Functional selectivity of natural and synthetic prostaglandin EP₄ receptor ligands

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**Non-standard abbreviations:** AC (adenylate cyclase); BRET (Bioluminescence Resonance Energy Transfer); GFP (Green Fluorescent Protein); GPCRs (G protein-coupled receptors); HEK (Human Embryonic Kidney); PGE₂ (Prostaglandin E₂); PTX (Pertussis toxin); Rluc (Renilla luciferase); YFP (Yellow Fluorescent Protein).

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Abstract

Classically, the prostaglandin E$_2$ receptor EP$_4$ has been classified as coupling to the $\mathrm{G}_\alpha_s$ subunit, leading to intracellular cAMP increases. However EP$_4$ signaling has revealed more complex and also involves coupling to PTX-sensitive $\mathrm{G}_\alpha_i$ proteins and $\beta$-arrestin mediated effects. There are now many examples of selective activation of independent pathways by G-protein coupled receptor (GPCR) ligands, a concept referred to as functional selectivity. Since most EP$_4$ ligands had thus far only been functionally characterized by their ability to stimulate cAMP production, we systematically determined the potencies and efficacies of a panel of EP$_4$ ligands for activation of $\mathrm{G}_\alpha_s$, $\mathrm{G}_\alpha_i$ and $\beta$-arrestin relative to the endogenous ligand PGE$_2$. For this purpose, we adapted three bioluminescence resonance energy transfer (BRET) assays to evaluate the respective pathways in living cells. Our results suggest considerable functional selectivity among the tested, structurally related agonists. PGE$_2$ was the most selective in activating $\mathrm{G}_\alpha_s$, whereas PGF$_{2\alpha}$ and PGE$_1$ alcohol were the most biased for activating $\mathrm{G}_\alpha_{i1}$ and $\beta$-arrestin, respectively. We observed reversal in order of potencies between $\beta$-arrestin 2 and $\mathrm{G}_\alpha_{i1}$ functional assays when comparing PGE$_1$ alcohol and either PGF$_{2\alpha}$, PGD$_2$ or M&B28767. Most ligands were full agonists for the three pathways tested. Our results have implications for the use of PGE$_2$ analogues in experimental and possibly clinical settings, as their activity spectra on EP$_4$ differ from that of the native agonist. The BRET-based methodology used for this first systematic assessment of a set of EP$_4$ agonists should be applicable for the study of other GPCRs.
**Introduction**

Prostanoids (prostaglandins and thromboxane) are lipid hormone mediators derived from cyclooxygenase-catalyzed metabolism of arachidonic acid. Among prostanoids, prostaglandin E₂ (PGE₂) is the most widely produced in the body. Its various effects include the contraction or relaxation of smooth muscle, modulation of immune responses, and the regulation of the production of a variety of cytokines. PGE₂ is also implicated in several pathologies including cancer, inflammation and hypertension. Four G protein-coupled receptors (GPCRs) designated subtypes EP₁-₂-₃-₄, which activate different G protein-dependent signaling pathways but are often expressed in the same cell types, mediate the effects of PGE₂. The biology of responses to PGE₂ is correspondingly complex, with however increasing evidence that EP₄ is a critical determinant for the role of PGE₂ in carcinogenesis and cancer progression (Fulton et al., 2006).

Accordingly, studies using mice lacking the EP₄ receptor (EP₄⁻/⁻) and EP₄-selective ligands have revealed a role of this receptor in progression of colon carcinogenesis, in addition to closure of the ductus arteriosus at birth, bone formation, protection against inflammatory bowel disease and progression of rheumatoid arthritis (Regan, 2003; Sugimoto and Narumiya, 2007). These studies suggest that EP₄ agonism or antagonism could find clinical applications such as in the treatment of cancers. The EP₄ receptor was initially shown to couple to Ga₃, leading to stimulation of adenylate cyclase and increases in intracellular cAMP concentrations (Coleman et al., 1994). More recently, it was demonstrated that EP₄ could also couple to a PTX-sensitive inhibitory G protein (Ga₁ᵢₒ family), resulting in activation of phosphatidylinositol 3-kinase (PI3K) (Fujino and Regan, 2006). EP₄-mediated PI3K signaling then leads to extracellular signal-regulated kinase (ERK)-dependent induction of functional expression of early growth response factor-1 (EGR-1), which may play a role in cancer, as well as phosphorylation of cAMP response
element binding protein (CREB) and inhibition of protein kinase A (PKA) activity (Fujino et al., 2003; Fujino et al., 2005). Moreover, activation of EP_4 by PGE_2 leads to rapid desensitization (Nishigaki et al., 1996), as well as recruitment of β-arrestins 1 and 2 and internalization of the receptor (Desai and Ashby, 2001). Accumulating evidence indicates that β-arrestins can also serve as scaffolds to activate signaling cascades independently of G protein coupling for many GPCRs (DeWire et al., 2007). This was shown to be the case for EP_4 in colorectal cancer cells, where β-arrestin 1-dependent c-Src activation, and not G-protein activation, is responsible for PGE_2/EP_4-mediated increased cellular migration and metastasis (Buchanan et al., 2006). Therefore, specific subsets of EP_4 signaling are involved in the clinically relevant effects of PGE_2.

Growing evidence suggests that GPCRs can exist in multiple active conformations (Kenakin, 2003). Different ligands can stabilize distinct active receptor conformations that are only permissive to a subset of the receptor’s complete repertoire of behaviors, or activate distinct pathways with different potency and intrinsic activity (efficacy). Thus, individual agonists lead to differential and independent coupling of the receptor to different G proteins or intracellular effectors, a concept referred to as functional selectivity (Galandrin et al., 2007; Urban et al., 2007). To date, prostanoid receptor ligands are mainly characterized by their relative binding affinity to the different receptor subtypes as determined by radioligand competition binding assays (Abramovitz et al., 2000; Davis and Sharif, 2000). The evaluation of the functional pharmacology of EP_4 receptor ligands is often limited to their ability to induce cAMP production (Wilson et al., 2004), since the development of these drugs occurred before other EP_4 signaling pathways were characterized. Information about activation of other EP_4 pathways by these compounds is still lacking, and systematic investigation of the effect of these ligands on other EP_4 signaling pathways is therefore warranted. Knowledge about biased agonism of these ligands
will be relevant for their use as pharmacological tools, with potential clinical relevance (Buchanan et al., 2006).

In this study, we use three different BRET assays for functional pharmacological characterization of various EP₄ receptor ligands in living HEK293 cells by evaluating the relative potency and intrinsic activity of these ligands on three distinct signaling pathways relevant for EP₄ biology, namely the activation of Gaᵢ and Gaₛ subunits, as well as the recruitment of β-arrestin.
Methods

Reagents – M&B28767 (7-[(1R,2R)-2-[(E,3R)-3-hydroxy-4-(phenoxy)but-1-enyl]-5-oxocyclopentyl]heptanoic acid) was a gift from Rhone-Poulenc Rorer, Dagenham Essex, UK; L-902688 (5R-[(1E)-4,4-difluoro-3R-hydroxy-4-phenylbut-1-en-1-yl]-1-{6-(1H-tertrazol-5-yl)hexyl]pyrrolidin-2-one) was a gift from Merk Frosst (Pointe-Claire, QC, Canada); all other EP4 ligands were purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and transfections – Human embryonic kidney (HEK) 293E cells (passage number ≈10-30; Invitrogen) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Wisent), 100 units/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen) and 200 μg/ml G418. Transient transfections were performed in 6-well or 10 cm dishes using the polyethylenimine (Polysciences, PA) method (Boussif et al., 1995).

Plasmids – The expression vectors containing human EP4 receptor and Gγ2 were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). EP4-YFP was constructed by ligating the PCR-amplified coding sequence of EP4 (lacking its stop codon) into the pGFP-N1-Topaze backbone of CXCR4-YFP (Issafras et al., 2002) at HindIII/AgeI sites. The mutations A55T, C124A and M185V were inserted in Rluc to generate Rluc3, a variation of the Rluc8 mutant described by Loening et al. (2006). The GFP10-Epac-Rluc3 BRET2-cAMP biosensor was constructed in 3 steps. First, the green fluorescent protein variant GFP10 (Hamdan et al., 2006), the linker GSAGT-(Acc65I/HindIII)-KLPAT and Rluc3 were inserted in pcDNA3.1/Zeo (Invitrogen). Part of the human Epac1 (residues 144-881) was then amplified by PCR, digested by Acc65I-HindIII and inserted using the same restriction sites between GFP10-GSAGT and
KLPAT-Rluc3 in pCDNA3.1/Zeo. Finally, the substitutions T781A and F782A were introduced by PCR to remove the Rap1 binding site of Epac1. YFP-\(\beta\) expression vector was obtained from GFP10-\(\beta\) (Galés et al., 2005) by replacing GFP10 with the coding sequence of eYFP (pIRES-eYFP, Clontech). Plasmids encoding \(\alpha_{i1}-91Rluc\) and Rluc-\(\beta\)-arrestin 2 have been described previously (Perroy et al., 2003; Galés et al., 2006). All generated constructs were confirmed by sequencing.

**BRET measurement** – Transiently transfected HEK293 cells were seeded in 96-well white clear bottom microplates (ViewPlate, PerkinElmer) coated with poly-D-lysine and left in culture for 24h. Cells were washed once with PBS and the Rluc substrates coelenterazine h (for BRET\(^1\) experiments; NanoLight Technology) or coelenterazine 400A (for BRET\(^2\) experiments; Biotium) added at a final concentration of 5\(\mu\)M in BRET buffer (PBS, 0.5 mM MgCl\(_2\), 0.1% glucose). BRET readings were collected using a Mithras LB940 plate reader (Berthold) and MicroWin2000 software. BRET\(^1\) measurement between Rluc and YFP was obtained by sequential integration of the signals detected in the 460-500 nm (luciferase) and 510-550 nm (YFP) windows, whereas BRET\(^2\) readings between Rluc3 and GFP10 were collected by sequential integration of the signals detected in the 365-435 nm (Rluc3) and 505-525 nm (GFP10) windows. The BRET signal was calculated as the ratio of light emitted by acceptor (YFP or GFP10) over the light emitted by donor (Rluc or Rluc3). The values were corrected to net BRET by subtracting the background BRET signal obtained in cells transfected with Rluc (BRET\(^1\)) or Rluc3 (BRET\(^2\)) constructs alone. Ligands were incubated at room temperature for 3 (\(\alpha_{i1}\)), 10 (Epac) or 25 (\(\beta\)-arrestin) min, except for kinetics studies. For experiments with GW627368X or PTX, cells were treated for 10 min at room temperature or 16 hours at 37°C,
respectively, with the inhibitor prior to agonist exposure. For kinetic analysis, ligands were injected using the Mithras LB940 injector 10 min after coelenterazine addition and BRET readings collected at 1 (Gαi), 17 (Epac) or 50 s (β-arrestin) intervals.

Radioligand saturation binding – Transiently transfected HEK293 cells were incubated for 90 min at 4°C in PBS/0.5% BSA (w/v) buffer with different concentrations of [3H]PGE2, in the presence or absence of 1000-fold excess unlabelled PGE2 to determine specific binding. Cells were washed three times with PBS/0.5% BSA and lysed with 0.1 N NaOH/0.1% Triton X-100. Bound radioactivity was measured on cell lysates with a liquid scintillation counter.

Data analysis – Data from BRET assays were the mean of independent experiments each performed in triplicate. Curve-fitting and statistical analysis was conducted by using GraphPad Prism 4 software. Statistical significance of the differences between more than two groups was calculated using one-way ANOVA, followed by Tukey’s post-test.
**Results**

*BRET assay allows monitoring of EP₄-dependent Gαₛ activation.* Intracellular cAMP accumulation following stimulation of adenylate cyclase (AC) is the best characterized EP₄ signaling pathway. To monitor this Gαₛ-dependent pathway in living cells, we generated a BRET² version of the previously described CAMYEL Epac-based BRET sensor (Jiang et al., 2007). The inactive cytosolic mutant form of human Epac1 was inserted between GFP10 and Rluc3, a mutant form of *Renilla* luciferase with increased stability and light output. Binding of cAMP to GFP10-Epac-Rluc3 induces a conformational change in Epac that results in a decrease of the BRET² signal between the Rluc3 donor and GFP10 acceptor. Stimulation of HEK293 cells co-expressing this Epac biosensor and human EP₄ receptor with PGE₂ lead to a strong concentration-dependent cAMP response (Fig. 1A) with an average EC₅₀ of 0.3 nM (Table 1). Addition of forskolin (100 μM) to maximally stimulate AC lead to a BRET² variation greater than that obtained with PGE₂, indicating that the maximal PGE₂ response is within the dynamic range of the Epac sensor. Stimulation of Epac/EP₄-transfected cells with the cell-permeable cAMP analog 8-bromo-cAMP also lead to a sigmoidal concentration-response BRET² variation (Fig. 1B); stimulation of cells with a saturating (1 μM) dose of PGE₂ indicated that the maximal PGE₂-induced response was within the linear range of the Epac sensor response. The cAMP levels induced by PGE₂ stimulation rose rapidly and peaked after ~5-10 minutes, after which they slowly decreased due to the action of phosphodiesterases and the desensitization of the receptor and cAMP production system (Fig. 1C). To make certain that the measured BRET² signal was specific to EP₄ receptor activation, we pre-treated cells with the EP₄-specific antagonist GW627368X; this resulted in abrogation of the PGE₂-mediated cAMP level increase (Fig. 1D).
similar effect was observed when cells were transfected with Epac sensor in the absence of the
EP₄ receptor (Fig. 1D). To further ensure that other endogenously expressed prostanoid receptors
were not activating the Epac sensor, we also treated cells that were transfected with the sensor,
but not EP₄, with 1 µM of either PGD₂ (the natural DP receptor ligand), PGF₂α (the natural FP
receptor ligand), carlaprostacyclin (a stable analog of PGI₂ and agonist of IP receptor) or U-
46619 (a TP receptor agonist). Average BRET variations of less than 3% of the basal signal were
detected in all cases, as opposed to variations of 21.5% induced by PGE₂ in cells co-transfected
with EP₄ (results not shown).

**BRET assay to monitor activated EP₄-mediated Ga₁β₁ rearrangement.** In addition to coupling to
Ga_s, EP₄ can also couple to the PTX-sensitive Gaᵢ/o family of G proteins, and we detected an
increase in cAMP response by EP₄ following treatment with PTX (Fig. 1D), as previously
reported (Fujino and Regan, 2006) However, since this Gaᵢ/o-dependent inhibition of AC is
masked by the Ga_s coupling to EP₄, we used a recently developed BRET assay that allows
monitoring of receptor activation-dependant structural rearrangements within the heterotrimeric
Ga₁β₁γ₂ complex in live cells (Galés et al., 2006). Specifically, the Rluc donor was inserted
within the loop connecting helices A and B of the Gaᵢ helical domain, and the YFP acceptor was
fused to the N-terminus of Gβ₁. Under basal conditions, a strong BRET signal between Gaᵢ₁-Rluc
and YFP-Gβ₁ was measured (Fig. 2A), indicative of pre-association of Gaᵢ₁ and Gβ₁. Kinetic
analysis of the BRET signal upon stimulation of EP₄ by PGE₂, but not vehicle alone (Fig. 2A,
inset), revealed a very rapid decrease in the BRET signal (within 1s); this decrease was sustained
for at least 3 minutes. This signal was dose-dependent, with an average EC₅₀ of 4.08 nM (Table 1
and Fig. 2B). No response was detected in cells pre-treated with PTX or the EP₄ antagonist
GW627368X, or in cells in which empty vector was co-transfected in place of EP₄ (Fig. 2C),
demonstrating that the measured BRET variation specifically reflected EP4 receptor-mediated activation of Gαi1. To ascertain that other endogenously expressed prostanoid receptors were not implicated in Gαi1 activation, we also treated cells in which empty vector was co-transfected in place of EP4 with 1 µM of PGD2, PGF2α, carbaprostacyclin or U-46619. An average BRET variation of less than 2% of the basal level was detected after stimulation with either of these ligands (results not shown), as opposed to 41% variation induced by PGE2 when EP4 was co-transfected (Fig. 2C). The ligand-induced BRET variation most likely reflects G protein activation based on the following observations: 1) it is completely abrogated by PTX, 2) it is conserved for a large panel of Gαi-coupled GPCRs (Galés et al., 2006), 3) BRET variations correspond to the signaling efficacy of α2-adrenergic receptor ligands (Galés et al., 2006) and 4) the potencies of the δ-opioid receptor agonist D-pen-2,5-enkephalin to induce BRET variation and to promote [35S]GTPγS binding in cells transfected with δ-opioid receptors were very similar (Audet et al., 2008).

**BRET assay to monitor recruitment of β-arrestin to activated EP4 receptor.** A third EP4 receptor signaling pathway, relevant to cancer, is the recruitment of β-arrestin to EP4. This was measured by following the ligand-induced BRET signal between Rluc-β-arrestin 2 and EP4-YFP receptors in live HEK293 cells. BRET-based measurement of β-arrestin recruitment to monitor receptor activation has been used for a multitude of GPCRs, and has also been used as a high throughput screening method for the identification of ligands (Hamdan et al., 2005). Fusion of YFP at the C-terminus of EP4 had no effect on binding affinity of [3H]PGE2 for the receptor (Kd = 5.5 ± 3.1 nM for EP4-YFP and 5.0 ± 1.9 nM for EP4). BRET kinetics showed a rapid PGE2-induced recruitment of β-arrestin 2 to EP4 (Fig. 3A) that lead to a prolonged interaction (over 30 min), representative of a class B receptor-β-arrestin interaction (Oakley et al., 2000). Exposure of cells
to PGE₂ resulted in a significant concentration-dependent increase in the BRET signal detected over basal level (Fig. 3B), with an average EC₅₀ of 2.01 nM (Table 1). PGE₂-induced recruitment of Rluc-β-arrestin 1 to EP₄-YFP showed similar kinetics and potency as for β-arrestin 2 (data not shown). Pre-treatment of cells with PTX had no effect on the PGE₂-induced recruitment of β-arrestin 1 or 2 to EP₄ (results not shown), suggesting that this process was not Gαᵢ/o-dependent.

Potency of EP₄ ligands for Gαₛ, Gαᵢ₁ and β-arrestin 2 signaling pathways. Using the BRET assays described above, we compared the potencies of various EP₄ ligands to induce Gαₛ, Gαᵢ₁ and β-arrestin 2 responses. The chemical structure and previously reported binding affinity (in competition assays with [³H]PGE₂ to recombinant EP₄ receptors) of these ligands are shown in Table 2. In principle, it is problematic to interpret differences in ligand potencies between different assays systems, assessing different functional readouts. While different potencies might indeed reflect agonist bias, they might also be due to different coupling efficacies of the target receptor to the respective effectors, or simply to differences in parameters that determine the sensitivity of a given assay. However, the relative comparison of EC₅₀s obtained for a set of ligands with respect to a reference ligand permits to draw conclusions on their respective set of relative potencies. Moreover, the reconstituted systems used in this study are similar with respect to the expressed quantities of the transfected receptor, β-arrestin, and Gαᵢ₁ (Supplementary Figs. 1 and 2, Supplementary Table 1). Therefore, as a prospective limitation, our study does not address physiological or pathophysiological conditions of primary cells, which may be different from our model system due to availability of the respective signaling molecules.

Overall, the observed potency of all ligands for cAMP generation was greater than for β-arrestin recruitment, except for PGE₁ alcohol, for which no significant difference of pEC₅₀ values was observed between these two assays (Table 1, Fig. 4A). Similarly, potency of ligands in the
Ga\(_{i1}\) assay was equal or greater compared to their potency in β-arrestin recruitment, again with the exception of PGE\(_1\) alcohol, which was significantly more potent for recruitment of β-arrestin 2 than for Ga\(_{i1}\)β\(_1\) rearrangement. Overall, the potencies that we obtained for cAMP production were slightly lower (approximately half a log) than those previously reported in the study of Wilson and colleagues (2004), possibly due to variations in receptor density. However, relative potencies (compared to PGE\(_2\)) of all agonists tested by us match those reported by Wilson et al. (2004).

Comparing the rank orders of the set of EP\(_4\) agonists, we found that the rank orders of potency for Ga\(_{i1}\) and Ga\(_s\) activation were similar: L-902688 ≥ PGE\(_1\) ≥ PGE\(_2\) = 11-deoxy PGE\(_1\) >> 16,16-dimethyl PGE\(_2\) = Misoprostol = 17-phenyl-trinor PGE\(_2\) = M&B28767 >> PGE\(_1\) alcohol = PGF\(_{2\alpha}\) = PGD\(_2\). The rank order for the recruitment of β-arrestin 2 differed slightly: L-902688 ≥ PGE\(_1\) = PGE\(_2\) = 11-deoxy PGE\(_1\) >> 16,16-dimethyl PGE\(_2\) ≥ Misoprostol = 17-phenyl-trinor PGE\(_2\) = M&B28767 > PGE\(_1\) alcohol >> PGF\(_{2\alpha}\) = PGD\(_2\). Thus, unlike for the G-protein rank orders of potency, PGE\(_1\) was not superior to PGE\(_2\)/11-deoxy PGE\(_1\) (Ga\(_{i1}\)) or inferior to L-902688 (Ga\(_s\)), misoprostol was inferior to 16,16-dimethyl PGE\(_2\) but was not superior to PGE\(_1\) alcohol and PGE\(_1\) alcohol was clearly more potent than PGF\(_{2\alpha}\)/PGD\(_2\) (Table 1). We observed a reversal in the rank order of potencies between β-arrestin 2 and Ga\(_{i1}\) functional assays when comparing PGE\(_1\) alcohol and either PGF\(_{2\alpha}\), PGD\(_2\) or M&B28767 (Fig. 4A, Table 1). Other agonists showed important differences of relative potency between signaling pathways without however showing reversal of relative potencies. For example, pEC\(_{50}\) values for cAMP production and Ga\(_{i1}\) activation were not significantly different for 16,16-dimethyl PGE\(_2\) but differed by over a log for PGE\(_2\) (Fig. 4A); similarly, pEC\(_{50}\) values for cAMP production and β-arrestin recruitment were nearly identical for
PGE$_1$ alcohol, but differed by over a log for L-902688, M&B28767, Misoprostol and PGF$_{2\alpha}$ (Figs. 4A and 4B, Table 1).

When comparing relative potency ratios, a bias for $G_{\alpha_{i1}}$ over $G_{\alpha_s}$ signaling could be observed for all ligands compared to PGE$_2$, with 16,16-dimethyl PGE$_2$ being the most $G_{\alpha_{i1}}$-biased ligand (Fig. 5A). Six out of the ten ligands had a relative bias towards β-arrestin recruitment over $G_{\alpha_s}$ compared to PGE$_2$, with PGE$_1$ alcohol as the most β-arrestin-biased agonist (Fig. 5B). PGE$_1$ alcohol was also the only ligand that had an arrestin over $G_{\alpha_{i1}}$ bias (Fig. 5C).

**Intrinsic activity of EP$_4$ ligands for $G_{\alpha_s}$, $G_{\alpha_{i1}}$ and β-arrestin 2 signaling pathways.**

We then compared the relative intrinsic activities of the different ligands in $G_{\alpha_s}$, $G_{\alpha_{i1}}$ and β-arrestin 2 signaling pathways (Table 1), using PGE$_2$ as a reference “full agonist” set at 100%. Most ligands proved to be full agonists for the three pathways tested. One exception was PGD$_2$, which was a partial agonist for recruitment of β-arrestin 2 (intrinsic activity of 77.9 ± 4.4%; Table 1, Fig. 6), but a full agonist for activation of $G_{\alpha_s}$ and $G_{\alpha_{i1}}$ pathways (93.2 ± 4.2% and 96.1 ± 1.6%, respectively). Inversely, L-902688 had (slightly) lower intrinsic activity in cAMP production and the $G_{\alpha_{i1}}$ pathway (94.4 ± 2.6% and 89.9 ± 2%, respectively), while being full agonist for β-arrestin (102.6 ±1.1%).
Discussion

During the past years, it became increasingly clear that G-protein coupled receptors can activate independently a variety of signaling effectors, and that distinct receptor ligands can do so with different potencies and efficacies (intrinsic activities). This selective activation of independent pathways by ligands has been termed functional selectivity (Urban et al., 2007). The conceptual basis for this is that GPCRs do not have merely “inactive” and “active” conformations, but that ligands can stabilize distinct receptor conformations, which are more or less potent and efficient in activating a given readout (Kenakin, 2003; Kenakin, 2007). The significance of functional selectivity obviously raises the question whether previously reported properties of synthetic or natural ligands were based on the “right” (that is, clinically relevant) readout, especially for compounds that have therapeutic use. Moreover, for most clinical contexts the relevant signaling pathway of any given receptor remains often as yet elusive (Bosier and Hermans, 2007). Experiments using synthetic ligands with known functional selectivity profiles in animal models will be required to pin down the clinically relevant receptor signaling pathways, and might also identify drug candidates or leads for further development. However, functional selectivity profiles of drugs and synthetic ligands are only beginning to be identified (Galandrin and Bouvier, 2006; Audet et al., 2008; Gao and Jacobson, 2008; Masri et al., 2008). Reassessment of ligand-induced signaling activity of known GPCR ligands is therefore warranted, taking functional selectivity into account.

Classically, the prostaglandin receptor EP\textsubscript{4} had been classified as coupling to the G\textsubscript{a\textsubscript{s}} subunit as an effector. However, in recent years, EP\textsubscript{4} signaling has revealed more complex and was shown to also involve coupling to PTX-sensitive G\textsubscript{a\textsubscript{i}} proteins as well as \textbeta-arrestin mediated effects. Importantly, \textbeta-arrestin recruitment to EP\textsubscript{4} rather than G\textsubscript{a\textsubscript{s}}–mediated signaling has been
associated with colorectal cancer progression (Buchanan et al., 2006). Additionally, the $G_\alpha_\text{i}$ pathway could also be implicated in the cAMP/PKA-independant role of EP$_4$-mediated increased phosphorylation of PI3K and ERK1/2 and EGR-1 induction in colon cancer (Pozzi et al., 2004; Cherukuri et al., 2007). Our study set out to systematically characterize the response of a wide range of EP$_4$ agonists for the downstream effectors $G_\alpha_\text{s}$, $G_\alpha_\text{i1}$, and $\beta$-arrestin, thereby addressing functional selectivity.

By adapting three different BRET-based assays, we monitored receptor signaling in real time in live cells. $\beta$-arrestin recruitment was assessed by direct interaction between tagged EP$_4$ and $\beta$-arrestin 2 proteins. Activation of $G_\alpha_\text{s}$ and $G_\alpha_\text{i}$ pathways was determined by measuring BRET signal variations resulting from structural rearrangements within Epac and heterotrimeric $G_\alpha_\text{i}1\beta_1\gamma_2$ proteins, respectively. These energy transfer variations were practically absent in cells treated with the EP$_4$-specific antagonist GW627368X or in cells that were not transfected with EP$_4$, indicating that BRET modulations were indeed specific to EP$_4$ receptor and did not result from the activation of endogenously expressed prostanoid receptors.

Our results suggest considerable functional selectivity among the tested, structurally related agonists. Interestingly, all compounds showed a relative bias towards $G_\alpha_\text{i1}$ over $G_\alpha_\text{s}$ activation when compared to PGE$_2$, this effect being the strongest with 16,16-dimethyl PGE$_2$, which activated both alpha subunits with almost equal potency (while PGE$_2$ activates $G_\alpha_\text{s}$ with tenfold potency over $G_\alpha_\text{i1}$) (Figures 4A, 5A, and Table 1). Similarly, the profiles of six (PGE$_1$ alcohol, 16,16-dimethyl PGE$_2$, PGE$_1$, PGD$_2$, 17-phenyl-trinor PGE$_2$ and 11-deoxy PGE$_1$) out of the ten analogs show relatively more potent activation of $\beta$-arrestin recruitment than $G_\alpha_\text{s}$ responses relative to PGE$_2$. When comparing the relative potency of a ligand to induce each of the three measured signaling pathways, PGE$_2$ was the most selective in activating $G_\alpha_\text{s}$, whereas PGF$_{2\alpha}$ and PGE$_1$ alcohol were the most selective for activating $G_\alpha_\text{i1}$ and $\beta$-arrestin, respectively.
When all tested pathways were considered, the profiles of PGE₁ alcohol and 16,16-dimethyl PGE₂ were most distinct from PGE₂ (Fig. 4B). PGE₁ alcohol activated both Ga₁₄ and β-arrestin relatively more potently than Ga₃, as compared to PGE₂, whereas 16,16-dimethyl PGE₂ was a relatively weak activator of β-arrestin recruitment, but a relatively better activator of Ga₁₄ than PGE₂ (Fig. 5). The order of potency for PGE₁ alcohol and PGF₂α, PGD₂ or M&B28767 was reversed for β-arrestin recruitment and Ga₁₄ activation: PGE₁ alcohol was more potent for the β-arrestin pathway than for the Ga₁₄ pathway, while the opposite was true for PGF₂α, PGD₂ and M&B28767 (Fig. 4A, Table 1). This reversal of potency ratio for these signaling pathways is incompatible with a single receptor active state, but in line with the existence of ligand-specific receptor states that result in differential activation of signaling pathways, as suggested by the concept of functional selectivity (Urban et al., 2007).

Interestingly, we observed that most agonists tested had full intrinsic activity in all pathways, as reported earlier for cAMP production (Wilson et al., 2004). An exception was L-902688 that had slightly but significantly reduced efficacy in activation of both tested G-proteins. PGD₂ was also less efficacious in β-arrestin recruitment as detected by BRET. It is important to consider that maximal responses from BRET assays may relate to either more interacting molecules or to closer donor-acceptor distances. In this case, lower intrinsic activity for β-arrestin/EP₄ BRET induced by PGD₂ as compared to the reference ligand PGE₂ thus suggests that either less arrestin molecules are recruited to the receptor-fused energy acceptor, or that the energy transfer is less efficient in the receptor/arrestin complex induced by PGD₂ due to distinct conformational changes. Indeed, recently reported results obtained with the angiotensin receptor AT₁aR, the β₂-adrenergic receptor, and the parathyroid hormone receptor type 1 (PTH₁R), revealed different arrestin conformations depending on the ligand that was applied (Shukla et al., 2008). Accordingly, it might be expected that such different arrestin conformations also translate
in different BRET between receptor and arrestin. We interpret the lower efficacy of L-902688 in the Gαi1/Gβ1 BRET as a distinct conformational rearrangement in the Gαi1β1γ2 heterotrimer. Indeed, the PGE2-induced BRET decrease between Gαi1-Rluc and YFP-β1 could reflect dissociation of αi1 from the β1γ2 dimer, but overall results obtained by Galés et al. (2006) strongly suggest that this BRET variation rather corresponds to Gαi1β1γ2 structural rearrangements within a stable heterotrimer that results in G-protein activation. This is, however, not true for the cAMP Epac BRET sensor, where signal intensity only relates to cAMP levels, and not to different conformations of the sensor.

The paucity of differences in intrinsic activity between ligands is in line with previous reports on these ligand’s efficacies in cAMP production (Wilson et al., 2004), but somewhat surprising when considering results obtained in other receptor systems. Indeed, large differences in β-arrestin recruitment efficacy (also measured by BRET) were observed in studies with synthetic agonists of the dopamine D2 receptor (Klewe et al., 2008) or natural peptide agonists of the glucagon-like peptide receptor (Jorgensen et al., 2007). We speculate that the relative stability in intrinsic efficacy that we observed might be linked to the structural similarity of the here employed ligands (Table 2). In principle, differences in intrinsic ligand activity might also be masked by receptor overexpression, leading to increased receptor reserve and underestimation of the maximal response. This clearly is not the case for the Epac-based cAMP assay where the maximal response lies within the dynamic response range as shown with a saturating concentration of forskolin. In the intermolecular BRET systems between Gαi1β1 or EP4-β-arrestin, in turn, maximal response cannot be evaluated, as they may depend on both quantitative and qualitative differences (see above).
The activation of the Ga\textsubscript{i1} and β-arrestin recruitment pathways by EP\textsubscript{4} has been reported much later than its responses in the classical Ga\textsubscript{s} pathway. Accordingly, our study is the first to systematically report these pathways for a series of EP\textsubscript{4} agonists. The finding that a substantial number of the tested ligands are relatively stronger activators of these non-canonical EP\textsubscript{4} pathways than the natural ligand PGE\textsubscript{2} must be taken into account when using these compounds as tools in experiments designed to further dissect the biological roles of EP\textsubscript{4}, for example in cancer genesis and progression.

Taken together, our study is the first to systematically characterize the response of a set of EP\textsubscript{4} agonists for the downstream effectors β-arrestin and Ga\textsubscript{i1}, using BRET-based methodology that should be applicable for the study of other GPCRs. We find significant functional selectivity among the studied ligands. While more work will be required to examine the bearing of our observations in a more complex native context including the presence of other PGE\textsubscript{2} receptors, our EP\textsubscript{4}-limited study is the first step for assessing the consequences of functional selectivity in physiology and drug treatment.

**Acknowledgments**

We thank Hendrika Fernandez for expert technical assistance.
References


Footnotes

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Legends for Figures

Figure 1. Epac-based BRET biosensor monitors EP₄-mediated Gα₅ activation in living cells. A) Cells co-expressing Epac sensor and EP₄ were exposed to increasing concentrations of PGE₂ (solid triangles) or forskolin (100 μM; open circle) for 10 min at RT and cAMP levels were evaluated by the emission (Rluc₃/GFP10) ratios. Data are expressed as 1/BRET² signal so that an increase in ordinate values correlates with an increase in cAMP levels. Data from one representative experiment are shown (n=9); B) cells co-expressing Epac sensor and EP₄ were treated with increasing concentrations of 8-bromo-cAMP (open squares) or PGE₂ (1 μM; solid triangle) for 10 min at RT and emission ratios (Rluc₃/GFP10) measured. Data from one representative experiment are shown (n=2); C) kinetics of PGE₂ (solid triangles) or vehicle (open circles)-induced cAMP production in cells co-expressing EP₄ and Epac sensor. Data from one representative experiment are shown (n=4); D) specificity of PGE₂-promoted BRET signal. Cells transfected with Epac sensor in the presence or absence of EP₄ receptor were pre-treated or not with pertussis toxin (PTX; 0.1μg/ml, 16 h, 37ºC) or EP₄ antagonist GW627368X (10 μM, 10 min, RT) before addition of 10 nM PGE₂. Data represent the mean ± S.E.M. of four experiments, each performed in triplicate; *** indicates statistical difference compared to cells transfected with EP₄ and treated with PGE₂ alone (repeated measures ANOVA and Tukey’s post-test, P < 0.001).

Figure 2. BRET assay to monitor EP₄-mediated Gα₁i activation in living cells. BRET was measured in cells co-transfected with Gα₁i-Rluc, YFP-GBP₁, Gγ₂ and EP₄. A) BRET kinetics between Gα₁i-Rluc and YFP-GBP₁ was measured before and after addition (arrows) of PGE₂ (1 μM) or vehicle (inset). Data from one representative experiment are shown (n=3); B) cells were exposed to increasing concentrations of PGE₂ and BRET variation compared to basal condition.
was measured. Data from one representative experiment are shown (n=9); C) 1 μM PGE2-promoted BRET variation in the presence or absence of co-transfected EP4 receptor, pre-treated or not with pertussis toxin (PTX; 0.1μg/ml, 16 h, 37ºC) or EP4 antagonist GW627368X (10 μM, 10 min, RT). Data represent the mean ± S.E.M. of four experiments, each performed in triplicate; *** indicates statistical difference compared to cells transfected with EP4 and treated with PGE2 alone (one-way ANOVA and Tukey’s post-test, P < 0.001).

**Figure 3.** BRET assay to monitor β-arrestin recruitment to EP4 receptors in living cells. BRET was measured in cells co-expressing EP4-YFP and Rluc-β-arrestin 2. A) BRET kinetics between EP4-YFP and Rluc-β-arrestin 2 was measured after addition of PGE2 (1 μM). Data from one representative experiment are shown (n=3); B) cells were exposed for 25 min at RT to increasing concentrations of PGE2 and BRET measured. Data from one representative experiment are shown (n=11).

**Figure 4.** Comparison of the potency of EP4 agonists to activate distinct functional pathways. Cells were exposed to increasing concentrations of agonist and BRET measured as described in methods to determine concentration-response effects on cAMP production, Gαi1 activation and β-arrestin recruitment. A) The fitted curve pEC50 values obtained for the three functional assays are illustrated for selected agonists. Note that the pEC50 of PGE1 alcohol for Gαi1 pathway was significantly lower than for β-arrestin 2 recruitment, whereas the potency ratio is reversed for PGF2α, indicative of receptor-based functional selectivity. Data represent the mean ± S.E.M. of 5-11 experiments, each performed in triplicate; ***, P<0.001; **, P<0.01; *, P<0.05; ns, P>0.05 (one way ANOVA and Tukey’s post-test). B) Relative potency of each agonist for the activation
of $\alpha_s$, $\alpha_i$ or $\beta$-arrestin 2. The data are expressed as the log of the ratio between the EC$_{50}$ value (in nanomolars) of a ligand for $\alpha_s$ activation and the EC$_{50}$ value of the same ligand for $\alpha_s$, $\alpha_i$ or $\beta$-arrestin 2 pathways. Ligands with decreased relative potency for a function will thus have a negative change compared to another function, while ligands with increased potency for a function will show a positive change compared to the other function. 17-PT PGE$_2$: 17-phenyl-trinor PGE$_2$; 16,16-dm PGE$_2$: 16,16-dimethyl PGE$_2$.

**Figure 5.** Preferential activation of $\alpha_s$, $\alpha_i$ or $\beta$-arrestin 2 pathways compared to PGE$_2$. Potencies of agonists relative to PGE$_2$ (relative potency = EC$_{50}$ PGE$_2$ / EC$_{50}$ agonist) were compared for the three functional pathways. The data represent the relative potency (RP) ratio for each pair of assays: ($RP_{\text{assay 1}} / RP_{\text{assay 1}} + RP_{\text{assay 2}}$) and vice versa. A greater bar area represents a smaller EC$_{50}$ relative to PGE$_2$ and thus a greater potency for a given signaling pathway. Arrows indicate ligand bias (relative to PGE$_2$) for each signaling pathway examined: A) $\alpha_s$ and $\alpha_i$; B) $\alpha_s$ and $\beta$-arrestin 2; C) $\alpha_i$ and $\beta$-arrestin 2. 17-PT PGE$_2$: 17-phenyl-trinor PGE$_2$; 16,16-dm PGE$_2$: 16,16-dimethyl PGE$_2$.

**Figure 6.** Reversal of relative maximal response of agonists for $\alpha_s$/$\alpha_i$ and $\beta$-arrestin 2 pathways. Intrinsic activity (relative to PGE$_2$ maximal response) of L-902688 was significantly greater for $\beta$-arrestin 2 recruitment than for $\alpha_i$ ($P<0.01$) or $\alpha_s$ ($P<0.05$) functions, while intrinsic activity of PGD$_2$ was lower for $\beta$-arrestin 2 than for $\alpha_s$ or $\alpha_i$ ($P<0.05$) pathways ($n = 4-6$, one way ANOVA and Tukey’s post-test).
Table 1. Agonist concentration-response fitted curve parameters of EP₄ receptor-mediated cAMP production, Gᵢ₁ activation and ß-arrestin 2 recruitment.

Values represent means ± S.E.M. of 4 to 11 independent experiments, each performed in triplicate. Agonist efficacies (Eₘₐₓ) are expressed relative to that of a saturating concentration of PGE₂.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>cAMP</th>
<th>Gᵢ₁</th>
<th>ß-arrestin 2</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀ pEC₅₀ ± SEM</td>
<td>Eₘₐₓ ± SEM</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>(nM) (%)</td>
<td>(nM) (%)</td>
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<td>PGE₂</td>
<td>0.30 9.63 ± 0.12</td>
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<tr>
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<td>105.3 ± 6.5</td>
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<td>PGD2</td>
<td>1664</td>
<td>93.2</td>
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*a-b pEC50 values in the same row designated with a different letter are significantly different, \( P < 0.05 \) (one-way ANOVA followed by Tukey’s post-test)

†,‡ Emax values in the same row designated with a different symbol are significantly different, \( P < 0.05 \) (one-way ANOVA followed by Tukey’s post-test)

* Emax value is significantly different from PGE2 reference, \( P < 0.05 \) (one-way ANOVA followed by Tukey’s post-test)
Table 2. Chemical structure and binding affinity of ligands competing with \[^{3}H\]PGE\textsubscript{2} for binding to recombinant EP\textsubscript{4} receptors. \(K_i\) values of all ligands represent binding affinity for human EP\textsubscript{4} receptors, with the exception of 16,16-dimethyl PGE\textsubscript{2} and PGE\textsubscript{1} alcohol for which data for binding to murine EP\textsubscript{4} receptors are presented.

<table>
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<th>Ligand</th>
<th>(K_i) (nM)</th>
<th>Reference</th>
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<td>(Abramovitz et al., 2000)</td>
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<td></td>
<td>0.75</td>
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<td>(Young et al., 2004)</td>
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<td>PGE\textsubscript{1}</td>
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<td>(Davis and Sharif, 2000)</td>
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<td>11-deoxy PGE\textsubscript{1}</td>
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<td>(Davis and Sharif, 2000)</td>
</tr>
<tr>
<td>M&amp;B28767</td>
<td>10</td>
<td>(Abramovitz et al., 2000)</td>
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<tr>
<td>Misoprostol free acid</td>
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<td>(Abramovitz et al., 2000)</td>
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</table>
17-phenyl-trinor PGE$_2$ 34.5 (Davis and Sharif, 2000)

$\text{PGF}_2\alpha$

16,16-dimethyl PGE$_2$ 43 (Kiriyama et al., 1997)

PGE$_1$ alcohol 190 (Kiriyama et al., 1997)

PGD$_2$ 1483 (Abramovitz et al., 2000) 2139 (Davis and Sharif, 2000)
Figure 3

A

PGE₂-promoted BRET

Time (min)

B

PGE₂-promoted BRET

Log PGE₂ (M)
Figure 4

A

![Graphs showing pEC50 values for PGE₂, 16,16-dimethyl PGE₂, PGE₁ alcohol, and PGF₂α.](image)

B

![Graphs showing log [EC₅₀, Gₛ₀, Gₐ₁₁, or β-arrestin] for various compounds.](image)
Figure 6

L-902688

PGD₂

Eₘₐₓ (% of PGE₂ maximal response)

cAMP  Gᵢ₁  β-arrestin

cAMP  Gᵢ₁  β-arrestin

*  **

*  *