Analysis of agonist function at fusion proteins between the IP prostanoid receptor and cognate, unnatural and chimaeric G-proteins

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Direct measures of G-protein activation based on guanine nucleotide exchange and hydrolysis are frequently impossible to monitor for receptors which interact predominantly with G_{α} . An isolated FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Lys)-epitope-tagged human IP prostanoid receptor and fusion proteins generated between this form of the receptor and the α subunits of its cognate G-protein G_s , G_{11} , a G-protein which it fails to activate in co-expression studies, and a chimaeric G_{11} - G_s6 (a form of G_{11} in which the C-terminal six amino acids were replaced with the equivalent sequence of G_s) were stably expressed in HEK293 cells. These were detected by [³H]ligand-binding studies and by immunoblotting with both an anti-FLAG antibody and with appropriate antisera to the G-proteins. Each construct displayed similar affinity to bind the agonist iloprost. Iloprost stimulated adenylate cyclase activity in clones expressing both IP prostanoid receptor and the IP prostanoid receptor- $G_s\alpha$ fusion protein, and both constructs were shown to interact with and activate endogenously expressed $G_s\alpha$. Addition of iloprost to membranes of cells expressing the isolated receptor resulted in

a small stimulation of high-affinity GTPase activity. Iloprost produced no stimulation of GTPase activity which could be attributed to the IP prostanoid receptor- $G_{11}\alpha$ fusion. However, the fusion proteins containing either $G_s\alpha$ or G_{11} - $G_s6\alpha$ produced substantially greater stimulation of GTPase activity than the isolated IP prostanoid receptor. Treatment of cells expressing the IP prostanoid receptor- G_{11} - $G_s6\alpha$ fusion protein with a combination of cholera and pertussis toxins allowed direct measurement of agonist activation of the receptor-linked G-protein. Normalization of such results for levels of expression of the IP prostanoid receptor constructs demonstrated a 5-fold higher stimulation of GTPase activity when using the $G_s\alpha$ -containing fusion protein and a 9-fold improvement when using the fusion protein containing G_{11} - $G_s6\alpha$ to detect G-protein activation compared with expression of the isolated receptor.

Key words: adenylate cyclase, cAMP, GTPase activity, signal transduction.

INTRODUCTION

Signal-transduction cascades involving seven transmembrane element, G-protein-coupled receptors (GPCRs) require the obligate activation of heterotrimeric G-proteins [1,2]. Interactions between GPCRs and G-proteins have been assessed and measured by a variety of means, but amongst the most widespread and popular are assays which examine GPCR-induced exchange of GDP for GTP in the nucleotide-binding pocket of the G-protein [3], and the subsequent timer-controlled hydrolysis [4] of the terminal phosphate of GTP by the GTPase activity intrinsic to the α subunits of all G-proteins [1,2]. Although such approaches are relatively easy to perform, they have generally only been useful for GPCRs which interact predominantly with the G_i -like subfamily of pertussis toxin-sensitive G-proteins [5]. This reflects a combination of the higher intrinsic guanine nucleotide exchange and GTPase activity of these G-proteins and their relatively high levels of expression. Thus, although agonist-induced turnover of GTP is accepted to occur on other G-proteins, direct demonstration of this is often difficult or impossible without purification of the components and their reconstitution in artificial vesicles [6]. This is despite one of the earliest pieces of evidence for an obligate role for GTP hydrolysis in signal transmission being derived from analysis of β -adrenoceptor activation of G_s in avian erythrocytes [7,8].

We have recently generated a fusion protein between the porcine α_{2A} -adrenoceptor and a pertussis toxin-resistant (Cys³⁵¹

→ Gly) mutant of the α subunit of the G-protein G_{11} [9–11]. Expression of this fusion protein in COS-7 cells, which were treated with pertussis toxin to prevent any potential interactions between the introduced protein and the endogenously expressed G_i -like G-proteins, allowed agonist stimulation of high-affinity GTPase activity [9–11]. Such results demonstrated direct and productive interactions between the two elements of the fusion protein.

The human IP prostanoid receptor (hIPR) [12] is a typical example of a GPCR which stimulates adenylate cyclase activity via activation of $G_s\alpha$. We have recently demonstrated that co-expression of a FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Lys)-tagged form of hIPR (FhIPR) and a chimaeric G-protein (G_{11} - $G_s6\alpha$) in which the C-terminal six amino acids of $G_{11}\alpha$ were replaced with the equivalent sequence from $G_s\alpha$, allows robust measures of agonist-stimulation of guanine nucleotide exchange and hydrolysis [13]. In the present study we have generated fusion proteins between FhIPR and the α subunits of its cognate G-protein G_s , G_{11} and the chimaeric G_{11} - G_s6 . After stable expression of these constructs (FhIPR- $G_s\alpha$, FhIPR- $G_{11}\alpha$, FhIPR- G_{11} - $G_s6\alpha$) in HEK293 cells, we demonstrate that FhIPR- $G_s\alpha$ binds agonist and stimulates adenylate cyclase with the same affinity as the isolated receptor. However, this fusion construct allows substantially greater agonist-dependent stimulation of guanine nucleotide exchange and GTPase activity compared with an equivalent level of expression of the isolated FhIPR in the same cells. Measuring agonist-stimulation of GTPase activity

Abbreviations used: FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; GPCR, G-protein-coupled receptor; hIPR, human IP prostanoid receptor; hIPR- $G_s\alpha$, human IP prostanoid receptor- $G_s\alpha$ fusion protein; FhIPR, FLAG-tagged form of the human IP prostanoid receptor; G_{11} - G_s6 , a form of G_{11} in which the C-terminal six amino acids were replaced with the equivalent sequence of G_s .

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via GPCRs which couple to $G_{s\alpha}$ has traditionally been impractical. These results demonstrate that the use of GPCR–G-protein fusion proteins may provide a useful strategy to overcome this. Enzyme kinetic analysis provided quantitative measurement of the excess agonist-induced G-protein activation derived by maintaining and enforcing close proximity between the two elements of the fusion protein. Simple physical proximity of a GPCR and G-protein is, however, not sufficient to allow effective agonist-mediated information transfer, as FhIPR– $G_{11\alpha}$ was not activated by agonist. However, the benefit of using a chimaeric G-protein in co-expression studies [13] may be translated directly to the fusion protein context as FhIPR– G_{11} – $G_{s6\alpha}$ produced a substantially greater output than FhIPR– $G_{s\alpha}$.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Scotland, U.K.). The IP-prostanoid agonist [3H]iloprost (15.3 Ci/mmol) was purchased from Amersham Pharmacia Biotech. [γ - ^{32}P]GTP (30 Ci/mmol) was obtained from DuPont/NEN. Cholera toxin and pertussis toxin were purchased from Sigma. Iloprost was a gift from Schering AG, Burgess Hill, West Sussex, U.K. All other chemicals were from Sigma and Boehringer–Mannheim. Oligonucleotides were purchased from Genosys (Cambridge, U.K.).

Construction of FhIPR–G-protein fusion constructs

hIPR cDNA [12] was modified to incorporate the FLAG epitope at the N-terminal of the cDNA [13]. Using FhIPR cDNA, PCR amplification was used to remove the stop codon. At the same time, the last amino acid, cysteine, was changed to glutamic acid due to incorporation of an *XhoI* restriction site to allow fusion of the $G_{s\alpha}$ cDNA. This fragment of the FhIPR was cloned into the *EcoRI/XhoI* site of pcDNA3. Rat HA– $G_{s\alpha}(L)$ cDNA (a haemagglutinin-epitope-tagged form of the long isoform of rat $G_{s\alpha}$) [14,15] was obtained from Dr. M. J. Levis and Dr. H. R. Bourne, University of California at San Francisco, CA, U.S.A. To enable fusion with the FhIPR cDNA, bases 5' to the open reading frame were changed using PCR to generate an *XhoI* restriction site, while an *XbaI* site was incorporated after the stop codon. The sense oligonucleotide used was: 5'-CCGCTCGAGATGGGCTGCCTCGGCAACAG-3'; the underlined bases refer to the *XhoI* digestion site. The antisense oligonucleotide used was: 5'-TGCTCTAGATTAGAGCAGCTCGTATTGGC-3'; the underlined bases refer to the *XbaI* site. The PCR-amplified fragment was digested with *XhoI* and *XbaI* and ligated to the FhIPR in pcDNA3 at the *XhoI/XbaI* sites. G_{11} – $G_{s6\alpha}$ was constructed as described previously [13] and generation of the fusion proteins between the FhIPR and both $G_{11\alpha}$ and G_{11} – $G_{s6\alpha}$ was achieved as above.

Cell culture and stable expression

HEK293 cells were seeded into 100 mm culture dishes and grown to 60–80% confluency (18–24 h) before transfection with 5 μ g of FhIPR, FhIPR– $G_{s\alpha}$, FhIPR– $G_{11\alpha}$ or FhIPR– G_{11} – $G_{s6\alpha}$ cDNAs using DOTAP reagent {*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate} (Boehringer Mannheim) [13]. Cell lines stably expressing a G418 sulphate (Calbiochem) resistance marker were picked and transferred into 24-well plates. About 40 clones of each cDNA were amplified. Membrane preparations were made [13] and their specific binding of [3H]iloprost was determined.

[3H]iloprost binding studies

Binding assays were performed as in [13] by the addition of 20 μ g of membrane protein to an assay buffer [50 mM Tris/HCl (pH 7.5)/5 mM $MgCl_2$] containing [3H]iloprost. Non-specific binding was determined in the presence of 10 μ M iloprost.

Immunological studies

The antisera used in this study have been fully described previously [13]. Membrane samples (30 μ g/lane) were loaded on to 10% SDS/PAGE and run overnight at 60 V, 20 mA. Proteins were transferred to nitrocellulose (Schleicher and Schuell), and blocked overnight with 5% non-fat milk in PBS (0.27 mM KCl/0.15 mM KH_2PO_4 /137 mM NaCl/4 mM Na_2HPO_4 , pH 7.5). The blot was probed with primary antibody/antiserum in 3% non-fat milk in PBS for 1 h at room temperature with shaking, washed extensively with PBS containing 0.2% Nonidet P40 and incubated with secondary antibody in a similar manner.

High-affinity GTPase assays

The assays were performed as described by McKenzie and Milligan [16] with modifications as described by Wise et al. [9] to allow measurement of V_{max} . In a range of studies, cells were pretreated with either cholera toxin (100 ng·ml $^{-1}$), pertussis toxin (25 ng·ml $^{-1}$) or a combination of these toxins for 24 h prior to cell harvest.

Intact cell adenylate cyclase assays

Intact cell adenylate cyclase assays were performed as described by Merkouris et al. [17]. Cells were split into wells of a 24-well plate and incubated in medium containing [3H]adenine at 0.5 μ Ci/well for 16–24 h. The generation of [3H]cAMP in response to iloprost was assessed. Results are expressed as the ratio of [3H]cAMP to total [3H]adenine nucleotides.

RESULTS

A cDNA encoding FhIPR [13] was modified to remove the stop codon and introduce an *XhoI* linker sequence at the C-terminal end. The modified cDNA was subsequently ligated to a variant of the long isoform of the α subunit of the G-protein G_s [14,15]. The novel cDNA so generated was designed to encode a 792 amino acid open reading frame consisting of an FhIPR– $G_{s\alpha}$ fusion protein. Equivalent fusion constructs were made between the FhIPR and both full-length $G_{11\alpha}$, a G-protein which this GPCR fails to interact with and activate in co-expression studies [13], and a chimaeric G-protein, G_{11} – $G_{s6\alpha}$ [13], in which the last six amino acids of $G_{11\alpha}$ were replaced with the equivalent sequence from $G_{s\alpha}$, with which this GPCR has been shown to interact effectively [13].

Both the isolated FhIPR and FhIPR– $G_{s\alpha}$ fusion protein cDNAs were expressed stably in HEK293 cells. Initial detection of positive clones was based on [3H]ligand-binding studies with the IPR-selective ligand [3H]iloprost (Table 1). A number of clones expressing each construct displayed good levels of specific binding of this ligand. Non-specific binding, as defined by the presence of 10 μ M iloprost, was less than 5% of the measured total binding when using 10 nM [3H]iloprost. Confirmation of expression of the full-length FhIPR– $G_{s\alpha}$ and the FhIPR was

Table 1 Detection of expression of FhIPR and FhIPR–G-protein fusion proteins: [3 H]iloprost binding studies

Membranes from clones isolated following stable expression of FhIPR, FhIPR– $G_{s\alpha}$, FhIPR– $G_{11\alpha}$ or FhIPR– $G_{11}\text{-}G_{s6\alpha}$ in HEK293 cells were used to measure the specific binding of 10 nM [3 H]iloprost. Results represent means $\pm$ S.E.M. from $\geq$ five experiments. The above clones were those subsequently used for all further studies.

Cell line	Receptor expression levels (fmol $\cdot$ mg protein $^{-1}$)
FhIPR	2960 $\pm$ 140
FhIPR– $G_{s\alpha}$	1360 $\pm$ 140
FhIPR– $G_{11\alpha}$	900 $\pm$ 110
FhIPR– $G_{11}\text{-}G_{s6\alpha}$	920 $\pm$ 120

Figure 1 Expression of FhIPR and FhIPR–G-protein fusion proteins: immunological detection

Top: membranes from clones expressing FhIPR– $G_{s\alpha}$ (lanes 1) or FhIPR (lanes 2) were resolved by SDS/PAGE and immunoblotted with either antiserum CS, which identifies the C-terminal decapeptide of forms of $G_{s\alpha}$ (left-hand panel) or with an anti-FLAGTM antibody (right-hand panel) as described in the Materials and methods section. Bottom: membranes expressing the FhIPR– $G_{s\alpha}$ (lanes 1), FhIPR– $G_{11\alpha}$ (lanes 2) or FhIPR– $G_{11}\text{-}G_{s6\alpha}$ (lanes 3) fusion proteins were resolved by SDS/PAGE and immunoblotted with an anti-FLAGTM antibody (left-hand panel), antiserum CS which identifies the C-terminus of $G_{s\alpha}$ (centre panel) or antiserum IIC which identifies an epitope between amino acids 160–169 of $G_{11\alpha}$ (right-hand panel). (L) and (S) refer to the long and short isoforms of $G_{s\alpha}$ respectively.

demonstrated in immunoblots. The anti-FLAG antibody M5 specifically identified a band of some 90 kDa in membranes of clones expressing FhIPR– $G_{s\alpha}$, but not in cells expressing FhIPR where the predominant band identified was of some 60 kDa (Figure 1, top). A polypeptide of the same size as the anti-FLAG M5 antibody-detected FhIPR– $G_{s\alpha}$ was also identified, only in appropriate clones, by antiserum CS which is directed against the final ten amino acids of $G_{s\alpha}$ [18] (Figure 1, top). Antiserum CS also identified two polypeptides of 47 and 45 kDa which represent long and short splice variant forms of endogenous $G_{s\alpha}$ (Figure 1, top). These forms of $G_{s\alpha}$ were also present in membranes of cells expressing the FhIPR (Figure 1, top) and in parental HEK293

Figure 2 Expression and prostanoid binding affinity for stably expressed FhIPR- and FhIPR-containing fusion proteins: binding studies

The specific binding of [3 H]iloprost (3.4 nM) to membranes of clones expressing FhIPR (●) (IC_{50} = 6.1 ± 0.7 nM), FhIPR– $G_{s\alpha}$ (○) (IC_{50} = 4.8 ± 0.5 nM), FhIPR– $G_{11\alpha}$ (◆) (IC_{50} = 4.9 ± 0.6 nM), or FhIPR– $G_{11}\text{-}G_{s6\alpha}$ (△) (IC_{50} = 11.9 ± 2.2 nM) (means $\pm$ S.E.M.) was displaced by varying concentrations of iloprost. The Figure is representative of three independent experiments which were performed on separate membrane preparations from each clone.

cells (results not shown). Both FhIPR– $G_{11\alpha}$ and FhIPR– $G_{11}\text{-}G_{s6\alpha}$ were also expressed stably in HEK293 cells (Table 1). Immunoblotting studies with the anti-FLAG M5 antibody detected appropriately sized polypeptides (Figure 1, bottom). Antiserum CS and antiserum IIC, which identifies an internal epitope that is specific to $G_{11\alpha}$, also identified these polypeptides in membranes from clones expressing the appropriate fusion constructs (Figure 1, bottom). Specific binding of [3 H]iloprost to membranes of cells expressing each of FhIPR, FhIPR– $G_{s\alpha}$, FhIPR– $G_{11\alpha}$ and FhIPR– $G_{11}\text{-}G_{s6\alpha}$ could be effectively competed by unlabelled iloprost. Although the measured IC_{50} for iloprost was not different among FhIPR, FhIPR– $G_{s\alpha}$ and FhIPR– $G_{11\alpha}$ it was slightly higher for FhIPR– $G_{11}\text{-}G_{s6\alpha}$ (Figure 2). The competition curve in iloprost self-competition binding assays was more shallow in the membranes expressing the isolated FhIPR (0.64 ± 0.05) than in those expressing the various fusion proteins (0.85 ± 0.09) ($P < 0.01$).

The IPR is an activator of adenylate cyclase. Iloprost caused a concentration-dependent stimulation of cAMP production in cells expressing either FhIPR or FhIPR– $G_{s\alpha}$. The EC_{50} for iloprost was similar (FhIPR = 0.14 ± 0.03 nM, FhIPR– $G_{s\alpha}$ = 0.11 ± 0.03 nM) (Figure 3). Interestingly, both FhIPR– $G_{11\alpha}$ (EC_{50} = 0.55 ± 0.08 nM) and FhIPR– $G_{11}\text{-}G_{s6\alpha}$ (EC_{50} = 0.21 ± 0.03 nM) were also able to mediate stimulation of adenylate cyclase in response to iloprost. As we have recently demonstrated the capacity of a stably transfected α_{2A} -adrenoceptor– $G_{11\alpha}$ fusion protein to activate endogenously expressed forms of G_i , as well as the fusion protein partner G-protein [19], we wished to ascertain whether the FhIPR–G-protein fusion proteins could activate endogenously expressed $G_{s\alpha}$ as effectively as did the isolated FhIPR. Sustained occupancy of many GPCRs, including the IPR expressed endogenously by neuroblastoma $\times$ glioma hybrid NG108-15 cells [20], can result in downregulation of the G-protein(s) which interact with the receptor (see [21] for review). Cells expressing either FhIPR or FhIPR– $G_{s\alpha}$ were exposed to iloprost (1 μ M) for periods up to

Figure 3 Iloprost-stimulated cAMP formation in cells expressing each of FhIPR and FhIPR- $G_{s\alpha}$

Cells expressing FhIPR (●) and FhIPR- $G_{s\alpha}$ (○) were prelabelled with [3 H]adenine and the capacity of various concentration of iloprost to stimulate cAMP generation was subsequently assessed. The results are means $\pm$ S.E.M. of three independent experiments. In one example, basal cAMP/total adenine nucleotides ($\times 100$) was 0.38 for FhIPR- and 0.47 for FhIPR- $G_{s\alpha}$ -expressing cells and 30.5 for FhIPR- and 14.4 for FhIPR- $G_{s\alpha}$ -expressing cells which were treated with 10 μ M iloprost.

Figure 4 Sustained treatment with iloprost causes downregulation of $G_{s\alpha}$ but not FhIPR- $G_{s\alpha}$

Cells expressing either the FhIPR (lanes 1–6) or the FhIPR- $G_{s\alpha}$ fusion protein (lanes 7–12) were exposed to iloprost (1 μ M) for 0 (lanes 1 and 7), 0.5 (lanes 2 and 8), 2 (lanes 3 and 9), 4 (lanes 4 and 10), 8 (lanes 5 and 11) or 16 h (lanes 6 and 12). Membranes were prepared, resolved by SDS/PAGE and immunoblotted with antiserum CS to detect $G_{s\alpha}$. (L) and (S) refer to the long and short isoforms of $G_{s\alpha}$ respectively. Similar results were produced in three further experiments.

16 h. Membranes were prepared and immunoblotted. In both clones very large reductions in $G_{s\alpha}$ immunoreactivity were noted over time (Figure 4). Both the long and short splice variants were downregulated with similar time courses. Iloprost had no effect on cellular levels of $G_{s\alpha}$ in parental HEK293 cells (results not shown). In contrast with the effects on cellular $G_{s\alpha}$ levels, sustained iloprost treatment did not result in a downregulation of FhIPR- $G_{s\alpha}$ (Figure 4). Indeed, with prolonged exposure to

Table 2 Agonist stimulation of high-affinity GTPase activity in cells expressing FhIPR or FhIPR- $G_{s\alpha}$ fusion proteins

Experiments were performed at 0.5 μ M GTP. Results represent the means $\pm$ S.E.M. from three independent experiments.

Cell line	High-affinity GTPase activity ($\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)	
	Basal	Iloprost (10 μ M)
HEK293	11.6 $\pm$ 0.8	11.4 $\pm$ 0.7
FhIPR	12.2 $\pm$ 0.3	14.8 $\pm$ 0.4
FhIPR- $G_{s\alpha}$	13.7 $\pm$ 0.4	19.7 $\pm$ 0.1
FhIPR + transfected $G_{s\alpha}$	11.7 $\pm$ 0.6	14.1 $\pm$ 0.6
FhIPR- $G_{i1\alpha}$	10.0 $\pm$ 0.3	12.8 $\pm$ 0.5
FhIPR- $G_{i1\alpha}$ - $G_{s6\alpha}$	10.8 $\pm$ 0.6	21.1 $\pm$ 0.5

Figure 5 Effects of cholera and pertussis toxins on iloprost stimulation of high-affinity GTPase activity

Cells expressing FhIPR (1), FhIPR- $G_{s\alpha}$ (2), FhIPR- $G_{i1\alpha}$ (3), or FhIPR- $G_{i1\alpha}$ - $G_{s6\alpha}$ (4) were either untreated (open bars), treated with cholera toxin (100 $\text{ng} \cdot \text{ml}^{-1}$, 24 h) (stippled bars), treated with pertussis toxin (25 $\text{ng} \cdot \text{ml}^{-1}$, 24 h) (hatched bars) or with a combination of the two toxins (filled bars) prior to harvest and membrane preparation. The capacity of iloprost (1 μ M) to stimulate high-affinity GTPase activity was then measured at 0.5 μ M GTP as described in the Materials and methods section. Results represent means $\pm$ S.E.M. from three independent experiments performed on separate membrane preparations.

iloprost, levels of FhIPR- $G_{s\alpha}$ actually increased somewhat (Figure 4).

The capacity of GPCRs to increase guanine nucleotide exchange and hydrolysis by $G_{s\alpha}$ is notoriously difficult to measure [4]. Addition of iloprost (1 μ M) failed to alter high-affinity GTPase activity of membranes of parental HEK293 cells but did produce a limited stimulation in membranes of clones expressing the FhIPR (Table 2 and Figure 5). This was not increased by transient co-expression of $G_{s\alpha}$ (Table 2). In contrast, in membranes expressing the FhIPR- $G_{s\alpha}$ fusion protein, iloprost caused a robust stimulation of high-affinity GTPase activity (Table 2 and Figure 5). The capacity of iloprost to stimulate high-affinity GTPase activity in membranes expressing FhIPR- $G_{i1\alpha}$ was no

Figure 6 Iloprost stimulation of the GTPase activity of FhIPR- $G_s\alpha$ and FhIPR- $G_{s6}\alpha$

Basal high-affinity GTPase activity measured at $0.5 \mu\text{M}$ GTP and its regulation by various concentrations of iloprost was measured in membranes expressing either FhIPR- $G_s\alpha$ ($\circ$) or FhIPR- $G_{s6}\alpha$ ($\bullet$). In the case of FhIPR- $G_{s6}\alpha$ the cells were pretreated with a combination of pertussis toxin ($25 \text{ ng} \cdot \text{ml}^{-1}$) and cholera toxin ($100 \text{ ng} \cdot \text{ml}^{-1}$) for 24 h before cell harvest. The results represent means $\pm$ S.E.M. of triplicate assays of the stimulation above basal activity from one experiment of three performed.

greater than in those expressing the isolated FhIPR (Table 2 and Figure 5), whereas for FhIPR- $G_{s6}\alpha$, $1 \mu\text{M}$ iloprost again produced a robust stimulation which was substantially greater than that observed for FhIPR- $G_s\alpha$ (Table 2 and Figure 5). Pretreatment of cells with pertussis toxin did not substantially modify the effect of iloprost at the isolated FhIPR nor any of the receptor-containing fusion proteins (Figure 5), excluding the possibility that these GTPase signals derived from inappropriate activation of the endogenous cellular G_i population. Cholera toxin treatment causes ADP-ribosylation of $G_s\alpha$ and consequently prevents GTP hydrolysis by this G-protein. After cholera toxin treatment of cells expressing the isolated FhIPR, iloprost was no longer able to stimulate high-affinity GTPase activity (Figure 5). The same was true for the FhIPR- $G_{s6}\alpha$ fusion protein (Figure 5). As this protein is not a substrate for cholera toxin these results imply that the iloprost-mediated signal observed in these cells in the absence of toxin treatment must derive from activation of the endogenous $G_s\alpha$ population and not from activation of the fusion protein-linked $G_{s6}\alpha$. The large iloprost-mediated stimulation of high-affinity GTPase activity in cells expressing the FhIPR- $G_{s6}\alpha$ fusion protein was essentially unaffected by treatment with pertussis toxin, cholera toxin or a combination of the two toxins (Figure 5). The sequence of this chimaeric G-protein defines that it should be a substrate for neither cholera toxin nor pertussis toxin [13]. As such, the lack of effect of pertussis toxin was anticipated and the minor reduction in function following cholera toxin treatment can be attributed to interaction with endogenous $G_s\alpha$ (Figure 5). Therefore the high level of iloprost stimulation of high-affinity GTPase activity following treatment of the cells with a combination of cholera and pertussis toxins must represent activation only of the fusion protein-linked chimaeric G-protein (Figure 5). Cholera toxin treatment also essentially abolished the iloprost-mediated signal in cells expressing the FhIPR- $G_s\alpha$ fusion protein (Figure 5). However, as this protein should be a direct substrate for cholera

Figure 7 Effects of iloprost on the enzymic properties of FhIPR and FhIPR-G-protein fusion proteins

Basal high-affinity GTPase activity ($\circ$) and its regulation by iloprost ($1 \mu\text{M}$) ($\bullet$) were measured at various concentrations of GTP in membranes expressing FhIPR (top), FhIPR- $G_{s6}\alpha$ (middle) or FhIPR- $G_s\alpha$ (bottom). Results are presented as Eadie-Hofstee transformations from representative experiments of three performed for each construct.

Table 3 FhIPR-G_sα-containing fusion proteins allow substantially greater activation of high-affinity GTPase activity than the isolated FhIPR

Membranes of cells expressing the FhIPR, the FhIPR-G_sα fusion protein or the FhIPR-G₁₁-G_s6α fusion protein were used to measure both iloprost (1 μM)-stimulated high-affinity GTPase activity at $V_{\max}$, as in Figure 7, and the levels of expression of each construct detected by the specific binding of 10 nM [³H]iloprost. Values were calculated as: iloprost-stimulated GTPase activity at $V_{\max}$ (pmol · min⁻¹ · mg of protein⁻¹)/expression level measured at 10 nM [³H]iloprost (pmol · mg of protein⁻¹). These values allowed comparison of the effectiveness of iloprost to generate a GTPase signal at the individual GPCR constructs. Results represent means ± S.E.M. from three experiments performed on separate membrane preparations.

	GTPase activity at $V_{\max}$ /expression level
FhIPR	2.5 ± 0.2
FhIPR-G _s α	13.3 ± 1.6
FhIPR-G ₁₁ -G _s 6α	21.1 ± 2.5

toxin, abolition of signal does not indicate that all of the agonist signal derives via the endogenous G_sα population rather than from the fusion protein-linked G-protein. Iloprost had an EC_{50} = 4.7 ± 1.2 nM to stimulate the GTPase activity of FhIPR-G_sα (Figure 6). Equivalent concentration-effect curves (performed after combined cholera and pertussis toxin treatment to allow only agonist activation of the fusion protein to be recorded) in cells expressing FhIPR-G₁₁-G_s6α indicated an EC_{50} = 76.5 ± 6.6 nM (Figure 6). The effects of iloprost on the isolated FhIPR were sufficiently small to preclude generation of acceptable values for agonist EC_{50} (results not shown).

All of the above GTPase activity experiments were performed using a single concentration of GTP (0.5 μM) as substrate. When GTPase activity and its regulation by iloprost (1 μM) was measured at various GTP concentrations, the increase in activity in membranes expressing FhIPR was shown to represent a small increase in $V_{\max}$, without alteration in the K_m for GTP (K_m basal activity = 0.49 ± 0.03 μM, K_m in the presence of iloprost = 0.48 ± 0.04 μM) (Figure 7, top). The same was true for the much more robust iloprost stimulation of FhIPR-G₁₁-G_s6α recorded after combined pretreatment with pertussis and cholera toxins (K_m basal activity = 0.64 ± 0.03 μM, K_m in the presence of iloprost = 0.63 ± 0.03 μM) (Figure 7, middle). In contrast, iloprost stimulation of high-affinity GTPase activity in membranes expressing FhIPR-G_sα represented a combination of an increase in $V_{\max}$ and a small increase in the estimated K_m for GTP (K_m basal activity = 0.52 ± 0.02 μM, K_m in the presence of iloprost = 0.67 ± 0.03 μM) (Figure 7, bottom). When the increase in GTPase at $V_{\max}$ was normalized for expression levels of FhIPR, the FhIPR-G_sα and the FhIPR-G₁₁-G_s6α fusion proteins, the fusion proteins were demonstrated to provide 5.3 ± 0.6 -fold (for FhIPR-G_sα) and 8.5 ± 1.0 -fold (for FhIPR-G₁₁-G_s6α) greater GTPase response to iloprost than the isolated FhIPR (Table 3).

DISCUSSION

Prostaglandins (PG) and thromboxanes (TX) are locally acting mediators produced from arachidonic acid by the action of cyclo-oxygenases. Five primary active prostanoid metabolites, PGD₂, PGE₂, PGF_{2α}, PGI₂ and TXA₂ function via a family of GPCRs to produce a range of physiological actions. Receptors from all of these groupings have now been cloned [22]. In the case of the IPR, the recent production of mice lacking this receptor [22] has confirmed its central role in the regulation of platelet

aggregation and vasodilation, and hinted at contributions to the regulation of inflammation and pain perception.

Detailed analysis of the interactions of a GPCR with G-proteins is often performed by examining the capacity of receptor agonists to promote guanine nucleotide exchange and subsequent hydrolysis by the cellular G-protein population [3–5]. Although relatively easy to measure for the G_i-family G-proteins, such approaches are often hindered by the fact that other G-proteins, such as G_s, have low rates of guanine nucleotide exchange. Recent studies have generated fusion proteins between GPCRs and G-protein α subunits because the defined stoichiometry of expression of the GPCR to G-protein as 1:1 allows direct measurement of the catalytic efficiency of an agonist at a GPCR to promote the binding and hydrolysis of GTP by the G-protein [9].

We have suggested that the physical proximity of the receptor and G-protein, which is an inherent feature of the fusion protein approach, might enhance their interaction and thus generate a robust signal in these assays [23]. This appears true, as a fusion protein between the β₂-adrenoceptor and G_sα, which incorporates a proteolytic cleavage site between the two, produces markedly greater agonist stimulation of GTPase activity before proteolytic cleavage than after [24]. Furthermore, building on the studies of Conklin et al. [25], which indicated that switching of the extreme C-terminal region of G-protein α subunits can allow alterations in GPCR coupling selectivity, we generated a chimaeric G-protein in which the last six amino acids of G₁₁α were replaced by those from G_sα and showed effective stimulation of the GTPase activity of this protein by agonist upon co-expression with the FhIPR [13]. Given that Medici et al. [26] have indicated that the predominance of the G-protein C-terminal in GPCR interactions is lost in a fusion protein between the *Saccharomyces cerevisiae* alpha factor receptor Ste2 and a chimaeric yeast-mammalian G-protein, we wished to assess both whether direct linkage of a G-protein not normally activated by the FhIPR would now result in functional coupling, and the importance of the C-terminal region in such interactions.

Following stable expression of the FhIPR in these cells, concentration-dependent stimulation of adenylate cyclase could be recorded upon addition of iloprost (Figure 3). This was also the case for the FhIPR-G_sα fusion protein (Figure 3). It is unclear from such experiments whether this activity of the fusion protein was derived only from activation of the GPCR-associated G-protein or via activation of the endogenous G_sα pool. To assess this we took advantage of previous demonstrations that sustained occupancy of GPCRs can often result in a down-regulation of the G-protein(s) α subunits which interact with and are activated by the GPCR [21]. The maintained presence of iloprost on cells expressing either the FhIPR or the FhIPR-G_sα fusion protein resulted in a large, time-dependent down-regulation of both the long and short splice isoforms of G_sα (Figure 4), indicative of activation of these G-proteins by both GPCR constructs [21].

Addition of iloprost to membranes expressing the FhIPR did result in a detectable increase in high-affinity GTPase activity (Table 2). Moreover, this did not result from a limited interaction of the expressed GPCR with the endogenous G_i pool, as the effect was not attenuated by prior pertussis toxin treatment of the cells (Figure 5). It did, however, result from activation of G_sα, as iloprost stimulation of high-affinity GTPase was prevented by pretreatment with cholera toxin. Interestingly, although the levels of specific binding of [³H]iloprost indicated the FhIPR-G_sα fusion protein to be expressed at only some 50% of the levels of the isolated FhIPR, addition of iloprost resulted in a larger stimulation of high-affinity GTPase. When these values were

normalized for GPCR construct expression levels, the effectiveness of iloprost to induce GTPase activity measured at $V_{\max}$ was some 5-fold higher for the FhIPR– $G_{s\alpha}$ fusion protein than for FhIPR (Table 3). Seifert et al. [27] have also noted a marked benefit of using a β_2 -adrenoceptor– $G_{s\alpha}$ fusion protein to generate agonist-stimulated GTPase activity compared with the isolated GPCR.

Iloprost also caused a small stimulation of GTPase activity in membranes expressing the FhIPR– $G_{11\alpha}$ fusion protein (Figure 5). However, this did not reflect activation of the G-protein contained within the fusion protein. Cholera toxin pretreatment abolished this effect, an outcome not expected for G_i -family G-proteins. Furthermore, the effect of iloprost in these membranes was not blocked by prior pertussis toxin treatment (Figure 5). As such, simple physical proximity resulting from the fusion construction is not sufficient to allow the FhIPR to activate an unnatural partner G-protein in an agonist-dependent manner. The greatest stimulation of activity by iloprost was produced in membranes expressing the fusion protein between the FhIPR and the chimaeric G_{11} – $G_{s6\alpha}$ G-protein. This stimulation was little reduced by combined treatment with cholera and pertussis toxins and, as the chimaeric G-protein is designed to be resistant to both these toxins [13], this indicated the agonist signal to be derived from activation of this GPCR-linked G-protein. Such results are in accord with our recent co-transfection studies [13], but it is both important and gratifying to note that the basic features of FhIPR activation of each of the natural, unnatural and chimaeric G-proteins appear to be preserved in the fusion proteins used herein. Furthermore, unlike the work of Medici et al. [26], these studies support a maintained central role for the G-protein C-terminus in GPCR interactions within the fusion-protein context.

The current experiments offer a novel insight into a way in which to improve detection of activation of specific G-proteins by GPCRs. The capacity to enhance effective guanine nucleotide exchange and hydrolysis in response to agonist ligands not only provides a strategy by which potentially novel agonist ligands can be detected at one of the earliest steps in GPCR-mediated signal transduction, but also indicates that within the FhIPR– $G\alpha$ fusion proteins both the GPCR and the G-protein fold effectively and retain their capacity to interact appropriately in the presence of ligands.

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