0026-895X/98/020249-09$3.00/0

MOLECULAR PHARMACOLOGY, **54:**249–257 (1998).

Selective Activation of a Chimeric G_{i1}/G_s G Protein α Subunit by the Human IP Prostanoid Receptor: Analysis Using Agonist Stimulation of High Affinity GTPase Activity and [^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate Binding

CHEE WAI FONG, DALJIT S. BAHIA, STEPHEN REES, and GRAEME MILLIGAN

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G12 8QQ, Scotland (C.W.F., D.S.B., G.M.) and Receptor Systems Unit, Glaxo Wellcome Research and Development, Stevenage, Hertfordshire, SG1 2NY, England (S.R.)

Received February 24, 1998; Accepted April 14, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

A FLAG-tagged form of the human IP prostanoid receptor was expressed stably in HEK 293 cells. This bound [^{3}H]iloprost with high affinity and stimulated cAMP production when exposed to agonist. Iloprost produced weak stimulation of GTPase activity and [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding in membranes of these cells. Pretreatment of cells with pertussis toxin did not modify iloprost-mediated stimulation, but this was blocked by cholera toxin. The effects of iloprost were not increased by coexpression of either $G_{s\alpha}$ or $G_{i1\alpha}$. In contrast, coexpression of a chimeric G protein α subunit in which the carboxyl-terminal six amino acids of $G_{i1\alpha}$ were altered to those of $G_{s\alpha}$ resulted in robust stimulation by iloprost. Because the chimeric G protein α subunit ($G_{i1}/G_{s6\alpha}$) is not a substrate for either pertussis or cholera toxin, pretreatment of cells coexpressing the IP prostanoid receptor and $G_{i1}/G_{s6\alpha}$ with a mixture of these toxins resulted in resolution of the signal derived from activation of the chimeric G protein. Agonist-stimulated [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding and GTPase activity assays are the most commonly used strategies to examine interactions between G protein-coupled receptors and G proteins. These usually are not appropriate for receptors such as the IP prostanoid receptor that interact with G proteins with low rates of guanine nucleotide exchange and hydrolysis. Chimeric G proteins such as $G_{i1}/G_s6\alpha$ that allow appropriate receptor contacts to be converted to the higher nucleotide turnover rates typical of the G_i family G proteins can overcome this and offer a novel means to examine agonist function at such receptors.

Signal transduction cascades involving seven-transmembrane element, GPCRs require the obligate activation of heterotrimeric guanine nucleotide binding proteins (G proteins) (Birnbaumer *et al.*, 1990; Bourne *et al.*, 1990) Interactions between GPCRs and G proteins have been assessed and measured by a variety of means. Among the most widespread and popular are assays that examine GPCR-induced exchange of GDP for GTP in the nucleotide binding pocket of the G protein and the subsequent timer-controlled hydrolysis (Gierschik *et al.*, 1994) of the terminal phosphate of GTP by the GTPase activity intrinsic to the α subunits of all G proteins (Milligan, 1988) or the binding of a poorly hydrolysed analogue of GTP such as [^{35}S]GTPγS (Wieland and Jakobs, 1994). Such approaches have been particularly useful for GPCRs that interact predominantly with the subfamily of pertussis toxin-sensitive G_i-like G proteins (Milligan, 1988; Gierschik *et al.*, 1994; Wieland and Jakobs, 1994). 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 compared with other G proteins. As such, although agonist-induced turnover of GTP is accepted to occur on other G proteins, direct demonstration of this often is difficult in membrane systems (Koski and Klee, 1981). This occurs despite some of the earliest evidence for a obligate role for GTP hydrolysis in signal transmission and termination being derived from analysis of β-adrenoceptor activation of G_s in avian erythrocytes (Cassel and Selinger, 1976, 1977).

Prostaglandins and thromboxanes are locally acting mediators that are produced from arachidonic acid by the action of

This work was supported by the Medical Research Council and the Biotechnology and Biosciences Research Council (UK). C.W.F. received a studentship from the National Science and Technology Board of Singapore. D.S.B. received a CASE studentship from the Biotechnology and Biosciences Research Council.

ABBREVIATIONS: FhIPR, FLAG-tagged human IP prostanoid receptor; hIPR, human IP prostanoid receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PBS, phosphate-buffered saline; GPCR, G protein-coupled receptor; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate.

cyclooxygenases. Five primary active prostanoid metabolites, the prostaglandins D_2, E_2, $F_{2\alpha}$, and I_2 and thromboxane A_2, function via a family of GPCRs to produce a range of physiological actions. Receptors from all of these groupings have been cloned (Pierce *et al.*, 1995), including the human IP prostanoid receptor (Boie *et al.*, 1994). Agonist activation of the IP prostanoid receptor results in stimulation of adenylyl cyclase via activation of the G protein $G_{s\alpha}$ (McKenzie and Milligan, 1990b). Although not studied as extensively as certain other $G_{s\alpha}$-linked adenylyl cyclase stimulatory receptors, such as the β-adrenoceptors, the IP prostanoid receptor is of major potential therapeutic significance. Indeed, the recent production of mice lacking the IP prostanoid receptor (Murata *et al.*, 1997) 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. Furthermore, its endogenous expression by platelets (Armstrong, 1996) and NG108–15 neuroblastoma X glioma hybrid (Kelly *et al.*, 1990; McKenzie and Milligan, 1990b; Williams and Kelly, 1994) and other related cell lines (Kelly *et al.*, 1990) resulted in substantial characterization of its regulation and desensitization before the availability of the receptor cDNA.

Chimeric G protein α subunits have been generated that allowed certain agonist activated GPCRs to switch output direction. For example, the alteration of as few as the last three amino acids of $G_{q\alpha}$ with those from $G_{i1\alpha}/G_{i2\alpha}$ can allow certain GPCRs that normally mediate inhibition of adenylyl cyclase to stimulate phosphoinositidase C (Conklin *et al.*, 1993). A similar chimeric strategy involving substitution of the carboxyl-terminal amino acids of $G_{13\alpha}$ with those from $G_{z\alpha}$ has been used to define a role for $G_{13\alpha}$ in Na^+-H^+ exchange (Voyno-Yasenetskaya *et al.*, 1994).

Equally, conversion of the carboxyl-terminal five amino acids of $G_{s\alpha}$ to that of $G_{i1\alpha}/G_{i2\alpha}$ has been shown to allow the somatostatin SSTR3 receptor to mediate stimulation of cAMP production, although this receptor normally is considered to mediate inhibition of adenylyl cyclase activity (Komatsuzaki *et al.*, 1997). Generic G protein α subunit carboxyl-terminal switching also has been applied to attempt to define the specificity of GPCR/G protein interactions (Liu *et al.*, 1995; Komatsuzaki *et al.*, 1997).

In the current study, we adapted this basic approach to generate a chimeric G protein α subunit ($G_{i1}/G_{s6\alpha}$), which has the backbone of $G_{i1\alpha}$ except that the carboxyl-terminal six amino acids have been converted to those of $G_{s\alpha}$. We predicted that this G protein should allow predominantly $G_{s\alpha}$-coupled receptors to produce robust agonist-dependent regulation of high affinity GTPase activity and binding of [^{35}S]GTPγS. Using transient transfection of the chimeric G protein into a cell line stably expressing a FLAG-tagged form of the human IP prostanoid receptor, we demonstrate the usefulness of this system and compare it with the effectiveness of the authentic G proteins.

Experimental Procedures

Materials. All materials for tissue culture were supplied by Life Technologies Europe (Paisley, Scotland, UK). The IP prostanoid receptor agonist [^{3}H]iloprost (15.3 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). [γ-^{32}P]GTP (30 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont-New England Nuclear. Pertussis toxin (240 μg/ml) and all other chemicals were from Sigma Chemical (Poole, Dorset, UK) and Boehringer-Mannheim (Mannheim, Germany). Oligonucleotides were purchased from Genosys (Cambridge, UK).

Construction of a FLAG epitope-tagged human IP prostanoid receptor cDNA. An hIPR cDNA was obtained from Dr. Mark Abramovitz (Department of Biochemistry and Molecular Biology, Merck Frosst Center for Therapeutic Research, Quebec, Canada). To incorporate the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the amino terminus of the cDNA, a set of PCR oligonucleotide primers was used. The sense oligonucleotide was 5′-AAGGATCCGCCACCATG(GACTACAAGGACGACGATGATAAG)GCGGATTCGTGCAGGAACC-3′; the underlined bases refer to restriction sites for *Bam*HI and *Nco*I, respectively, with FLAG epitope bases in parenthesis. The antisense oligonucleotide was 5′-AAGAATTCTCAGCTTGAAATG(TCA)GCAGAG-3′; the underlined bases refer to *Eco*RI restriction site, with the stop codon in parenthesis. The PCR-amplified fragment was digested with *Bam*HI and *Eco*RI and ligated to pcDNA3 (InVitrogen, San Diego, CA) through these restriction sites. Introduction of the *Nco*I site at the start codon allowed the selection of positive clones on *Nco*I digestion and agarose gel electrophoresis. The cDNA construct was fully sequenced.

Construction of $G_{i1}/G_{s6\alpha}$ cDNA. $G_{i1}/G_{s6\alpha}$ was generated from a pertussis toxin-resistant form of rat $G_{i\alpha1}$ in which the Cys351 was mutated to aspartic acid rat $G_{i\alpha1}$ by PCR amplification using the primers 5′-ACGT-GAA-TTC-GCC-ACC-ATG-GGC-TGC-ACA-CTG-AGC-GC-3′ (primer 1) and 5′-CCA-CGT-GAA-TTC-TTA-TAA-GAG-TTC-ATA-TTG-CCT-TAG-GTT-ATT-CTT-TAT-3′ (primer 2).

The PCR consisted of 50 ng of template, 100 pmol of a consensus 5′ primer (primer 1) and 100 pmol of a 3′ primer (primer 2), dNTPs (0.2 mM concentration of each dATP, dCTP, dGTP, and dTTP; Pharmacia, Piscataway, NJ), 1 unit of *Pyrococcus furiosus* (*PFu*) DNA polymerase (Stratagene, Cambridge, UK), and 1**PFu* buffer in a total volume of 50 μl. The reaction conditions were as follows: 1 cycle at 95° for 2 min and 30 cycles consisting of a 95° and 45-sec denaturation step, a 55° and 45-sec annealing step, and a 72° and 3-min extension step. The reaction was completed by a single 5-min extension at 72°. All reactions were performed on a Hybaid OmniGene temperature cycler. Purified amplification product was subcloned into *Eco*RV (Boehringer-Mannheim) linearized, phosphatase-treated pcDNA3 (InVitrogen). The correct sequence of the modified cDNA was confirmed by sequencing, and the immunological characteristics of the expressed protein (see Results) were used as further conformation of the validity of the construct.

Cell culture and stable transfection. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum and 2 mM L-glutamine. Cells were seeded into 100-mm culture dishes and grown to 60–80% confluency (18–24 hr) before transfection with 5 μg of FhIPR cDNA using DOTAP reagent (Boehringer-Mannheim). At 48 hr after transfection, the cells were split 1:4 into 800 μg/ml geneticin (G418; Calbiochem, San Diego, CA)-containing medium. A 100-mm dish of untransfected HEK 293 cells also was split into the same medium as a control dish.

About 1 week later, after all the cells in the control dish had died, geneticin-resistant cells in the transfected dishes were picked and transferred into 24-well plates using autoclaved pipette tips. About 40 clones were selected and grown in 1 ml/well of geneticin (400 μg/ml)-containing medium. When the cells were confluent, they were trypsinized and transferred into 25-cm^2 culture flask and subsequently into 2 × 75-cm^2 flasks. Membrane preparations were made, and their binding of [^{3}H]iloprost was determined.

Transient transfections. Transient transfections into HEK 293 and cells stably expressing the FhIPR (clone 13) were essentially similar to that described. DNA at 5 μg/100-mm culture dish was used. At 48–72 hr after transfection, cells were rinsed and harvested in PBS (0.27 mM KCl, 0.15 mM KH_2PO_4, 137 mM NaCl, 4 mM Na_2HPO_4, pH 7.5), followed by centrifugation at 1000 × *g* in a

refrigerated centrifuge. The cell pastes obtained were kept at −80° until ready for membrane preparation.

Preparation of membranes. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80° after harvesting. Cell pellets were resuspended in Tris/EDTA buffer (10 mM Tris·HCl, pH 7.5, 0.1 mM EDTA), and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-on-glass homogenizer. Unbroken cells and nuclei were removed by centrifugation at low speed (2,000 rpm) in a refrigerated microcentrifuge. The supernatant fraction then was centrifuged at 75,000 rpm for 30 min in a Beckman Instruments (Palo Alto, CA) Optima TLX Ultracentrifuge with a TLA100.2 rotor. The pellets were resuspended in Tris/EDTA buffer to a final protein concentration of 1–3 mg/ml and stored at −80° until required.

[^{3}H]Iloprost binding studies. Binding assays were initiated by the addition of 20 μg of membrane protein to an assay buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$) containing [^{3}H]iloprost (0.1–50 nM in saturation assays and 10 nM in competition assays). Nonspecific binding was determined in the presence of 10 μM unlabeled iloprost. Reactions were incubated for 30 min at 30°, and bound ligand was separated from free ligand by vacuum filtration through GF/C filters. The filters were washed three times with ice-cold wash buffer (50 mM Tris·HCl, pH 7.5, 0.25 mM EDTA), and bound ligand was determined by liquid scintillation counting.

Immunological studies. M5 anti-FLAG antibody (IB13091; Kodak IBI) was used to blot for the FLAG epitope. It was raised in mouse and specifically recognizes the amino-terminal FLAG (MDYKDDDDK) epitope. Antiserum CS was produced in a New Zealand White rabbit using conjugates of synthetic peptide (RMHLRQYELL) and keyhole-limpet hemocyanin (Calbiochem) as antigen. It was used to blot for both authentic G_s and $G_{i1}/G_{s6\alpha}$ because it recognizes the carboxyl-terminal decapeptide of $G_{s\alpha}$ (both the short and long isoforms) (Milligan and Unson, 1989). Antisera I1C and SG were produced in similar fashion using peptides LDRIAQPNYI (I1C), which corresponds to amino acids 159–168 of $G_{i1\alpha}$, and KENLKDCGLF (SG), which corresponds to amino acids 341–350 of transducin 1α and differs by a single amino acid from the carboxyl-terminal decapeptide of $G_{i1\alpha}$ and $G_{i2\alpha}$ (Green *et al*., 1990).

Membrane samples (30 μg/lane) were loaded onto 10% SDS-PAGE and run overnight at 60 V and 25 mA. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) and blocked overnight with 5% nonfat milk in PBS. The blot was probed with primary antibody in 3% nonfat milk in PBS for 1 hr at room temperature with shaking, washed extensively with PBS containing 0.2% Nonidet P40, and incubated with secondary antibody in a similar manner. M5 anti-FLAG antibody was used at 1:500 dilution, whereas the secondary antibody peroxidase-conjugated anti-mouse IgG (A4416; Sigma) was used at 1:2000 dilution. Antisera CS, I1C, and SG were used at 1:2000 dilution, and the peroxidase-conjugated donkey anti-rabbit IgG also was used at 1:2000 dilution. After extensive washing, the blots were visualized by enhanced chemiluminescence. High affinity GTPase assays were performed as described by McKenzie and Milligan (1990a).

[^{35}S]GTPγS binding studies. [^{35}S]GTPγS binding studies were performed according to Wieland and Jakobs (1994). Briefly, 25 μg of membranes was incubated at 25° for 60 min in a final assay volume of 100 μl. The assay mix consisted of 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, and 5 μM GDP. [^{35}S]GTPγS was used at 50 nCi/assay tube, giving a final assay concentration of 0.3–0.5 nM. Agonist-driven binding of [^{35}S]GTPγS was determined in the presence of 1 μM iloprost, whereas nonspecific binding was performed in the presence of 20 μM unlabeled GTPγS. The binding was stopped by the addition of 2.5 ml of ice-cold wash buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$), filtered through Whatman GF/C filters, and followed by three quick washes of 5 ml of wash buffer. Filters were air-dried; 5 ml of ULTIMA-Gold XR scintillant was added and left overnight before liquid scintillation counting.

Whole-cell adenylate cyclase assays. Whole-cell adenylate cyclase assays were performed as described by Merkouris *et al*. (1997). Clone 13 cells were split into the wells of a 24-well plate and incubated in medium containing [^{3}H]adenine at 0.5 μCi/well for 16–24 hr. The generation of [^{3}H]cAMP in response to iloprost or forskolin was assessed. Results are expressed as the ratio of [^{3}H]cAMP to total [^{3}H]adenine nucleotides (×100).

Results

The hIPR was amino-terminally modified by the addition of a FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) epitope. The modified IP prostanoid receptor cDNA (FhIPR) was expressed stably in HEK 293 cells. A number of individual antibiotic resistant clones were expanded and analyzed. Specific binding of [^{3}H]iloprost was observed in membranes derived from a number of the clones (data not shown), whereas no detectable specific binding was observed in the membranes of parental HEK 293 cells. Clone 13 expressed the highest levels of specific [^{3}H]iloprost binding. The specific binding of [^{3}H]iloprost (5 nM) to membranes of clone 13 cells could be competed for with high affinity by unlabeled iloprost, under the assay conditions used, in an apparently monophasic manner ($n_H = 1.00 \pm 0.12$) (Fig. 1a) with an IC_{50} value of 9.5 ± 1.1 nM. Applying the formalisms of DeBlasi *et al*. (1989), this would correspond to a K_d value for [^{3}H]iloprost of 4.5 nM under these assay conditions. Immunoblotting membranes of clone 13 cells with the anti-FLAG monoclonal antibody M5 confirmed expression of a FLAG-tagged protein (Fig. 1b), which was not present in parental HEK 293 cells. The predominant FLAG antibody reactive species migrated through SDS-PAGE with an apparent molecular mass of 45 kDa. There was some evidence for heterogeneity in that a somewhat more slowly migrating polypeptide also was observed (Fig. 1b). These may represent differentially glycosylated forms of the receptor.

The functionality of the expressed FhIPR was demonstrated by measuring the ability of iloprost to stimulate cAMP production in intact cells. The capacity of forskolin to stimulate cAMP production was measured in parallel; based on a combination of levels of expression of the FhIPR and because iloprost and forskolin gave similar maximal levels of cAMP production, clone 13 was selected for detailed analysis. Concentration-response curves to iloprost indicated an EC_{50} value of $1.5 \pm 0.4 \times 10^{-10}$ M for stimulation of cAMP production (Fig. 2).

The addition of iloprost to membranes of clone 13 cells resulted in a small stimulation of high affinity GTPase activity (see below). However, although this increase in activity in response to the agonist was not observed in membranes of parental HEK 293 cells, it was too limited for detailed pharmacological analysis. Historically, it has been much easier to measure agonist stimulation of high affinity GTPase activity when studying GPCRs that interact with members of the G_i family of pertussis toxin-sensitive G proteins (Koski and Klee, 1981; Milligan, 1988; Gierschik *et al*., 1994), and, for example, we recently examined the interaction of the α_{2A}-adrenoceptor and the α subunit of G_{i1} (Wise *et al*., 1997) using this approach.

Because the extreme carboxyl terminus of G protein α subunits is a key element for GPCR activation of these transducing proteins (Conklin *et al*., 1993; Liu *et al*., 1995) and modification of as few as three amino acids from the carboxyl

terminus of the α subunit of the phosphoinositidase C-linked G protein G_q has been reported to be sufficient to cause alteration in the nature of GPCRs able to activate these G proteins (Conklin *et al*., 1993), we used a PCR-based strategy to alter the last six amino acids of $G_{i1\alpha}$ to those of $G_{s\alpha}$ (Fig. 3a). Confirmation of the success of this strategy was obtained both by direct sequencing of the modified G protein ($G_{i1}/G_{s6\alpha}$) cDNA and by a series of immunoblotting strategies. $G_{i1}/G_{s6\alpha}$ was expressed transiently in COS-7 cells, and membranes from each of these cells, mock transfected cells, and rat brain cortex (which expresses high levels of $G_{i1\alpha}$) were immunoblotted with either antiserum I1C, which was generated against a peptide corresponding to amino acids 159–168 of $G_{i1\alpha}$ and is specific for this G protein (Green *et al*., 1990), or with antiserum SG, which was generated against a peptide corresponding to the carboxyl-terminal 10 amino acids of $G_{i1\alpha}$ and which identifies both $G_{i1\alpha}$ and $G_{i2\alpha}$ (this epitope is identical in these two G proteins) (Green *et al*., 1990). When blotting with antiserum I1C, a low level of authentic $G_{i1\alpha}$ was detected as a 41-kDa protein in mock transfected COS-7 cells, as we reported previously (Galbiati *et al*., 1996). Successful expression of $G_{i1}/G_{s6\alpha}$ was recorded as a marked increase in I1C reactivity, which essentially comigrated with authentic rat brain $G_{i1\alpha}$ (Fig. 3b, *top*). In contrast, immunoblotting of the same membranes with antiserum SG failed to identify $G_{i1}/G_{s6\alpha}$, although a combination of endogenous $G_{i1\alpha}$ and $G_{i2\alpha}$ was detected in both mock and transfected COS-7 cells and in rat brain (Fig. 3b, *bottom*). Although these results clearly demonstrated the loss of carboxyl-terminal $G_{i1\alpha}$ immunoreactivity in $G_{i1}/G_{s6\alpha}$, the positive appearance of $G_{s\alpha}$ carboxyl-terminal immunoreactivity in this protein was obtained by immunoblotting such transfected cells with antiserum CS, which was generated against a peptide corresponding to the carboxyl-terminal 10 amino acids of the splice variants of $G_{s\alpha}$. Now, in addition to authentic, endogenously expressed $G_{s\alpha}$, transient transfection with $G_{i1}/G_{s6\alpha}$ resulted in the appearance of a more rapidly migrating CS immunoreactive polypeptide that comigrated with $G_{i1\alpha}$ (Fig. 3c).

Clone 13 cells subsequently were either mock transfected or transiently transfected with $G_{i1}/G_{s6\alpha}$, $G_{i1\alpha}$, or $G_{s\alpha}$. Membranes were prepared, and basal and iloprost stimulation of high affinity GTPase activity was measured. As noted, a small stimulation over the basal high affinity GTPase activity was observed in mock transfected clone 13 cells (Fig. 4). This was markedly increased in cells transiently transfected with $G_{i1}/G_{s6\alpha}$ but not after transfection with either $G_{i1\alpha}$ or $G_{s\alpha}$ (Fig. 4). In contrast, transient transfection of parental HEK 293 cells with $G_{i1}/G_{s6\alpha}$ did not result in any measurable iloprost stimulation of high affinity GTPase activity (Fig. 4).

We wanted to assess whether the increased agonist-stimulated high affinity GTPase activity produced by transfection with $G_{i1}/G_{s6\alpha}$, but not with authentic $G_{i1\alpha}$, simply reflected substantially greater levels of expression of $G_{i1}/G_{s6\alpha}$. How-

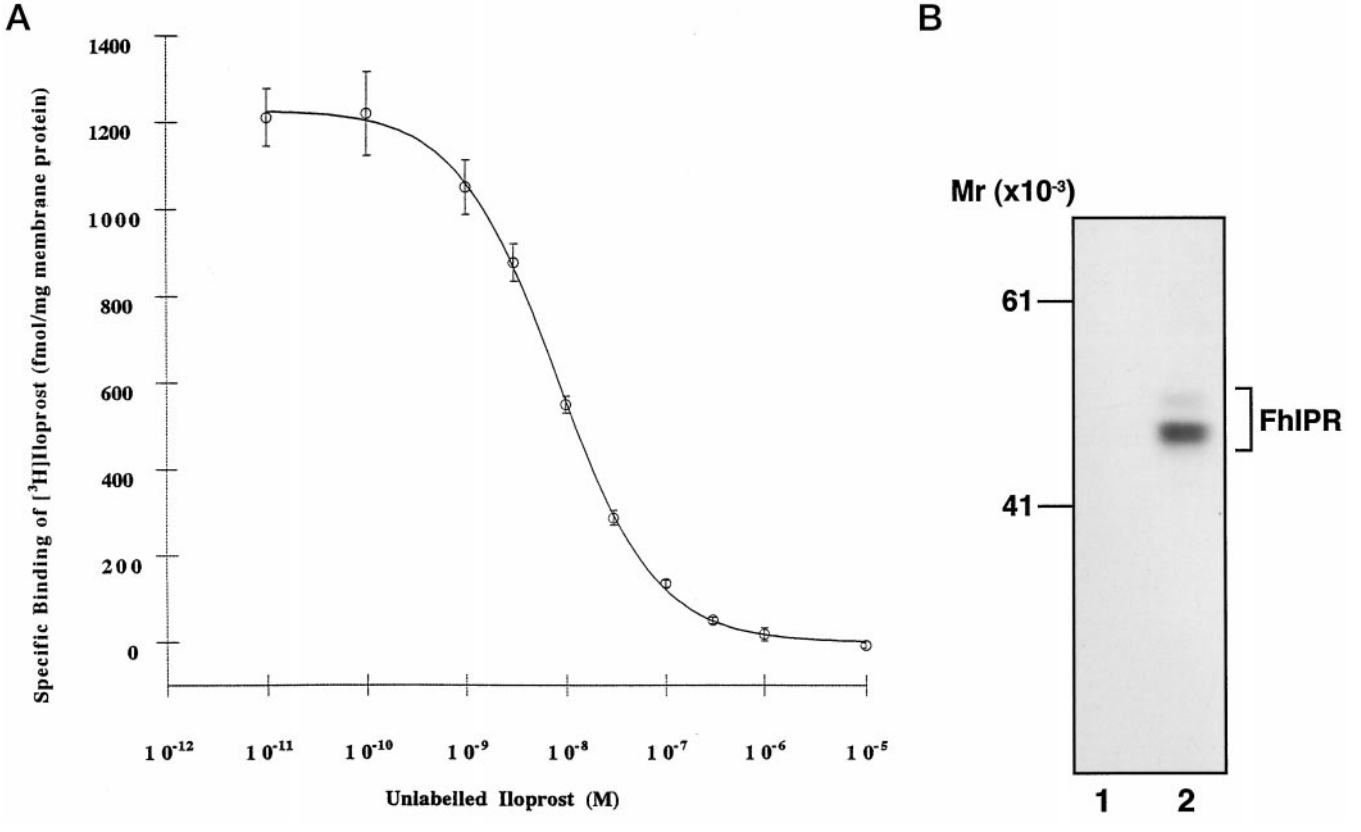

Fig. 1. Stable expression of the FLAG-tagged human IP prostanoid receptor in clones of HEK 293 cells. a, Competition for the specific binding of [^{3}H]iloprost in membranes of clone 13 by unlabeled iloprost. The binding of [^{3}H]iloprost (5 nM) to membranes of clone 13 cells was measured in the presence of varying concentrations of unlabeled iloprost. Data are taken from a representative experiment. Through the use of the formalisms of De Blasi *et al*. (1989), these data estimate a K_d value of 4.5 nM for iloprost in these assay conditions. b, Immunodetection of the FLAG-tagged human IP prostanoid receptor in clone 13 cells. Membranes from parental HEK 293 cells (*1*) or clone 13 cells (*2*) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using the anti-FLAG monoclonal antibody M5.

ever, immunoblotting membranes of clone 13 with antiserum I1C after transient transfection with either of the two G proteins demonstrated these to be expressed to equivalent levels (Fig. 5).

It was possible that the iloprost stimulation of high affinity GTPase activity in membranes of clone 13 reflected an interaction of the FhIPR with endogenously expressed G_i-like proteins. However, the effect of iloprost was not prevented by pretreatment of the cells with pertussis toxin (25 ng/ml, 16 hr) (Fig. 6). In contrast, pretreatment of the cells with cholera toxin (200 ng/ml, 16 hr) essentially attenuated iloprost-stimulated high affinity GTPase activity (Fig. 6). Because cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ attenuates the GTPase activity of this G protein, such results indicated the signal to be derived from activation of endogenous G_s.

An alternative assay for GPCR-mediated activation of G proteins is to measure agonist-induced binding of [^{35}S]GTPγS. Basal specific binding of [^{35}S]GTPγS was not increased by iloprost in the membranes of parental HEK 293 cells. As with the GTPase assay, iloprost did cause stimulation of [^{35}S]GTPγS binding in membranes of clone 13 cells. However, again, although transient expression of either $G_{s\alpha}$ or $G_{i1\alpha}$ into clone 13 cells did not result in any greater level of iloprost-mediated stimulation (see below), the binding of [^{35}S]GTPγS in response to iloprost was increased substantially in the presence of G_{i1}/$G_{s6\alpha}$ (Fig. 7).

G_{i1}/$G_{s6\alpha}$ would be anticipated to be a substrate for neither cholera toxin nor pertussis toxin. Clone 13 cells were either mock transfected or transfected with $G_{s\alpha}$, $G_{i1\alpha}$, or G_{i1}/$G_{s6\alpha}$ and then treated for the last 16 hr before cell harvest with a combination of cholera toxin (200 ng/ml) and pertussis toxin (25 ng/ml). This treatment essentially attenuated iloprost-stimulation of the binding of [^{35}S]GTPγS in each of mock transfected, $G_{s\alpha}$-transfected, and $G_{i1\alpha}$-transfected clone 13 cells (Fig. 8). In contrast, in cells transiently transfected with G_{i1}/$G_{s6\alpha}$, a substantial stimulation of binding of [^{35}S]GTPγS by iloprost was still achieved (Fig. 8). This was not as great as in the absence of pretreatment with cholera and pertussis toxins; however, the degree of reduction in iloprost-stimulated binding of [^{35}S]GTPγS herein was similar to the maximal effect of iloprost in mock-transfected clone 13 cells, suggesting that this fraction was derived from activation of the endogenous $G_{s\alpha}$ pool.

Immunoblotting membranes from such transfected cells with antiserum I1C demonstrated expression of G_{i1}/$G_{s6\alpha}$ and $G_{i1\alpha}$ (Fig. 9, *top*). The small reduction in mobility of the transfected $G_{i1\alpha}$ through SDS-PAGE in the membranes from

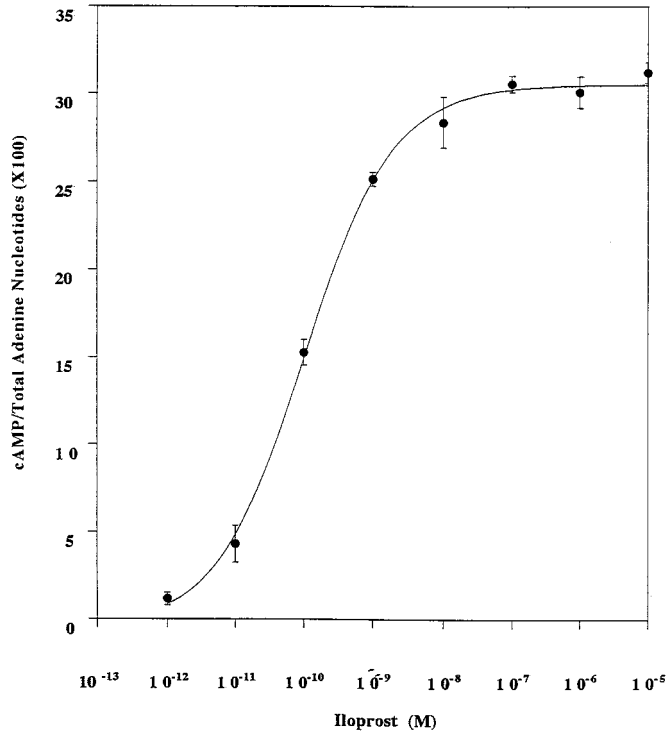

Fig. 2. Agonist occupation of the FLAG-tagged human IP prostanoid receptor results in stimulation of cAMP production. Basal cAMP levels and their regulation by varying concentrations of iloprost were measured in intact clone 13 cells. Data represent mean ± standard deviation of quadruplicate assays from a typical experiment. Forskolin (50 μM) produced a similar stimulation of adenylyl cyclase activity in clone 13 cells as maximally effective concentrations of iloprost (data not shown).

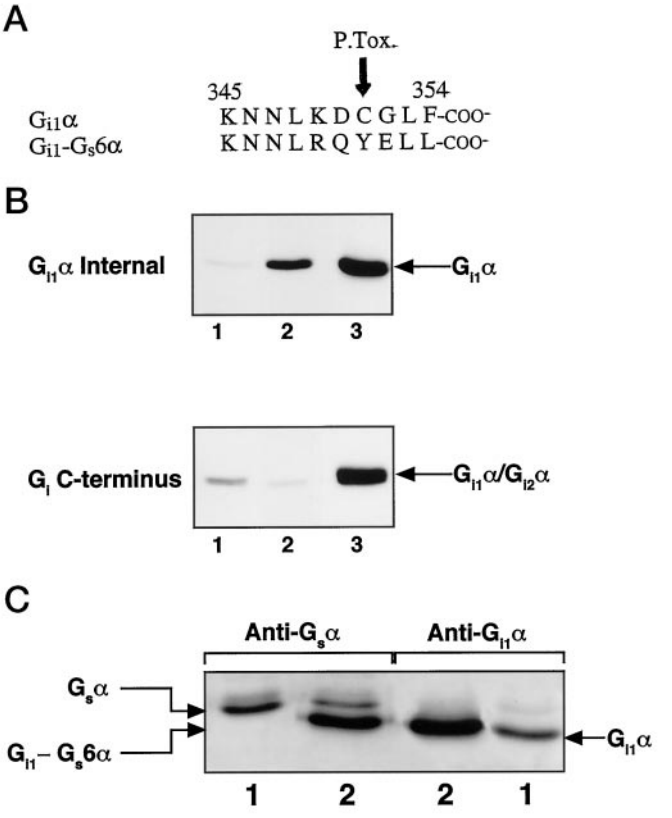

Fig. 3. Generation and expression of G_{i1}/$G_{s6\alpha}$. a, Carboxyl-terminal sequence of $G_{i1\alpha}$ and G_{i1}/$G_{s6\alpha}$. The carboxyl-terminal decapeptides of $G_{i1\alpha}$ and G_{i1}/$G_{s6\alpha}$ are shown using standard one-letter codes. Unlike $G_{i1\alpha}$, G_{i1}/$G_{s6\alpha}$ is not a substrate for pertussis toxin-catalyzed ADP-ribosylation because the marked C, which is the target for modification in $G_{i1\alpha}$, is absent from the chimeric G protein. b, Immunological characterization. G_{i1}/$G_{s6\alpha}$ has lost the carboxyl terminus of $G_{i1\alpha}$. COS-7 cells were mock transfected (*1*) or transiently transfected to express G_{i1}/$G_{s6\alpha}$ (*2*). Membranes from these cells or from rat brain cortex (*3*) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiserum I1C (internal epitope of $G_{i1\alpha}$) (*top*) or with antiserum SG (carboxyl terminus of $G_{i1\alpha}$ and $G_{i2\alpha}$) (*bottom*). c, G_{i1}/$G_{s6\alpha}$ has an internal epitope derived from $G_{i1\alpha}$ but the carboxyl-terminal epitope derived from $G_{s\alpha}$. COS-7 cells were mock transfected (*1*) or transiently transfected to express G_{i1}/$G_{s6\alpha}$ (*2*). Membranes prepared from these cells were resolved by SDS-PAGE and immunoblotted with antiserum CS (carboxyl-terminal epitope of $G_{s\alpha}$) or I1C (internal epitope of $G_{i1\alpha}$). Mock and positively transfected cells endogenously express $G_{s\alpha}$ (upper immunoreactive bands, *left*), whereas only the positively transfected cells display a $G_{s\alpha}$-immunoreactive polypeptide (G_{i1}/$G_{s6\alpha}$), which comigrates with $G_{i1\alpha}$.

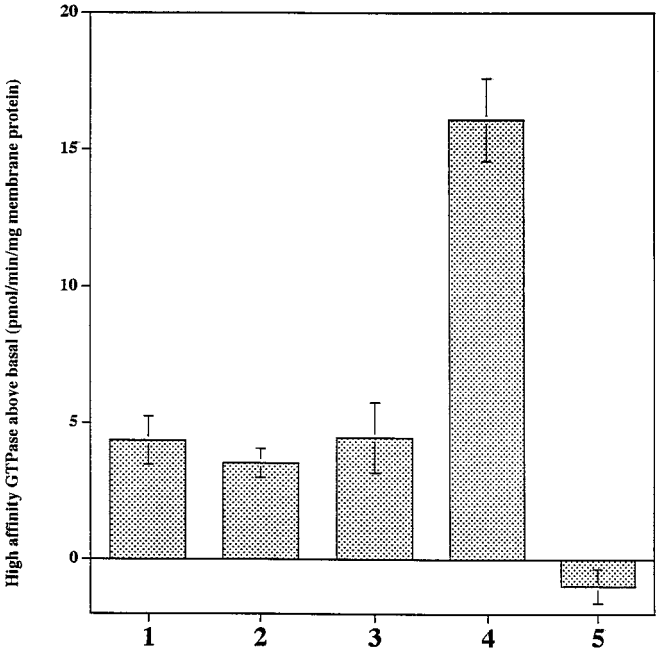

Fig. 4. Expression of $G_{i1}/G_{s6\alpha}$ with the FLAG-tagged human IP prostanoid receptor showing the effects on iloprost stimulation of high affinity GTPase activity. Clone 13 cells were either mock transfected (*1*) or transiently transfected to express $G_{s\alpha}$ (*2*), $G_{i1\alpha}$ (*3*), or $G_{i1}/G_{s6\alpha}$ (*4*). Parental HEK 293 cells also were transiently transfected to express $G_{i1}/G_{s6\alpha}$ (*5*). Membranes from these cells then were used to measure basal and iloprost (1 μM)-stimulated high affinity GTPase activity. The stimulation above basal produced by iloprost is shown. The experiment displayed is representative of four performed on membranes derived from separate transient transfections.

pertussis toxin-treated cells (Fig. 9, compare *lanes 3 and 4*) is indicative of toxin-catalyzed ADP-ribosylation of this protein. Very low levels of endogenous $G_{i1\alpha}$ could be detected in these immunoblots (Fig. 9, see *lanes 5–8*). Reduced mobility of endogenous $G_{i1\alpha}$ also was observed after pertussis toxin treatment. In contrast, the mobility of $G_{i1}/G_{s6\alpha}$ was unaffected by such treatment because it is not a substrate for pertussis toxin. As anticipated from previous results (Milligan *et al.*, 1989), cholera toxin treatment of clone 13 cells resulted in a substantial reduction in total membrane levels of authentic $G_{s\alpha}$, even in cells transiently transfected to express excess $G_{s\alpha}$ (Fig. 9, *bottom*, *lanes 5 and 6*). However, this treatment did not alter membrane levels of $G_{i1}/G_{s6\alpha}$ (Fig. 9, *bottom*, *lanes 1 and 2*) because it also is not a substrate for cholera toxin.

Discussion

Studies on GPCR activation of heterotrimeric G proteins are performed in a number of ways. Probably the two most widely applied methods are to measure agonist-enhanced binding of [^{35}S]GTPγS (Wieland and Jakobs, 1994) or the hydrolysis of the terminal phosphate of [γ^{32}P]GTP (Gierschik *et al.*, 1994). Despite the widespread use of such assays, they have been applied predominantly to GPCRs that couple selectively with members of the pertussis toxin-sensitive family of G_i-like G proteins. For other GPCRs, the effect of agonist often is small compared with the basal signal, resulting in poor specific signal-to-noise ratios. Systems that improve the response from such GPCRs would provide an attractive way to study agonist pharmacology.

Substantial evidence indicates that the extreme carboxyl terminus of a G protein α subunit is a key site for functional contacts with GPCRs. Pertussis toxin-catalyzed ADP-ribosylation of G_i-like G proteins at a conserved cysteine residue four amino acids from the carboxyl terminus of these α subunits (Milligan, 1988) prevents effective functional interactions between GPCRs and these G proteins. The *unc* mutation of $G_{s\alpha}$, which represents a single arginine-to-proline alteration six amino acids from the carboxyl terminus, disallows signal transduction from the β_2-adrenoceptor (Sullivan *et al.*, 1987). Furthermore, anti-peptide antibodies directed toward this region of G proteins often are able to interfere with agonist activation of G proteins (McKenzie and Milligan, 1990a; Shenker *et al.*, 1991). Given the high sequence, and thus probable structural, similarities between individual G protein α subunits, a large number of chimeras have been generated between different G proteins to determine elements of G protein function. Indeed, in recent times, detailed analyses of such chimeras has indicated that only small elements derived from the extreme carboxyl terminus of the G protein α subunit can be sufficient to define receptor specificity (Conklin *et al.*, 1993, 1996; Liu *et al.*, 1995; Gomeza *et al.*, 1996; Ikezu *et al.*, 1996; Komatsuzaki *et al.*, 1997; Tsu *et al.*, 1997). This information has resulted in the use of such chimeric G proteins to transduce signals from GPCRs to second messenger effector systems that may not be their usual ones. This can provide a means to use sensitive assay systems to examine the agonist pharmacology of GPCRs.

We used a chimeric G protein in which the last six amino acids of $G_{i1\alpha}$ were converted to those of $G_{s\alpha}$ ($G_{i1}/G_{s6\alpha}$) to allow effective agonist stimulation of high affinity GTPase activity and [^{35}S]GTPγS binding by the IP prostanoid receptor. Platelets and neuroblastoma X glioma hybrid NG108–15 cells

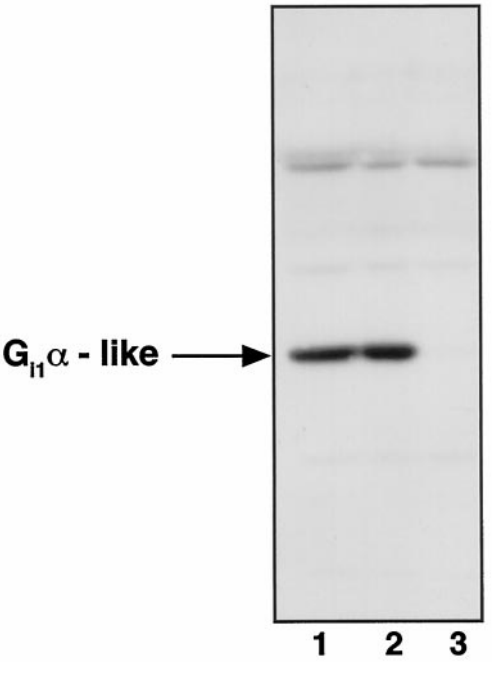

Fig. 5. The enhanced stimulation of high affinity GTPase by iloprost after transfection of $G_{i1}/G_{s6\alpha}$ compared with $G_{i1\alpha}$ is not related to the level of expression. Membranes of clone 13 cells transfected as in Fig. 4 to express $G_{i1\alpha}$ (*1*), $G_{i1}/G_{s6\alpha}$ (*2*), or $G_{s\alpha}$ (*3*) were resolved by SDS-PAGE and immunoblotted with antiserum I1C. $G_{i1\alpha}$ and $G_{i1}/G_{s6\alpha}$ are identical in the region identified by this antiserum and displayed similar levels of expression.

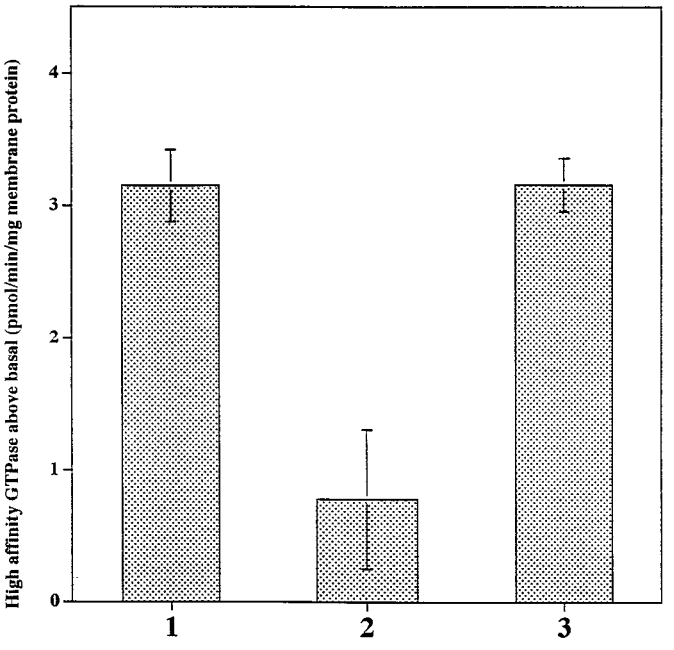

Fig. 6. Cholera toxin pretreatment abolishes iloprost stimulation of high affinity GTPase activity in membranes of clone 13. Clone 13 cells were treated with vehicle (*1*) cholera toxin (200 ng/nl, 16 hr) (*2*), or pertussis toxin (25 ng/ml, 16 hr) (*3*) before harvest. Membranes from these cells then were used to measure basal high affinity GTPase activity and its stimulation by iloprost. Iloprost stimulation of activity was lost in the cholera toxin-treated cells. Data represent mean ± standard deviation of quadruplicate assays from a representative experiment.

have been two of the most studied systems that express this receptor. Although this GPCR is expressed to levels of >1 pmol/mg membrane protein in NG108–15 cells, agonists at this GPCR are unable to stimulate high affinity GTPase activity, although they strongly activate adenylyl cyclase. In contrast, agonists at the δ-opioid receptor, which is expressed in considerably lower levels in these cells, produce a robust GTPase response (Koski and Klee, 1981; McKenzie and Milligan 1990a).

A cDNA encoding the hIPR was FLAG-tagged and then stably expressed in HEK 293 cells. In clone 13, there were good levels of specific [^{3}H]iloprost binding. Because this ligand is an agonist (and no radiolabeled antagonists are currently available), the measured levels of binding can represent only a best estimate. However, using a concentration of [^{3}H]iloprost close to twice its estimated K_d value (Fig. 1a), ~2–3 pmol/mg of membrane protein of the GPCR was detected. In these membranes, an iloprost-mediated stimulation of high affinity GTPase could be measured. However, it represented a small incremental increase compared with the basal activity. This did, however, seem to represent activation of $G_{s\alpha}$ because it was eliminated by pretreatment of the cells with cholera toxin. This conclusion reflects that this toxin eliminates the GTPase activity of G_s by catalyzing the ADP-ribosylation of a key arginine residue, a modification that also can lead to accelerated degradation of this G protein (Milligan *et al.*, 1989). However, by contrast, iloprost-stimulated high affinity GTPase activity and binding of [^{35}S]GTPγS were unaffected by pertussis toxin treatment, indicating the GPCR-stimulated GTPase activity did not result from weak activation of the endogenous pool of G_i-like G proteins.

Transient expression of $G_{i1}/G_{s6\alpha}$ into clone 13 cells allowed effective stimulation of both high affinity GTPase activity and binding of [^{35}S]GTPγS on the addition of iloprost. In contrast, expression of $G_{i1\alpha}$ was unable to mimic these effects. The availability of an antiserum (I1C) that identifies an internal epitope present in both $G_{i1\alpha}$ and $G_{i1}/G_{s6\alpha}$ demonstrated clearly that these were expressed to equal levels and thus that although the FhIPR displayed no capacity to activate $G_{i1\alpha}$, interaction with the expressed $G_{i1}/G_{s6\alpha}$ could be converted into a simple guanine nucleotide exchange-based assay readout.

Cholera toxin treatment would be anticipated to prevent any GPCR-mediated stimulation of the GTPase activity of $G_{s\alpha}$, and pertussis toxin is known to prevent functional contacts between GPCRs and the G_i-like G proteins. Because $G_{i1}/G_{s6\alpha}$ would not be anticipated to act as a substrate for either pertussis or cholera toxin, pretreatment of clone 13 cells transiently transfected to express $G_{i1}/G_{s6\alpha}$ with a combination of both toxins defined that the agonist-induced signal must be generated by the chimeric G protein. Although as noted, in this system no evidence was obtained for an effective interaction of the FhIPR with endogenously expressed G_i-family G proteins and thus the combined cholera and pertussis toxin treatment was not required to isolate and define receptor activation of the chimeric G protein (Fig. 8), this is not always the case. In a number of examples, agonist at GPCRs that do not appear to mediate their primary effector function via activation of pertussis toxin-sensitive G proteins do catalyze measurable GTPase activity or stimulate the binding of [^{35}S]GTPγS. In such studies, pertussis toxin pretreatment indicates much of the signal to have been derived from a minor interaction with G_i-like G proteins (Akam *et al.*, 1997).

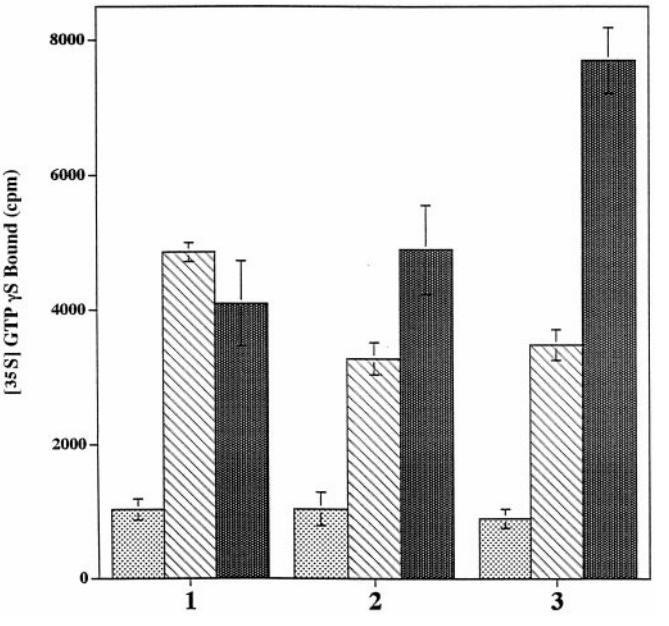

Fig. 7. Coexpression of the FLAG-tagged human IP prostanoid receptor and $G_{i1}/G_{s6\alpha}$ promotes iloprost-stimulated binding of [^{35}S]GTPγS. Membranes were prepared from parental HEK 293 cells (*1*), clone 13 cells (*2*), and clone 13 cells that had been transiently transfected to express $G_{i1}/G_{s6\alpha}$ (*3*). Nonspecific, measured in the presence of 10 μM unlabeled GTPγS (*dots*), basal (*hatched*), and 1 μM iloprost-stimulated (*filled*) binding of [^{35}S]GTPγS was measured as described in Experimental Procedures.

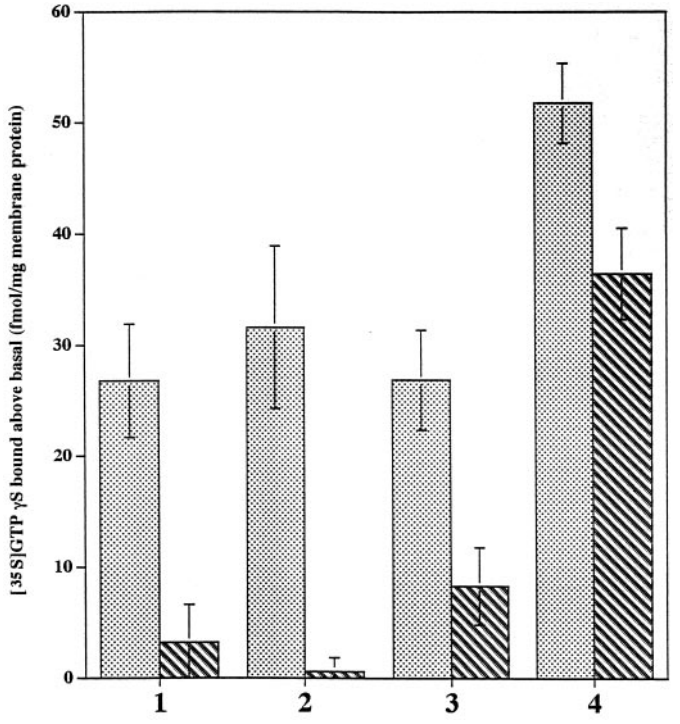

Fig. 8. The effects of cholera and pertussis toxin on iloprost-stimulated [^{35}S]GTPγS binding. Cells of clone 13 stably expressing the FhIPR were mock transfected (*1*) or transiently transfected to express $G_{i1\alpha}$ (*2*), $G_{s\alpha}$ (*3*), or $G_{i1}/G_{s6\alpha}$ (*4*). For the last 16 hr, these cells were exposed to vehicle (*dotted bars*) or a combination of cholera toxin (200 ng/ml) and pertussis toxin (25 ng/ml) (*lined bars*). Membranes were prepared from the cells, and basal and iloprost (1 μM) stimulation of basal [^{35}S]GTPγS binding was assessed. The stimulation above basal produced by iloprost is shown.

The current results demonstrate an effective interaction between the IP prostanoid receptor and the chimeric $G_{i1}/G_{s6\alpha}$ G protein. It remains to be established whether this chimeric G protein also will be effectively activated by other predominantly $G_{s\alpha}$-coupled GPCRs. If so, it should provide a sensitive means to analyze agonist pharmacology at such GPCRs using the type of assays that are widely used in both basic research and screening systems designed to detect novel agonists.

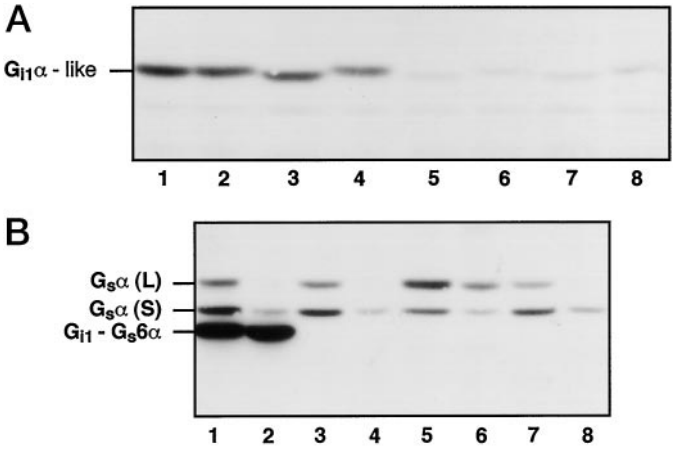

Fig. 9. Sustained treatment of clone 13 cells with cholera and pertussis toxin down-regulates levels of $G_{s\alpha}$ but not of $G_{i1\alpha}$ or $G_{i1}/G_{s6\alpha}$. Clone 13 cells were transfected to express either $G_{i1}/G_{s6\alpha}$ (*1, 2*), $G_{i1\alpha}$ (*3, 4*), or $G_{s\alpha}$ (*5, 6*) or mock transfected (*7, 8*). During the final 16 hr, these cells were exposed to either a combination of cholera toxin and pertussis toxin, as in Fig. 7 (*2, 4, 6,* and *8*), or to vehicle (*1, 3, 5,* and *7*). Membranes prepared from these cells then were immunoblotted with antiserum I1C (*top*) or antiserum CS (*bottom*).

Acknowledgments

We thank Dr. M. Abramovitz (Merck Frosst, Quebec, Canada) for the gift of the human IP prostanoid receptor cDNA.

References

Akam EC, Carruthers AM, Nahorski SR, and Challiss RA (1997) Pharmacological characterization of type 1α metabotropic glutamate receptor-stimulated [^{35}S]-GTPγS binding. *Br J Pharmacol* **121:**1203–1209.
Armstrong RA (1996) Platelet prostanoid receptors. *Pharmacol Ther* **72:**171–191.
Birnbaumer L, Abramowitz J, and Brown AM (1990) Receptor-effector coupling by G proteins. *Biochim Biophys Acta* **1031:**163–224.
Boie Y, Rushmore TH, Darmon-Goodwin A, Grygorczyk R, Slipetz DM, Metters KM, and Abramovitz M (1994) Cloning and expression of a cDNA for the human prostanoid IP receptor. *J Biol Chem* **269:**12173–12178.
Bourne HR, Sanders DA, and McCormick F (1990)The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (Lond)* **348:** 125–132.
Cassel D and Selinger Z (1976) Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochim Biophys Acta* **452:**538–551.
Cassel D and Selinger Z (1977) Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc Natl Acad Sci USA* **74:**3307–3311.
Conklin BR, Farfel Z, Lustig KD, Julius D, and Bourne HR (1993) Substitution of three amino acids switches receptor specificity of Gq *alpha* to that of Gi *alpha*. *Nature (Lond)* **363:**274–276.
Conklin BR, Herzmark P, Ishida S, Voyno-Yasenetskaya TA, Sun Y, Farfel Z, and Bourne HR (1996) Carboxyl-terminal mutations of $G_{q\alpha}$ and $G_{s\alpha}$ that alter the fidelity of receptor activation. *Mol Pharmacol* **50:**885–890.
DeBlasi A, O'Reilly K, and Motulsky HJ (1989) Calculating receptor number from binding experiments using same compound as radioligand and competitor. *Trends Pharmacol Sci* **10:**227–229.
Galbiati F, Guzzi F, Magee AI, Milligan G, and Parenti M (1996) Chemical inhibition of myristoylation of the G-protein Gi1 *alpha* by 2-hydroxymyristate does not interfere with its palmitoylation or membrane association: evidence that palmitoylation, but not myristoylation, regulates membrane attachment. *Biochem J* **313:**717–720.
Gierschik P, Bouillon T, and Jakobs KH (1994) Receptor-stimulated hydrolysis of guanosine 5′-triphosphate in membrane preparations. *Methods Enzymol* **237:**13–26.
Gomeza J, Mary S, Brabet I, Parmentier ML, Restituito S, Bockaert J, and Pin JP (1996) Coupling of metabotropic glutamate receptors 2 and 4 to $G_{\alpha 15}$, G_{α}, and chimeric $G_{\alpha q/i}$ proteins: characterization of new antagonists. *Mol Pharmacol* **50:** 923–930.
Green A, Johnson JL, and Milligan G (1990) Down-regulation of G_i subtypes by prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. *J Biol Chem*. **265:**5206–5210.
Ikezu T, Okamoto T, Komatsuzaki K, Matsui T, Martyn JA, and Nishimoto I (1996) Negative transactivation of cAMP response element by familial Alzheimer's mutants of APP. *EMBO J* **15:**2468–2475.
Kelly E, Keen M, Nobbs P, and MacDermot J (1990) Segregation of discrete GS *alpha*-mediated responses that accompany homologous or heterologous desensitization in two related somatic hybrids. *Br J Pharmacol* **99:**309–316.
Komatsuzaki K, Murayama Y, Giambarella U, Ogata E, Seino S, and Nishimoto I (1997) A novel system that reports the G-proteins linked to a given receptor: a study of type 3 somatostatin receptor. *FEBS Lett* **406:**165–170.
Koski G and Klee WA (1981) Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. *Proc Natl Acad Sci USA* **78:**4185–4189.
Liu J, Conklin BR, Blin N, Yun J, and Wess J (1995) Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation *Proc Natl Acad Sci USA* **92:**11642–11646.
McKenzie FR and Milligan G (1990a) *Delta*-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi2. *Biochem J* **267:**391–398.
McKenzie FR and Milligan G (1990b) Prostaglandin E1-mediated, cyclic AMP-independent, down-regulation of Gs *alpha* in neuroblastoma X glioma hybrid cells *J Biol Chem* **265:**17084–17093.
Merkouris M, Mullaney I, Georgoussi Z, and Milligan G (1997) Regulation of spontaneous activity of the δ opioid receptor: studies of inverse agonism in intact cells. *J Neurochem* **69:**2115–2122.
Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem J* **255:**1–13.
Milligan G and Unson CG (1989) Persistent activation of the α subunit of Gs promotes its removal from the plasma membrane. *Biochem J* **260:**837–841.
Milligan G, Unson CG, and Wakelam MJ (1989) Cholera toxin treatment produces

down-regulation of the *alpha*-subunit of the stimulatory guanine-nucleotide-binding protein (Gs). *Biochem J* **262:**643–649.
Mullaney I and Milligan G (1994) Equivalent regulation of wild type and an epitope-tagged variant of Gs *alpha* by the IP prostanoid receptor following expression in neuroblastoma X glioma hybrid, NG108–15, cells. *FEBS Lett* **353:**231–234.
Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S, and Narumiya S (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature (Lond)* **388:**678–682.
Pierce KL, Gil DW, Woodward DF, and Regan JW (1995) Cloning of human prostanoid receptors. *Trends Pharmacol Sci* **16:**253–256.
Shenker A, Goldsmith P, Unson CG, and Spiegel AM (1991) The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem* **266:**9309–9313.
Sullivan KA, Miller RT, Masters SB, Beiderman B, Heideman W, and Bourne HR (1987) Identification of receptor contact site involved in receptor-G protein coupling. *Nature (Lond)* **330:**758–760.
Tsu RC, Ho MK, Yung LY, Joshi S, and Wong YH (1997) Role of amino- and carboxyl-terminal regions of $G_{\alpha Z}$ in the recognition of G_i-coupled receptors. *Mol Pharmacol* **52:**38–45.
Voyno-Yasenetskaya T, Conklin BR, Gilbert RL, Hooley R, Bourne HR, and Barber DL (1994) G *alpha* 13 stimulates Na-H exchange. *J Biol Chem* **269:**4721–4724.
Wieland T and Jakobs KH (1994) Measurement of receptor-stimulated guanosine 5′-O-(γ-thio)triphosphate binding by G proteins. *Methods Enzymol* **237:**3–13.
Williams RJ and Kelly E (1994) Gs *alpha*-dependent and -independent desensitisation of prostanoid IP receptor-activated adenylyl cyclase in NG108–15 cells. *Eur J Pharmacol* **268:**177–186.
Wise A, Watson-Koken MA, Rees S, Lee M, and Milligan G (1997) Interactions of the *alpha*2A-adrenoceptor with multiple Gi-family G-proteins: studies with pertussis toxin-resistant G-protein mutants. *Biochem J* **321:**721–728.

Send reprint requests to: Dr. Graeme Milligan, Davidson Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, Scotland. E-mail: g.milligan@bio.gla.ac.uk