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Regulation of the avidity of ternary complexes containing the human 5-HT_{1A} receptor by mutation of a receptor contact site on the interacting G protein α subunit

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1 Fusion proteins were constructed between the human 5-HT_{1A} receptor and pertussis toxin-resistant forms of both $G_{i1}\alpha$ and $G_{o1}\alpha$ mutated at residue[351] from cysteine to either glycine or isoleucine. Each of these was expressed stably in HEK293 cells.

2 Increasing concentrations of GDP inhibited binding of the agonist [^{3}H]-8-OH-DPAT but not the antagonist [^{3}H]-MPPF to each construct.

3 The IC_{50} for GDP was greater for constructs containing isoleucine at residue[351] of the G proteins compared to those with glycine at this position.

4 The G protein antagonist suramin had similar effects to GDP on the binding of [^{3}H]-8-OH-DPAT.

5 The proportion of 5-HT_{1A} receptor binding sites detected by [^{3}H]-MPPF that displayed high affinity for 8-OH-DPAT was significantly greater when the interacting G protein contained isoleucine rather than glycine at residue[351].

6 The 5-HT_{1A} receptor displayed similar avidity of interaction with $G_{i1}\alpha$ and $G_{o1}\alpha$.

7 These results indicate that a higher avidity ternary complex is formed between 8-OH-DPAT, the 5-HT_{1A} receptor and G proteins when isoleucine rather than glycine is located at residue[351] of the interacting G protein.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(N″-pyridinyl)-p-fluorobenzamido]ethyl-piperazine

Introduction

The 5-HT_{1A} receptor plays important roles in the regulation of anxiety and depression (Heisler *et al*., 1998; Ramboz *et al*., 1998; Middlemiss *et al*., 2002). This has resulted in it being one of the most studied G protein-coupled receptors (GPCRs) that interacts selectivity with the G_i-family of pertussis toxin-sensitive G proteins.

The ternary complex model (Wreggett and De Lean, 1984) and extensions of it (Samama *et al*., 1993; Weiss *et al*., 1996) is central to our understanding of interactions between GPCRs and G proteins and to analysis of signal transduction *via* these proteins. Predictions based on these models have been robust (Kenakin *et al*., 2000). However, simulations of the behaviour of such models are based on circumstances in which GPCR and G protein concentrations are equivalent. This is generally not the situation in cell membranes where levels of G proteins are routinely in marked excess over any given GPCR (Milligan, 2000a). It is also well appreciated that the characteristics of ligand binding and agonist efficacy can vary markedly with alterations in GPCR-G protein stoichiometry (Kenakin, 1997). Although designed predominantly to allow detailed enzyme kinetic analysis of agonist activation of G proteins (Wise *et al*., 1997a) and its regulation by interacting proteins (Cavalli *et al*., 2000) GPCR-G protein fusion proteins offer attractive models for detailed pharmacological analysis (Seifert *et al*., 1999; Milligan, 2000b; Wurch & Pauwels, 2001). This reflects their defined 1 : 1 stoichiometry that is unchanged by mutation of the protein sequence or exposure to ligands. However, when such constructs are generated using wild type forms of the G proteins it is at least possible that the signal measured reflects GPCR-mediated activation of endogenously expressed G proteins as well as the G protein physically linked to the receptor. Indeed, in certain cases this has been demonstrated directly (Burt *et al*., 1998; Holst *et al*., 2001). As pertussis toxin-catalysed ADP-ribosylation eliminates functional contacts between GPCRs and G_i-family G proteins, such fusions are frequently generated using variant forms of the G protein mutated to be resistant to the actions of this toxin (Milligan, 2002c). Thus, following pertussis toxin treatment of cells, signals generated must reflect only activation of the GPCR-linked G protein. However, the identity of the amino acid used to

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replace the cysteine that is the target for pertussis toxin can alter the effectiveness of information transfer between GPCR and G proteins as it is located within a key contact interface (Moon *et al*., 2001a, b). For example, fusion proteins between the 5-HT$_{1A}$ receptor and G$_{i1}\alpha$ display substantially higher levels of constitutive activity when cysteine^{351} of the G protein is replaced with isoleucine than when it is replaced by glycine (Kellett *et al*., 1999). The implication is that, even in the absence of agonist, the avidity of interaction between the 5-HT$_{1A}$ receptor and G$_{i1}\alpha$ is greater with a more hydrophobic amino acid at this site. Agonists, but not antagonists, bind with higher affinity to a GPCR associated with a G protein than following their dissociation. Furthermore, agonist occupation of a GPCR results in reduction in the affinity of binding of GDP to the G protein α subunit to allow guanine nucleotide exchange and G protein activation (Gilman, 1987). It should thus also be expected that increasing concentrations of GDP reduce the binding affinity of agonists but not antagonists for the GPCR (see Birnbaumer *et al*., 1990 for review) and limit the potency of agonists to stabilize the ternary complex and produce guanine nucleotide exchange (McLoughlin & Strange, 2000). Thus, herein we have used measurements of the regulation of [^{3}H]-agonist binding to fusion proteins incorporating the 5-HT$_{1A}$ receptor and glycine and isoleucine containing forms of both G$_{i1}\alpha$ and G$_{o1}\alpha$ to gain direct information on the relative stability of such ternary complexes that differ simply in a single amino acid. The interactions between the GPCR and the forms of the G protein with isoleucine at residue^{351} are noted to be of substantially higher avidity than for the equivalent G proteins with glycine at this location. It is thus not surprising that the 5-HT$_{1A}$ receptor-G protein fusions containing the isoleucine mutation displays significantly greater information transfer in the absence of agonist, i.e. constitutive activity, than those containing the glycine mutation (Kellett *et al*., 1999; Welsby *et al*., 2002). A similar approach has recently been adopted to monitor interactions between the A1 adenosine receptor and various G proteins (Waldhoer *et al*., 1999; Kudlacek *et al*., 2002).

Methods

All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Strathclyde, U.K.). The 5-HT$_{1A}$ receptor antagonist [^{3}H]-MPPF (70.5 Ci/mmol) was obtained from DuPont/NEN and the agonist [^{3}H]8-OH-DPAT (221 Ci/mmol) from Amersham Pharmacia Biotech. Pertussis toxin was purchased from Sigma. Oligonucleotides were purchased from Cruachem (Glasgow, Strathclyde, U.K.). All other chemicals were from Sigma or Boehringer Mannheim.

Construction of plasmids encoding 5-HT$_{1A}$-G$_{i1}\alpha$ and 5-HT$_{1A}$-G$_{o1}\alpha$ fusion proteins

The human 5-HT$_{1A}$ receptor clone in pSP64 (a gift from Glaxo SmithKline, Stevenage, U.K.) was digested with *Xba*I/*Bam*HI and the resulting 1.5 Kb fragment ligated to pcDNA3. To obtain the reading frame of 1.3 Kb PCR was carried out using the following primers to introduce a *Hind*III 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′-CTGAAGCTTATGGATGTGCTCAGCCC-TGGTC-3′; 5′-CTGGGATCCCTGGCGGCAGAAGTTA-CACTTAATG-3′ (restriction enzyme sites underlined). The PCR fragment was digested with *Hind*III 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′-CTGGGATCCGGCTGCACACTGAGCGCT-GAG-3′ at the 5′ end and 5′-GAGAATTCTTAGAAAGA-GACCACAGTC-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 5-HT$_{1A}$-(Gly^{351}) and (Ile^{351})G$_{i1}\alpha$ fusion proteins plasmid (Gly or Ile^{351})G$_{i1}\alpha$ in pBS (Bahia *et al*., 1998) was digested with *Sac*II/*Eco*RI and the 730 bp fragment used to replace the corresponding fragment in p5HTG$_{i1}$. Equivalent strategies were used to produce the 5-HT$_{1A}$-(Gly or Ile^{351})G$_{o1}\alpha$ fusions using pertussis toxin-resistant forms of G$_{o1}\alpha$ (Moon *et al*., 2001a). 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^{-1}) newborn calf serum, 2 mM L-glutamine. Cells were seeded into 100 mm culture dishes and grown to 60–80% confluency (18–24 h) before transfection with 5 μg of appropriate cDNAs using DOTAP reagent (Boehringer Mannheim). Forty-eight hours after transfection, the cells were split 1 : 4 into 800 μg ml^{-1} G418 sulphate (Calbiochem) containing medium. A 100 mm dish of untransfected HEK293 cells was also split into the same medium as a control dish. About one 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^{-1} of G418 medium (400 μg ml^{-1}). Clones were amplified, membrane preparations made and their binding of [^{3}H]-MPPF determined (Kellett *et al*., 1999).

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 (1600 r.p.m.) in a refrigerated microcentrifuge. The supernatant fraction was then centrifuged at 50 000 r.p.m. 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 mg/ml^{-1} and stored at −80°C until required.

[^{3}H]-MPPF binding studies

Binding assays were performed by adding 5 μg membrane protein to an assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.2% ascorbic acid, 10 μM pargyline, pH 7.4) containing [^{3}H]-

4-(2′-methoxy)-phenyl-1-2′-[N-2″-pyridinyl)-p-fluorobenzamido]ethyl-piperazine. ([^{3}H]-MPPF) (0.25–9 nM). Non-specific 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 three times with 5 ml ice-cold wash buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) and then counted. In competition binding assays [^{3}H]-MPPF was present at 1 nM.

[^{3}H]-8-OH DPAT binding studies

Binding in triplicate reaction mixtures containing 2.5 μg membrane protein and either suramin or GDP (1 nM–1 mM) was initiated by the addition of [^{3}H]-8-OH-DPAT (0.6 nM) and incubated at 30°C for 30 min in binding buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1% ascorbic acid, 10 μM pargyline, pH 7.4). Reaction was terminated by rapid filtration through GF/C filters subsequently washed three times with 5 ml ice-cold wash buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) and then counted. Non-specific binding was determined in parallel in the presence of 100 μM 5-HT.

Adenylyl cyclase

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^{-1} for 24 h. The generation of [^{3}H]-cAMP in response to forskolin or 5-HT was assessed in the presence of the non-selective cAMP phosphodiesterase inhibitor isobutylmethylxanthine (1 mM).

Results

Fusion proteins between the human 5-HT$_{1A}$ receptor and forms of both $G_{i1}\alpha$ and $G_{o1}\alpha$ that are insensitive to the ADP-ribosyltransferase activity of pertussis toxin because cysteine^{351} had been mutated to either glycine or isoleucine (Welsby *et al.*, 2002) were each expressed stably in HEK293 cells. Clones expressing similar levels (some 6 pmol mg^{-1} membrane protein) of each construct as measured by the specific binding of the antagonist [^{3}H]-MPPF were expanded and used in these studies. The K_d for [^{3}H]-MPPF (1.5–2.0 nM) was similar for each construct and for the wild type 5-HT$_{1A}$ receptor and this was not altered by pertussis toxin pre-treatment of the cells (data not shown). In all cases the binding isotherms were consistent with this ligand interacting with a single population of sites.

The binding of a single concentration (1 nM) of the agonist [^{3}H]-8-OH-DPAT to membranes of HEK293 cells expressing the isolated 5-HT$_{1A}$ receptor was reduced by some 90% by pre-treatment of the cells with pertussis toxin (Figure 1). Addition of increasing concentrations of GDP to membranes of untreated cells also resulted in a reduction in the binding of [^{3}H]-8-OH-DPAT to the 5-HT$_{1A}$ receptor (Figure 1) with an EC_{50} for GDP of 13.7±2.4 μM (mean±s.e.m., $n=4$). By contrast no further reduction in [^{3}H]-8-OH-DPAT binding was produced by adding GDP to membranes from pertussis toxin-treated cells (Figure 1). When equivalent experiments were performed on membranes expressing the 5-HT$_{1A}$ receptor-Gly^{351}$G_{i1}\alpha$ fusion protein increasing concentrations of GDP again reduced the specific binding of [^{3}H]-8-OH-DPAT (Figure 2 and Table 1). However, pertussis toxin pre-treatment altered neither the binding of [^{3}H]-8-OH-DPAT in the absence of GDP nor the potency of GDP to reduce binding of [^{3}H]-8-OH-DPAT to this construct (Figure 2).

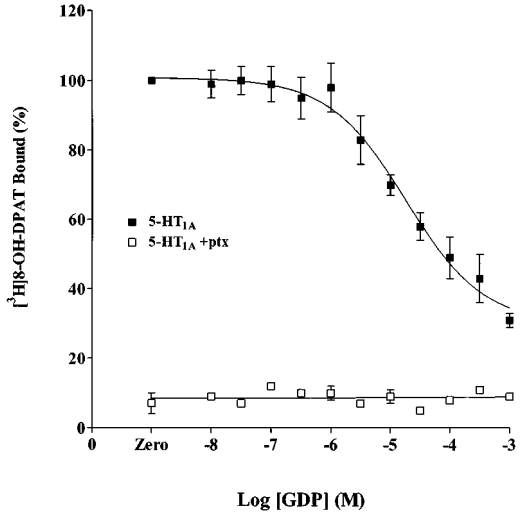

Figure 1 GDP and pertussis toxin pre-treatment reduces the binding of [^{3}H]-8-OH-DPAT to the 5-HT$_{1A}$ receptor. Membranes were prepared from untreated and pertussis toxin-pre-treated (25 ng ml^{-1}), 16 h) HEK293 cells stably expressing the human 5-HT$_{1A}$ receptor. The specific binding of [^{3}H]-8-OH-DPAT (1 nM) was then assessed in the presence of increasing concentrations of GDP.

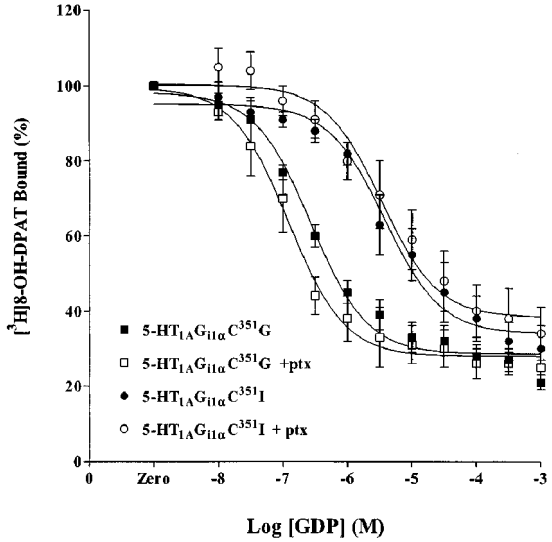

Figure 2 GDP but not pertussis toxin pre-treatment reduces the binding of [^{3}H]-8-OH-DPAT to pertussis toxin-resistant 5-HT$_{1A}$ receptor-$G_{i1}\alpha$ fusion proteins. Membranes were prepared from untreated and pertussis toxin-pretreated (25 ng ml^{-1}, 16 h) HEK293 cells stably expressing 5-HT$_{1A}$ receptor-$G_{i1}\alpha$ fusion proteins in which the pertussis toxin-sensitive cysteine was replaced by either glycine or isoleucine. The specific binding of [^{3}H]-8-OH-DPAT was assessed in the presence of varying concentrations of GDP as in Figure 1.

Although the same was true when membranes were generated from cells expressing the 5-HT$_{1A}$ receptor-Ile^{351}G$_{i1}\alpha$ fusion protein the potency of GDP to reduce the binding of [^{3}H]-8-OH-DPAT was markedly lower than for the 5-HT$_{1A}$ receptor-Gly^{351}G$_{i1}\alpha$ construct (Table 1 and Figure 2). Exactly the same was observed for the fusion proteins incorporating the Gly^{351} and Ile^{351} forms of G$_{o1}\alpha$ (Figure 3 and Table 1). Although pertussis toxin treatment was without effect, the potency of GDP to limit binding of [^{3}H]-8-OH-DPAT was significantly lower ($P<0.05$) for the Ile^{351} containing constructs. In contrast to these results, GDP had little effect on the binding of [^{3}H]-MPPF to any of these constructs (data not shown).

Suramin is sometimes described as a 'G protein antagonist' (Freissmuth *et al*., 1999). Addition of increasing concentrations of suramin to [^{3}H]-8-OH-DPAT binding assays employing membranes expressing the isolated 5-HT$_{1A}$ receptor also resulted in reduction of agonist binding (Figure 4). Suramin did not produce this effect by acting as a competitive inhibitor at the ligand binding site as it did not reduce the binding of [^{3}H]-MPPF (Figure 4). Increasing concentrations of suramin also reduced the binding of [^{3}H]-8-OH-DPAT to the 5-HT$_{1A}$ receptor-G$_{i1}\alpha$ fusion proteins (Figure 5). The concentrations of suramin required to reduce binding of [^{3}H]-8-OH-DPAT to the 5-HT$_{1A}$ receptor-Ile^{351}G$_{i1}\alpha$ construct were significantly greater than for the 5-HT$_{1A}$ receptor-Gly^{351}G$_{i1}\alpha$ fusion protein in membranes from both untreated ($P=0.012$) and pertussis toxin treated ($P=0.002$) cells (Figure 5a). An equivalent significant difference ($P=0.002$) in the potency of suramin to reduce binding of [^{3}H]-8-OH-DPAT to the fusion proteins containing Ile^{351} and Gly^{351}G$_{o1}\alpha$ was also observed (Figure 5b).

Competition for the specific binding of [^{3}H]-MPPF to the 5-HT$_{1A}$ receptor-Gly^{351}G$_{i1}\alpha$ fusion protein by increasing concentrations of 8-OH-DPAT was unaffected by prior pertussus toxin treatment of cells (Figure 6 and data not shown) and was best described by a 2-site model in which the high affinity sites for the agonist comprized 30.9±0.1% and the low affinity sites 69.1±0.2% of the total. This was also true when the 5-HT$_{1A}$ receptor-Ile^{351}G$_{i1}\alpha$ construct was examined, except that the proportion of high affinity sites for agonist represented 60.3±11.0% of the binding sites and those with low affinity 39.7±5.5% (Figure 6). This difference was highly significant ($P=0.004$). To explore whether the 5-HT$_{1A}$ receptor displayed different affinities of interaction with G$_{i1}\alpha$ and G$_{o1}\alpha$ we constructed fusion proteins between the receptor and the wild type (Cys^{351}) version of these G proteins. As anticipated, 5-HT$_{1A}$ was able to mediate inhibition of forskolin-stimulated adenylyl cyclase activity in cells expressing either of these constructs (data not shown). However, as these constructs are sensitive to pertussis toxin-catalysed ADP-ribosylation, such treatment largely eliminated agonist regulation of cAMP levels (data not shown). This is distinct from the fusion proteins containing the

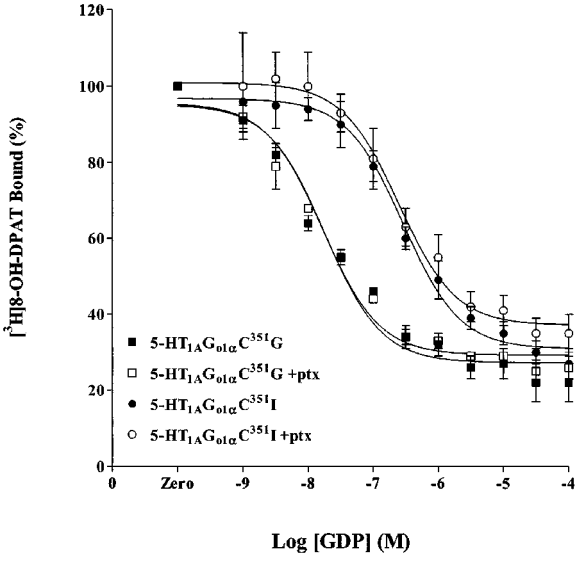

Figure 3 The nature of the pertussis toxin-insensitive mutation alters the potency of GDP to reduce the binding of [^{3}H]-8-OH-DPAT to pertussis toxin-resistant 5-HT$_{1A}$ receptor-G$_{o1}\alpha$ fusion proteins. Membranes were prepared from untreated and pertussis toxin-pretreated (25 ng ml^{-1}, 16 h) HEK293 cells stably expressing 5-HT$_{1A}$ receptor-G$_{o1}\alpha$ fusion proteins in which the pertussis toxin-sensitive cysteine was replaced by either glycine or isoleucine. The specific binding of [^{3}H]-8-OH-DPAT was assessed in the presence of varying concentrations of GDP as in Figure 2.

Table 1 GDP inhibits the binding of [^{3}H]-8-OH-DPAT to pertussis toxin-resistant 5-HT$_{1A}$ receptor-G$_{i1}\alpha$ and G$_{o1}\alpha$ fusion proteins

	EC$_{50}$ GDP (μM)	
	5-HT$_{1A}$-G$_{i1}\alpha$	*5-HT$_{1A}$-G$_{o1}\alpha$*
Gly^{351}	0.3±0.05 (3)	0.10±0.04 (3)
Ile^{351}	10.0±3.1 (5)	3.0±0.6 (3)

The ability of GDP to inhibit the specific binding of [^{3}H]-8-OH-DPAT to fusion proteins between the 5-HT$_{1A}$ receptor and pertussis toxin-resistant forms of G$_{i1}\alpha$ or G$_{o1}\alpha$ in which Cys^{351} was replaced by Gly or Ile is recorded. Data represent means±s.e.m. Numbers in parentheses indicate the number of experiments performed.

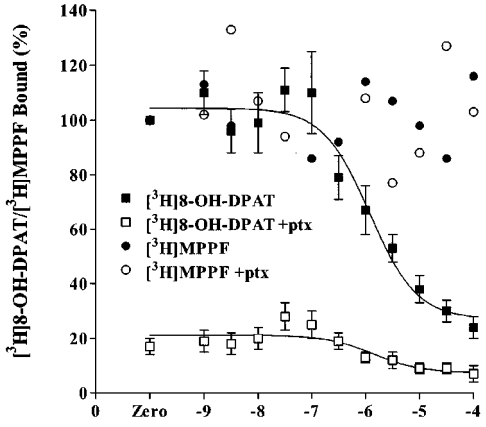

Figure 4 Suramin reduces the binding of [^{3}H]-8-OH-DPAT but not [^{3}H]-MPPF to the 5HT$_{1A}$ receptor. Membranes were prepared from untreated and pertussis toxin pre-treated (25 mg ml^{-1}, 16 h) HEK293 cells stably expressing the human 5-HT$_{1A}$ receptor. The specific binding of [^{3}H]-8-OH-DPAT (1 nM) or [^{3}H]-MPPF (1 nM) was then assessed in the presence of increasing concentrations of suramin.

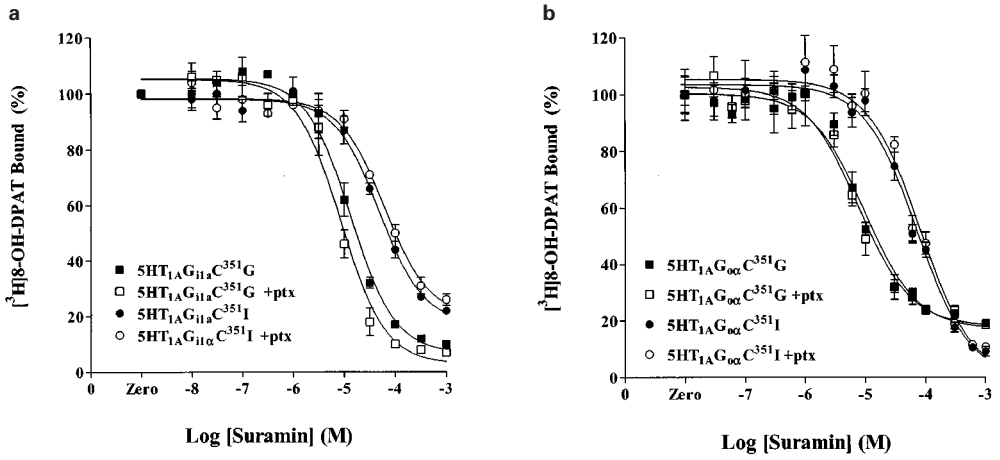

Figure 5 The nature of the pertussis toxin-insensitive mutation alters the potency of suramin to reduce the binding of [^{3}H]-8-OH-DPAT to pertussis toxin-resistant 5-HT$_{1A}$ receptor-G$_{i1}\alpha$ and $-$G$_{o1}\alpha$ fusion proteins. Membranes were prepared from untreated and pertussis toxin-pre-treated (25 ng ml^{-1}, 16 h) HEK293 cells stably expressing 5-HT$_{1A}$ receptor-G$_{i1}\alpha$ (**a**) or 5-HT$_{1A}$ receptor-G$_{o1}\alpha$ (**b**) fusion proteins in which the pertussis toxin sensitive cysteine was replaced by either glycine or isoleucine. The specific binding of [^{3}H]-8-OH-DPAT was assessed in the presence of varying concentrations of suramin as in Figure 4.

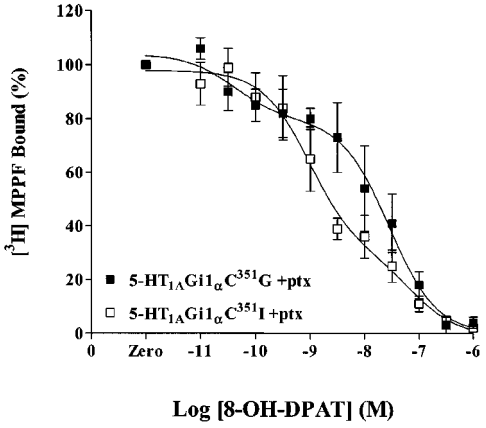

Figure 6 A 5HT$_{1A}$-(Cys^{351}Ile)G$_{i1}\alpha$ fusion protein displays a greater proportion of high affinity binding sites for 8-OH-DPAT than a 5HT$_{1A}$-(Cys^{351}Gly)G$_{i1}\alpha$ fusion protein. Membranes were prepared from pertussis toxin pre-treated HEK293 cells expressing either the 5HT$_{1A}$-(Cys^{351}Ile)G$_{i1}\alpha$ fusion protein or the 5HT$_{1A}$-(Cys^{351}Gly)G$_{i1}\alpha$ fusion protein. The ability of varying concentrations of 8-OH-DPAT to compete with [^{3}H]-MPPF (1 nM) for the binding site was then assessed.

pertussis toxin-resistant forms of the G proteins where agonist-inhibition of adenylyl cyclase activity is unaffected by pertussis toxin treatment (Kellett *et al*., 1999). The binding of [^{3}H]-8-OH-DPAT to both of the constructs containing the wild type G proteins was also reduced by increasing concentrations of either GDP or suramin. However, there were no significant differences in the IC$_{50}$ values for these two compounds when examining 5-HT$_{1A}$ receptor-Cys^{351}G$_{i1}$ (GDP 2.2$\pm$0.5 μM, suramin 1.7$\pm$0.5 μM, means$\pm$s.e.m., $n=4$) and 5-HT$_{1A}$ receptor-Cys^{351}G$_{o1}$ (GDP 5.9$\pm$3.8 μM, suramin 1.3$\pm$0.6 μM, means$\pm$s.e.m., $n=4$). This indicates that the avidity of ternary complexes between 8-OH-DPAT, the 5-HT$_{1A}$ receptor and the wild type forms of these two G proteins is similar.

Discussion

GPCR agonists function by reducing the affinity and hence enhancing the dissociation of GDP from the nucleotide binding pocket of the G protein α subunit. This increases guanine nucleotide exchange (Gilman, 1987). Thus, a corollary is that increasing concentrations of GDP should reduce the affinity of agonist binding to a receptor. Indeed, previous studies on the 5-HT$_{1A}$ receptor expressed in CHO cells has shown the capacity of GDP to reduce the potency of agonists at this receptor to cause activation of endogenously expressed G proteins. This was modelled to reflect a reduced ability of agonist to stabilize the ternary complex in the presence of GDP (McLoughlin & Strange, 2000). As the complex with high affinity for agonist is between GPCR and G protein denuded of guanine nucleotide, increasing concentration of GDP must limit production of this state. In initial studies the binding of a single concentration of the agonist [^{3}H]-8-OH-DPAT to the isolated human 5-HT$_{1A}$ receptor was assessed in membranes of HEK293 cells. Binding was reduced by GDP in a concentration-dependent manner and the extent of binding remaining in the presence of a maximally effective concentration of GDP was similar to that observed following pertussis toxin pre-treatment of the cells to eliminate interactions with G proteins. When equivalent experiments were performed on cell membranes expressing fusion proteins between the 5-HT$_{1A}$ receptor and pertussis toxin-resistant forms of either G$_{i1}\alpha$ and G$_{o1}\alpha$ increasing concentrations of GDP again reduced binding of the agonist ligand. By contrast with the isolated receptor, however, pertussis toxin pre-treatment had no effect on ligand binding and did not alter the effectiveness of GDP to reduce binding of the agonist. Such results clearly indicated that [^{3}H]-8-OH-DPAT binding was a useful monitor of the 1 : 1 : 1 ternary complex of agonist, receptor and G protein. A substantial difference in potency of GDP to reduce the binding of [^{3}H]-8-OH-DPAT to fusion proteins containing the single isoleucine or glycine substitutions at position 351 of the G proteins was observed. The IC$_{50}$ for GDP was

significantly higher for the fusions containing the isoleucine substitution in the G proteins and this was true for both $G_{i1}\alpha$ and $G_{o1}\alpha$. These differences must indicate that the 8-OH-DPAT-5-HT$_{1A}$ receptor-G protein ternary complex is of significantly higher avidity when the isoleucine substituted G protein is present. By contrast, antagonists do not modulate GDP release and guanine nucleotide exchange and thus the binding of [^{3}H]-MPPF to the fusion proteins was unaffected by the presence of GDP or the nature of amino acid[351] of the G protein.

We also wished to address whether there were marked differences in the avidity of interactions between the 5-HT$_{1A}$ receptor and $G_{i1}\alpha$ and $G_{o1}\alpha$. As we could not be certain that the glycine and isoleucine substitutions functioned equivalently for the two G proteins we also constructed fusion proteins between the wild type forms of each G protein and the 5-HT$_{1A}$ receptor. As we did not observe large differences in the effectiveness and potency of GDP to limit the binding of [^{3}H]-8-OH-DPAT to these two constructs we conclude that interactions between the 5-HT$_{1A}$ receptor- and $G_{i1}\alpha$ and $G_{o1}\alpha$ are of similar affinity. Similar conclusions have been reached using co-expression studies in insect Sf9 cells (Butkerait *et al*., 1995).

We extended these studies by the use of suramin. Although suramin is a polyanion with a wide range of pharmacological functions it has been used as a G protein antagonist (Freissmuth *et al*., 1999) as it is able to interfere with G protein activation by GPCRs, at least partially by interacting directly with G protein. As concentrations up to 10 μM did not interfere with the binding of [^{3}H]-MPPF to the pertussis toxin-resistant fusion proteins suramin is thus not a direct competitor at the ligand binding pocket. Despite this, increasing concentrations of suramin were able to reduce the binding of [^{3}H]-8-OH-DPAT and this effect of suramin was also unaffacted by prior pertussis toxin pre-treatment of cells. Suramin was again less potent in limiting agonist binding to the fusion proteins containing the isoleucine replacement G proteins than to those with glycine at this position. Such data is also consistent with the ternary complexes containing the isoleucine containing G proteins possessing higher avidity. Suramin did not display marked differences in potency to disrupt binding of [^{3}H]-8-OH-DPAT to fusion proteins in which the receptor was linked to either wild type $G_{i1}\alpha$ or $G_{o1}\alpha$. However, it is interesting to note that an analogue of suramin has recently been shown to selectively interfere with interactions between the A1 adenosine receptor and $G_{i1}\alpha$ but not between this receptor and $G_{o1}\alpha$ (Kudlacek *et al*., 2002). It thus appears that the detailed contacts between GPCRs and closely related G proteins can differ and thus may be an interface that can be targeted effectively by ligands with therapeutic potential.

As a further test of the hypothesis that the avidity of GPCR-G proteins contacts can be altered significantly by single point mutations we compared the ability of non-radioactive 8-OH-DPAT to compete with [^{3}H]-MPPF for binding to fusion proteins containing the pertussis toxin-resistant forms of $G_{i1}\alpha$. The competition curves were clearly biphasic demonstrating both high and low affinity sites for the agonist. Although this was expected from the ability of GDP to reduce the binding of [^{3}H]-8-OH-DPAT it is important to note that, given the physical link between GPCR and G protein imbued by the fusion, one hypothesis is that this would create a single set of non-interconverting binding sites. However, such fusion proteins display both low and high affinity binding sites for agonists, a feature that has also been noted for fusions incorporating, amongst others, the β_2-adrenoceptor (Seifert *et al*., 1999) and the δ-opioid receptor (Moon *et al*., 2001b). They thus function in the manner expected for separated but co-expressed polypeptides. Given that GDP was less potent in reducing agonist binding to the isoleucine containing G proteins it was gratifying to note that the 5-HT$_{1A}$ receptor-$G_{i1}\alpha$ fusion protein containing isoleucine displayed a significantly greater proportion of high affinity agonist binding sites than the variant incorporating the glycine containing form of $G_{i1}\alpha$. These data are entirely in accord with the results from the other approaches and further support the contention that introduction of isoleucine at this position in the G protein results in the interaction with the 5-HT$_{1A}$ receptor being of greater avidity than when glycine is present.

Since their initial introduction by Bertin *et al*. (1994) GPCR-G protein fusions have become increasingly popular tools to study details of the interactions between these two classes of proteins. They have proved to be ideal reagents to study aspects of ligand efficacy (Wise *et al*., 1997b; Dupuis *et al*., 1999), the selectivity of interactions of GPCRs with different G proteins (Fong & Milligan, 1999; Wenzel-Seifert & Seifert, 2000; Holst *et al*., 2001), the concept that different ligands induce distinct receptor conformations (Seifert *et al*., 2001), the role and regulation of post-translational acylation of both GPCRs and G proteins (Loisel *et al*., 1999; Stevens *et al*., 2001) and the role of G protein β/γ subunits in information transfer between GPCRs and G proteins (Liu *et al*., 2002). In recent times they have also been used effectively in drug discovery programmes, both to overcome the widespread distribution pattern of Edg receptor subtypes (McAllister *et al*., 2000), to help identify ligands for orphan GPCRs (Hosoi *et al*., 2002) and to screen for inverse agonists (Carrillo *et al*., 2002). In all these cases, the defined 1 : 1 stochiometry of the partner polypeptides has provided distinct advantages. This has been of particular value as saturation ligand binding studies thus provide a direct measure of the expression level of not only the GPCR but also the G protein. In concert with measures of agonist stimulated GTPase activity this has allowed measurement of the turnover member for GTP induced by agonists (Wise *et al*., 1997a) and the regulation of the lifetime of activated G proteins by Regulators of G protein Signalling (Cavalli *et al*., 2000). Because mutation of either GPCR or G protein does not alter the stoichiometry of the two polypeptides this has also allowed quantitative analysis of the effects of such mutations on signal initiation (Ward & Milligan, 2002) and the extent of constitutive activity imbued by such mutations (Carrillo *et al*., 2002). The current data further define the usefulness of GPCR-G protein fusions in allowing analysis of pharmacological details inherent in the ternary complex model.

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References

BAHIA, D.S., WISE, A., FANELLI, F., LEE, M., REES, S. & MILLIGAN, G. (1998). Hydrophobicity of residue^{351} of the G-protein $G_{i1}\alpha$ determines the extent of activation by the α_{2A}-adrenoceptor. *Biochemistry,* **37,** 11555 – 11562.

BERTIN, B., FREISSMUTH, M., JOCKERS, R., STROSBERG, A.D. & MARULLO, S. (1994). Cellular signaling by an agonist-activated receptor/Gs alpha fusion protein. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 8827 – 8831.

BIRNBAUMER, L., ABRAMOWITZ, J. & BROWN, A.M. (1990). Receptor-effector coupling by G-proteins. *Biochim. Biophys. Acta,* **1031,** 163 – 224.

BURT, A.R., SAUTEL, M., WILSON, M.A., REES, S., WISE, A. & MILLIGAN, G. (1998). Agonist-occupation of an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein results in activation of both receptor-linked and endogenous G proteins. Comparisons of their contributions to GTPase activity and signal transduction and analysis of receptor-G protein activation stoichiometry. *J. Biol. Chem.,* **273,** 10367 – 10375.

BUTKERAIT, P., ZHENG, Y., HALLAK, H., GRAHAM, T.E. MILLER, H.A., BURRIS, K.D., MOLINOFF, P.B. & MANNING, D.R. (1995). Expression of the human 5-hydroxytryptamine1A receptor in Sf9 cells. Reconstitution of a coupled phenotype by co-expression of mammalian G protein subunits. *J. Biol. Chem.,* **270,** 18691 – 18699.

CARRILLO, J.J., STEVENS, P.A. & MILLIGAN, G. (2002). Measurement of agonist-dependent and-independent signal initiation of α_{1b}-adrenoceptor mutants by direct analysis of guanine nucleotide exchange on the G protein $G\alpha_{11}$. *J. Pharmacol. Exp. Ther.* (in press).

CAVALLI, A., DRUEY, K.M. & MILLIGAN, G. (2000). The regulator of G protein signaling RGS4 selectively enhances α_{2A}-adrenoceptor stimulation of the GTPase activity of $G_{o1}\alpha$ and $G_{i2}\alpha$. *J. Biol. Chem.,* **275,** 23693 – 23699.

DUPUIS, D.S., TARDIF, S., WURCH, T., COLPAERT, F.C., PAUWELS, P.J. (1999). Modulation of 5-HT1A receptor signalling by point-mutation of cysteine351 in the rat Galpha(o) protein. *Neuropharmacology,* **38,** 1035 – 1041.

FONG, C.W. & MILLIGAN, G. (1999). Analysis of agonist function at fusion proteins between the IP prostanoid receptor and cognate, unnatural and chimaeric G proteins. *Biochem. J.,* **342,** 457 – 463.

FREISSMUTH, M., WALDHOER, M., BOFILL-CARDONA, E. & NANOFF, C. (1999). G protein antagonists. *Trends Pharmacol. Sci.,* **20,** 237 – 245.

GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.,* **56,** 615 – 649.

HOLST, B., HASTRUP, H, RAFFETSEDER, U., MARTINI, L. & SCHWARTZ, T.W. (2002). Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis. *J. Biol. Chem.,* **276,** 19793 – 19799.

HOSOI, T., KOGUCHI, Y., SUGIKAWA, E., CHIKADA, A., OGAWA, K., TSUDA, N., SUTO, N., TSUNODA, S., TANIGUCHI, T. & OHNUKI, T. (2002). Identification of a novel eiconsanoid receptor coupled to Gi/o. *J. Biol. Chem.,* (in press) PMID: 12065583.

HEISLER, L.K., CHU, H.-M., BRENNAN, T.J., DANAO, J.A., BAJWA, P., PARSONS, L.R. & TECOTT, L.H. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT$_{1A}$ receptor mutant mice. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 15049 – 15054.

KELLETT, E., CARR, I.C. & MILLIGAN, G. (1999). Regulation of G protein activation and effector modulation by fusion proteins between the human 5-HT$_{1A}$ receptor and the α subunit of G_{i1}. Differences in receptor constitutive activity imparted by single amino acid substitutions in $G_{i1}\alpha$. *Mol. Pharmacol.,* **56,** 684 – 692.

KENAKIN, T. (1997). Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol. Sci.,* **18,** 456 – 464.

KENAKIN, T., MORGAN, P., LUTZ, M. & WEISS, J. (2000). The evolution of drug-receptor models: The cubic ternary complex for G protein-coupled receptors. In: *The pharmacology of functional, biochemical, and recombinant receptor systems. Handbook of Experimental Pharmacology*. Vol. 148. ed. Kenakin, T. and Angus, J.A. pp. 147 – 165. Berlin: Springer-Verlag.

KUDLACEK, O., WALDHOER, M., KASSACK, M.U., NICKEL, P., SALMI, J.I., FREISSMUTH, M. & NANOFF, C. (2002). Biased inhibition by a suramin analogue of A1-adenosine receptor/G protein coupling in fused receptor/G protein tandems: the A1-adenosine receptor is predominantly coupled to Goalpha in human brain. *Naunyn Schmiedebergs Arch Pharmacol.,* **365,** 8 – 16.

LOISEL, T.P., ANSANAY, H., ADAM, L., MARULLO, S., SEIFERT, R., LAGACE, M. & BOUVIER, M. (1999). Activation of the beta(2)-adrenergic receptor-Galpha(s) complex leads to rapid depalmitoylation and inhibition of repalmitoylation of both the receptor and Galpha(s). *J. Biol. Chem.,* **274,** 31014 – 31019.

LIU, S., CARRILLO, J.J., PEDIANI, J. & MILLIGAN, G. (2002). Effective information transfer from the α_{1b}-adrenoceptor to $G_{11}\alpha$ requires both β/γ interactions and an aromatic group 4 amino acid from the C-terminus of the G protein. *J. Biol. Chem.,* **277,** 25707 – 25714.

MCALLISTER, G., STANTON, J.A., SALIM, K., HANDFORD, E.J. & BEER, M.S. (2000). Edg2 receptor functionality: Giα1 coexpression and fusion protein studies. *Mol. Pharmacol.,* **58,** 407 – 412.

MCLOUGHLIN, D.J. & STRANGE, P.G. (2000). Mechanisms of agonism and inverse agonism at serotonin 5-HT$_{1A}$ receptors. *J. Neurochem.,* **74,** 347 – 357.

MERKOURIS, M., MULLANEY, I., GEORGOUSSI, Z & MILLIGAN, G. (1997). Regulation of spontaneous activity at the delta opioid receptor: Studies of inverse agonism in intact cells. *J. Neurochem.,* **69,** 2115 – 2122.

MIDDLEMISS, D.N., PRICE, G.W. & WATSON, J.M. (2002). Serotonergic targets in depression. *Curr. Opin. Pharmacol.,* **2,** 18 – 22.

MILLIGAN, G. (2000a). Altering the relative stoichiometry of receptors, G-proteins and effectors: effects on agonist function. In: *The pharmacology of functional, biochemical, and recombinant receptor systems. Handbook of Experimental Pharmacology*. Vol. 148. ed. Kenakin, T. and Angus, J.A pp. 363 – 389. Berlin: Springer-Verlag.

MILLIGAN, G. (2000b). Insights into ligand pharmacology using receptor-G protein fusion proteins. *Trends Pharmacol. Sci.,* **21,** 24 – 28.

MILLIGAN, G. (2002). Construction and analysis of function of GPCR-G protein fusion proteins. *Methods Enzymol.,* **343,** 260 – 273.

MOON, H.E., CAVALLI, A., BAHIA, D.S., HOFFMANN, M., MASSOTTE, D. & MILLIGAN, G. (2001a). The human δ opioid receptor activates $G_{i1}\alpha$ more efficiently than $G_{o1}\alpha$. *J. Neurochem.,* **76,** 1805 – 1813.

MOON, H.-E., BAHIA, D.S., CAVALLI, A., HOFFMAN, M. & MILLIGAN, G. (2001b). Control of the efficiency of agonist-induced information transfer and stability of the ternary complex containing the δ opioid receptor and the α subunit of G_{i1} by mutation of a receptor/G protein contact interface. *Neuropharmacology,* **41,** 321 – 330.

RAMBOZ, S., OOSTING, R., AIT AMARA, D., KUNG, H.F., BLIER, P., MENDELSOHN, M., MANN, J.J., BRUNNER, D. & HEN, R. (1998). Serotonin receptor 1A knockout: An animal model of anxiety-related disorder. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 14476 – 14481.

SAMAMA, P., COTECCHIA, S., COSTA, T. & LEFKOWITZ, R.J. (1993). A mutation-induced activated state of the β2-adrenergic receptor; extending the ternary complex model. *J. Biol. Chem.,* **268,** 4625 – 4636.

SEIFERT, R., WENZEL-SEIFERT, K., GETHER, U. & KOBILKA, B.K. (2001). Functional differences between full and partial agonists: evidence for ligand-specific receptor conformations. *J. Pharmacol. Exp. Ther.,* **297,** 1218 – 1226.

SEIFERT, R., WENZEL-SEIFERT, K. & KOBILKA, B.K. (1999). GPCR-Galpha fusion proteins: molecular analysis of receptor-G-protein coupling. *Trends Pharmacol. Sci.,* **20,** 383 – 389.

STEVENS, P.A., PEDIANI, J., CARRILLO, J.J. & MILLIGAN, G. (2001). Co-ordinated agonist-regulation of receptor and G protein palmitoylation and functional rescue of palmitoylation-deficient mutants of the G protein $G_{11}\alpha$ following fusion to the α_{1b}-adrenoceptor. Palmitoylation of $G_{11}\alpha$ is not required for interaction with β/γ complex. *J. Biol. Chem.,* **276,** 35883 – 35890.

WALDHOER, M., WISE, A., MILLIGAN, G., FREISSMUTH, M. & NANOFF, C. (1999). Kinetics of ternary complex formation with fusion proteins composed of the A1-adenosine receptor and G protein α-subunits. *J. Biol. Chem.,* **274,** 30571 – 30579.

WARD, R.J. & MILLIGAN, G. (2002). Reciprocal mutations of highly conserved residues in transmembrane helices 2 and 7 of the α_{2A}-adrenoceptor restore agonist activation of $G_{i1}\alpha$. *Cell Signalling,* **14,** 139 – 144.

WEISS, J.M., MORGAN, P.H., LUTZ, M.W. & KENAKIN, T.P. (1996). The cubic ternary complex receptor occupancy model. I Model description. *J. Theoret. Biol.,* **178,** 151 – 167.

WELSBY, P.J., KELLETT, E., WILKINSON, G. & MILLIGAN, G. (2002). Enhanced detection of receptor constitutive activity in the presence of Regulators of G protein Signalling: Applications to the detection and analysis of inverse agonists and low efficacy partial agonists. *Mol. Pharmacol.,* **61,** 1211 – 1221.

WENZEL-SEIFERT, K. & SEIFERT, R. (2000). Molecular analysis of beta(2)-adrenoceptor coupling to G(s)-, G(i)-, and G(q)-proteins. *Mol. Pharmacol.,* **58,** 954 – 966.

WISE, A., CARR, I.C. & MILLIGAN, G. (1997a). Measurement of agonist-induced guanine nucleotide turnover by the G protein $G_{i1}\alpha$ when constrained within an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein. *Biochem. J.,* **325,** 17 – 21.

WISE, A., CARR, I.C., GROARKE, D.A. & MILLIGAN, G. (1997b). Measurement of agonist efficacy using an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein. *FEBS Lett.,* **419,** 141 – 146.

WREGGETT, K.A. & DE LEAN, A. (1984). The ternary complex model: its properties and applications to ligand interactions with the D2-dopamine receptor and the anterior pituitary gland. *Mol. Pharmacol.,* **26,** 214 – 227.

WONG, Y.G. (1994). G_i assays in transfected cells. *Methods Enzymol.,* **238,** 81 – 94.

WURCH, T. & PAUWELS, P.J. (2001). Analytical pharmacology of G protein-coupled receptors by stoichiometric expression of the receptor and G(alpha) protein subunits. *J. Pharmacol. Toxicol. Meth.,* **45,** 3 – 16.

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