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Molecular pharmacology of the human prostaglandin D_2 receptor, CRTH2

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1 The recombinant human prostaglandin D_2 (PGD_2) receptor, hCRTH2, has been expressed in HEK293(EBNA) and characterized with respect to radioligand binding and signal transduction properties. High and low affinity binding sites for PGD_2 were identified in the CRTH2 receptor population by saturation analysis with respective equilibrium dissociation constants (K_D) of 2.5 and 109 nM. This revealed that the affinity of PGD_2 for CRTH2 is eight times less than its affinity for the DP receptor.

2 Equilibrium competition binding assays revealed that of the compounds tested, only PGD_2 and several related metabolites bound with high affinity to CRTH2 (K_i values ranging from 2.4 to 34.0 nM) with the following rank order of potency: $PGD_2 > 13,14$-dihydro-15-keto $PGD_2 > 15$-deoxy-$\Delta^{12,14}$-$PGJ_2 > PGJ_2 > \Delta^{12}$-$PGJ_2 > 15$(S)-15 methyl-$PGD_2$. This is in sharp contrast with the rank order of potency obtained at DP : $PGD_2 > PGJ_2 > \Delta^{12}$-$PGJ_2 > 15$-deoxy-$\Delta^{12,14}$-$PGJ_2 > > > 13,14$-dihydro-15-keto-$PGD_2$.

3 Functional studies demonstrated that PGD_2 activation of recombinant CRTH2 results in decrease of intracellular cAMP in a pertussis toxin-sensitive manner. Therefore, we showed that CRTH2 can functionally couple to the G-protein $G_{\alpha i/o}$. PGD_2 and related metabolites were tested and their rank order of potency followed the results of the membrane binding assay.

4 By Northern blot analysis, we showed that, besides haemopoietic cells, CRTH2 is expressed in many other tissues such as brain, heart, thymus, spleen and various tissues of the digestive system. In addition, *in situ* hybridization studies revealed that CRTH2 mRNA is expressed in human eosinophils. Finally, radioligand binding studies demonstrated that two eosinophilic cell lines, butyric acid-differentiated HL-60 and AML 14.3D10, also endogenously express CRTH2.
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Keywords: CRTH2; prostaglandin D_2; prostanoid receptor; DP; recombinant; pharmacology; tissue distribution; eosinophils; binding assay; intracellular signalling

Abbreviations: 13,14-dihydro-15-keto-PGD_2, DK-PGD_2; cAMP, cyclic AMP; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; i[cAMP], intracellular cyclic AMP; i[Ca^{2+}], intracellular calcium; NSAID, non-steroidal anti-inflammatory drug; PGD_2, prostaglandin D_2; PTX, pertussis toxin

Introduction

Prostaglandin D_2 (PGD_2) is a prostanoid derived from arachidonic acid *via* the action of PGHS I & II and specific PGD synthases (Narumiya *et al*., 1999). PGD_2 has been shown to bind and activate two G-protein-coupled receptors, CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells) and DP (Boie *et al*., 1995; Hirai *et al*., 2001). Interestingly, the two PGD_2 receptors share little sequence homology. The highest amino acid sequence identity for CRTH2 is found with members of the leukocyte chemoattractant receptor subfamily which includes the FMLP receptor, C3a receptor and C5a receptor (Nagata *et al*., 1999). In fact, PGD_2 was reported to have chemotactic/chemokinetic activity on T-helper type 2 cells, eosinophils and basophils (Gervais *et al*., 2001; Hirai *et al*., 2001; Monneret *et al*., 2001). DP shares the highest sequence identity with the other prostanoid receptors, TP, FP, EP_{1-4} and IP (Hirai *et al*., 2001). Through the activation of the DP receptor, PGD_2 has been implicated in different physiological events including sleep induction, mucus production, cell survival, control of intraocular pressure and allergic responses (Gervais *et al*., 2001; Matsumura *et al*., 1994; Matsuoka *et al*., 2000; Woodward *et al*., 1993; Wright *et al*., 1999). The physiological role of CRTH2 is not as well characterized. However, it has been demonstrated that its activation by PGD_2 can increase eosinophil and Th2 cell motility and can also modulate eosinophil morphology and degranulation (Gervais *et al*., 2001; Hirai *et al*., 2001; Monneret *et al*., 2001).

DP activation by PGD_2 leads to the stimulation of adenylyl cyclase activity and an increase in intracellular cAMP levels (i[cAMP]) in a G-protein $G_{\alpha s}$-dependent manner (Boie *et al*., 1995). In contrast, CRTH2 intracellular signalling is less well defined. CRTH2 activation by PGD_2 does not lead to a stimulation of cAMP production (Hirai *et al*., 2001). On the other hand, activation of CRTH2 by PGD_2

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has been shown to lead to a pertussis toxin-sensitive increase in intracellular calcium (i[Ca^{2+}]) mobilization (Hirai *et al*., 2001). This toxin sensitivity suggests that CRTH2 couples to the G-protein $G_{\alpha i/o}$ class which results in inhibition of adenylyl cyclase activity and, as a consequence, decreases intracellular cAMP levels. However, CRTH2-mediated decreases in i[cAMP] has not yet been reported.

In contrast to DP, CRTH2 expression profile, signalling properties and pharmacology is poorly defined. Hirai *et al*. have established the expression of CRTH2 in various immune cells and its capacity to signal through increase in i[Ca^{2+}]. They have also determined the affinity of PGD_2 ($K_i = 61 \pm 23$ nM) and a limited number of ligands for the recombinant human CRTH2 using a whole cell radioligand binding assay (Hirai *et al*., 2001).

In the present study, we extensively characterized CRTH2 at the pharmacological level. We evaluated ligand : receptor interactions of recombinant CRTH2 using a membrane-based radioligand binding assay. The effect of CRTH2 activation on i[cAMP] and i[Ca^{2+}] levels was also assessed. Finally, we determined CRTH2 expression profile by Northern blot analysis. This study provides new important information that should prove useful in understanding the role for the PGD_2 receptor CRTH2 *in vivo*.

Methods

Construction of pCEP4-hCRTH2 mammalian expression vector

RT–PCR was performed on human (h) eosinophil total RNA using oligo dT (Perkin Elmer RNA PCR core kit). The cDNA was amplified using Advantage cDNA PCR (Clontech) with gene specific primers containing restriction sites for cloning into pcDNA3.1neo(+) vector (Invitrogen). Sense primer 5′-GCATAAGGTACCATGTCGGCCAACGCCACACTG-3′. Reverse primer 5′-CGCTAGTCTAGACTAACTCGAGGTGCTGCTCA-3′. The nucleotide sequence of the cloned cDNA was identical to the previously published sequence (Genbank accession number AB008535) (Nagata *et al*., 1999). The human CRTH2 (hCRTH2) cDNA was subsequently subcloned in Kpn1/Nhe1 in the mammalian episomal expression vector pCEP4 for the generation of a cell line stably expressing CRTH2.

pCEP4-hCRTH2 expression in HEK293(EBNA) cells

The cell line HEK293(EBNA) was chosen based on its absence of endogenous expression of CRTH2. This was determined by *in situ* hybridization, [^{3}H]-PGD_2 membrane binding assays and in functional cell-based assays (i.e changes in levels of cAMP and calcium following challenge with PGD_2) (data not shown; Figure 2C). HEK293(EBNA) cells were maintained in culture in a humidified atmosphere at 37°C (6% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units ml^{-1} of penicillin, 100 μg ml^{-1} streptomycin and 250 μg (active) ml^{-1} G418 (for EBNA selection). Stable expression of the hCRTH2 receptor in HEK293(EBNA) cells was achieved by transfection of the pCEP4-hCRTH2 plasmid using Lipofectamine-PLUS™ reagent according to the manufacturer's instructions (Invitrogen). Cells were maintained in culture for 48 h post-transfection and then grown in the presence of 250 μg ml^{-1} hygromycin B (Calbiochem) for 2 weeks to select for resistant cells expressing the hCRTH2 receptor. Clonal cell lines were then generated by serial dilution. A clonal cell line, HEK-hCRTH2 no. 4, was selected for its high level of expression of recombinant hCRTH2 and the largest window of maximal inhibition of cAMP level achievable, as determined by receptor binding assays and cAMP functional assays, respectively.

Differentiation of HL60 cells and AML14.3D10 cells

HL-60/MF211 no. 7 is a pro-eosinophilic substrain of the human pro-myelocytic cell line that had been subcultured under alkaline conditions with the continuous selection of subtypes displaying eosinophilic properties when cultured with butyric acid (Scoggan *et al*., 1996). AML14.3D10 is an eosinophilic subline of the AML14 leukaemic cell line that does not require cytokine supplementation for eosinophilic differentiation (Paul *et al*., 1995). These cell lines were grown in a humidified atmosphere at 37°C (6% CO_2) in RPMI-1640 media (Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum, 2 mM glutamine and 100 units ml^{-1} of penicillin and 100 μg ml^{-1} streptomycin. On day 1 of differentiation, cells were cultured at a cell density off 2×10^5 cells ml^{-1} and supplemented with 0.4 mM butyric acid. On day 4, the cells were diluted to 2×10^5 cells ml^{-1} and the concentration of butyric acid was adjusted to 0.4 mM. The cells were harvested on day 7.

Preparation of membranes

HEK-hCRTH2 cells were rinsed with phosphate buffered saline (PBS), detached using dissociation buffer (Specialty Media) and collected by centrifugation at 500 g for 10 min at 4°C. HL-60 and AML cells were collected by centrifugation for 15 min at 500 g at 4°C, washed with PBS and recovered by centrifugation. In all cases, the final cell pellet was resuspended in 10 mM HEPES/KOH pH 7.4, 1 mM EDTA, at approximately 10^7 cells ml^{-1} and frozen in liquid nitrogen or immediately processed for membrane preparation. The cells were disrupted by nitrogen cavitation (Parr) on ice (800 p.s.i. for 30 min) in the presence of protease inhibitors (2 mM AEBSF, 10 μM E-64, 100 μM leupeptin and 0.05 mg ml^{-1} pepstatin). Cell membranes were isolated by differential centrifugation at 4°C, first at $1000 \times$ g for 10 min, then 160 000 g for 30 min. After centrifugation the pellet was resuspended in 10 mM HEPES/KOH pH 7.4, 1 mM EDTA, using Dounce homogenization (ten strokes), frozen in liquid nitrogen and stored at −80°C.

Radioligand binding assay

Radioligand binding assays were performed at room temperature in 10 mM HEPES/KOH pH 7.4, 1 mM EDTA containing 10 mM $MnCl_2$ and 0.4 nM [^{3}H]PGD_2 (NEN, 172 Ci mmol^{-1}), in a final volume of 0.2 ml. Competing ligands (from Biomol and Cayman) were diluted in dimethylsulphoxide (Me_2SO) that was kept constant at 1% (v v^{-1}) of the final incubation volume. The reaction was

initiated by the addition of 150 μg (for HL-60 no. 7 and AML14.3D10 cells) or 23 μg (for HEK-hCRTH2) of membrane protein. Total and non-specific binding were determined in the absence and the presence of 10 μM PGD_2, respectively. Under these conditions, specific binding (total minus non-specific) of the radioligand to the receptor reached equilibrium within 50 min and was stable up to 180 min. The reaction was routinely conducted for 60 min at room temperature and terminated by rapid filtration through GF/C filters (Brandel), prewetted in cold 10 mM HEPES/KOH pH 7.4, using a Brandel™ harvester (for HL-60 and AML cells) or through prewetted Unifilters GF/C (Packard), using a Tomtec MachIII semi-automated harvester (for HEK-hCRTH2). The filters were then washed with 4 ml of the same buffer and residual radioligand bound to the filter was determined by liquid scintillation counting following equilibration in 5 ml Ultima Gold™ (GF/C) or 50 μl Ultima Gold F™ (Unifilter) (Packard). Radioligand binding assays with HEK-hDP were performed as described (Abramovitz *et al*., 2000).

i[cAMP] measurements

HEK-hCRTH2 cells were grown to confluency on the day of the assay. The cells were washed with PBS, incubated for 3 min in cell dissociation buffer, harvested by centrifugation at 300 g for 6 min at room temperature and resuspended at 10^6 cells ml^{-1} in Hanks' balanced salt solution containing 25 mM HEPES pH 7.4 (HBSS/HEPES). The assay was performed in 0.2 ml HBSS/HEPES containing 5 μM forskolin (Sigma), 100 μM RO 20-1724 (Biomol) and 2 μl of test compound (from Biomol and Cayman). The reaction was initiated by the addition of 100 000 cells and left to proceed for 10 min at 37°C. The reaction was stopped by a 3 min incubation in a boiling water bath. The samples were centrifuged for 10 min at 500 g and the cAMP content in the supernatant was determined using a [^{125}I]-cAMP scintillation proximity assay (Amersham). Maximal inhibition of forskolin stimulated cAMP production by activation of CRTH2 was determined in the presence of 1 μM PGD_2. All compounds were prepared in Me_2SO kept constant at 1% (v v^{-1}) of the final incubation volume.

Construction of pCDNA3-$G_{\alpha 15}$ mammalian expression vector

$G_{\alpha 15}$ cDNA (Aurora Biosciences) was subcloned into the *Hind*III and *Xba*I sites of pCDNA3.1-Zeo(+) (Invitrogen). The resulting plasmid was transfected into HEK-hCRTH2 no. 4 using Lipofectamine™ reagent. Cells were selected in presence of 250 μg (active) ml^{-1} G-418 and hygromycin B and 10 μg ml^{-1} Zeocin (InVitrogen) (for $G_{\alpha 15}$ selection). The clonal cell line HEK-hCRTH2-$G_{\alpha 15}$ no. 14 was selected for further characterization based on release of intracellular calcium (i[Ca^{2+}]) following PGD_2 challenge.

i[Ca^{+2}] measurements

Cells were seeded onto Biocoat poly-D lysine 96-well plates (Becton Dickinson) at 40 000 cells per well and incubated overnight in supplemented DMEM as indicated above. Cells were washed once with HBSS supplemented with 20 mM HEPES pH 7.4 and then incubated for 50 min at 37°C (6% CO_2) in the same buffer containing the cytoplasmic Ca^{+2} indicator (Calcium assay kit; Molecular Devices). Changes in fluorescence were monitored before and after the addition of various agonists using a FLIPR™ (Molecular Devices) at $\lambda_{ex} = 488$ nM and $\lambda_{em} = 540$ nM.

Northern blot analysis

Human multiple tissue Northern (MTN™) blot I, III and IV (Clontech) were probed with a [^{32}P]-dCTP radiolabelled probe generated from full length CRTH2 using the T7 QuickPrime kit according to the manufacturer's specifications (Pharmacia). The blots were first incubated with 10 ml of ExpressHyb solution (Clontech) for 30 min at 68°C. Hybridization of the probe was carried out in ExpressHyb solution (1×10^6 c.p.m. per ml) at 68°C overnight. Following hybridization, Northern blots were washed twice with 2 × SSC-0.1% (w v^{-1}) SDS at room temperature for 10 min and then twice with 0.2 × SSC-0.1% (w v^{-1}) SDS at 50°C for 20 min. Autoradiography was carried out overnight using MR Kodak film. β-actin was used as a control probe.

In situ *hybridization*

Freshly isolated eosinophils (2×10^5 cells) were layered onto a poly-lysine coated glass slide by centrifugation (Cytospin). Cells were then fixed in 4% (v v^{-1}) paraformaldehyde solution prepared in PBS, pH 7.4, for 20 min at room temperature. The slides were then processed as follows: 2 min in 3 × PBS, 2 min in 1 × PBS twice and incubations of 5 min in 50, 70, 95 and 100% ethanol : water (v v^{-1}) solutions. Slides were air dried and stored at −80°C. The slides were equilibrated to room temperature and washed for 5 min in diethylpyrocarbonate (DEPC)-treated water and twice in PBS. The purity of the eosinophil population was assessed by means of flow cytometry (CELL-DYN 3700 system) on the basis of size, complexity, granularity and lobularity and found to be >95% pure. A CRTH2 cDNA fragment representing nucleotides 360 to 676 (corresponding to amino acids 120 to 225; Genbank accession no. AB008535) was amplified by PCR and subcloned into the PCR II dual promoter vector (Invitrogen). DIG-labelled riboprobes were synthesized using DIG-RNA labelling kit from Boehringer Mannheim. *In situ* hybridization was carried out as previously described (Wright *et al*., 1999). Colorimetric detection of the hybridized riboprobe was performed using an alkaline phosphatase-linked anti-DIG antibody (Boehringer Mannheim). All subsequent steps were carried out at room temperature. The slides were washed in detection buffer (DB; 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v v^{-1}) Tween), incubated with SuperBlock buffer (Biogenex) for 10 min and then incubated for 2 h with anti-DIG antibody (1 : 75 dilution) in DB and then washed three times in DB. The chromagen solution Fast Red (Sigma Biochemicals) was then added and the slides were left to incubate for 30 min. The reaction was stopped by washing in 10 mM Tris pH 8.0, 1 mM EDTA. The cells were mounted using SlowFade (Molecular Probes) and examined on a fluorescent microscope connected to a CCD camera.

Results

Saturation analyses of [^{3}H]PGD_2 specific binding to HEK-hCRTH2

Radioligand binding assays to recombinant hCRTH2 were initially optimized with respect to divalent cation, pH dependence and incubation time as described in the Methods section. Under optimal conditions, [^{3}H]PGD_2 binding to CRTH2 was at equilibrium in 50 min and stable for 3 h, and could be fully dissociated by excess unlabelled PGD_2. Saturation analysis of [^{3}H]PGD_2 specific binding to hCRTH2 was then performed and revealed the presence of two populations of specific binding sites (Figure 1). Tritiated PGD_2 bound with high and low affinity to hCRTH2 with equilibrium dissociation constants (K_D) of 2.5 ± 1.1 nM ($n=3$; mean $\pm$ s.e.mean throughout) and 109 ± 68.4 nM ($n=3$) respectively. The maximal number of high and low affinity binding sites (B_{max}) detected were 7.8 ± 2.9 pmol mg^{-1} ($n=3$) and 29.5 ± 9.5 pmol mg^{-1} ($n=3$) of membrane protein respectively.

Competition for [^{3}H]PGD_2 specific binding to HEK-hCRTH2

Five groups of ligands were evaluated for their ability to compete with [^{3}H]PGD_2 specific binding to HEK293(EBNA) cell membranes expressing hCRTH2 in equilibrium competition assays (Table 1). The first group comprised the preferred ligands for the prostanoid receptors: PGD_2 for DP, PGE_2 for the EP subtypes 1 to 4, $PGF_{2\alpha}$ for FP, the stable prostacyclin analogue iloprost for IP and the thromboxane mimetic U46619 for TP (Coleman *et al*., 1994). Out of all the prostanoid receptor preferred ligands, only PGD_2 competed with high affinity with [^{3}H]PGD_2 for CRTH2 specific binding with a K_i value of 2.4 ± 0.2 nM ($n=17$). The overall rank order of affinities for this group of ligands was: $PGD_2 > PGF_{2\alpha} > U46619 > PGE_2 >$ iloprost. Since PGD_2 binds with the highest affinity to hCRTH2, we evaluated various PGD_2 metabolites. Most of the metabolites tested retain high affinity for CRTH2. Specifically, 13,14-dihydro-15-keto PGD_2 (DK-PGD_2) and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were comparable to PGD_2 in binding affinities to hCRTH2 with K_i values of 2.91 ± 0.29 nM ($n=10$) and 3.15 ± 0.32 nM ($n=3$), respectively. The third group of ligands consisted of selective compounds that are routinely used in pharmacological studies to classify the prostanoid receptors (Coleman *et al*., 1994), while the fourth group included various eicosanoid ligands. These ligands did not display appreciable affinity. It is important to note that DP selective agonists such as BW245C and L-644698 (Wright *et al*., 1998) were inactive at hCRTH2. Finally, the fifth group of compounds tested were a selection of NSAIDS. Only indomethacin showed high affinity ($K_i = 25.0 \pm 3.6$ nM, $n=4$) for hCRTH2.

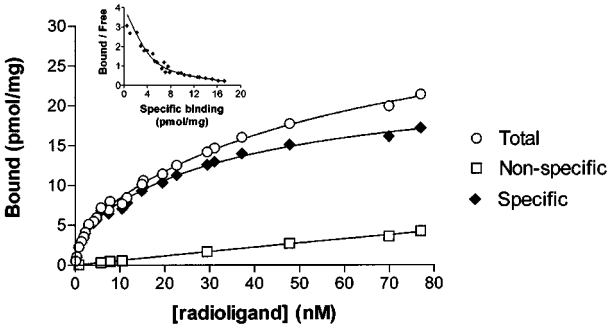

Figure 1 Saturation analysis of [^{3}H]PGD_2 specific binding to recombinant HEK293(EBNA) cell membranes expressing hCRTH2. The radioligand binding assay was performed essentially as described in Methods. The incubation also contained increasing concentrations of radioligand from 0.2 to 77 nM, and 100 μM of unlabelled PGD_2 for non-specific determination. The experiment was conducted over 90 min. Inset: Non-linear transformation of the deduced specific binding isotherm showing the presence of two affinity binding sites. Analysis was done using GraphPad Prism (GraphPad Software Inc.). This is representative of three independent experiments where each data point was determined in duplicate.

Cyclic AMP functional assay with HEK-hCRTH2

The signal transduction properties resulting from activation of recombinant CRTH2 in HEK293(EBNA) were investigated. PGD_2 was unable to stimulate cAMP production (data not shown). PGD_2, however, reduces forskolin-elevated i[cAMP] levels in a dose-dependent manner with an $EC_{50} = 1.8$ nM ± 0.4 nM (Figure 2A and Table 2, $n=16$). Activation of CRTH2 by PGD_2 reduced forskolin-stimulated i[cAMP] levels from 16.3 ± 1.9 pmol to 3.0 ± 0.5 pmol ($n=16$). Maximal potency was achieved with 100 nM PGD_2. There was no PGD_2-mediated reduction in i[cAMP] in forskolin-treated HEK293(EBNA) wild-type cells (data not shown). Moreover, the decreased cAMP response mediated by 100 nM of PGD_2 was reduced in the presence of 100 ng ml^{-1} pertussis toxin (PTX) (Figure 2B). This suggests that CRTH2 couples through the PTX-sensitive $G_{\alpha i/o}$ class of G-proteins. Additional prostanoid-like ligands with high affinity for hCRTH2 were tested for their potency to stimulate the receptor to reduce forskolin-stimulated i[cAMP]. All the ligands shown in Table 2 are full agonists at hCRTH2, as compared with PGD_2. Overall, the EC_{50} for these ligands followed the same rank order as their affinities determined in radioligand binding assays, with the highest potencies observed for PGD_2 and its metabolites. Indomethacin was the only NSAID tested in the radioligand binding assay that had high affinity for CRTH2. Indomethacin was also a full agonist at hCRTH2 and decreased forskolin-stimulated i[cAMP] with an EC_{50} of 14.9 ± 4.9 nM ($n=3$). The other NSAIDS tested in membrane binding assays were unable to efficiently reduce cAMP under the same assay conditions (data not shown).

Calcium functional assay with HEK-hCRTH2-Gα_{15}

We also investigated whether activation of CRTH2 resulted in mobilization of i[Ca^{2+}]. PGD_2 stimulated a slight increase in intracellular calcium in HEK-hCRTH2 cells compared to the HEK parental cell line (Figure 2C). This increase in intracellular calcium was fully inhibited by 100 ng ml^{-1} of PTX. The release of i[Ca^{2+}] provoked by PGD_2 was dramatically increased in HEK-hCRTH2 cells stably transfected with the promiscuous G-protein $G_{\alpha 15}$. Using the HEK-hCRTH2-$G_{\alpha 15}$ cell line, the EC_{50} value for PGD_2 in mobilizing i[Ca^{2+}] was determined as 22.1 ± 4.4 nM ($n=6$, Table 2). This is approximately 10 fold higher than the EC_{50}

Table 1 Affinities of eicosanoid ligands and related compounds for recombinant hCRTH2 and hDP expressed in HEK293(EBNA) cell membranes

	K_i (nM)	
	hCRTH2	*hDP**
Prostaglandin receptor preferred agonists and related analogs		
PGD_2	2.4±0.2 (17)	1.7±0.3 (12)
PGE_2	4730±64 (3)	307±106 (5)
$PGF_{2\alpha}$	395±77 (3)	861±139 (4)
Iloprost	>8000 (2)	1035±171 (3)
U46619	3333±971 (3)	3970±390 (3)
PGD_2 metabolites		
13,14-dihydro-15-keto PGD_2	2.9±0.3 (10)	6374±1208 (3)
15(S)-15-methyl PGD_2	34±11 (3)	nd
15-deoxy-$\Delta^{12,14}$-PGJ_2	3.2±0.3 (3)	280±30 (3)
9α,11β-PGF_2	315±92 (3)	nd
PGJ_2	6.6±0.3 (3)	0.9±0.1 (4)
Δ^{12}-PGJ_2	6.8±3.5 (3)	100±13 (3)
Prostaglandin receptor selective ligands		
19(R)-OH PGE_2	>8000 (2)	nd
AH 23848	2847±759 (3)	1380±176 (3)
AH 6809	>80 000 (2)	1415±104 (4)
Butaprost (free acid)	>80 000 (2)	12 097±2253 (3)
BW245C	>80 000 (2)	0.4±0.1 (4)
Carbacyclin	>80 000 (2)	132±16 (3)
Cloprostenol	8143±745 (3)	15 493±5983 (3)
Fluprostenol	39 333±6929 (3)	>100 000 (2)
GR63799	>80 000 (2)	>10 000 (3)
L-644698	>20 000 (2)	0.9±0.2 (3)
Latanoprost (free acid)	1141±162 (3)	54 567±3757 (3)
Misoprostol (free acid)	73 000±4139 (3)	>15 000 (2)
SC51089	>80 000 (2)	>100 000 (2)
SC51322	>80 000 (2)	11 007±1281 (3)
SQ29548	>80 000 (2)	>100 000 (3)
Sulprostone	36 633±4173 (3)	>100 000 (2)
Other eicosanoids		
12(R)-HETE	>2500 (2)	nd
13,14-dihydro-15-keto $PGF_{2\alpha}$	454±74 (3)	nd
15(S)-HETE	>2500 (2)	nd
5(S)-HETE	>2500 (2)	nd
5-oxo-ete	>2500 (2)	nd
LTB_4	>4330 (2)	nd
LTC_4	>1060 (2)	nd
LTD_4	>1010 (2)	nd
LTE_4	>3280 (2)	nd
LXA_4	>1100 (2)	nd
LXB_4	>1100 (2)	nd
PGA_2	23 000±1909 (3)	nd
PGB_2	29 767±6747 (3)	nd
PGD_2-methyl ester	319±73 (3)	nd
NSAIDS		
Indomethacin	25.0±3.6 (4)	10 539±4329 (3)
Sulindac sulfide	3450±607 (3)	350±44 (3)
Sulindac sulfone	18 567±4352 (3)	4038±371 (3)
Fenclofenac	4953±505 (3)	3238±476 (3)
Carprofen	13 167±2517 (3)	6669±298 (3)
Flufenamic acid	14 833±3060 (3)	8117±1212 (3)
Meclofenamic acid	17 833±1155 (3)	1458±100 (3)

Radioligand binding assays were conducted as described in Methods. K_i values were determined by equilibrium competition binding experiments against [^{3}H]PGD_2. Sigmoidal competition curves were analysed with a custom designed software employing a nonlinear least-squares curve fitting routine based on a four-parameter logistic equation. K_i values were calculated from K_i = Inflection point/$1+([\text{radioligand}]/K_D)$. Results are from at least three independent experiments performed in duplicate±s.e.mean with the number of determinations in parenthesis. nd = not determined. *From Abramovitz *et al*. (2000) and Wright *et al*. (1998), except for NSAIDS.

determined for reduction of i[cAMP]. A limited number of CRTH2 ligands were evaluated for their ability to mobilize i[Ca^{2+}]. These ligands were generally 10 fold less potent in this paradigm compared with their effect on i[cAMP], in

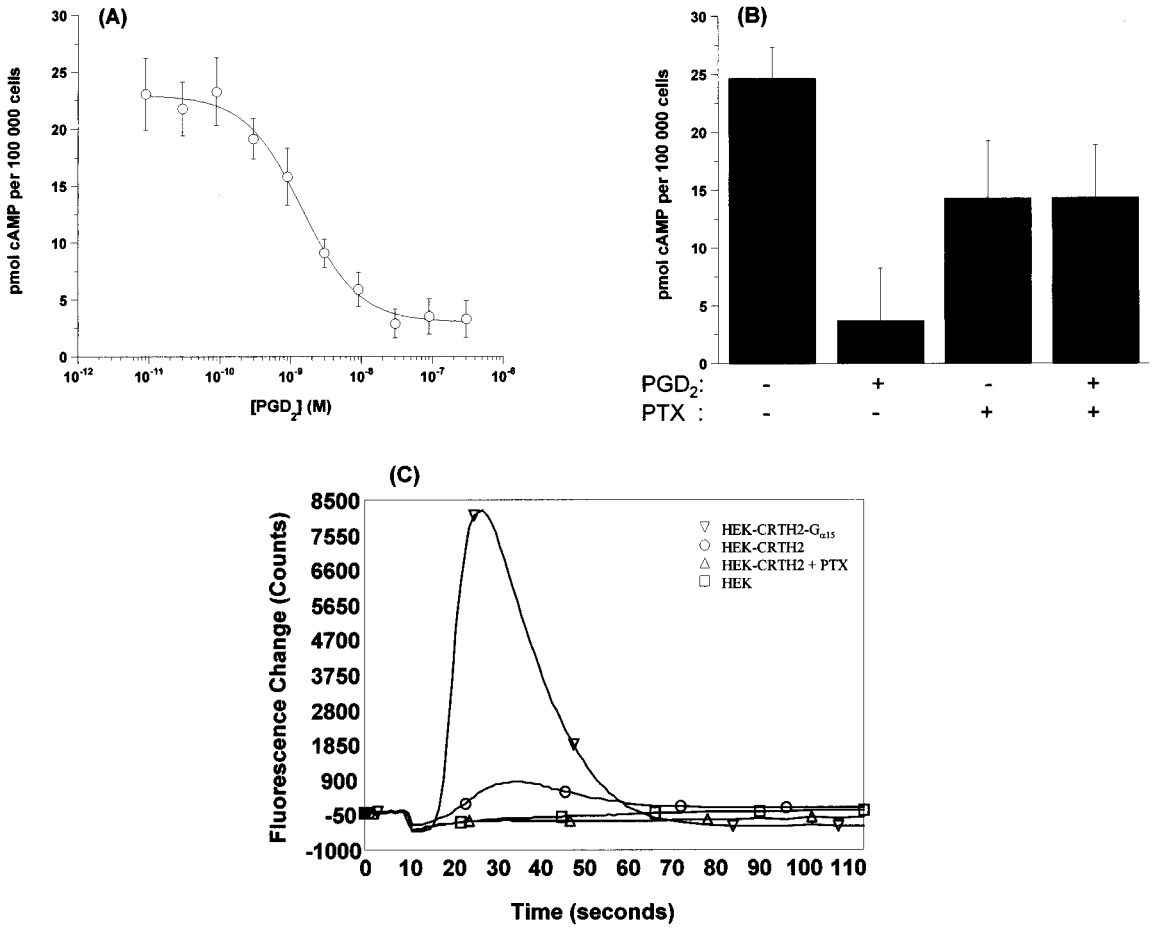

Figure 2 Inhibition of forskolin-stimulated i[cAMP] levels in HEK-hCRTH2 cells. (A) PGD_2 dose-response curve. (B) Pertussis toxin effect in the absence and presence of 100 nM PGD_2. Cells were treated overnight with 0 and 100 ng ml^{-1} of PTX and harvested the next day for cAMP assay as described in the Methods. Curves for (A) and (B) were generated in the same experiment and are from 4–5 independent experiments where each data point was done in duplicate. Sigmoidal dose response curves were constructed and analysed as in Table 1 to determine the half maximum effective concentration, EC_{50}. (C) The release of i[Ca^{2+}] was monitored over time by fluorometry in the cell lines indicated following a challenge with 100 nM of PGD_2 at time = 10 sec. PTX was used at 100 ng ml^{-1} to block the G_i/G_o-mediated response in HEK-CRTH2 cells. A representative experiment is shown.

Table 2 Determination of agonist potency in inhibiting i[cAMP] production and stimulating i[Ca^{2+}] release in HEK-hCRTH2 and HEK-hCRTH2-$G_{\alpha 15}$ cells

	EC_{50} (nM)	
Ligand	*Inhibition of cAMP*	*Calcium mobilization*
PGD_2	1.6 ± 0.3 (16)	22.1 ± 4.4 (6)
13,14-dihydro-15-keto PGD_2	4.9 ± 1.1 (10)	54.4 ± 8.1 (3)
15(S)-15-methyl PGD_2	24.9 ± 8.9 (4)	nd
15-deoxy-$\Delta^{12,14}$-PGJ_2	11.6 ± 6.3 (4)	nd
9α,11β-PGF_2	855.7 ± 321.5 (3)	nd
PGJ_2	13.1 ± 5.36 (4)	479 ± 33 (3)
Δ^{12}-PGJ_2	10.8 ± 3.4 (4)	nd
BW 245C	nd	> 10 000 (3)
$PGF_{2\alpha}$	456 ± 169 (3)	3094 ± 342 (3)
PGD_2-methyl ester	540 ± 261 (3)	1599 ± 300 (3)
13,14-dihydro-15-keto $PGF_{2\alpha}$	548 ± 326 (3)	nd
Indomethacin	14.9 ± 4.9 (3)	nd

Signal transduction assays were conducted as described in Methods. Results were expressed in per cent maximal inhibition of cAMP and per cent maximal stimulation of peak calcium release achieved with 1 μM PGD_2. Sigmoidal dose response curves were constructed and analysed as described in Table 1 to determine the half maximum effective concentration, EC_{50}. Results are from at least three independent experiments performed in duplicate ± s.e.mean with the number of determinations in parenthesis. nd = not determined.

agreement with the result observed with PGD_2. However, the rank order of potency observed in the calcium assay was consistent with the ligand binding data (see Table 1).

Tissue distribution of hCRTH2

The tissue distribution of CRTH2 was determined by Northern blot analysis on RNA from 23 different human tissues. A single transcript of approximately 3.5 Kb was detected in a variety of tissues (Figure 3). Higher levels of CRTH2 expression were seen in stomach, small intestine, heart and thymus. Intermediate levels were detected in colon, spinal cord and peripheral blood. Lower levels were observed in brain, skeletal muscle and spleen.

Detection of CRTH2 mRNA in human eosinophils by in situ *hybridization*

It was previously shown, by RT–PCR, that human eosinophils express CRTH2 (Gervais *et al*., 2001; Hirai *et al*., 2001). *In situ* hybridization studies were performed on purified eosinophils to ensure that CRTH2 mRNA detected by means of RT–PCR was not produced by a non-eosinophil contaminating cell type. Human eosinophils (Figure 4a) but not neutrophils (Figure 4c) were positively stained with a CRTH2-specific antisense riboprobe. Staining of eosinophils was not observed with the negative control sense riboprobe (Figure 4b).

Expression of hCRTH2 on eosinophilic cell lines

The eosinophilic cell lines HL-60/MF211 no. 7 (Scoggan *et al*., 1996) and AML14.3D10 (Paul *et al*., 1995) were also evaluated for expression of CRTH2. Membranes prepared from HL-60 no. 7 showed negligible binding to [^{3}H]PGD_2 (Figure 5a). However, membranes prepared from AML14.3D10 showed significant binding to [^{3}H]PGD_2. Binding of [^{3}H]PGD_2 to membranes from both cell lines was increased by differentiation in the presence of butyric acid, 800 fold and 3 fold for HL-60 no. 7 and AML14.3D10 cells, respectively. Competition assays revealed that DK-PGD_2, a CRTH2-selective ligand, but not BW245C, a DP-selective ligand, efficiently competed for [^{3}H]PGD_2 from the eosinophilic cell line membranes (Figure 5b). These results indicate that CRTH2 is predominantly expressed by AML14.3D10 and that the expression of this receptor by both AML14.3D10 and HL-60/MF211 no. 7 can be significantly increased in the presence of the pro-eosinophilic differentiating agent, butyric acid. These cell lines represent valuable sources of endogenous CRTH2.

Discussion

We have characterized pharmacologically the recombinant human CRTH2 on HEK293(EBNA) cells. Two populations of hCRTH2 specific binding sites for PGD_2 were identified by saturation analysis: a high and low affinity site both expressed at high levels (Figure 1). The affinity of PGD_2 for hCRTH2 high affinity binding sites is significantly lower than the affinity of PGD_2 for hDP high affinity binding sites as determined in a similar recombinant system, with equilibrium dissociation constants (K_D) of 2.5 and 0.3 nM for hCRTH2 and hDP, respectively. The affinity of PGD_2 for CRTH2 low affinity binding sites is also significantly lower than for DP low affinity binding sites, with K_D values of 109 nM and 13 nM for hCRTH2 and hDP, respectively (Figure 1 and Wright *et al*., 1998). Saturation analysis reveals, therefore, that the affinity of PGD_2 for CRTH2 is approximately eight times inferior to its affinity for DP.

Equilibrium competition binding assays revealed that, of all compounds tested, only PGD_2, several of its metabolites and the NSAID indomethacin bind efficiently to CRTH2 with K_i values in the low nanomolar range and the following rank order of affinities: $PGD_2 \geqslant$ DK-$PGD_2 \geqslant$ 15-deoxy-$\Delta^{12,14}$-$PGJ_2 >$ $PGJ_2 \geqslant \Delta^{12}$-$PGJ_2 >$ indomethacin $\geqslant$ 15(s)-15 methyl-PGD_2. This is in sharp contrast with the rank order of affinities obtained for PGD_2 at DP: $PGD_2 > PGJ_2 > \Delta^{12}$-$PGJ_2 >$ 15-deoxy-$\Delta^{12,14}$-$PGJ_2 >>>$ DK-PGD_2 (Wright *et al*., 1998). DK-PGD_2 has previously been shown to be selective for CRTH2 over DP with K_i values of 160 and $>30\,000$ nM for CRTH2 and DP, respectively, as determined in a whole cell binding assay (Hirai *et al*., 2001). In our membrane-based binding assay, DK-PGD_2 shows a 2000 fold selectivity for CRTH2 over DP (Table 1). Whether this metabolite is stable *in vivo* and exerts important physiological effects remains to

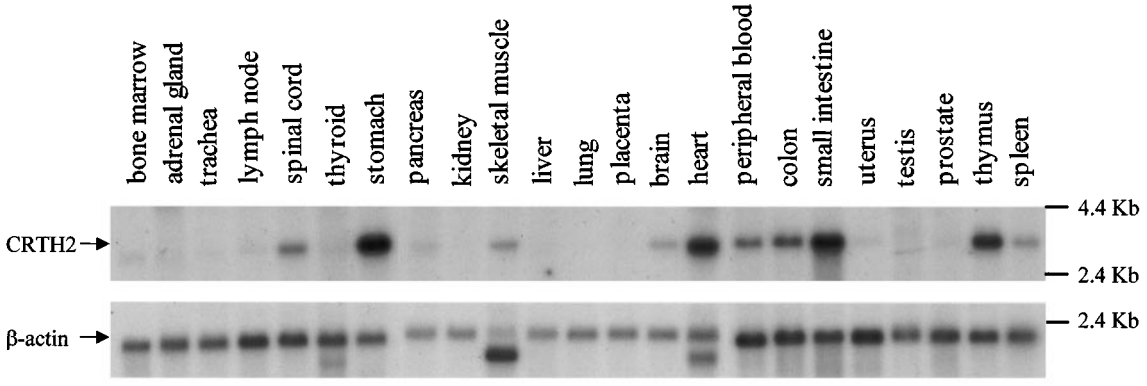

Figure 3 Distribution of CRTH2 mRNA in human tissues. A Northern blot of total human RNA from adult tissues shows an approximately 3.5-kb transcript hybridized with the CRTH2 probe (upper panel). The lower panel represents the same blot hybridized with a β-actin probe to confirm RNA integrity and relative loading. Molecular markers are indicated in kb on the right.

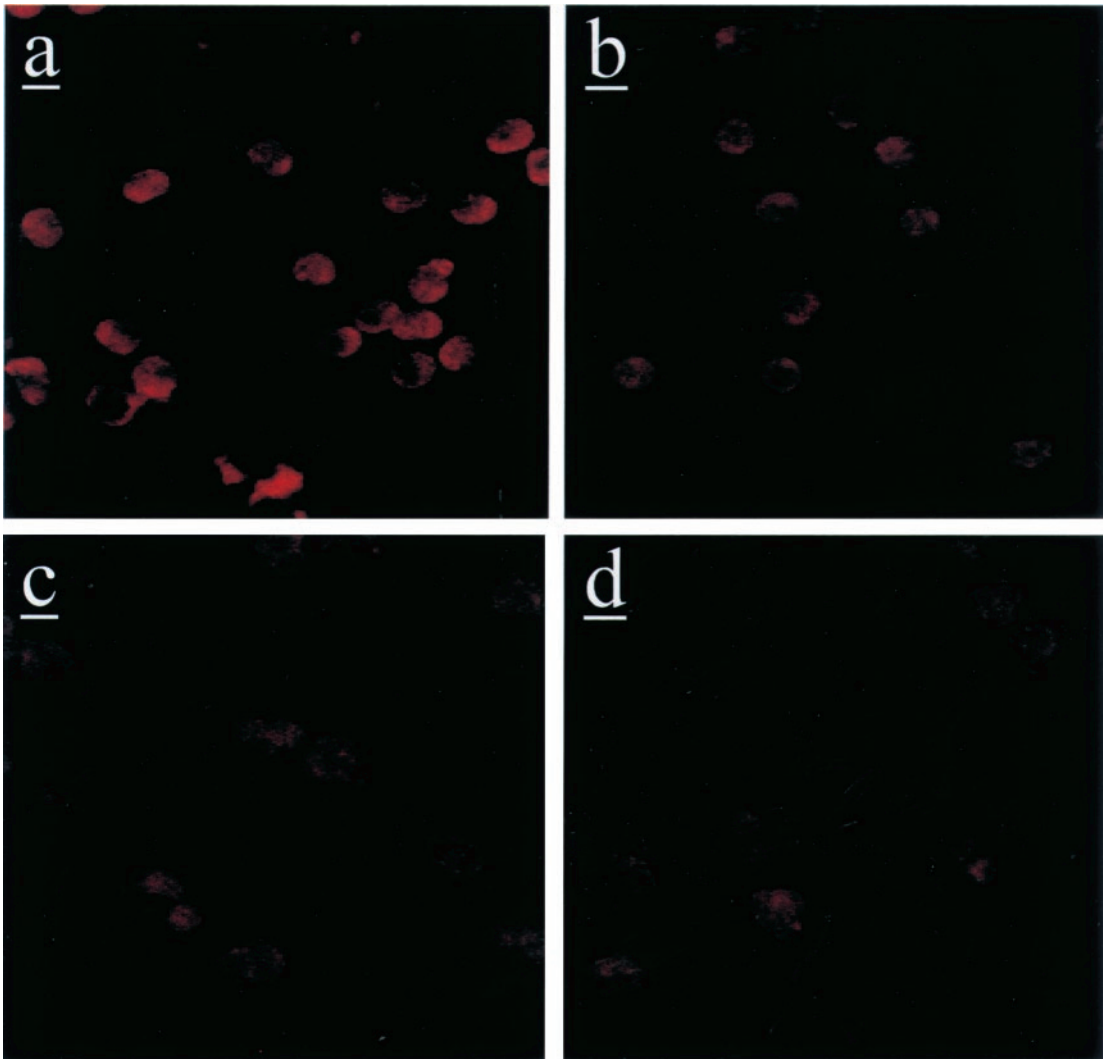

Figure 4 *In situ* hybridization of hCRTH2 in human eosinophils (a and b) and granulocytes (c and d). CRTH2 mRNA was revealed using CRTH2-specific antisense probe (a and c). A complementary sense probe was used as a negative control (b and d).

be determined. However, it represents a valuable tool to evaluate the role of endogenously expressed CRTH2. 15-deoxy-$\Delta^{12,14}$-PGJ_2 is another PGD_2 metabolite which preferentially binds CRTH2 over DP with 89 fold selectivity. It is a high affinity ligand for the nuclear receptor PPARγ through which it exerts anti-inflammatory effects (Straus & Glass, 2001). Recently generation of this metabolite has been shown *in vivo* during inflammatory processes using immunohistochemical methods (Shibata *et al*., 2002). It has also been shown to mediate both pro- and anti-inflammatory effects independently of PPARγ (Harris *et al*., 2002; Straus & Glass, 2001). Whether these effects are mediated through CRTH2 needs to be established. Finally, in contrast to other NSAIDS tested, indomethacin is a high affinity and potent agonist at hCRTH2, approximately 10 fold less potent than PGD_2 itself. Indomethacin was previously reported to have potent agonist effects on hCRTH2 in both recombinant and naturally expressing cell lines and it was suggested that some of the therapeutic and/or adverse effects of indomethacin could be mediated through CRTH2. Gastrointestinal injury is one example of such indomethacin-induced adverse effects (Shen & Winter, 1977). An early stage of indomethacin-induced intestinal injury was shown to be the infiltration and degranulation of eosinophils in the epithelium (Anthony *et al*., 1993). It is possible that agonism of CRTH2 by indomethacin contribute to the inflammation of the gastrointestinal tract through the recruitment and activation of inflammatory cells such as eosinophils. However, gastrointestinal toxicity is a property associated with a number of NSAIDs that are not ligands for CRTH2. The use of CRTH2-selective antagonists or CRTH2-deficient mice will be useful in evaluating the potential contribution of CRTH2 to this side effect of indomethacin. Hirai *et al*. (2002) reported that the K_i value for indomethacin at CRTH2 was 8.1 μM which differs significantly from the K_i of 20 nM that we determined (Table 1). A possible explanation for this discrepancy could be that Hirai *et al*. determined the binding affinity of PGD_2 using a cell-based binding assay in which both the radioligand and the competitor could potentially be internalized.

Receptors coupled to the PTX-sensitive $G_{\alpha i/o}$ subunit typically decrease i[cAMP] through the inhibition of adenylyl

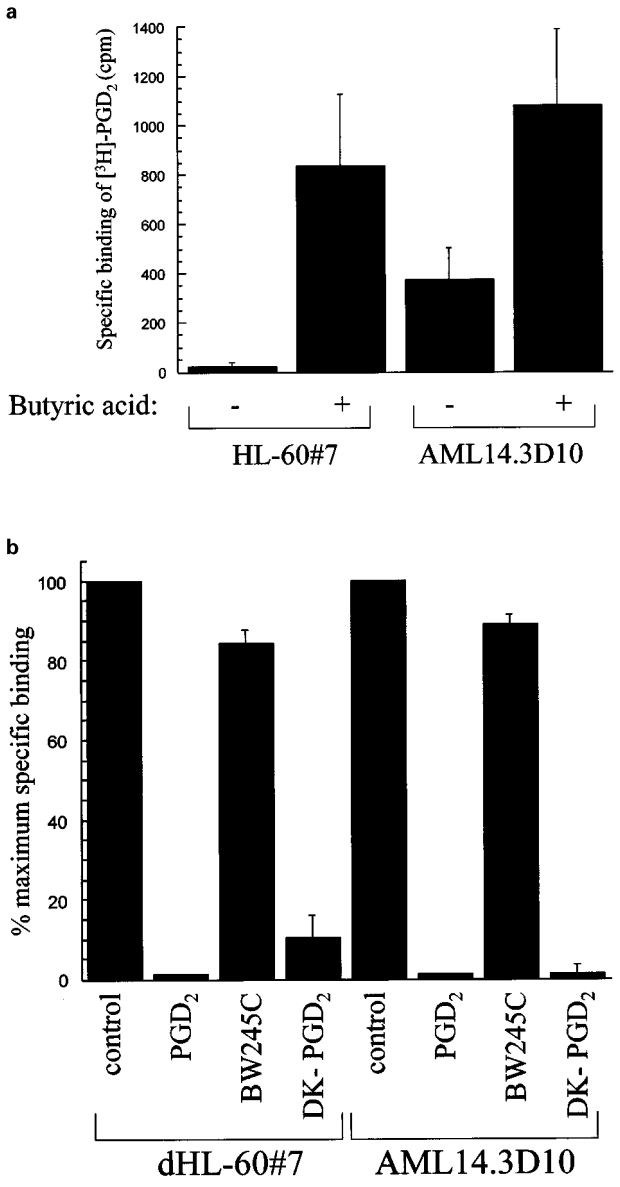

Figure 5 Tritiated PGD_2 specific binding to HL-60 no. 7 and AML14.3D10 eosinophilic cell lines. (A) Specific radioligand binding on HL-60 no. 7 and AML14.3D10 cell membranes before (−) and after (+) treatment with the eosinophilic differentiating agent butyric acid at 0.4 mM. Results are from three independent experiments performed in duplicate. (B) Specific radioligand binding to butyric acid-treated HL-60 no. 7 and untreated AML14.3D10 cell membranes in the presence of either vehicle (Me_2SO) only (control), unlabelled PGD_2, the DP-selective ligand BW245C and the CRTH2-selective ligand DK-PGD_2 at 1 μM. The results are expressed as a percentage of the maximal [^{3}H]PGD_2 specific binding observed in the absence of unlabelled competitor (control). Results are from three independent experiments done in duplicate.

cyclase. We have demonstrated that CRTH2 activation can lead to a pertussis toxin-sensitive decrease in i[cAMP] and, therefore, presumably couples to the $G_{\alpha i/o}$ subunit. We have also observed a PTX-sensitive mobilization of i[Ca^{2+}] in HEK-CRTH2 upon PGD_2 challenge. This slight mobilization of i[Ca^{2+}] may be generated by $G_{\beta\gamma}$ subunits that have previously been shown to activate phospholipase C when released from G_i-coupled receptors (Smrcka *et al*., 1993). A more robust mobilization of i[Ca^{2+}] was achieved when the promiscuous $G_{\alpha 15}$ subunit was co-expressed with CRTH2. $G_{\alpha 15}$ couples to many receptors that are not activators of the $G_{\alpha q}$ subunit and allows these receptors to strongly activate the phospholipase C/calcium pathway (Offermans *et al*., 1995; Zhu *et al*., 1996). The EC_{50} values for the various ligands tested for mobilization of i[Ca^{2+}] are approximately one log unit higher than the values obtained for these ligands in reducing i[cAMP]. This probably reflects a less efficient coupling of CRTH2 to the transfected $G_{\alpha 15}$ compared to endogenous $G_{\alpha i}$. However, the overall rank order of potencies of the various agonists tested for mobilization of i[Ca^{2+}] is comparable to the one obtained for reducing i[cAMP]. The coupling to $G_{\alpha 15}$ may prove useful for calcium-based high-throughput screening for CRTH2 ligands.

Our Northern blot analysis revealed that the expression of CRTH2 is not limited to cells of haemopoietic origin such as Th2 cells, eosinophils and basophils. Similarly to DP, CRTH2 is expressed in tissues of haemopoietic origin, but also in the digestive system and in the central nervous system. Data suggests that PGD_2 might differentially affect both receptors when expressed in the same tissue. For example, PGD_2 has been shown to produce both increases and decreases in short circuit current across the canine proximal colon. Interestingly, the DP selective agonist BW245C only produced increases in current while the CRTH2 selective agonist DK-PGD_2 elicited only decreases (Rangachari *et al*., 1995). We have previously shown that PGD_2 could increase or decrease the extent of eosinophil apoptotic cell death depending on the blood donor. Again, a DP-selective agonist increased eosinophil survival while a CRTH2-selective agonist mainly decreased it (Gervais *et al*., 2001). This suggests that DP and CRTH2 can antagonize each other in a given tissue. This agrees with the data showing that activation of CRTH2 can lead to a decrease in i[cAMP] whereas DP stimulation activates adenylyl cyclase and causes an accumulation of i[cAMP] in the cell (Boie *et al*., 1995). Thus, for a given cell type, the relative abundance of functional CRTH2 and DP might determine how this cell responds to PGD_2.

PGD2 has been reported to be a potent coronary vasoconstrictor agent in guinea pig isolated heart (Schror, 1978). Interestingly, CRTH2 mRNA was highly expressed in heart tissues (Figure 3), although the cell type expressing CRTH2 in the heart has not been identified and the physiological role for this receptor in the heart has not been defined. It would be of interest to determine whether the vasoconstrictor effect of PGD_2 on heart is mediated through CRTH2.

In summary, we have characterized the ligand binding and signal transduction properties of CRTH2, a novel PGD_2 receptor (also known as DP2). We have shown that CRTH2, a chemokine family member, has distinct pharmacological properties when compared to the classic PGD_2 receptor DP, a prostanoid family member, with respect to both ligand interactions and cell signalling. These data bring forward the intriguing possibility that these two receptors will have distinct and interactive functions when regulating PGD_2 effects *in vivo*.

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