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Regulation of G Protein Activation and Effector Modulation by Fusion Proteins between the Human 5-Hydroxytryptamine$_{1A}$ Receptor and the α Subunit of $G_{i1}\alpha$: Differences in Receptor-Constitutive Activity Imparted by Single Amino Acid Substitutions in $G_{i1}\alpha$

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ABSTRACT

Fusion proteins were generated between the human 5-hydroxytryptamine (5-HT)$_{1A}$ receptor and both wild-type (Cys^{351}) and pertussis toxin-resistant (Gly^{351} and Ile^{351}) forms of G_{i1}. These were expressed stably. Pertussis toxin treatment substantially reduced basal high-affinity GTPase activity in clones expressing the 5-HT$_{1A}$ receptor wild-type $G_{i1}\alpha$ construct but not in clones expressing 5-HT$_{1A}$ receptor (Gly^{351})$G_{i1}\alpha$ or (Ile^{351})$G_{i1}\alpha$. Spiperone functioned as an inverse agonist in membranes expressing the 5-HT$_{1A}$ receptor wild-type $G_{i1}\alpha$ fusion protein and in those expressing 5-HT$_{1A}$ receptor (Ile^{351})$G_{i1}\alpha$ but not the 5-HT$_{1A}$ receptor (Gly^{351})$G_{i1}\alpha$ fusion protein. The effect of spiperone at the 5-HT$_{1A}$ receptor wild-type $G_{i1}\alpha$ construct but not the 5-HT$_{1A}$ receptor (Ile^{351})$G_{i1}\alpha$ construct was blocked by pertussis toxin treatment. By contrast, agonists functioned with equal effectiveness at the three fusion proteins and were unaffected by pertussis toxin treatment of the (Ile^{351})$G_{i1}\alpha$- and (Gly^{351})$G_{i1}\alpha$-containing constructs. 5-HT resulted in strong inhibition of forskolin-amplified adenylyl cyclase in intact cells expressing the isolated 5-HT$_{1A}$ receptor. In fusion protein-expressing cells, 5-HT-mediated inhibition of adenylyl cyclase was also observed. Pertussis toxin treatment obliterated 5-HT-mediated inhibition in cells expressing the isolated receptor and the 5-HT$_{1A}$ receptor wild-type $G_{i1}\alpha$ fusion protein but not in those expressing the 5-HT$_{1A}$ receptor (Ile^{351}) or (Gly^{351})$G_{i1}\alpha$ fusion proteins. These studies demonstrate that alteration of a single amino acid in $G_{i1}\alpha$ located at a key contact site between the G protein and a G protein-coupled receptor can regulate agonist-independent constitutive activity of the G protein-coupled receptor and that fusion proteins can directly regulate adenylyl cyclase.

5-hydroxytryptamine (5-HT) mediates a wide range of physiological actions via activation of a large family of receptors. With the exception of the 5-HT$_3$ receptor, which is an intrinsic cation channel, all of the receptors for 5-HT are members of the superfamily of seven transmembrane element, G protein-coupled receptors (GPCRs). A highly studied member of this family is the 5-HT$_{1A}$ receptor, which is expressed both presynaptically on serotinergic nerve bodies, where it functions as an autoreceptor to dampen neuronal activity, and postsynaptically in many locations in the central nervous system to which serotinergic neurones project. Signal transduction from this GPCR is mediated predominantly via activation of members of the pertussis toxin-sensitive family of G_i-like G proteins and involves inhibitory regulation of adenylyl cyclase as well as modulation of the activity of a series of ion channels (Julius, 1998). Considerable interest has been accorded pharmacological studies indicating a likely role for this GPCR in the regulation of anxiety states, and the recent production of mice lacking expression of this GPCR has confirmed these ideas (Heisler et al., 1998; Ramboz et al., 1998).

In a series of studies designed to address details of the interactions of GPCRs and G proteins, we have recently constructed a number of fusion proteins in which the N terminus of a G protein α subunit was linked directly to the C-terminal tail of a GPCR (Wise and Milligan, 1997; Wise et al., 1997a,b, 1999). Some of the constructs we have examined in detail have utilized the α_{2A} adrenoceptor (Wise and Milligan, 1997; Wise et al. 1997a,b). This GPCR is well estab-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GPCR, G protein-coupled receptor; MPPF, 4(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; OH-DPAT, hydroxy-2-(di-*n*-propylamino)tetralin; PCR, polymerase chain reaction; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis.

lished to function predominantly via members of the G_i family of G proteins. After stable expression of an α_{2A} adrenoceptor-$G_{i1}\alpha$ fusion protein in which the G protein was altered by conversion of cysteine^{351} to glycine to render it insensitive to the actions of pertussis toxin, we noted that although inhibition of adenylyl cyclase could be produced by α_{2A} adrenoceptor agonists in untreated cells, this was abolished after pertussis toxin treatment and thus could not have been mediated via the G protein linked to the GPCR (Burt et al., 1998).

In the present study, we have generated fusion proteins between the human 5-HT$_{1A}$ receptor and both wild-type (Cys^{351}) and pertussis toxin-insensitive (Gly^{351} and Ile^{351}) forms of $G_{i1}\alpha$. After stable expression of these constructs in human embryonic kidney (HEK)293 cells, ligand-mediated modulation of their GTPase activity and regulation of adenylyl cyclase activity were examined. As point mutation of residue^{351} of $G_{i1}\alpha$ can alter both the maximal effectiveness of agonist ligands (Bahia et al., 1998; Carr et al., 1998) and the relative intrinsic activity of different agonists (Jackson et al., 1999) we wished to assess how agonist-independent, constitutive activity might be modified by mutation of this residue, which lies within a key GPCR-G protein contact domain. We note marked constitutive activity of the 5-HT$_{1A}$ receptor wild-type $G_{i1}\alpha$ fusion protein and the 5-HT$_{1A}$ receptor (Ile^{351})-$G_{i1}\alpha$ fusion protein, but not the 5-HT$_{1A}$ receptor (Gly^{351})-$G_{i1}\alpha$ fusion protein, which could be inhibited by the inverse agonist spiperone. Confirmation that the constitutive activity of the 5-HT$_{1A}$ receptor (Ile^{351}) $G_{i1}\alpha$ fusion protein was inherently derived from intramolecular interactions of the two elements of the fusion protein was provided by the inability of pertussis toxin treatment to prevent spiperone-mediated inhibition of GTPase activity at this construct. These studies provide the first demonstration that single amino acid alterations in a G protein can significantly alter agonist-independent constitutive activity of a GPCR. Furthermore, we also record clear agonist-induced inhibitory regulation of adenylyl cyclase activity, which proceeds via the G protein of the fusion constructs.

Experimental Procedures

Materials. All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Strathclyde, UK). Both the 5-HT$_{1A}$ receptor antagonist [^{3}H]4(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine (MPPF; 78.3 Ci/mmol) and [γ-^{32}P]GTP (30 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Cholera toxin and pertussis toxin were purchased from Sigma (St. Louis, MO). Oligonucleotides were purchased from Cruachem (Glasgow, Strathclyde, UK). All other chemicals were obtained from Sigma and Boehringer Mannheim (Mannheim, Germany).

Construction of Plasmids Encoding 5HT$_{1A}$, 5HT$_{1A}$-G$_{i1}\alpha$ Fusion Proteins. The human 5HT$_{1A}$ receptor clone in pSP64 (a gift from Glaxo-Wellcome, Stevenage, UK) was digested with *Xba*I/*Bam*HI and the resulting 1.5-kb fragment ligated to pcDNA3. To obtain the open reading frame of 1.3 kb, polymerase chain reaction (PCR) was carried out using the following primers to introduce a *Hin*dIII restriction site at the 5′ end and to remove the stop codon and introduce a *Bam*HI restriction site at the 3′ end, respectively: 5′-CTGAAGCTTATGGATGTGCTCAGCCCTGGTC-3′; 5′-CTGGGATCCCTGGCGGCAGAAGTTACACTTAATG-3′ (restriction enzyme sites underlined). The PCR fragment was digested with *Hin*dIII and *Bam*HI and ligated into pcDNA3 to make the plasmid p5HT. To link the $G_{i1}\alpha$ wild-type (Cys^{351}) cDNA to the 5HT$_{1A}$ receptor sequence, PCR was carried out on $G_{i1}\alpha$ to produce compatible restriction sites. The oligonucleotides used to do this were 5′-CTGGGATCCGGCTGCACACTGAGCGCTGAG-3′ at the 5′ end and 5′-GAGAATTCTTAGAAAGAGACCACAGTC-3′ for the 3′ end. The plasmid p5HT was digested with *Bam*HI/*Eco*RI as was the $G_{i1}\alpha$ PCR fragment and the two were ligated to give the plasmid p5HTGi1. To construct the 5HT$_{1A}$-(Gly^{351})$G_{i1}\alpha$ fusion plasmid (Gly^{351})$G_{i1}\alpha$ in PBS was digested with *Sac*II/*Eco*RI and the 730-bp fragment was used to replace the corresponding fragment in p5HTGi1. An equivalent strategy was used to produce 5HT$_{1A}$-(Ile^{351})$G_{i1}\alpha$. The constructs were then sequenced to verify the DNA sequence.

Cell Culture and Stable Expression. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum, and 2 mM L-glutamine. Cells were seeded into 100-mm culture dishes and grown to 60 to 80% confluence (18–24 h) before transfection with 5 μg of appropriate cDNAs using *N*-[1-(2,3-dideoxyloxy)propyl]-*N,N,N*-trimethyl ammonium methyl sulphate reagent (Boehringer Mannheim). Forty-eight hours after transfection, the cells were split 1:4 into 800 μg/ml G418 sulfate (Calbiochem, La Jolla, CA) containing medium. A 100-mm dish of untransfected HEK293 cells was also split into the same medium as a control dish. About 1 week later, after all the cells in the control dish had died, G418-resistant cells in the transfected dishes were picked and transferred into 24-well plates using autoclaved pipette tips. About 20 clones of each cDNA were picked and grown in 1 ml/well of G418 medium (400 μg/ml). Clones were amplified, membrane preparations made, and their binding of [^{3}H]MPPF determined.

Preparation of Membranes. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80°C after harvesting. Cell pellets were resuspended in TE 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 (2000 rpm) in a refrigerated microcentrifuge. The supernatant fraction was then centrifuged at 75,000 rpm for 30 min in a Beckman Optima TLX Ultracentrifuge with a TLA100.2 rotor. The pellets were resuspended in TE buffer to a final protein concentration of 1 to 3 mg/ml and stored at −80°C until required.

[^{3}H]MPPF Binding Studies. Binding assays were performed by adding 5 to 10 μg of membrane protein to an assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1% ascorbic acid, 10 μM pargyline, pH 7.4) containing [^{3}H]MPPF (0.25–20 nM). Nonspecific binding was determined in parallel in the presence of 100 μM 5-HT. Samples were incubated at 30°C for 40 min and then terminated by rapid filtration through GF/C filters. The filters were washed 3 times with 5 ml of ice-cold wash buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) and then counted.

High-Affinity GTPase Assays. The assays were performed as described by McKenzie and Milligan (1990) with modifications as described by Wise et al. (1997a,b) to allow measurement of V_{max} in a number of cases. In such studies concentrations of GTP used as substrate ranged from 22 nM to 2 μM.

Intact Cell Adenylyl Cyclase Assays. Intact cell adenylyl cyclase assays were performed essentially as described by Wong (1994) and Merkouris et al. (1997). Cells were split into wells of a 24-well plate and incubated in medium containing [^{3}H]adenine at 0.5 μCi/well for 16 to 24 h. The generation of [^{3}H]cAMP in response to ligands was then assessed in the presence of the nonselective cAMP phosphodiesterase inhibitor isobutylmethylxanthine (1 mM).

Miscellaneous. All experiments were performed on a minimum of three occasions using cells or membrane preparations derived from different cell passages. Where appropriate data are presented as means ± S.E.M.

Results

PCR was used to amplify a cDNA encoding the human 5-HT_{1A} receptor and in the process both remove the stop codon and introduce a *Bam*HI restriction site in this region. This amplified cDNA was ligated to cDNAs encoding full-length forms of either wild-type (Cys^{351})$G_{i1}\alpha$ or either Gly^{351} or Ile^{351}, pertussis toxin-resistant, forms of this G protein, which we have described previously (Wise et al., 1997c; Bahia et al., 1998). These had been amplified to remove the initiator codon and introduce a *Bam*HI restriction site at the 5′ end (Fig. 1). The ligated products were fully sequenced and were predicted to encode single open reading frames of 776 amino acids. Both these fusion constructs and the isolated 5-HT_{1A} receptor were expressed stably in HEK293 cells and a number of individual clones isolated based on their capacity to specifically bind the selective antagonist ligand [^{3}H]MPPF. Membrane preparations from cells either untreated or pretreated with pertussis toxin (25 ng/ml, 16 h) and expressing the isolated receptor or either wild-type- or Gly^{351} $G_{i1}\alpha$-containing fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with an antiserum, I1C (Green et al., 1990), which identifies an epitope between amino acids 159 and 168 of $G_{i1}\alpha$. All of these membranes expressed a 41-kDa polypeptide that comigrated with an I1C-reactive polypeptide from rat brain cortex, a particularly rich source of $G_{i1}\alpha$ (Fig. 2, bottom). Prior pertussis toxin treatment slightly slowed the mobility of this polypeptide through SDS-PAGE, as demonstrated for other systems (Wise et al., 1997a,c), a feature associated with the ADP-ribosylation of this and other pertussis toxin-sensitive G proteins (Fig. 2, bottom). Membranes expressing either of these 5-HT_{1A} receptor-containing fusion proteins, but not the isolated receptor, also contained I1C-reactive polypeptides of apparent M_r of 85 to 90 kDa, consistent with expression of the fusion proteins (Fig. 2, top). Alterations in mobility of the fusion protein containing the wild-type form of the G protein in response to pertussis toxin treatment could not be observed. We believe this to reflect the large size of the fusion construct and thus its mobility through SDS-PAGE rather than its inability to be modified by pertussis toxin (see below).

Membranes from clones expressing each of the three fusion proteins were used to perform saturation binding studies with [^{3}H]MPPF (Fig. 3 and data not shown). These indicated that each clone selected for detailed study expressed high levels of an apparently single population of [^{3}H]MPPF binding sites with B_{max} in the region of 10 pmol/mg membrane protein and a K_d for [^{3}H]MPPF of 2.7 ± 0.2 nM. Prior treatment with pertussis toxin did not modify these parameters substantially (Fig. 3).

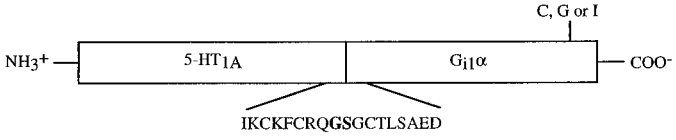

Fig. 1. Construction of human 5-HT_{1A} receptor-$G_{i1}\alpha$ fusion proteins. Fusion protein cDNAs were constructed between the human 5HT_{1A}-receptor and either wild-type (Cys^{351}) (C) or pertussis toxin-resistant (Gly^{351})(G) and (Ile^{351})(I) form of $G_{i1}\alpha$ as described in *Experimental Procedures*. For convenience of construction this resulted in the addition of two amino acids (in bold) as a linker which are not present in either the isolated receptor or G protein.

Addition of 100 μM 5-HT to membranes of cells expressing each of the fusion proteins allowed stimulation of high-affinity GTPase activity. To explore these processes in detail, 5-HT stimulation of high-affinity GTPase activity was examined at a range of GTP concentrations. In membranes expressing the 5-HT_{1A} receptor (Gly^{351})$G_{i1}\alpha$ fusion protein the agonist produced stimulation of high GTPase activity over a range of nucleotide concentrations (20 nM–2 μM) and transformation of such data to Eadie-Hofstee plots demonstrated the agonist to produce a large increase in V_{max} without alteration in K_m for GTP (Fig. 4A). Prior pertussis toxin treatment of these cells did not significantly reduce basal high-affinity GTPase activity and had little effect on 5-HT stimulation of activity (Fig. 4A). By contrast, pertussis toxin pretreatment greatly reduced basal high-affinity GTPase activity in the 5-HT_{1A} receptor wild-type $G_{i1}\alpha$ fusion protein-expressing membranes (Fig. 4B) and substantially inhibited, but did not fully block, the capacity of 5-HT to stimulate this activity (Fig. 4B). Such data infer that the signal observed

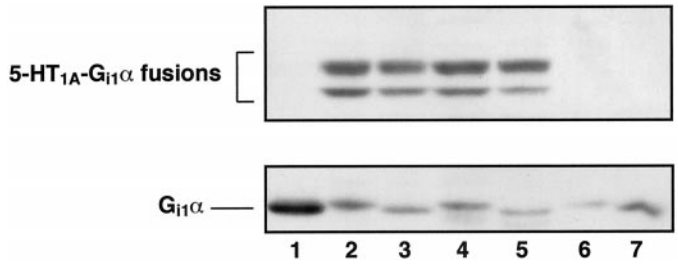

Fig. 2. Immunodetection of 5-HT_{1A} receptor-$G_{i1}\alpha$ fusion proteins. Membranes from rat brain cortex (1), untreated (3,5,7), or pertussis toxin-treated (2,4,6) cells stably expressing 5HT_{1A}-receptor wild-type $G_{i1}\alpha$ (2–3), 5HT_{1A}-receptor-(Gly^{351})$G_{i1}\alpha$ (4–5) or the isolated 5HT_{1A}-receptor (6–7) were resolved by SDS-PAGE and immunoblotted with antiserum I1C (Green et al., 1990), which identifies amino acids 159 to 168 of $G_{i1}\alpha$. Top, detection of the fusion proteins. Bottom, detection of endogenous $G_{i1}\alpha$.

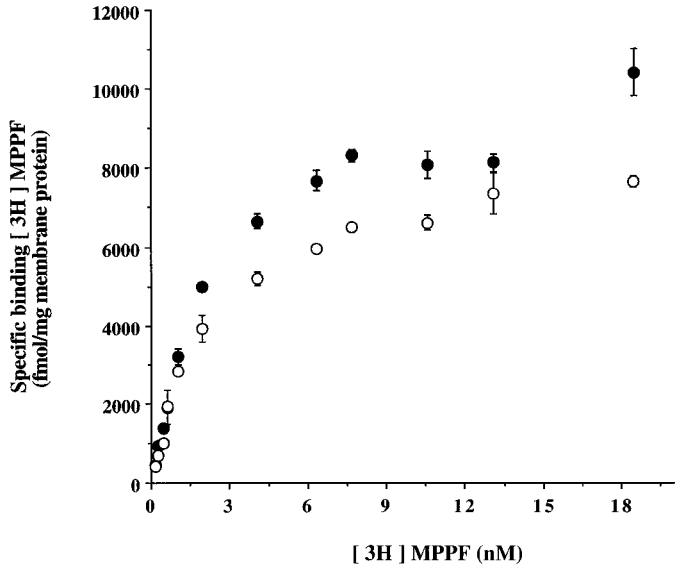

Fig. 3. Binding studies with [^{3}H]MPPF. The specific binding of varying concentration of [^{3}H]MPPF was assessed, as described in *Experimental Procedures*, in membranes of untreated (open symbols) and pertussis toxin-treated (filled symbols) cells expressing the fusion protein between the human 5HT_{1A}-receptor and (Gly^{351})$G_{i1}\alpha$. Similar experiments were performed in membranes of each of the clonal cell lines used in these studies to assess levels of expression of the recombinant proteins.

after pertussis toxin treatment in cells expressing the 5-HT$_{1A}$ receptor (Gly^{351})G$_{i1}\alpha$ fusion protein must represent activation of the linked (Gly^{351})G$_{i1}\alpha$ of the fusion protein and imply little or no activation of the endogenously expressed forms of G$_i$ by this fusion protein. By contrast, the effects of pertussis toxin in membranes derived from cells expressing the 5-HT$_{1A}$ receptor fusion protein containing the wild-type G protein suggest both constitutive activity of this construct and the maintenance of a level of 5-HT-mediated functional activation of the linked G protein even when ADP-ribose has been attached to Cys^{351}.

To explore both these differences in greater detail, membranes were prepared from cells expressing each of the fusion proteins, both with and without prior pertussis toxin treatment. The effects of toxin treatment on basal and ligand regulation of high-affinity GTPase activity were then explored using the agonists 5-HT, 7-hydroxy-2-(di-*n*-propylamino)tetralin (7-OH DPAT) and 8-OH DPAT. Each of the agonists stimulated high-affinity GTPase activity to similar extents at maximally effective concentrations in untreated cells (Fig. 5 and data not shown). As anticipated from the data of Fig. 4, the effects of each of the agonists were unaffected by pertussis toxin treatment of cells expressing the 5-HT$_{1A}$ receptor-(Gly^{351})G$_{i1}\alpha$ (Fig. 5) and -(Ile^{351})G$_{i1}\alpha$ (data not shown) fusion proteins and were blunted but not eliminated in cells expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein (Fig. 5).

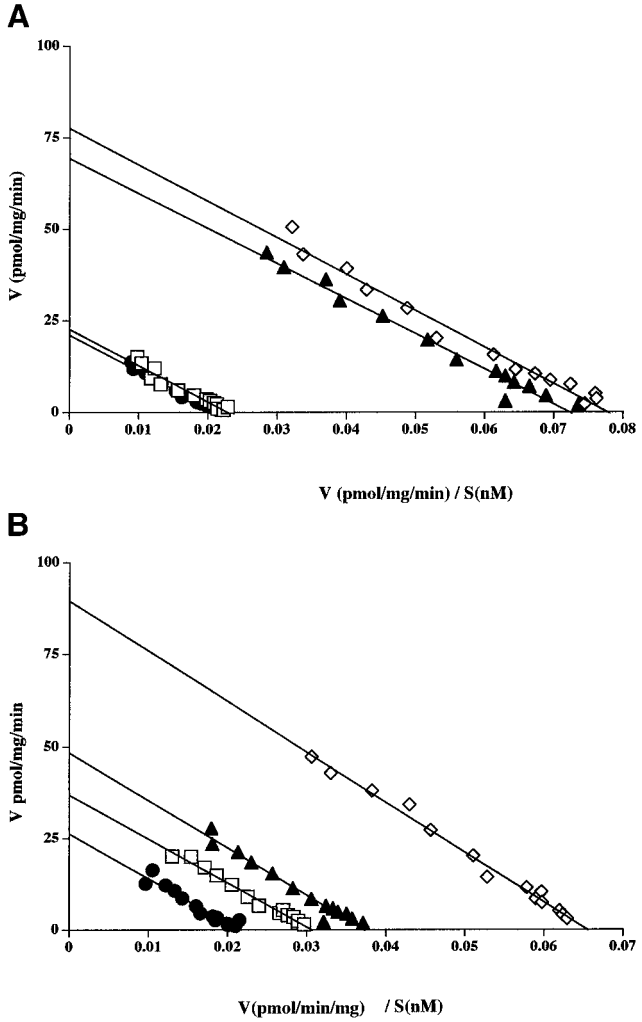

Fig. 4. 5-HT stimulation of GTPase activity. Effects of pertussis toxin. Membranes of either untreated (□, ◇) or pertussis toxin-pretreated (●, ▲) cells expressing fusion proteins between the human 5HT$_{1A}$ receptor and either (Gly^{351}) G$_{i1}\alpha$ (4A) or wild-type (Cys^{351}) G$_{i1}\alpha$ (4B) were analyzed for basal high-affinity GTPase activity (●, □) and its regulation by 100 μM 5-HT (◇, △) at varying concentrations of GTP. Data so generated were converted to Eadie-Hofstee plots to analyze effects on K_m for GTP hydrolysis and V_{max}.

The ligand spiperone has been described as an inverse agonist at the human 5-HT$_{1A}$ receptor (Barr and Manning, 1997; Newman-Tancredi et al., 1997a). At high concentrations spiperone clearly functioned as an inverse agonist in untreated membranes expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein (Fig. 6A). No further effect of spiperone could be recorded, however, after pertussis toxin treatment of these cells (Fig. 6A), which as noted also in Fig. 4B reduced basal GTPase activity significantly. By contrast, no effect of spiperone as an inverse agonist could be measured in either in untreated or pertussis toxin-treated membranes expressing the 5-HT$_{1A}$ receptor-(Gly^{351})G$_{i1}\alpha$ fusion protein (Fig. 6A), a feature consistent with the inability of pertussis toxin treatment to reduce basal high-affinity GTPase in membranes of these cells (Fig. 4A). In untreated membranes expressing the 5-HT$_{1A}$ receptor-(Ile^{351})G$_{i1}\alpha$ fusion protein spiperone again displayed marked inverse agonism (Fig. 6A). As the basal GTPase activity of these membranes was unaffected by pertussis toxin treatment (Fig. 6A), spiperone still functioned as an inverse agonist after toxin treatment (Fig. 6A). These results confirmed that the constitutive GTPase activity in the 5-HT$_{1A}$ receptor-(Ile^{351})G$_{i1}\alpha$ fusion protein expressing membranes, which was suppressed by spiperone, directly reflected activity derived from intramolecular interactions between the GPCR and G protein elements of the fusion protein. As with the stimulatory effects of 5-HT on the fusion proteins described above, the inhibitory effect of spiperone on the basal GTPase activity of the 5-HT$_{1A}$ receptor-(Ile^{351})G$_{i1}\alpha$ fusion protein reflected a decrease in V_{max} (Fig. 6B). Further analysis of these effects were produced by use of a range of concentrations of these ligands in membranes expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein (Fig. 7). 5-HT (EC_{50} = 1.0 ± 0.4 × 10^{-8} M, nH = 0.60) and 8-OH DPAT (EC_{50} = 3.3 ± 1.1 × 10^{-8} M, nH = 0.57) displayed similar potencies to activate the high-affinity GTPase activity of this construct, whereas 7-OH DPAT displayed a significantly lower potency (EC_{50} = 1.8 ± 0.8 × 10^{-6} M, nH = 0.47). The inverse agonist effects of spiperone were produced with EC_{50} of 7.1 ± 2.4 × 10^{-8} M and nH = 1.09. After pertussis toxin treatment not only were the maximal effects of the agonists blunted but required substantially higher ligand concentrations (Fig. 7). Because of the differences in effects of both pertussis toxin treatment and spiperone on the high-affinity GTPase activity of membranes expressing the various GPCR-G protein fusion proteins, equivalent experiments were performed on separate clones expressing each construct. Equivalent results to those described above were recorded in each case (data not shown).

Comparison of the effects of 5-HT to stimulate high-affinity GTPase activity via the isolated 5-HT$_{1A}$ receptor and each of the fusion proteins demonstrated highly similar potencies (EC_{50} = 6 × 10^{-8} M − 2 × 10^{-7} M) and slope coefficients (nH = 0.52–0.64) of the agonist (Fig. 8). Although there was no detectable ability of 5-HT to stimulate high-affinity GT-

Pase activity via the isolated receptor after pertussis toxin treatment (Fig. 8), and no alteration in function of 5-HT was recorded at the 5-HT_{1A} receptor-(Gly^{351})$G_{i1}\alpha$ fusion protein (EC_{50} = 6.5 ± 2.0 × 10^{-8} M, nH = 0.62), a clear reduction in effectiveness and potency (EC_{50} = 1.2 ± 0.2 × 10^{-6} M) was recorded for 5-HT at the 5-HT_{1A} receptor wild-type $G_{i1}\alpha$ fusion protein (Fig. 8).

The 5-HT_{1A} receptor is well established as a member of the family of GPCRs able to cause inhibition of adenylyl cyclase activity. In intact cells expressing the isolated 5-HT_{1A} recep-

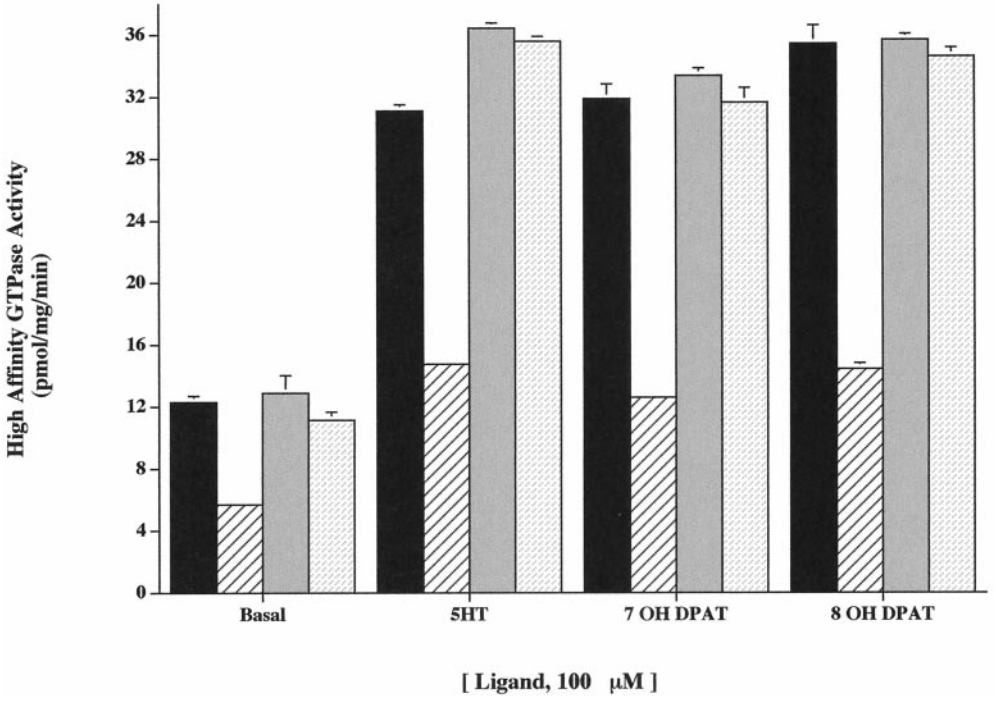

Fig. 5. Pharmacology of regulation of GTPase activity. Basal high-affinity GTPase activity in membranes of untreated (filled columns) or pertussis toxin treated (hatched columns) cells and its regulation by a range of ligands (all at 100 μM) was assessed for fusion proteins between the human 5HT_{1A} receptor and either wild-type $G_{i1}\alpha$ (dark and bold hatched columns) or (Gly^{351})$G_{i1}\alpha$ (light and light hatched columns).

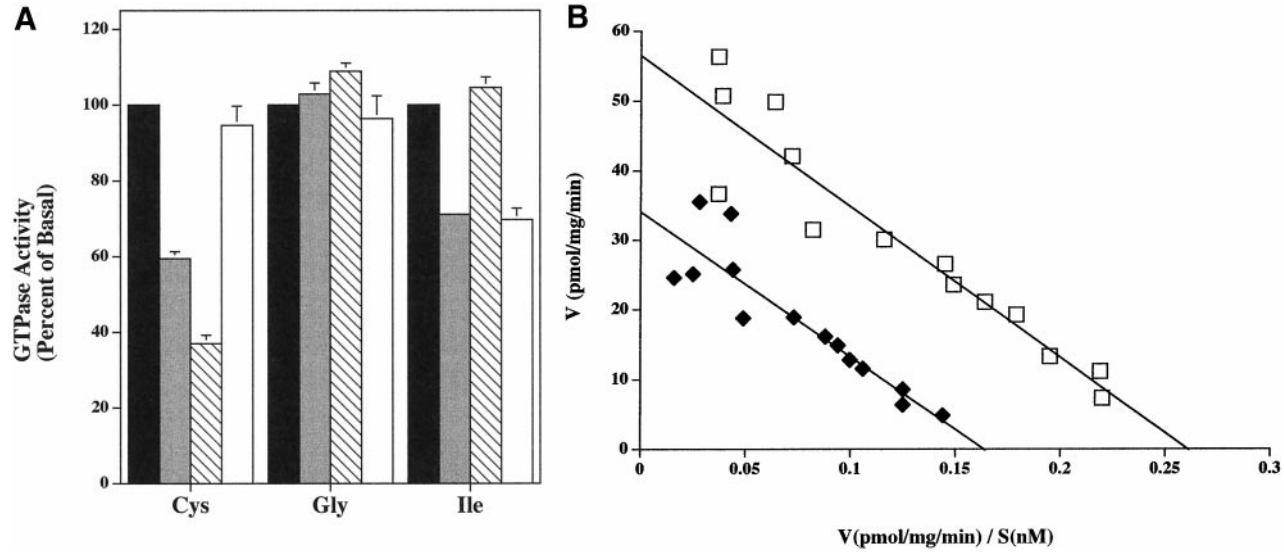

Fig. 6. Differential regulation of basal GTPase activity by pertussis toxin and spiperone in membranes expressing various 5-HT_{1A}-$G_{i1}\alpha$ fusion proteins. A, membranes were prepared from untreated (filled columns) and pertussis toxin-pretreated (25 ng/ml, 24 h) (open columns) cells expressing 5-HT_{1A}-$G_{i1}\alpha$ fusion proteins in which residue^{351} of the G protein was Cys, Gly, or Ile. Basal GTPase activity and its regulation by 10 μM spiperone was measured. Basal activity in untreated membranes expressing each construct is displayed as 100% (dark filled columns). The effects of spiperone (light filled columns) and pertussis toxin treatment (hatched columns) on basal activity are presented as a percentage of the activity remaining. The effects of spiperone after pertussis toxin treatment (open columns) are presented as a percentage of the basal activity after pertussis toxin treatment, i.e., of the data in cross hatched columns. There was no significant effect of spiperone in membranes expressing the Gly-containing fusion, whereas the inverse agonist effect of spiperone was still observed after pertussis toxin treatment of cells expressing the Ile containing fusion. By contrast the effect of spiperone was abolished by pertussis toxin treatment in membranes expressing the Cys-containing fusion. Data represent means ± S.E. from three individual experiments. In a representative experiment basal high-affinity GTPase activity (measured at 0.5 μM GTP) was 27.8 ± 0.6 pmol/min/mg protein in the (Cys^{351})$G_{i1}\alpha$ fusion protein expressing membranes, 10.3 ± 0.6 pmol/min/mg protein in those expressing the (Gly^{351})$G_{i1}\alpha$ fusion protein, and 20.6 ± 0.6 pmol/min/mg protein in those expressing the (Ile^{351})$G_{i1}\alpha$ fusion protein. Equivalent results were observed in membranes from either two (Cys, Gly) or three (Ile) individual clones expressing each construct. B, basal high-affinity GTPase activity (□) in untreated membranes expressing the 5-HT_{1A}-(Ile^{351})-$G_{i1}\alpha$ fusion protein and its regulation by 10 μM spiperone (♦) were measured at varying concentrations of GTP as in Fig. 4. Data were converted to Eadie-Hofstee plots. Three additional experiments produced similar results.

tor, forskolin (50 μM)-amplified adenylyl cyclase activity was inhibited in a concentration-dependent manner with EC_{50} of $1.2 \pm 0.6 \times 10^{-9}$ M. This was obliterated after pertussis toxin treatment of the cells (Fig. 9). In cells expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein, 5-HT produced a profound, virtually complete, inhibition of forskolin-amplified adenylyl cyclase activity with $EC_{50} = 6.4 \pm 0.4 \times 10^{-9}$ M, which again was fully prevented by pertussis toxin treatment (Fig. 10A). 5-HT displayed similar potency ($EC_{50} = 3.7 \pm 0.2 \times 10^{-9}$ M) to mediate inhibition of forskolin-amplified adenylyl cyclase activity via the 5-HT$_{1A}$ receptor-(Ile^{351})G$_{i1}\alpha$ fusion protein. However, now prior pertussis toxin treatment did not modify the effect of the agonist (Fig. 10B). Similar results were produced in cells expressing the 5-HT$_{1A}$ receptor-(Gly^{351})G$_{i1}\alpha$ fusion protein except that the potency of 5-HT was some 10-fold lower ($EC_{50} = 5.5 \pm 0.6 \times 10^{-8}$ M) but again pertussis toxin treatment did not substantially modify the inhibition (Fig. 10C). A surprising feature of adenylyl cyclase inhibition in cells expressing the fusion proteins containing both (Ile^{351})G$_{i1}\alpha$ and (Cys^{351})G$_{i1}\alpha$ was the apparent cooperativity of the effects of 5-HT. In both cases the concentration-effect curves had pseudo Hill coefficients between 1.5 and 2.0.

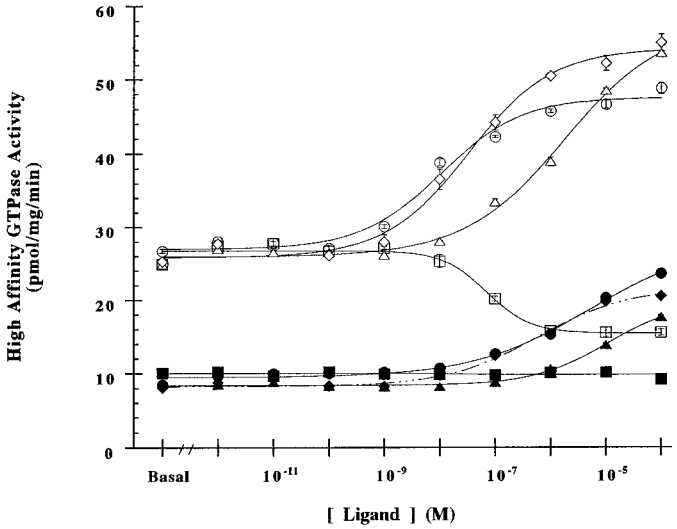

Fig. 7. Concentration-response curves for ligand regulation of GTPase activity of a 5-HT$_{1A}$ wild-type G$_{i1}\alpha$ fusion protein. The capacity of varying concentrations of 5-HT (circles), spiperone (squares), 7-OH DPAT (triangles), and 8-OH DPAT (diamonds) to regulate basal high-affinity GTPase activity was assessed in membranes of untreated (open symbols) and pertussis toxin-treated (filled symbols) cells expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein.

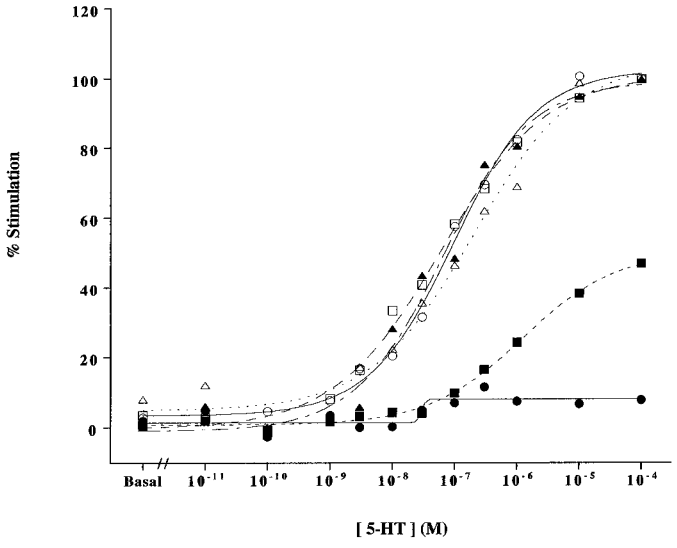

Fig. 8. Potency of 5-HT to stimulate high-affinity GTPase in the various constructs. The potency of 5-HT to stimulate high-affinity GTPase activity was assessed in membranes of untreated (open symbols) and pertussis toxin-treated (filled symbols) cells stably expressing the isolated 5-HT$_{1A}$-receptor (circles) and fusion proteins with either wild-type (squares) or pertussis toxin-resistant (Gly^{351}) (triangles) G$_{i1}\alpha$. Data are presented as the percentage of the effect produced by 100 μM 5-HT in untreated cells expressing each form of the receptor.

Discussion

In the current study, we have used the strategy of constructing and stably expressing fusion proteins between a GPCR and a G protein α subunit to promote and explore interactions between the human 5-HT$_{1A}$ receptor and forms of the G protein G$_{i1}\alpha$. To do so we used both wild-type G$_{i1}\alpha$ and mutationally modified (Gly^{351}, Ile^{351}) forms of the G protein, which have previously been characterized extensively (Wise et al., 1997a; Bahia et al., 1998; Jackson et al., 1999), and are resistant to ADP ribosylation catalyzed by pertussis toxin. Each of these three fusion proteins, as well as the isolated 5-HT$_{1A}$ receptor, were expressed stably in HEK293 cells before functional analysis. In part, these studies were designed to build on our recent analyses of a stably expressed α_{2A} adrenoceptor-(Gly^{351}) G$_{i1}\alpha$ fusion protein (Burt et al., 1998). However, the current studies have produced a significant range of differences from, as well as some similarities with, the previous studies. The most important of these is the clear demonstration that alteration of a single amino acid in the G protein can result in substantial differences in the constitutive activity of the GPCR-G protein fusion protein.

Identification of expression of the 5-HT$_{1A}$ receptor-G$_{i1}\alpha$ fusion proteins was achieved in two ways, first in ligand-binding studies using the selective 5-HT$_{1A}$ receptor antagonist [^{3}H]MPPF. Such studies allowed selection for detailed

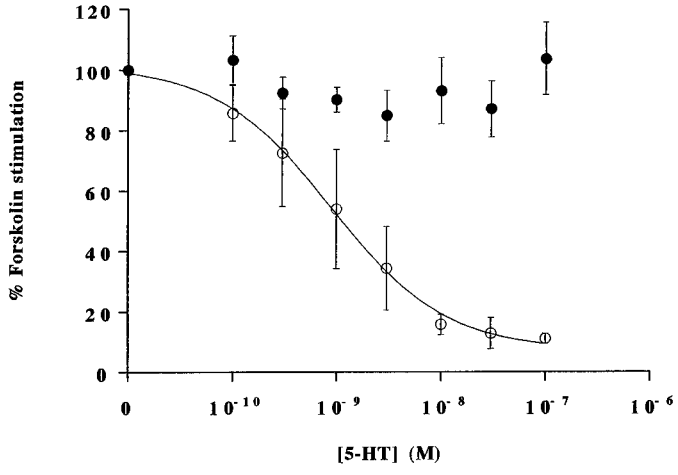

Fig. 9. Regulation of adenylyl cyclase activity by the isolated 5-HT$_{1A}$ receptor. The capacity of varying concentrations of 5-HT to modulate intact cell adenylyl cyclase activity amplified with 50 μM forskolin (100%) was assessed in untreated (○) and pertussis toxin-pretreated (●) cells stably expressing the isolated human 5-HT$_{1A}$ receptor. In the experiment displayed, 100% represented 56,575 ± 18,555 disintegrations per min in the untreated cells and 24,498 ± 4,255 disintegrations per min in the pertussis toxin-pretreated cells.

analysis of clones expressing good levels of the fusion proteins and clones expressing similar levels of the 5-HT_{1A} receptor wild-type $G_{i1}\alpha$ and 5-HT_{1A} receptor-pertussis toxin-resistant $G_{i1}\alpha$ forms. Second, immunoblots of membrane fractions of these clones using an antiserum with an epitope between amino acids 159 and 168 of $G_{i1}\alpha$ identified polypeptides of apparent M_r between 85 and 90 kDa, whereas a clone expressing the isolated 5-HT_{1A} receptor did not, although it too specifically bound [^{3}H]MPPF.

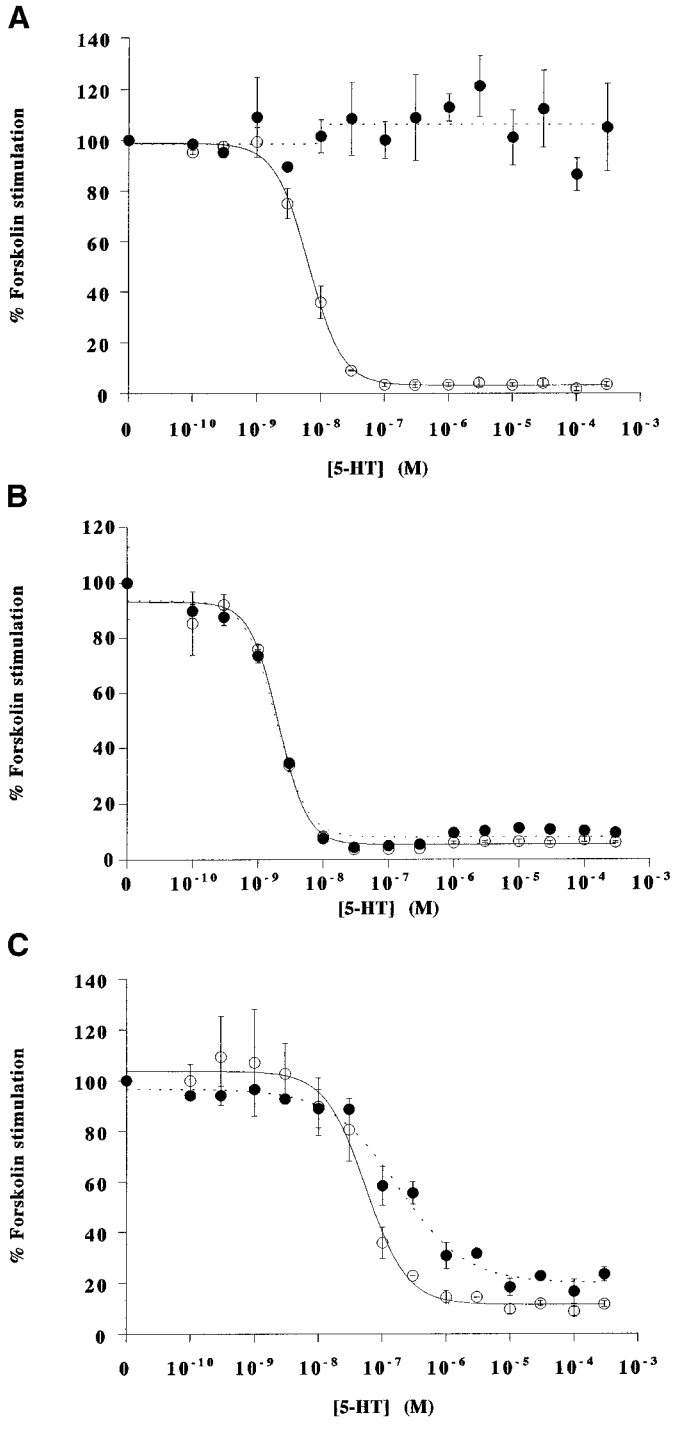

Fig. 10. Adenylyl cyclase regulation in cells expressing the fusion proteins: effects of pertussis toxin. The regulation of 50 μM forskolin-amplified adenylyl cyclase activity was measured in untreated (○) and pertussis toxin-pretreated (●) intact cells expressing fusion proteins between the human 5-HT_{1A} receptor and either wild-type (A), Ile^{351} (B), or Gly^{351} (C) $G_{i1}\alpha$.

One attraction of the fusion protein strategy has been to allow detailed analysis of the receptor constructs as agonist-regulated high-affinity GTPases (Wise et al., 1997b; Carr et al., 1998). At a maximally effective concentration of 5-HT, each of the 5-HT_{1A} receptor $G_{i1}\alpha$ fusion proteins caused a large stimulation of membrane high-affinity GTPase activity. In previous studies of an α_{2A} adrenoceptor-$(Gly^{351})G_{i1}\alpha$ fusion protein stably expressed in Rat-1 fibroblasts (Burt et al., 1998), analysis of agonist function in membranes of untreated and pertussis toxin-pretreated cells indicated that a large fraction of the agonist-stimulated signal derived from activation of the endogenously expressed forms of G_i. As such, pertussis toxin pretreatment was required to isolate and analyze GPCR interaction with the linked G protein. However, in the current studies, pertussis toxin pretreatment, under conditions which clearly caused ADP ribosylation of essentially the entire pool of endogenous G_i as measured by the reduced mobility of the modified protein through SDS-PAGE (Fig. 2), had essentially no effect on agonist-stimulated GTPase activity in membranes expressing the 5-HT_{1A} receptor-$(Gly^{351})G_{i1}\alpha$ or -$(Ile^{351})G_{i1}\alpha$ fusion proteins (Fig. 4). These results indicate a lack of functional access of the expressed 5-HT_{1A} receptor pertussis toxin-resistant $G_{i1}\alpha$ fusion proteins to a significant pool of endogenous G_i. Pertussis toxin treatment of these cells also had little effect on basal high-affinity GTPase activity, an observation that could be interpreted to suggest little constitutive activity (Samama et al., 1993; Lefkowitz et al., 1993) of these fusion proteins. However, because these fusion proteins cannot act as substrates for pertussis toxin-catalyzed ADP-ribosylation these are not clear-cut experiments. To address this issue we made use of previous reports that spiperone acts as an inverse agonist at the human 5-HT_{1A} receptor, able to reduce basal, agonist-independent, signal transduction (Barr and Manning, 1997; Newman-Tancredi et al., 1997a,b). This ligand had little or no effect on the basal GTPase activity of membranes expressing the 5-HT_{1A} receptor-$(Gly^{351})G_{i1}\alpha$ (Fig. 6A). However, in membranes expressing 5-HT_{1A} receptor-$(Ile^{351})G_{i1}\alpha$ spiperone functioned as an effective inverse agonist. This effect of spiperone was not prevented by prior treatment of these cells with pertussis toxin (Fig. 6A), demonstrating convincingly that the effect of spiperone could not have been produced by interaction of the fusion construct with endogenously expressed G_i family proteins. This confirmed that the constitutive GTPase activity must result from intramolecular interactions between the GPCR and G protein of the fusion protein. Furthermore, the effect of spiperone was shown to represent a decrease in V_{max} of the intrinsic constitutive activity of this fusion protein (Fig. 6B). Unlike the situation described above, pertussis toxin pretreatment produced a substantial reduction in basal high-affinity GTPase activity in membranes expressing the 5-HT_{1A} receptor wild-type $G_{i1}\alpha$ fusion protein, a feature at least consistent with constitutive activity of this construct. Addition of spiperone to membranes expressing the 5-HT_{1A} receptor wild-type $G_{i1}\alpha$ fusion protein resulted in a concentration-dependent inhibition of basal high-affinity GTPase activity with EC_{50} entirely in accord with that reported previously for the isolated 5-HT_{1A} receptor (Newman-Tancredi et al., 1997a). These observations, in parallel with those of

the other fusion proteins, confirmed the constitutive activity of the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein. Furthermore, as the inverse agonist activity of spiperone was completely lacking in membranes after pertussis toxin pretreatment, this indicates that the effectiveness of agonist-independent (as well as agonist-induced) interactions between the GPCR and G protein elements of the fusion protein had been substantially reduced or ablated by attachment of ADP-ribose to Cys^{351}.

The current studies also bear comparison with the demonstration that a fusion protein between the β_2 adrenoceptor and the long splice variant of G$_s\alpha$ displayed greater constitutive activity than an equivalent fusion protein containing the short splice variant of this G protein (Seifert et al., 1998). However, in the current case this alteration in measurable constitutive activity is even more dramatic, resulting from a single amino acid alteration in the G protein sequence. This provides a unique example of regulation of GPCR constitutive activity.

Previous studies have shown that the 5-HT$_{1A}$ receptor is able to inhibit adenylyl cyclase activity. In cells expressing the isolated 5-HT$_{1A}$ receptor inhibition of forskolin-amplified activity by 80 to 90% could be achieved with low concentrations of 5-HT. Pertussis toxin treatment essentially attenuated this effect. It has previously been noted that after stable expression of an α_{2A} adrenoceptor-(Gly^{351})G$_{i1}\alpha$ fusion protein agonists could mediate highly effective inhibition of forskolin-amplified adenylyl cyclase activity in intact, untreated cells. However, after pertussis toxin treatment this effect was completely attenuated (Burt et al., 1998). Such results argued that the fusion protein-linked G protein was unable to produce the effect and that regulation of effector function reflected activation of the endogenous G$_i$ protein. In cells expressing both the 5-HT$_{1A}$ receptor-(Gly^{351})G$_{i1}\alpha$ and -(Ile^{351})G$_{i1}\alpha$ fusion proteins, 5-HT was able to produce robust and concentration-dependent inhibition of forskolin-amplified adenylyl cyclase activity. However, and in agreement with the lack of effect of pertussis toxin on the capacity of 5-HT to activate high-affinity GTPase in membranes from these cells, pertussis toxin had little effect on agonist-mediated inhibition of adenylyl cyclase. In contrast, the ability of 5-HT to inhibit adenylyl cyclase activity in cells expressing the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion protein was fully attenuated by pertussis toxin treatment.

Pertussis toxin-treatment was not able to fully attenuate agonist stimulation of high-affinity GTPase activity in cells expressing the fusion protein containing the wild-type G protein (Fig. 4B). Although this may initially appear surprising, these observations are entirely in accord with previous studies on an α_{2A} adrenoceptor wild-type G$_{i1}\alpha$ fusion protein (Carr et al., 1998). Although the extreme C terminus of G protein α subunits is a key GPCR contact site it is clearly not the only one (Bourne, 1997; Hamm, 1998). Therefore, although pertussis toxin-catalyzed ADP-ribosylation of G$_i$-like proteins appears to fully uncouple GPCRs from coexpressed but resolved G proteins, now for two separate receptor systems, it is not sufficient to fully eliminate agonist-mediated information transfer between the two proteins given their enforced proximity in the fusion construction. With this in mind it might then also be considered surprising that pertussis toxin treatment resulted in a complete attenuation of agonist-mediated inhibition of adenylyl cyclase. However, the lack of signal amplification defined by the 1:1 molar ratio of GPCR and G protein in the fusion protein means that high levels of fusion protein expression are required to produce downstream signaling. Indeed in the case of an A$_1$ adenosine receptor-(Gly^{351})G$_{i1}\alpha$ fusion protein, agonist activation of mitogen-activated protein kinase activity can be observed with high level of expression of the construct but this is rapidly lost with diminishing levels of the fusion protein (N. Bevan, A. Wise, G.M. and S. Rees, in preparation). Furthermore, based on our analysis of the interactions between point mutants of G$_{i1}\alpha$ at Cys^{351} and the α_{2A} adrenoceptor, where greater hydrophilicity both limited functional activation by agonist and moved the response curve for agonist to higher concentrations (Bahia et al., 1998; Jackson et al., 1999), it is not surprising that ADP-ribosylation of the fusion protein at this position should have been observed to reduce the potency of 5-HT to stimulate GTPase activity (Fig. 8). However, it should also be noted that 5-HT displayed equivalent potency to stimulate the GTPase activity of the 5-HT$_{1A}$ receptor-(Gly^{351})G$_{i1}\alpha$ and the 5-HT$_{1A}$ receptor wild-type G$_{i1}\alpha$ fusion proteins (Fig. 8) and thus substitution of Cys^{351} by Gly in G$_{i1}\alpha$ does not appear to be as deleterious to agonist function at the 5-HT$_{1A}$ receptor as for the α_{2A} adrenoceptor (Bahia et al., 1998; Jackson et al., 1999).

These studies have provided a wealth of information on the details of interactions between the 5-HT$_{1A}$ receptor and G$_{i1}\alpha$, not least relating to the appearance and detection of constitutive activity and inverse agonism. We predict that further analysis of the pharmacology of regulation of these and related fusion constructs will provide additional novel insights.

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