in luciferase reporter gene assays. Cells were transiently transfected with 200 ng of the plasmid pCREB-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2, DK-PGD_2 or BW245c. Data were normalized to vehicle and are shown as means ± S.E.M. of n = 3-4 independent experiments.

To specifically determine the involvement of each receptor in HEK-CRTH2+DP cells in CREB responses, I applied selective antagonists (Fig. 21). Cells were pre-treated with 100 pM to 10 µM of Cay10471, BWA868c or vehicle for 20 min at 37 °C followed by stimulation with 1 µM of PGD_2 or BW245c. BWA868c completely blocked PGD_2- and BW245c-mediated CREB activation (Fig. 21B and D). In contrast, Cay10471 had no inhibitory effect on PGD_2- or BW245c-induced CREB activation (Fig. 21A and C).

Therefore, although CRTH2 does not mediate appreciable CREB activation, it seems to profoundly amplify the agonist-mediated effect transduced by DP (see Fig. 20A and C, ■).

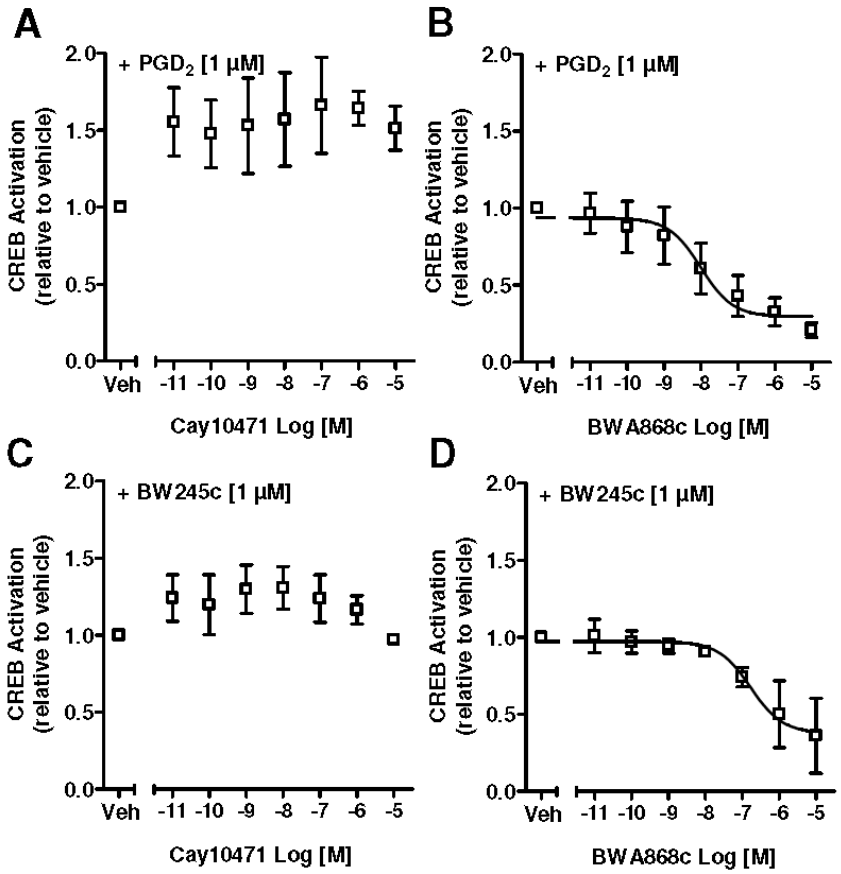

Figure 21. The DP antagonist BWA868c, but not the CRTH2 antagonist Cay10471 inhibits agonist-induced CREB activation in HEK-CRTH2+DP cells. Agonist-induced CREB activation was measured in luciferase reporter gene assays. Cells were transiently transfected with 200 ng of the plasmid pCREB-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2 or BW245c following pre-treatment with antagonist for 20 min. Data were normalized to vehicle and are shown as means ± S.E.M. of n = 3-4 independent experiments.

4.11.3. The DP receptor cooperates in CRTH2-mediated activation of NFAT

NFAT is involved in the development of allergic inflammation and regulates the cytokine expression during the early immune response[188]. The NFAT proteins are located in the cytoplasm and are translocated into the nucleus upon activation. This nuclear transport can be induced for instance by Ca^{2+}- controlled calcineurin-mediated dephosphorylation[189] as well as by Th2 cytokines[190]. Here, I set out to investigate the involvement of PGD_2 receptors in NFAT activation (Figs. 22 and 23). Agonist-induced NFAT activation was measured as described above (4.10.1). PGD_2 induced NFAT activation (Fig. 22A) about three-fold over control levels, but with ten-fold higher potency in HEK-CRTH2+DP (■) cells as compared to HEK-DP (▼) cells (see also Table 5). No PGD_2-induced NFAT activation could be observed in HEK-CRTH2 (▲) cells (Fig. 22A). Likewise, the CRTH2 agonist DK-PGD_2 failed to invoke NFAT activation in HEK-CRTH2 (▲) and HEK-DP (▼) cells (Fig. 22B). However, in HEK-CRTH2+DP (■) cells DK-PGD_2 was capable of activating NFAT about three-fold as

compared to vehicle. Similar to PGD_2, the DP agonist BW245c mediated NFAT activation three to four-fold as compared to vehicle in both, HEK-DP (▼) and HEK-CRTH2+DP (■) cells (Fig. 22C).

These data closely reflect our findings from the Ca^{2+} signaling assays (see Fig. 8) and suggest that CRTH2 becomes responsive to its agonist in the presence of the DP receptor.

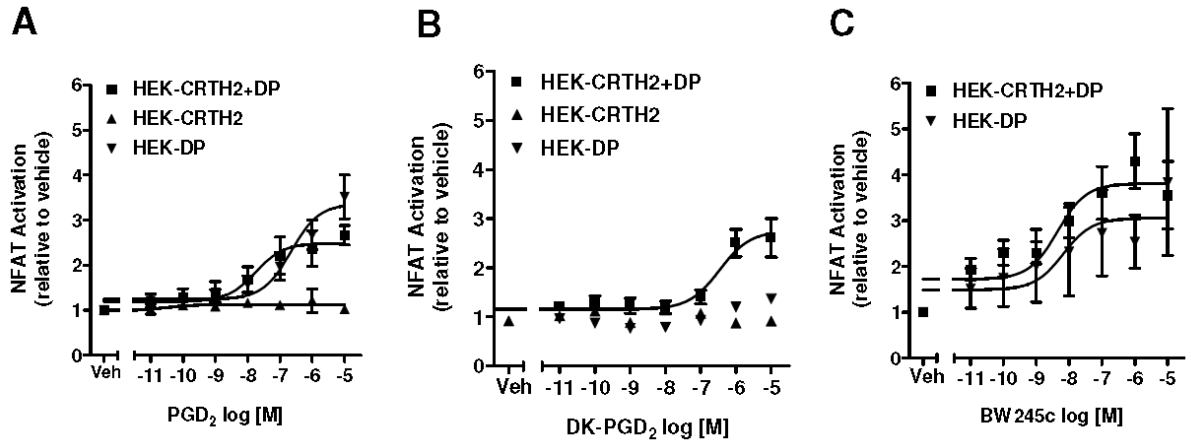

Figure 22. The DP receptor cooperates in CRTH2-mediated activation of NFAT in HEK293 cells. Agonist-induced NFAT activation was measured in HEK-CRTH2 (▲), HEK-DP (▼) and HEK-CRTH2+DP (■) cells in luciferase reporter gene assays. Cells were transiently transfected with 200 ng of the plasmid pNFAT-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2, DK-PGD_2 or BW245c. Data were normalized to vehicle and are shown as means ± S.E.M. of *n*=3-5 independent experiments.

In the ensuing experiments, I used selective antagonists to confirm the involvement of the individual PGD_2 receptors in NFAT activation (Fig. 23). HEK-CRTH2+DP cells were pre-treated with 100 pM to 10 µM of Cay10471, BWA868c, MK0524 or vehicle for 20 min at 37 °C followed by stimulation with 1 µM of PGD_2 or BW245c. I found that the PGD_2-induced NFAT activation was partially reduced by the CRTH2 antagonist Cay10471 (Fig. 23A). The DP antagonist BWA868c – in contrast to its effect on agonist-induced CREB activation – induced some NFAT activation on its own in HEK-CRTH2+DP cells (Fig. 23B). This was consistent with the purported partial agonism of the compound described in the literature (i.e. partial agonism of BWA868c on cAMP formation in chinese ovarian hamster cells expressing the DP receptor, or on Cl^- secretion by primary epithelial cells)[122;191]. For this reason I tested another selective DP antagonist, MK0524 (= laropiprant), which completely abrogated PGD_2-induced NFAT activation without inducing NFAT activation by itself (Fig. 23C). Equally, DK-PGD_2 – mediated NFAT activity was reduced by Cay10471 (Fig. 23D), while BWA868c had no effect (Fig. 23E). The combination of Cay10471 and MK0524 (Fig. 23F) was more

potent in NFAT inhibition in HEK-CRTH2+DP cells as reflected by the IC_{50} values (IC_{50} of Cay10471+MK0524 = 20 nM versus IC_{50} of Cay10471 = 2 µM). BW245c-induced NFAT activation in HEK-CRTH2+DP cells was inhibited by BWA868c, while Cay10471 had no effect (Fig. 23G and H).

In conclusion, this set of data further demonstrates a close interaction between CRTH2 and DP receptors in NFAT activation - i.e. a permissive effect of the DP receptor on the CRTH2 response - with clear parallels to the signaling pattern of CRTH2- and DP-mediated intracellular Ca^{2+} release (Fig. 8).

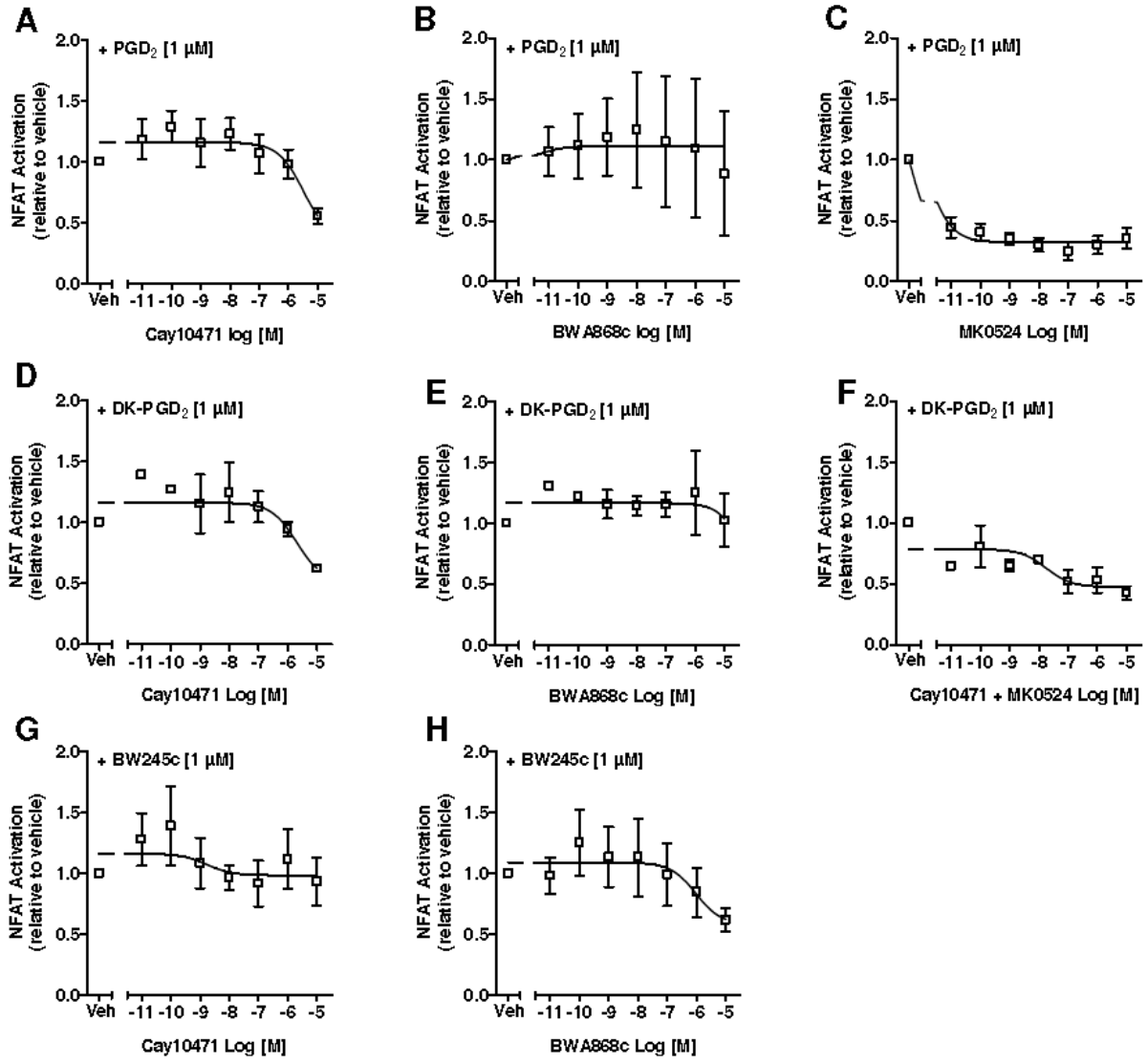

Figure 23. DP antagonists and the CRTH2 antagonist inhibit agonist-induced NFAT activation in HEK-CRTH2+DP cells. Agonist-induced NFAT activation was measured in luciferase reporter gene assays. Cells were transiently transfected with 200 ng of the plasmid pNFAT-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2, DK-PGD_2 or BW245c following pre-

treatment with antagonist for 20 min. Data were normalized to vehicle and are shown as means ± S.E.M. of *n* = 3-4 independent experiments.

4.11.4. The DP receptor, but not CRTH2, induces SRE activation

The serum response element (SRE) can be induced by the serum response factor (SRF). SRE is particularly involved in cytoskeletal rearrangement through a Rho family GTPase-dependent pathway[192]. Since activation of both CRTH2 and DP receptors induces actin polymerization in HEK293 cells (see Fig. 17), I aimed to determine subsequent SRE induction in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells.

Agonist-induced SRE induction was measured similar to experiments described above (4.10.1) and is shown in Figs. 24 and 25. Increasing doses of PGD_2 (Fig. 24A) failed to initiate SRE induction in HEK-CRTH2 (▲) cells, but induced SRE in HEK-DP (▼) and HEK-CRTH2+DP (■) cells. In a similar manner, the DP agonist BW245c (Fig. 24C) induced SRE activation up to ten-fold over control levels. Notably, the CRTH2 agonist DK-PGD_2 failed to induce SRE activation in any of the cell lines tested (Fig. 24B).

Therefore, these data clearly suggest a sole involvement of the DP receptor in signaling pathways leading to SRE induction.

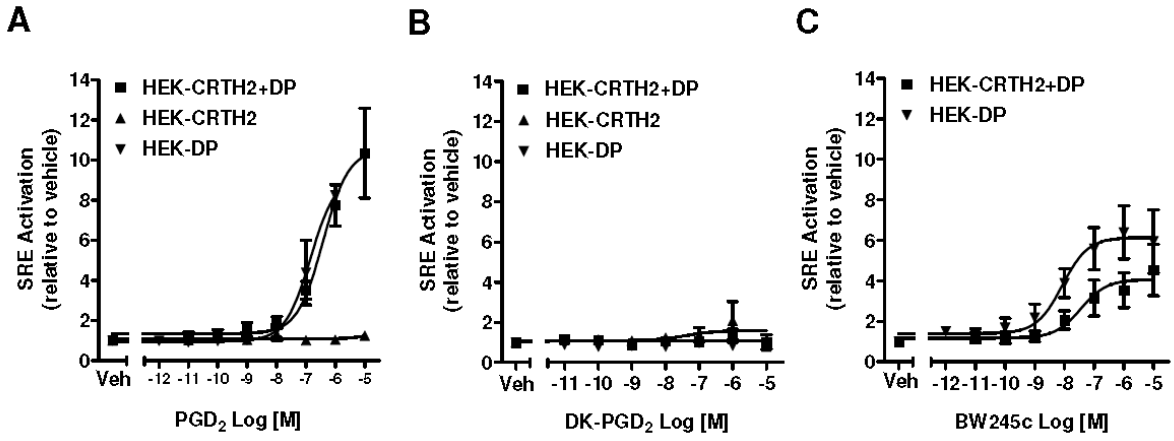

Figure 24. The DP receptor but not CRTH2 induce SRE activation in HEK293 cells. SRE activation was measured in HEK-CRTH2 (▲), HEK-DP (▼) and HEK-CRTH2+DP (■) cells in luciferase reporter gene assays. Cells were transiently transfected with 200 ng of the plasmid pSRE-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2, DK-PGD_2 or BW245c. Data were normalized to vehicle and are shown as means ± S.E.M. of *n* = 3-6 independent experiments.

To get further insights into the mechanism(s) of PGD_2-mediated SRE induction in cells co-expressing DP and CRTH2, I set out to modulate the respective receptor-mediated

reponses by using the selective antagonists Cay10471, BWA868c and MK0524. Hence, HEK-CRTH2+DP cells were pre-treated with 100 pM to 10 µM of antagonist or vehicle for 20 min at 37 °C (Fig. 25) followed by stimulation with 1 µM of PGD_2 or BW245c. Even though selective CRTH2 activation by DK-PGD_2 failed to induce SRE (see Fig 24B), the CRTH2 antagonist Cay10471 (Fig. 25A) efficiently abrogated PGD_2-mediated SRE activation in HEK-CRTH2+DP cells. Similarly, the DP antagonists BWA868c (Fig. 25B) and MK0524 (Fig. 25C) abrogated SRE activation in response to PGD_2. MK0524 was 10^5-fold more potent when compared to BWA868c. BW245c-induced SRE induction was reduced by Cay10471 and BWA868c and was completely prevented by MK0524 (Fig. 25D-F).

In summary, CRTH2 stimulation alone does not lead to SRE induction, but blocking CRTH2 in CRTH2/DP co-expressing cells prevents PGD_2- and BW245c-mediated SRE induction. Therefore, these results further corroborate the collaborative mode of action of CRTH2 and DP receptors exemplified by co-mediating SRE activation.

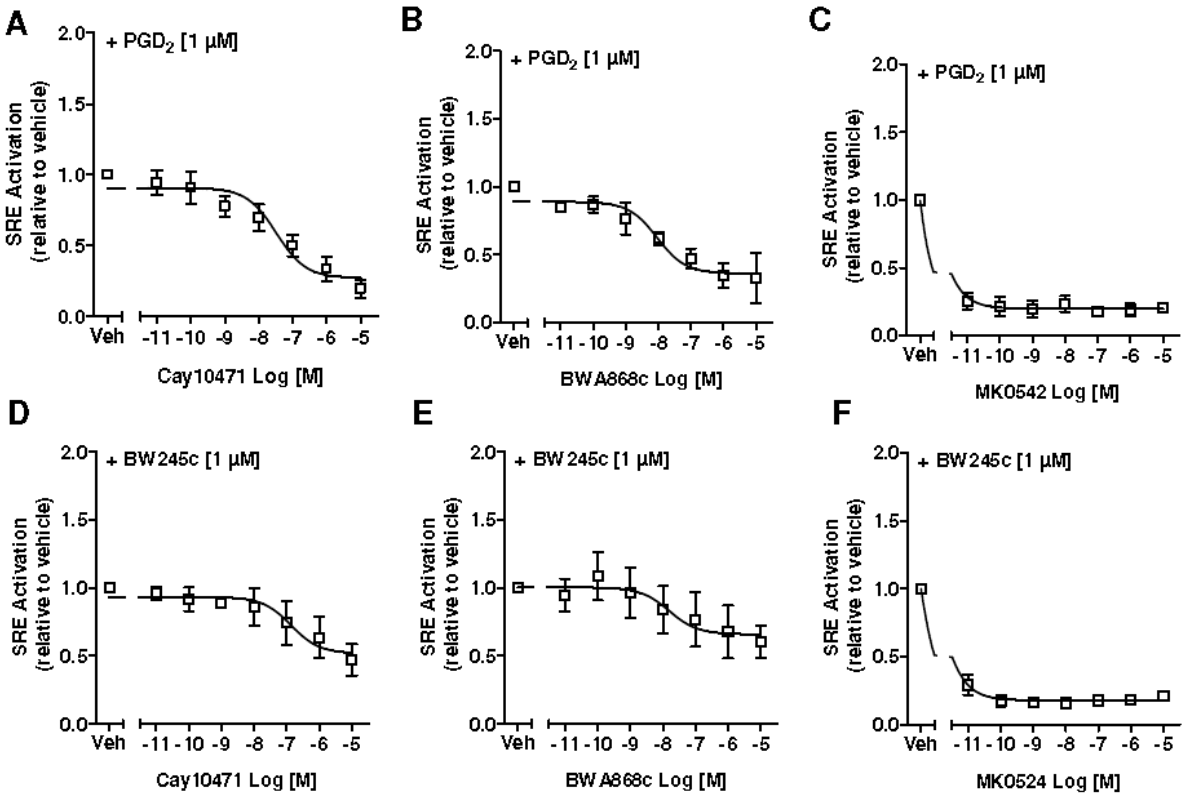

Figure 25. The DP antagonist BWA868c and the CRTH2 antagonist Cay10471 modulate agonist-induced SRE activation in HEK-CRTH2+DP cells. Agonist-induced SRE activation was measured in luciferase reporter gene assays. Cells were transisently transfected with 200 ng of the plasmid pSRE-Luc and luciferase activity was measured in relative light units after five hours of stimulation with PGD_2 or BW245c following pre-treatment with antagonist for 20 min. Data were normalized to vehicle and are shown as means ± S.E.M. of n = 3-4 independent experiments.

Table 5. EC_{50} values for transcription factor activation in HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells.

	EC_{50} CREB activation (nM)		
	CRTH2	DP	CRTH2+DP
PGD_2	-	0.01 ± 0.65	0.13 ± 0.37
DK-PGD_2	-	-	
BW245c	-	< 0.01	< 0.01

	EC_{50} NFAT activation (nM)		
	CRTH2	DP	CRTH2+DP
PGD_2	-	200 ± 0.22	18 ± 0.37
DK-PGD_2	-	-	340 ± 0.26
BW245c	-	6.6 ± 0.95	4.4 ± 0.44

	EC_{50} SRE induction (nM)		
	CRTH2	DP	CRTH2+DP
PGD_2	-	140 ± 0.20	380 ± 0.21
DK-PGD_2	-		
BW245c	-	8.4 ± 0.29	35 ± 0.46

Data are means ± S.E.M. of three to six independent experiments performed in triplicates. -, not calculated

The luciferase reporter gene assasys were partly conducted by Miriam Peinhaupt during her Master thesis under the supervision of Akos Heinemann and myself.

Part II: Signaling and trafficking properties of CRTH2 and DP receptors in human eosinophils

4.12. CRTH2 and DP receptors are expressed in human eosinophils

I confirmed CRTH2 and DP receptor expression in human eosinophils by quantitative real-time PCR at the mRNA level (relative to GAPDH, Fig. 26A) and by Western blot at the protein level (Fig. 26B). CRTH2 and DP mRNA was expressed three-fold and nine-fold as compared to GAPDH, the internal control. Using antibodies specifically targeting the respective receptors, I found that CRTH2 and DP receptors were expressed at the appropriate protein sizes, i.e. receptor monomers at ~37 kDa and higher order oligomers at ~70 kDa.

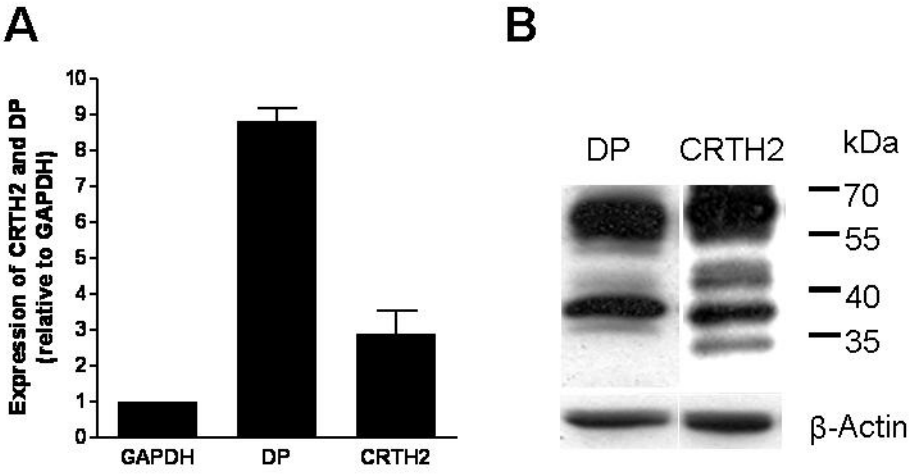

Figure 26. Endogenous expression of CRTH2 and DP receptors in human eosinophils. DP and CRTH2 expression was determined **(A)** at mRNA level relative to GAPDH (n=2) and **(B)** at protein level in lysates of eosinophils using polyclonal rabbit anti-PGD_2 receptor and polyclonal rabbit anti-human CRTH2 antibodies, respectively. Lysate samples immunoblotted for β-actin served as loading controls. Shown is a representative blot of 3 independent experiments.

4.13. CRTH2, but not DP receptors, induce intracellular Ca^{2+} release in eosinophils

I investigated the contribution of DP receptor signaling to CRTH2-mediated responses in human eosinophils to compare to my observations made in HEK-CRTH2+DP cells. Thus, I performed agonist-induced intracellular Ca^{2+} assays in a FLEXStation II (Fig. 27). Both, PGD_2 (◊) and the CRTH2-selective agonist DK-PGD_2 (□) induced a concentration-dependent intracellular Ca^{2+} flux in eosinophils. Notably, the DP-selective agonist BW245c (Δ) failed to evoke Ca^{2+} mobilization. Even though in HEK-CRTH2+DP cells (see results part I, Fig. 8C)

the Ca^{2+} response was not completely absent, the responsiveness to BW245c of this cell line as compared to HEK-DP cells was significantly reduced.

Based on the clear ability of the DP receptor to mobilize Ca^{2+} from intracellular stores in HEK-DP cells, it follows that co-expression of CRTH2 downregulates or – in the case of eosinophils - even completely abolishes DP-activity. In strong contrast, CRTH2 seems to rely on the presence of DP to initiate a cellular Ca^{2+} response.

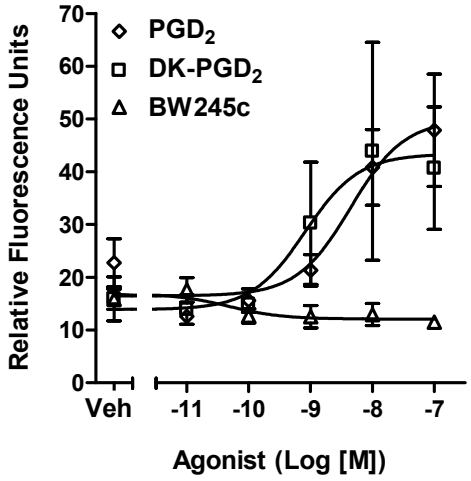

Figure 27. The DP receptor modulates CRTH2-induced intracellular Ca^{2+} release in eosinophils. Agonist-induced intracellular Ca^{2+} release in eosinophils was measured in a FLEXStation II and is shown in relative fluorescence units. Eosinophils were stimulated with PGD_2 (◊), DK-PGD_2 (□) or BW245c (Δ). Data are means ± S.E.M. of *n*=3.

4.14. CRTH2-mediated Ca^{2+} responses in eosinophils are affected by inhibition of DP receptor pathways

4.14.1 Both, CRTH2 and DP receptor antagonists block Ca^{2+} release in eosinophils

Next, I examined the effects of the CRTH2 and DP antagonists Cay10471 and BWA868c on PGD_2- and DK-PGD_2-mediated Ca^{2+} flux in eosinophils using a flow cytometric Ca^{2+} flux assay (Fig. 28). Cells were pre-treated with 1 µM of antagonist or vehicle for 10 min and Ca^{2+} flux was induced by 3 nM of agonist. Although the DP agonist BW245c did not induce a Ca^{2+} response, the DP antagonist BWA868c significantly attenuated the PGD_2- and DK-PGD_2-induced Ca^{2+} flux (Fig. 28, grey bars). This effect was reminiscent - although less pronounced – of the BWA868c-induced inhibition of Ca^{2+} responses in HEK-CRTH2+DP cells (see results part I, Fig. 12). The CRTH2 antagonist Cay10471 completely blocked the Ca^{2+} flux in response to DK-PGD_2 and PGD_2 (Fig. 28, black bars).

In conclusion, these results support my hypothesis that CRTH2 and DP receptors modulate each other's signaling.

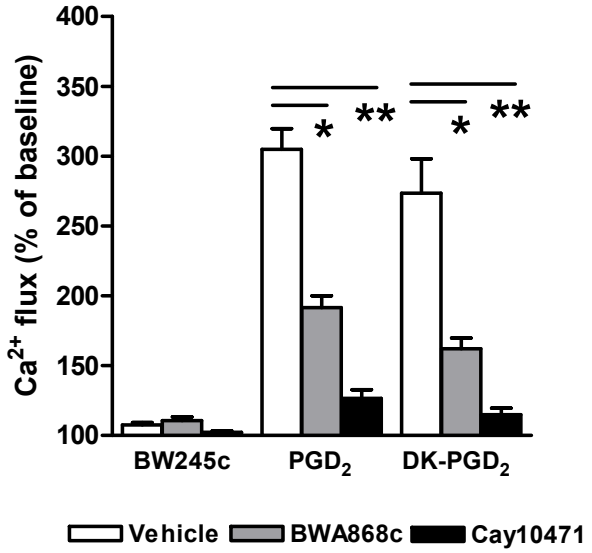

Figure 28. CRTH2 and DP receptor antagonists modulate Ca^{2+} flux in eosinophils. Agonist-induced intracellular Ca^{2+} release in eosinophils was measured by flow cytometry. Eosinophils were pre-treated with 1 μM of BWA868c (grey bars), Cay10471 (black bars) or vehicle (white bars) for 10 min followed by stimulation with 3 nM of agonist. Fluorescence changes were recorded in the FL-1 fluorescence channel. Data are means ± S.E.M. of n = 4-5 and are expressed as percent of baseline. * $P < 0.05$, ** $P < 0.001$.

4.14.2 Desensitization of DP receptors in eosinophils impairs CRTH2-Ca^{2+} signaling

Based on the clear-cut parallels between eosinophils and HEK-CRTH2+DP cells in Ca^{2+} signaling and the apparent regulatory role of the DP receptor, I tested whether DP desensitization in eosinophils would likewise lead to a reduction in CRTH2 signaling (Fig. 29). Thus, cells were pre-treated with 10 nM of the DP agonist BW245c or vehicle for 10 min and Ca^{2+} flux was induced by 3 nM of PGD_2 (Fig. 29A) or the CRTH2-selective agonist DK-PGD_2 (Fig. 29B). The responses to both agonists were significantly reduced upon pre-activation of the DP receptor.

This finding strongly indicates the dependence of CRTH2 on a functional DP response.

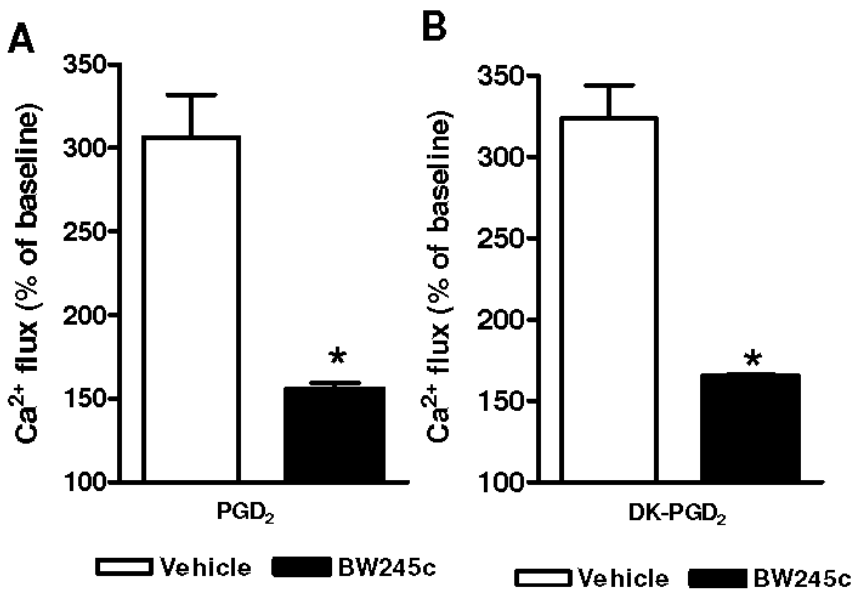

Figure 29. DP receptor desensitization curbs CRTH2-mediated Ca^{2+} flux in eosinophils. Agonist-induced intracellular Ca^{2+} release in eosinophils was measured by flow cytometry. Eosinophils were pre-treated with BW245c (10 nM, black bars) or vehicle (white bars) for 10 min followed by stimulation with 3 nM of **(A)** PGD_2 or **(B)** DK-PGD_2. Fluorescence changes were recorded in the FL-1 fluorescence channel. Data are means ± S.E.M. of n = 3-4 and are expressed as percent of baseline. * $P < 0.05$.

4.15. Both, $G\alpha_i$-and $G\alpha_{q/11}$-proteins contribute to CRTH2- and DP-induced Ca^{2+} signaling in eosinophils

To investigate the contribution of $G\alpha_i$- and $G\alpha_{q/11}$-proteins to CRTH2- and DP-mediated Ca^{2+} flux, eosinophils where pre-treated with 3 µg/mL of the $G\alpha_i$-protein inhibitor PTX or 1 µM of the $G\alpha_{q/11}$-protein inhibitor MH-362-63-8 for 1 hour (Fig. 30). DK-PGD_2-induced Ca^{2+} signals were sensitive to PTX, whereas PGD_2-induced responses were only partially reduced by the $G\alpha_i$-protein inhibitor (Fig. 30A). Selective inhibition of $G\alpha_{q/11}$-proteins completely abolished PGD_2- and DK-PGD_2-induced Ca^{2+} flux (Fig. 30B). PTX did not exhibit non-specific toxic effects in eosinophils as it had little effect on flow cytometric shape change responses to PGD_2 (Fig. 30C). Toxic and unspecific effects of MH-362-63-8 were excluded by Dynamic Mass Redistribution assays (performed by Evi Kostenis, Institute of Pharmaceutical Biology, Bonn, Germany; data not shown).

In summary, the comparison of the data derived from both, HEK293 cells and eosinophils, implicates that CRTH2 and DP receptors closely cooperate in mediating intracellular Ca^{2+} release in response to PGD_2 or its analogues.

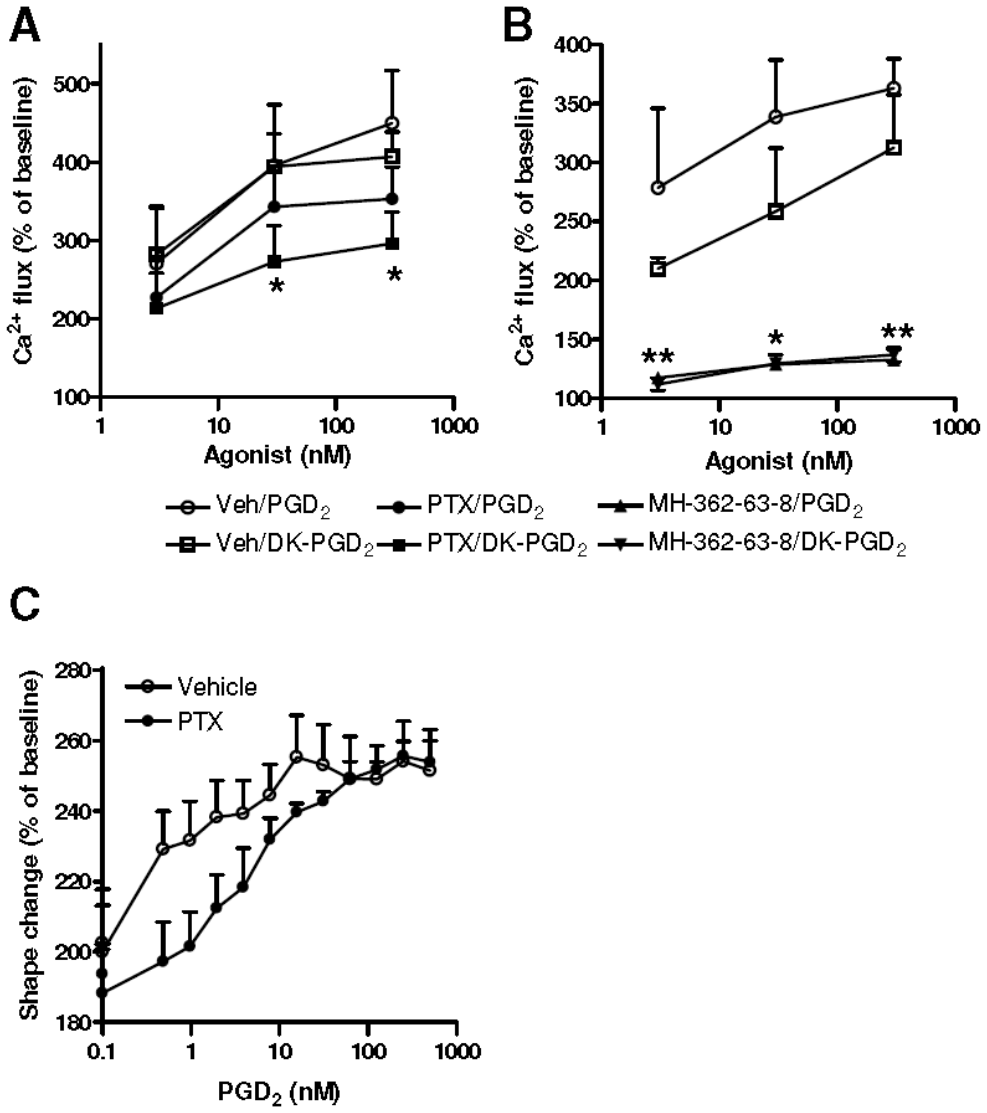

Figure 30. Effect of PTX and MH-362-63-8 on agonist-induced intracellular Ca^{2+} release in eosinophils. PGD_2- and DK-PGD_2-induced Ca^{2+} flux in eosinophils was measured by flow cytometry. Cells were treated with **(A)** PTX (3 µg/mL), **(B)** MH-362-63-8 (1 µM) or vehicle 1 h before agonist application. **(C)** PGD_2-mediated shape change of eosinophils was investigated upon pre-treatment with 1 µM of PTX. Fluorescence changes were recorded in the FL-1 fluorescence channel. Fluorescence units are shown as percent of the signal of untreated cells (set to 100 %). Data are means ± S.E.M. of n=3. * $P < 0.05$, ** $P < 0.001$.

4.16. CRTH2, but not DP receptors, undergo agonist-induced internalization in eosinophils

In HEK-CRTH2+DP cells CRTH2 receptors translocate to the cytosol in response to agonist stimulation, while the DP receptor remained on the cell surface upon agonist-stimulation (see results part I, Fig. 18). Here, I conducted receptor internalization assays in eosinophils endogenously expressing CRTH2 and DP (Fig. 31). Cell surface receptor expression was measured by flow cytometry upon stimulating eosinophils with 1 nM, 100 nM or 1 μM of PGD_2, DK-PGD_2 or BW245c for 1h. Eosinophils were stained with Alexa Fluor 647 rat anti-human CRTH2 or polyclonal goat anti-human DP and Alexa Fluor 647 rabbit anti-goat antibodies. I found that - corresponding to HEK293 cells - CRTH2 surface expression was significantly reduced following PGD_2 and DK-PGD_2 treatment (Fig. 31A), while DP failed to internalize in response to its agonists, PGD_2 and BW245c (Fig. 31B).

Hence, in cells co-expressing CRTH2 and DP, the CRTH2 receptor internalizes independently of DP. This conclusion was further supported by the observation that the CRTH2 antagonist Cay10471 (Fig. 31C, black bar) prevented internalization, while the DP antagonist BWA868c (Fig. 31C, grey bar) did not influence CRTH2 translocation to the cytoplasm. These findings were in good agreement with my results described in HEK293 cells (Fig. 18).

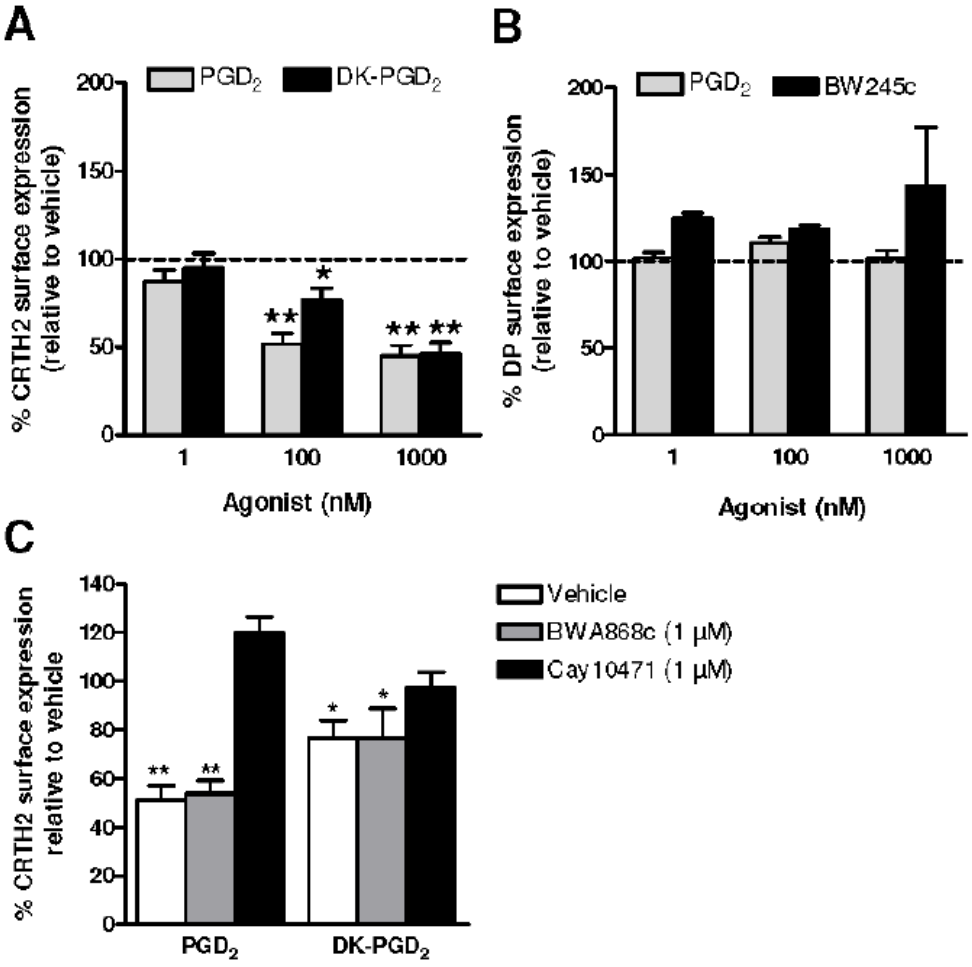

Figure 31. Agonist-mediated CRTH2 and DP receptor internalization in eosinophils. Agonist-induced CRTH2 and DP internalization in eosinophils was measured by flow cytometry and data were expressed as percent surface receptor expression relative to vehicle-treated cells. Eosinophils were stimulated with agonist for 60 min and stained with **(A)** Alexa Fluor 647 rat anti-human CRTH2 or **(B)** polyclonal goat anti-human DP and Alexa Fluor 647 rabbit anti-goat antibodies. **(C)** Eosinophils were treated with 1 µM of antagonist or vehicle for 15 min following stimulation with 100 nM of PGD_2 or DK-PGD_2 and labeled with Alexa Fluor 647 rat anti-human CRTH2. Data are means ± S.E.M. of n = 8-12 (A) or n = 3 (B, C). * $P < 0.05$, ** $P < 0.01$.

4.17. The role of PGD_2 receptors in actin dynamics and adhesion of eosinophils

Accumulation of eosinophils in the inflamed tissue is a cardinal feature of allergic inflammation. The extravasation of eosinophils from the blood vessel to the tissue is a highly coordinated process which involves rearrangement of the actin cytoskeleton, various chemoattractants and adhesion molecules expressed on both, eosinophils and endothelial cells[168]. However, the involvement of CRTH2 and DP receptors in this complex process is still not entirely clear.

4.17.1. PGD_2 receptor-mediated actin-polymerization in eosinophils

I determined the role of CRTH2 and DP receptors on actin polymerization in isolated eosinophils (Fig. 32). Prior to F-actin staining, eosinophils were stimulated with 100 nM of CRTH2 or DP agonists or vehicle for 10 min at 37 °C following pre-traetment with 1 μM of antagonist or vehicle for 10 min at 37 °C. I found that eosinophils respond to PGD_2, DK-PGD_2 and BW245c in a differential manner: Unlike vehicle-treated cells (i), PGD_2-stimulated cells (ii) developed thin protrusions (= fillopodia) and lamellopodia (= plane protrusions). In contrast, treatment with DK-PGD_2 (iii) and BW245c (iv) resulted in cells that were smaller, round-shaped and had a polarized tail, resembling a “tear drop”. The CRTH2 antagonist Cay10471 completely abolished PGD_2- (vi) and DK-PGD_2-induced (vii) actin rearrangement, but had no effect on BW245c-mediated (viii) shape change. In contrast, the DP antagonist BWA868c prevented BW245c-induced actin-polymerization (xii), but could not inhibit PGD_2- (x) and DK-PGD_2-induced (xi) formation of protrusions. Both antagonists had no effect *per se* on the eosinophil cytoskeleton ((v) and ix)).

Taken together, these data show that CRTH2 and DP act synergistically on the cytoskeleton, DP supporting polarization and gross F-actin polymerization while CRTH2 promoting the formation of protrusions along with localized F-actin. CRTH2 seems to exhibit a dominant function in this process.

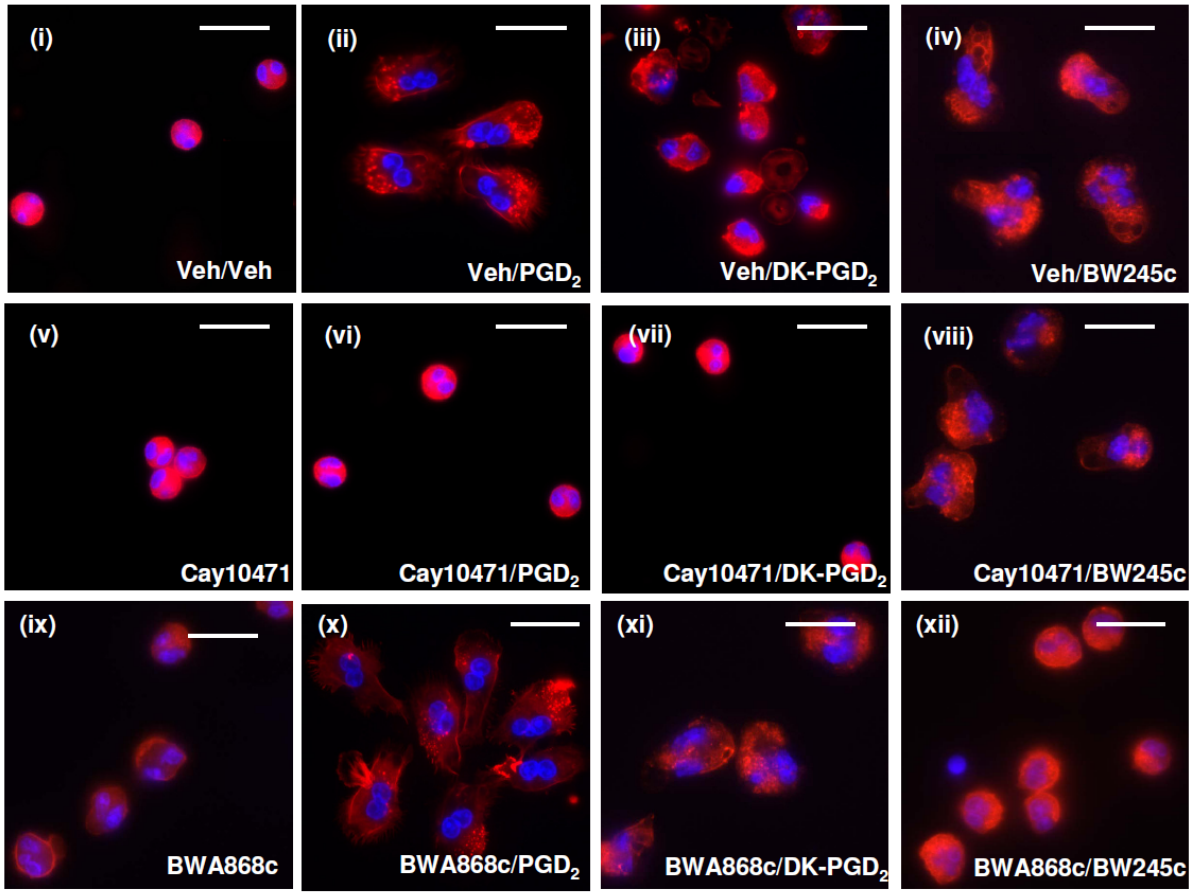

Figure 32. CRTH2 and DP receptors induce actin polymerization in eosinophils. Eosinophils were plated on fibronectin-coated glass cover slips and stimulated with 100 nM of agonist or vehicle for 10 min at 37 °C upon pre-treatment with 1 µM of antagonist or vehicle for 10 min at 37 °C. Actin polymerization was visualized by Phalloidin staining. F-actin was visualized with 0.4 U/ mL Texas Red Phalloidin. Cells were analysed with an Olympus IX70 fluorescence microscope and an Olympus DP50-CU digital camera. Shown are representative images of 4 independent experiments.

4.17.2. Role of PGD_2 receptors in adhesion of eosinophils to human pulmonary microvascular endothelial cells

The adhesion cascade of leukocytes embraces tethering, rolling, activation, firm adhesion and transmigration of the cells[193;194]. The latter two processes are less well understood, although involvement of α4- and β2-integrins as well as the platelet-endothelial cell adhesion molecule-1 (PECAM-1) are known to initiate firm adhesion to the endothelium[193;195].

I investigated the role of CRTH2 and DP receptors in eosinophil adhesion to human pulmonary microvascular endothelial cells (HMVEC-L) under laminar flow conditions (Fig. 33). This assay setup mimicks the hydrodynamic shear of a vessel occuring *in vivo*. The importance of shear stress for eosinophil transmigration has been reported previously[196]. Thus, I grew endothelial cells in an *in vitro* flow chamber[171;178] and perfused the isolated eosinophils across the endothelial monolayer. First, I compared PGD_2-induced eosinophil

adhesion to resting endothelial cells (vehicle, Fig. 33A) or endothelial cells activated with 10 pM TNF-α (Fig. 33B). Upon agonist stimulation for 10 min at 37 °C eosinophils were placed into the flow chamber channel and were perfused over the endothelial cells for 2.5 min at 2 dyn/cm^2. I found that 100 nM of PGD_2 compared to vehicle increased adhesion of eosinophils about four-fold or two-fold to resting and activated endothelial cells, respectively. Basal adhesion of eosinophils to TNF-α-treated human pulmonary microvascular endothelial cells was about five-fold higher as compared to non-treated, resting endothelial cells (Fig. 33C).

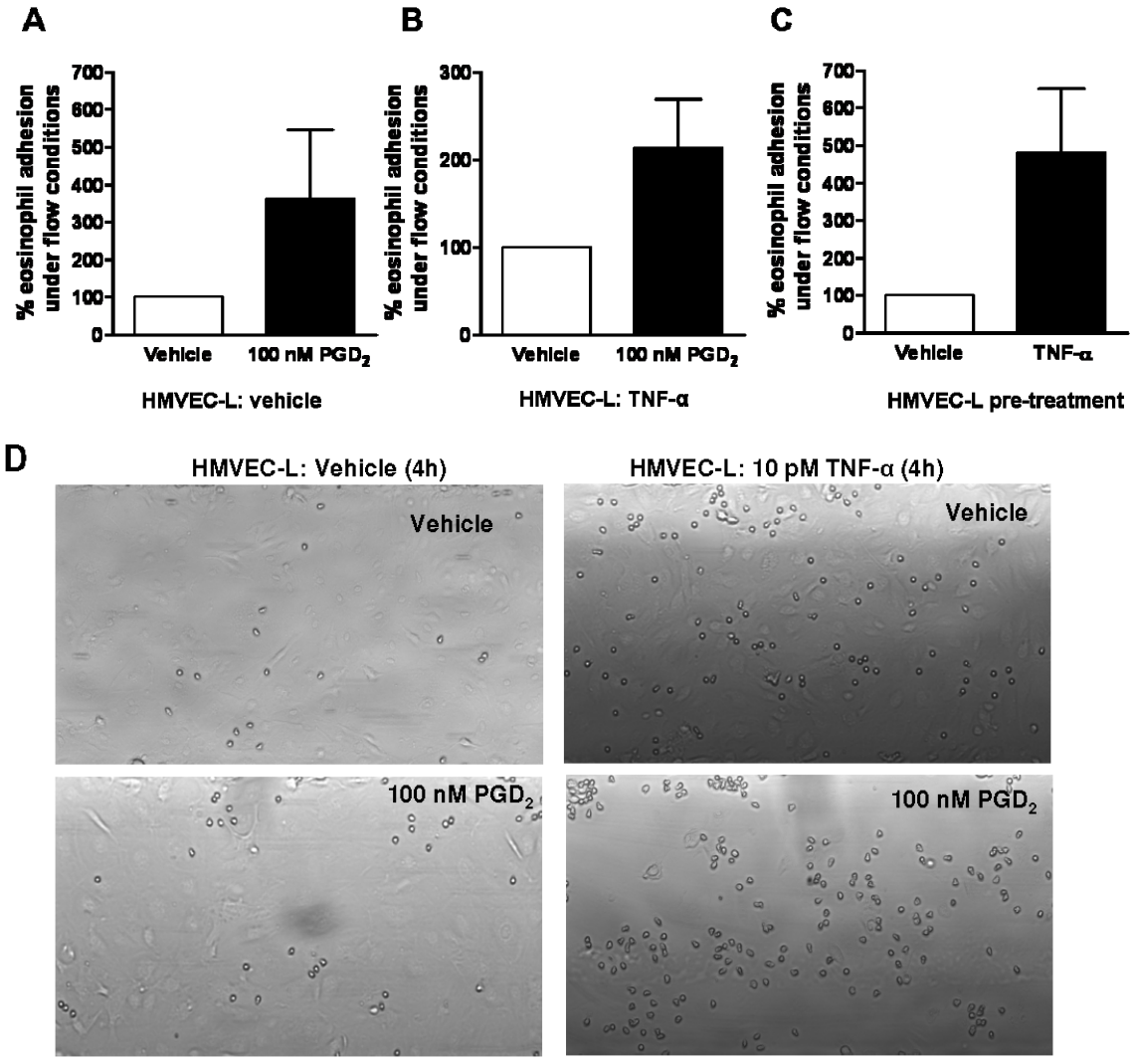

Figure 33. PGD_2 increases eosinophil adhesion to human pulmonary microvascular endothelial cells under flow conditions. Endothelial monolayers were pre-treated with **(A)** vehicle or **(B)** 10 pM of TNF-α for 4h at 37 °C. Eosinophils were stimulated with 100 nM of PGD_2 for 10 min and perfused across the endothelial cells for 2.5 min at 2 dyn/cm^2. **(C)** Basal adhesion of eosinophils to resting and activated endothelial cells **(D)** Representative pictures of vehicle- or 100 nM PGD_2-induced eosinophil accumulation on vehicle- or TNF-α-treated HMVEC-L. Movies were recorded using 10 x magnification via a CCD camera. Data are shown as mean ± S.E.M. of $n = 3$ independent experiments.

Next, I determined the participation of CRTH2 and DP receptors in the regulation of eosinophil adhesion to endothelial cells by using selective ligands (Fig. 34). Prior to stimulation with 100 nM of DK-PGD_2, BW245c or vehicle for 10 min at 37 °C eosinophils were pre-treated with 1 μM of antagonist or vehicle for 10 min at 37 °C. Subsequently, eosinophils were perfused across the activated endothelial monolayer, i.e. endothelial cells that had been pre-treated with 10 pM of TNF-α. The PGD_2-induced adhesion was significantly reduced to basal levels by the CRTH2 antagonist Cay10471, while there was only a slight non-significant reduction by the DP antagonist MK0524 (Fig. 34A). The CRTH2 agonist DK-PGD_2-induced adhesion was less pronounced and showd a higher degree of variation (Fig. 34B). Based on the fact, that the DP agonist BW245c had no effect on eosinophil adhesion (Fig. 34C), it can be speculated that only a concurrent activation of CRTH2 and DP receptors might efficiently stimulates eosinophil adhesion. Again, CRTH2 seems to be the dominant receptor in driving eosinophils adhesion, since the DP antagonist had only partial inhibitory effect on PGD_2-mediated adhesion. However, further experiments, such as simultaneous stimulation of CRTH2 and DP receptors with selective agonists are required to finally interpret the role of PGD_2 receptors in adhesion to the endothelium.

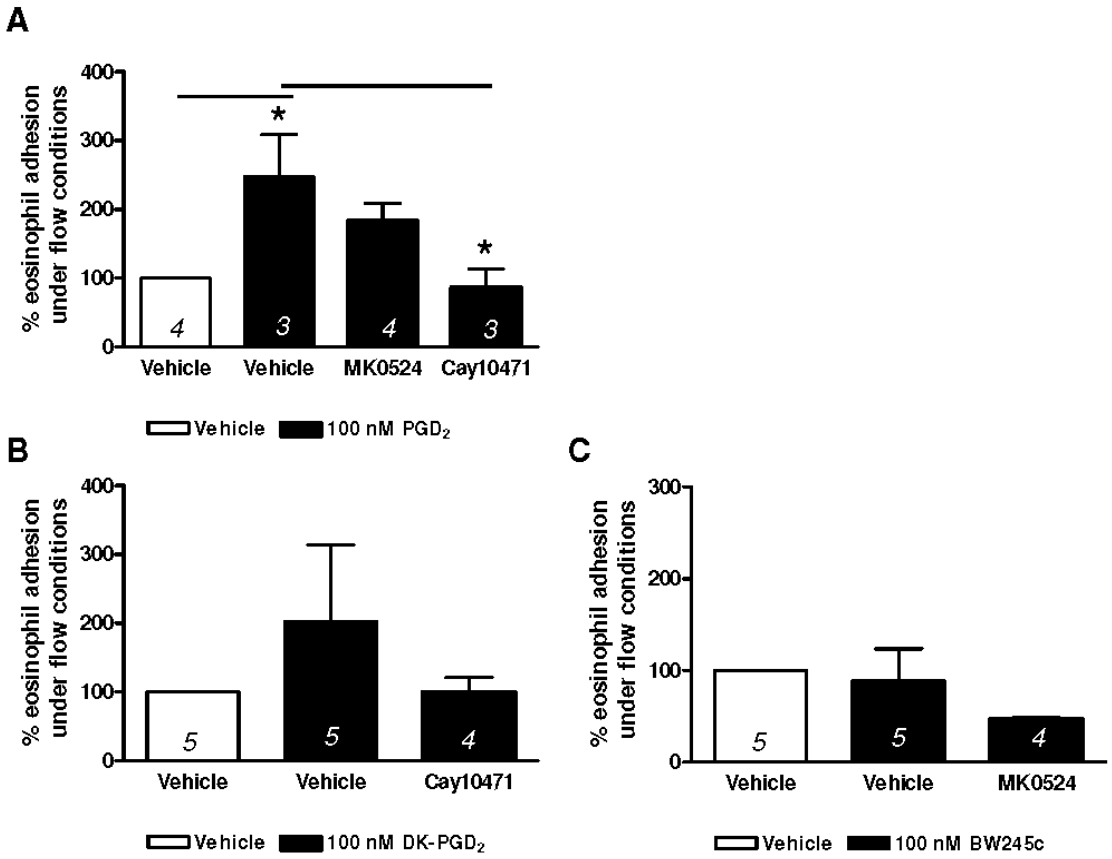

Figure 34. Involvement of PGD_2 receptors in eosinophil adhesion to HMVEC-L under flow conditions. Endothelial monolayers were activated with 10 pM of TNF-α for 4h at 37 °C. Eosinophils were pre-treated with 1 μM of Cay10471, MK0524 or vehicle for 10 min at 37 °C followed by stimulation with 100 nM of **(A)** PGD_2 **(B)** DK-PGD_2 or **(C)** BW245c for 10 min at 37 °C and were perfused across the endothelial cells for 2.5 min at 2 dyn/ cm^2. Data are shown as means ± S.E.M. of *n* = 3-5 independent experiments.

Miriam Peinhaupt assisted in the Cellix experiments during her Master thesis.

5. Discussion

My research aimed to investigate the signaling properties of the PGD_2 receptors CRTH2 and DP. The central hypothesis of my work was that the functional responses of the CRTH2 receptor essentially depend on a cross-talk with the DP receptor. I studied both receptors parallel in a HEK293 cell model and in human peripheral blood eosinophils. Thereby I could demonstrate that my cell model recombinantly expressing the PGD_2 receptors largely resembles human primary eosinophils endogenously expressing CRTH2 and DP. Overall, my results indicate that the CRTH2/DP receptor signaling unit represents a potential target for development of heteromer-directed therapies to treat inflammatory diseases.

In the first part of my thesis I show that the PGD_2 receptors CRTH2 and DP profoundly modulate the signaling properties of each other in a recombinant HEK293 cell model: While the DP-mediated intracellular Ca^{2+} mobilization was attenuated in the presence of CRTH2, the CRTH2-mediated signaling capacity was significantly dependent on the presence of the DP receptor. Previously, we demonstrated that the DP receptor is expressed on eosinophils, which I confirm here by Western blot analysis. In addition, we showed that DP is fundamentally involved in proinflammatory responses such as eosinophil migration, mobilization from bone marrow and oxidative burst[135;197]. My findings have thus corroborated previous observations which suggested a proinflammatory role of DP receptors: Mice deficient in DP receptors displayed reduced pulmonary inflammation in response to allergen[134], and antigen-induced rhinitis and conjunctivitis were reduced by pharmacological blockade of DP[133]. However, these findings were difficult to explain with the known intracellular signaling pathways of DP. In particular, the mechanisms by which the DP receptor might modulate intracellular signaling events in response to concomitant CRTH2 activation have remained elusive to date.

When HEK293 cells expressing the DP receptor alone were stimulated with PGD_2 or BW245c, I observed a strong intracellular Ca^{2+} flux, confirming earlier reports[123;198]. However, I found that Ca^{2+} responses to DP activation were markedly reduced when CRTH2 was expressed along with the DP receptor. Conversely, HEK293 cells expressing only CRTH2 were deficient in Ca^{2+} responses. These cells became responsive to DK-PGD_2 when DP receptors were coexpressed with CRTH2, with the magnitude of the Ca^{2+} response being closely correlated with the amount of the DP receptor co-transfected into HEK-CRTH2 cells. Moreover, pre-stimulation (i.e. desensitization) of the DP receptor in HEK-CRTH2+DP cells

with its agonist BW245c conferred CRTH2 into an unresponsive state. Finally, BWA868c, a highly selective antagonist at the human DP receptor with a 10,000-fold lower affinity for human CRTH2[197;198], prevented PGD_2- as well as DK-PGD_2-induced Ca^{2+} release in HEK-CRTH2+DP cells.

One potential mechanism of cross-talk between 7TM/GPCRs is by direct protein-protein interaction, i.e. forming functional heteromers and thereby altering the receptors' signaling properties[181]. Indeed, I observed a co-localization of CRTH2 and DP receptors on the surface of HEK293 cells and the formation of CRTH2/DP heteromers in co-immunoprecipitation studies. The impact of heteromerization between 7TM/GPCRs on receptor desensitization has been demonstrated, e.g. in the D1-D2 dopamine heteromer[199]. Here, the D1-D2 dopamine heteromer-specific Ca^{2+} signal was desensitized in a GRK-dependent manner by pretreatment of either receptor with selective agonists. Furthermore, heteromerization of 7TM/GPCRs can result in altered ligand binding properties, i.e. some ligands prefer the receptors in their homomeric versus heteromeric conformation[200]. However, we did not observe any changes in the binding affinities of ligands to CRTH2 or DP in the absence or presence of the other receptor. This suggests that the signal transduction rather than the binding properties are altered by CRTH2/DP heteromer formation.

Therefore, I investigated the G protein-coupling properties of the co-expressed PGD_2 receptors using the selective $G\alpha_i$-protein inhibitor PTX and the $G\alpha_{q/11}$-protein inhibitor MH-362-63-8[182]. In HEK293 cells coexpressing CRTH2 and DP, PTX had no effect on Ca^{2+} responses to the DP agonist BW245c while it completely blocked responses to the CRTH2 agonist DK-PGD_2. In contrast, MH-362-63-8 completely blocked Ca^{2+} responses to both agonists, which indicates that both $G\alpha_i$-and $G\alpha_{q/11}$-proteins are required for CRTH2-mediated Ca^{2+} release while the DP response solely relies on $G\alpha_{q/11}$-proteins. Consequently, responses to PGD_2 were partially blocked by PTX, which is in agreement with the ability of PGD_2 to bind to both CRTH2 and DP. From these data it may be inferred that activation of CRTH2 leads to $G\alpha_i$-protein-mediated transactivation of DP, which – notably in the absence of a DP ligand – elicits a Ca^{2+} response through $G\alpha_{q/11}$-proteins. When the DP receptor is absent, CRTH2 cannot access $G\alpha_{q/11}$-proteins and fails to induce Ca^{2+} mobilization when stimulated with DK-PGD_2 or PGD_2.

Together, these data suggest a possible transactivation of the DP receptor by CRTH2 that in turn enables signal transduction of CRTH2 via the DP pathway. This observation correlates with the finding that stimulation of $G\alpha_i$-coupled receptors has been shown to amplify the inositol phosphate signals derived from activation of $G\alpha_q$-coupled receptors, even

though agonist binding to the $G\alpha_i$-coupled receptors alone often fails to induce inositol phosphates[19;201;202]. I has been suggested, that $G\beta\gamma$-subunits might be responsible for this effect[19;202;203]. However, adequate experiments, such as generation of inositol phosphates and the impact of $G\beta\gamma$-subunits on Ca^{2+} signaling in the HEK293 cell model, would have to be performed in order to confirm this hypothesis.

The capacity of 7TM/GPCRs to internalize following agonist stimulation is considered a major regulatory mechanism to maintain the homeostasis of a cell. Importantly, even closely related 7TM/GPCRs - like the opioid receptor family[204;205] - display different trafficking properties in response to the same agonist. Here, I show that CRTH2 undergoes agonist-induced internalization in HEK-CRTH2 and HEK-CRTH2+DP cells thereby confirming previous results[6;158;184;206]. However, I observed no agonist-induced internalization of the DP receptor in any of the cell lines investigated by flow cytometry and immunofluorescence microscopy. This observation contradicts a previous study that suggested PGD_2-induced DP receptor internalization within 60 min in a HEK293 cell model[184], though with a different technical approach (i.e. ELISA).

The cellular background can profoundly influence the desensitization capacity of 7TM/GPCRs as demonstrated for the M3 muscarinic receptor[207]. Subtypes of this receptor are expressed in different cell types such as neurons, smooth muscle cells and salivary gland epithelial cells. Along with the different cellular responses controlled by the M3 receptor, the regulation of this receptor, such as phosphorylation by kinases, varies between the cell types. The DP receptor, however, behaves identically when overexpressed in HEK293 cells or endogenously expressed in eosinophils. Hence, despite the formation of CRTH2/DP heteromers these receptors do not appear to affect each other's trafficking properties. The classical understanding of 7TM/GPCR desensitization is constantly renewed and updated since novel mechanisms add to the complexity of this model. Sibley *et al.*[208] and others[61] have shown that receptors are dephosphorylated in endosomes, but not at the plasma membrane. However, the hypothesis that 7TM/GPCR internalization is absolutely required for de- and resensitization processes has recently been challenged. The β2-adrenergic receptor[209], for instance, was shown to be dephosphorylated at the PKA site within the plasma membrane. The thyreotropin releasing hormone (TRP) receptor[210] is rapidly desensitized by β-arrestin binding, which is required for receptor internalization but is not obligate for receptor dephosphorylation in the plasma membrane. Dephosphorylation of the D1 dopamine receptor[211] was not prevented by inhibitors of internalization, but the involved phosphatase

has not been identified. The detailed mechanism(s) underlying DP desensitization, e.g the involvement of kinases and phosphatases has yet to be elucidated.

The involvement of various transcription factors in allergic inflammation is evident. The contribution of PGD_2 receptors in migration and other functional aspects such as respiratory burst, degranulation or shape change of immune cells, in particular eosinophils, mostly has been studied in association with G-protein coupling and second messenger signaling such as cAMP or Ca^{2+}. However, little is known about CRTH2- and DP-mediated activation/modulation of transcription factors. Hence, I set out to gain insight into the PGD_2 receptor cross-talk with regard to modulation of NF-κB-, CREB-, NFAT- and SRE-activity.

NF-κB has been associated to the pathophysiology of asthma and allergic inflammation[185]. In mouse models of allergic airway inflammation NF-κB activity was found to be increased[212;213]. Poynter *et al*. demonstrated that NF-κB activation contributes to the adaptive immune response upon antigen exposure, but it is not involved in airway hyperresponsiveness, a characteristic feature of allergic airway inflammation[214].

The association of CREB with allergic inflammation was observed in asthmatic patients with inflamed bronchi who expressed elevated levels of active, phosphorylated CREB[215]. Further on, it was shown that several prostaglandins, PGE_2[129;187], $PGF_{2\alpha}$[216] and PGD_2[217] are linked to CREB-mediated mucin hyperproduction via the ERK/MAPK pathway.

NFAT expression has been described in T cells, mast cells, basophils, B cells, macrophages, natural killer cells, endothelial cells and eosinophils[189;190;218]. In eosinophils NFAT, is involved in proinflammatory cytokine production, differentiation, trafficking[219] and apoptosis[220]. The proinflammatory role of NFAT was further clarified by the observation that inhibition of NFAT in T cells prevents allergic pulmonary inflammation[221]. In most cell types, receptors and signaling pathways leading to NFAT activation are not well known. In T cells and mast cells NFAT activation is Ca^{2+}-dependent and regulated via the T cell receptor[222] and the FcεRI receptor[223], respectively. In eosinophils it has been found that nuclear translocation of NFAT proteins can be stimulated by the Th2 cytokines IL-4 and IL-5[190].

Serum response elements are found in the promoters of immediate early genes[224] and can be induced by Rho family GTPases[192] including RhoA, Rac1 and Cdc42, which regulate actin polymerization, F-actin bundling and focal adhesion formation[225], for instance. Formation of F-actin in turn progagates the activation of myocardin related transcription factors which are translocated to the nucleus and induce SRE activity in cooperation with the serum response factor.

My data show a considerable cross-talk between CRTH2 and DP receptors in NFAT and SRE activation. Both PGD_2 receptors seem to be a dynamic signaling unit whose functionality and dependence on each other might be determined by the activated G-protein pathway. While the $G\alpha_s$-protein-mediated activation of CREB is not modulated by the interference of CRTH2 and DP, other pathways leading to NFAT or SRE activation, possibly via $G\alpha_q$-or $G\alpha_{12/13}$-proteins obviously underlie that cross-regulation. From my studies regarding Ca^{2+} flux, and NFAT and SRE activation it has become obvious that the interaction between CRTH2 and DP receptors underlies the following rules: i) CRTH2 can become responsive in the presence of DP through a possible transactivation and ii) CRTH2 activity is not obligatory even when co-espressed with DP, but blockade of CRTH2 can transmit an inhibitory effect on DP signaling.

During allergic inflammation leukocytes, including eosinophils, extravasate from the blood vessels and accumulate at sites of inflammation within the tissue. The steps involved in this process of interaction with the endothelium and the extracellular matrix are well characterized such as rolling, activation, firm adhesion and transmigration[226]. However, the participation of PGD_2 and its receptors in regulation of cytoskeleton rearrangement and adhesion has not been studied yet. Still, it is well known that PGD_2 mediates eosinophil migration as mentioned in the introduction.

Actin polymerization is a cellular response involved in the directed migration of granulocytes. In my thesis I investigated the dynamics of the actin cytoskeleton in the PGD_2 receptor-HEK293 cell model as well as in eosinophils. In agreement with the ability of CRTH2 and DP receptors to induce eosinophil chemotaxis I found that both receptors induce actin polymerization, even though to a different extent and with varying patterns. The clear morphological variations between cells stimulated with PGD_2 or selective CRTH2 and DP agonists were found in both, HEK293 cells and eosinophils. In HEK293 cells, I observed a characteristic pattern of stress fiber formation upon CRTH2 and DP stimulation, which was present in cells expressing each receptor alone or in comination. In eosinophils, I found an enormous increase in formation of filopodia and lamellopodia following PGD_2 treatment, as opposed to a mild polarization following selective receptor activation. These results indicated a synergy of CRTH2 and DP receptors in mediating actin polymerization. The use of selective antagonists revealed a dominant role of CRTH2, since PGD_2-induced actin polymerization was solely inhibited by the CRTH2 antagonist Cay10471 in the HEK293 cell model as well as in eosinophils. A co-operative signaling of CRTH2 and DP receptors was also recently reported[121]. The authors showed that leukotriene C (4) synthesis in eosinophils requires

simultaneous activation of both PGD_2 receptors leading to concomitant stimulation of $G\alpha_s$- and $G\alpha_i$-protein. Hence, this so-called "alliance" of CRTH2 and DP receptors might also apply to other signaling pathways, e.g. cytoskeleton rearrangement. In addition, it has been described that actin polymerization in eosinophils mediated by the chemoattractants C5a, platelet-activating factor and RANTES was dependent on the intracellular Ca^{2+} level[227]. Scavenging of cytosolic calcium ions prevented formation of F-actin. Thus, the different ability of CRTH2 and DP to induce intracellular Ca^{2+} release might result in the diverse magnitude and pattern of CRTH2- and DP-mediated actin polymerization, i.e. the length and morphology of the fillopodia and lamellopodia that I observed in my experiments. As I have shown in Figs. 8 and 27, the DP-mediated Ca^{2+} release is impaired in the presence of CRTH2 in the HEK293 cell model and absent in eosinophils. This could explain the prominent DP-induced stress fiber formation in HEK-DP cells, which strongly show concomitant agonist-induced Ca^{2+} release, versus attenuated DP-induced actin polymerization in HEK-CRTH2+DP cells. Likewise, this hypothesis is supported by extensive PGD_2-induced shape change versus mild BW245c-mediated polarization in eosinophils. Other studies[228;229] that have demonstrated the involvement of G-proteins in actin polymerization, in particular the pertussis toxin-sensitive $G\alpha_i$-protein, further emphasize the importance of intracellular Ca^{2+} signaling for assembly and disassembly of the F-actin. It would be challenging to further investigate the role of Ca^{2+} signaling, and to characterize the G-proteins involved in PGD_2-mediated actin polymerization in eosinophils.

In addition to actin polymerization, I quantified adhesion of eosinophils to activated human pulmonary endothelial cells. This process is obligatory for the recruitment of eosinophils into the inflamed tissue. Equally to F-actin formation, I observed a significant increase in eosinophil adhesion to activated endothelial cells upon simultaneous stimulation of CRTH2 and DP receptors using PGD_2, while the selective CRTH2 agonist DK-PGD_2 was less effective. The DP agonist BW245c failed to promote significant eosinophil adhesion to the endothelial cells. Similar to the actin polymerization assay, selective antagonists revealed a prevailing role of CRTH2 in eosinophil adhesion.

Therefore, these results indicate that both PGD_2 receptors are possibly required for a maximal response in cytoskeletal rearrangement and firm adhesion to the vessel wall. Notably, CRTH2 seems to be the fundamental receptor in these processes since pharmacological blockade of DP receptors revealed no significant impact on the cellular response. It remains elusive which functions do $G\alpha_s$-protein-induced adenylyl cyclase and PKA activity or $G\alpha_i$-protein-mediated Ca^{2+} signaling have in these processes.

Collectively, HEK-CRTH2+DP cells in many respects resembled eosinophils which natively express both receptors: i) eosinophils responded with effective Ca^{2+} flux to CRTH2 but not DP activation; ii) eosinophil responses to CRTH2 activation were sensitive to both PTX (partial inhibition) and MH-362-63-8 (full inhibition); iii) CRTH2 readily internalized after stimulation whereas DP surface expression in eosinophils remained unaltered; iv) CRTH2 and DP synergistically activate cytoskeleton rearrangement; v) exposure to the DP agonist BW245c prevented eosinophil Ca^{2+} responses to DK-PGD_2; and vi) the DP antagonist BWA868c attenuated Ca^{2+} responses in eosinophils, both to PGD_2 and DK-PGD_2. These latter observations are in good agreement with our previous report that pre-treatment with either the DP agonist BW245c or the DP antagonist BWA868c inhibited chemotactic responses of human peripheral blood eosinophils to PGD_2 and abrogated the PGD_2-induced release of bone marrow eosinophils in the guinea pig[135].

Conclusions

In summary, my data demonstrate remarkable cooperativity between the PGD_2 receptors, CRTH2 and DP. Since several native cells expressing CRTH2, such as eosinophils, basophils and Th2 lymphocytes, also express the DP receptor[127;141;230], it is tempting to speculate that CRTH2 activity can be amplified by the DP receptor. In some signaling pathways, the CRTH2 activity might even essentially depend on functional DP receptors *in vivo,* by "hijacking" its signaling machinery. Future studies using RNA interference knock-down of DP receptors in eosinophils, or DP-deficient mice might provide final evidence that the presence of the DP receptors is a prerequisite for functional CRTH2 responses. Further research might be needed to establish whether changes in DP expression under pathologic conditions are involved in the regulation of biological responses to CRTH2 activation and might hence impact on the development of allergic inflammation. Moreover, drugs that have been designed to account for the cross-talk of these PGD_2 receptors might be more selective and/or effective than traditional receptor antagonists.

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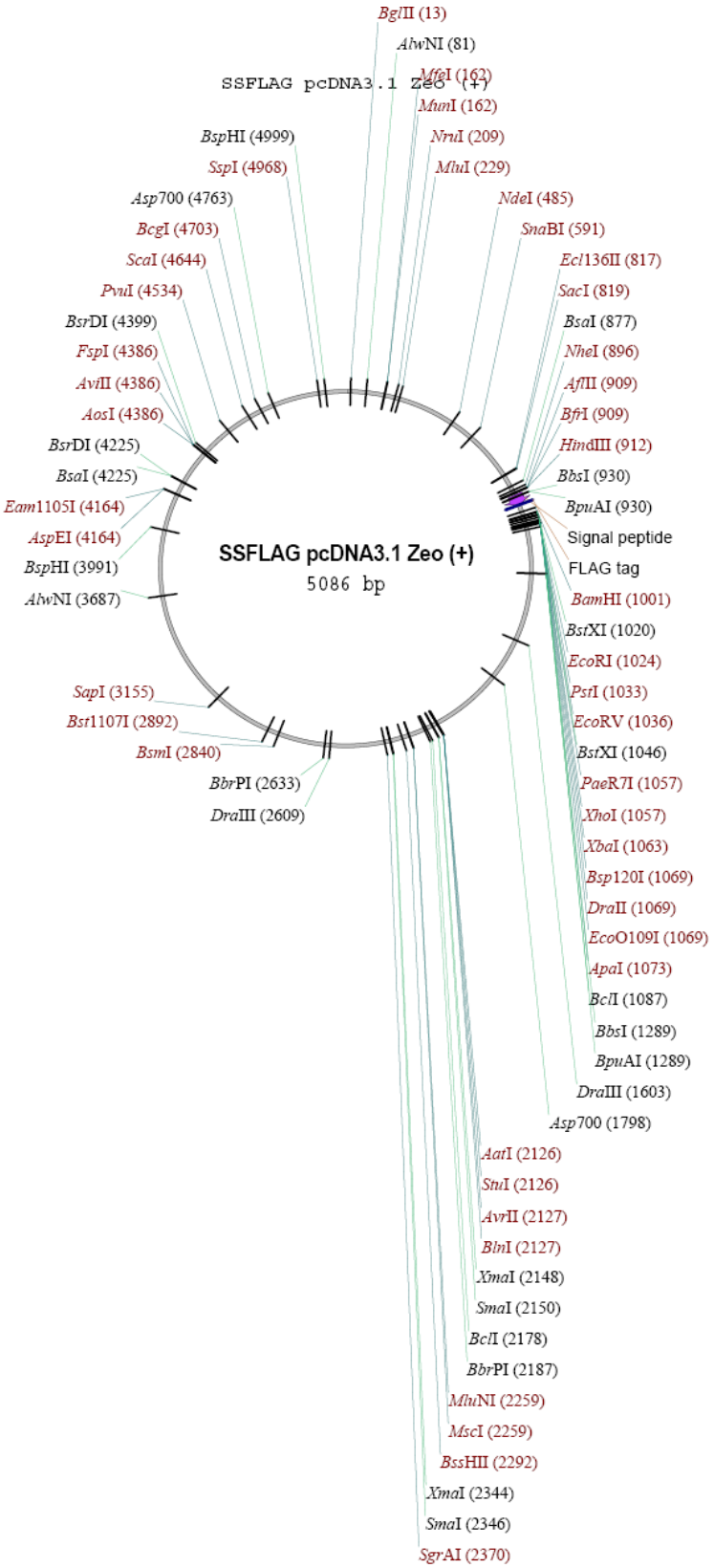
7. Appendices

Appendix I. Vector map of SSFLAG-pcDNA3.1 Zeo.



Appendix II. Published papers

Original article

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

Miriam Sedej, MSc,[a] Ralf Schröder, PhD,[b] Kathrin Bell, MSc,[b] Wolfgang Platzer, BSc,[a] Anela Vukoja, MSc,[a] Evi Kostenis, PhD,[b] Akos Heinemann, MD,[a] and Maria Waldhoer, PhD[a,c] *Graz, Austria, Bonn, Germany, and Gentofte, Denmark*

http://dx.doi.org/10.1016/j.jaci.2011.08.015

CRTH2 and D-Type Prostanoid Receptor Antagonists as Novel Therapeutic Agents for Inflammatory Diseases

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doi: 10.1159/000313836

Prostaglandin H_2 induces the migration of human eosinophils through the chemoattractant receptor homologous molecule of Th2 cells, CRTH2

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10/2007 - 05/2008	Master Thesis Medical University of Graz, Graz, Austria Institute of Experimental and Clinical Pharmacology Supervision: Prof. Akos Heinemann/ Dr. Maria Waldhoer
10/2006 – 05/2008	Master Candidate of Molecular Medicine Georg-August University, Göttingen, Germany

Scientific Publications

Sedej M, Schröder R, Bell K, Platzer W, Vukoja A, Kostenis E, Heinemann A, Waldhoer M
D-type prostanoid receptor enhances signaling of the chemoattractant receptor homologous molecule Expressed on Th2 cells. J Allergy Clin Immunol. **2011** Sep 17. [Epub ahead of print]

Schuligoi R, Sturm E, Luschnig P, Konya V, Philipose S, **Sedej M**, Waldhoer M, Peskar BA, Heinemann A CRTH2 and D-type prostanoid receptor antagonists as novel therapeutic agents for inflammatory diseases. Pharmacology **2010**; 85:372-82.

Schuligoi R, Sedej M, Waldhoer M, Vukoja A, Sturm EM, Lippe IT, Peskar BA, Heinemann A Prostaglandin H2 induces the migration of human eosinophils through the chemoattractant receptor homologous molecule of Th2 cells, CRTH2. J Leukoc Biol. **2009**; 85(1):136-145

Scientific Interests

Inflammation, eosinophils, signaling and trafficking of G-protein coupled receptors

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