Quantitative analysis of a cysteine^{351}glycine mutation in the G protein $G_{i1}\alpha$: effect on α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein activation

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Abstract Fusion proteins were constructed between the porcine α_{2A}-adrenoceptor and either wild-type (Cys^{351}) or a pertussis toxin-resistant (Gly^{351}) form of the G protein $G_{i1}\alpha$. Addition of adrenaline to membranes expressing the fusion proteins resulted in concentration-dependent stimulation of their high affinity GTPase activity. The α_{2A}-adrenoceptor-wild type $G_{i1}\alpha$ fusion protein produced substantially higher maximal stimulation of GTPase activity in response to adrenaline than that containing Gly^{351} $G_{i1}\alpha$. Treatment of the fusion proteins as agonist-regulated enzymes allowed measurement of V_{max} and turnover number for adrenaline-stimulation of the GTPase activity of each fusion construct. The turnover number of the α_{2A}-adrenoceptor-Cys^{351}Gly $G_{i1}\alpha$ fusion protein was only 44% of that for the α_{2A}-adrenoceptor-wild type $G_{i1}\alpha$ fusion protein. These data provide the first direct quantitative evaluation of the effects of a mutation of a G protein on the capacity of an agonist-occupied receptor to activate the mutant.

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Key words: Receptor; G protein; Agonist; Adrenaline

1. Introduction

The α subunits of members of the G_i-like family of heterotrimeric G proteins share the common feature of acting as substrates for ADP-ribosylation catalysed by pertussis toxin [1]. This is due to the presence of a conserved cysteine residue (Cys^{351} in $G_{i1}\alpha$) 4 amino acids from the C-terminus of these G proteins. Pertussis toxin-catalysed ADP-ribosylation prevents effective information transfer from appropriate receptors to the G_i-like G proteins [1]. Mutagenic alteration of this residue prevents pertussis toxin-catalysed ADP-ribosylation and, as certain mutations (e.g. Gly, Ser) have been reported to preserve receptor coupling, such modified G proteins have been used to examine the specificity of receptor interactions with different G_i-family G proteins [2–8]. Despite this, analysis of the quantitative effects of such mutations has not been considered. We have recently taken a novel approach to examine the details of interactions between receptors and G proteins. Following the initial demonstration that a fusion protein generated between the β_2-adrenoceptor and the α subunit of G_s could activate adenylyl cyclase in an agonist-dependent manner [9] we generated an equivalent fusion protein between the porcine α_{2A}-adrenoceptor and a pertussis toxin-insensitive (Cys^{351}Gly) mutant of $G_{i1}\alpha$ [10–12]. This resulted in expression of a single polypeptide containing both functions [10–12]. We demonstrated the utility of such an approach by measuring the capacity of this fusion protein to function as an agonist-activated GTPase following pertussis toxin treatment of cells [10–12]. Furthermore, fusion proteins between the receptor and acylation-resistant, mutationally modified forms of $G_{i1}\alpha$ which could not reach the plasma membrane when expressed independently, resulted in rescue of agonist activation of these G protein mutants [11]. Importantly for quantitative analyses, the fusion protein strategy defines a 1:1 stoichiometry of expression of the elements of the fusion protein and that they must be in proximity following expression [11]. Neither of these features can be easily defined and controlled during simple co-transfection experiments.

Potentially, the fusion protein approach should be particularly suitable to examine the effects of minor mutations in receptor or G protein which may alter their interactions. Herein we use this strategy to examine the quantitative effects of alteration of the pertussis toxin-sensitive cysteine^{351} of $G_{i1}\alpha$ to glycine on the capacity of the G protein to be activated by the α_{2A}-adrenoceptor.

2. Materials and methods

2.1. Materials

All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, Scotland, UK). [^{3}H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham International. [γ-^{32}P]GTP (30 Ci/mmol) was obtained from DuPont/NEN. Pertussis toxin (240 μg/ml) was purchased from Speywood. All other chemicals were from Sigma or Fisons plc and were of the highest purity available. Oligonucleotides were purchased from Genosys (Cambridge, UK).

2.2. Construction of the α_{2A}-adrenoceptor-Cys$^{351}G_{i1}\alpha$ fusion construct

The porcine α_{2A}-adrenoceptor [13] was obtained from Dr. L.E. Limbird, Vanderbilt University, TN, USA. Rat Cys^{351}Gly$G_{i1}\alpha$ was linked to the α_{2A}-adrenoceptor as described previously to generate α_{2A}R-Cys^{351}Gly$G_{i1}\alpha$ [10] and ligated into the *Kpn*I and *Eco*RI sites of the eukaryotic expression vector pcDNA3 (Invitrogen). Wild-type (Cys^{351}) rat $G_{i1}\alpha$ cDNA in pcDNA3 was digested with the restriction enzymes *Sac*II and *Eco*RI. This 1.3-kb fragment was recovered and ligated with α_{2A}R-Cys^{351}Gly$G_{i1}\alpha$ in pcDNA3 from which the equivalent 1.3-kb *Sac*II-*Eco*RI fragment had been removed. This generated α_{2A}R-Cys$^{351}G_{i1}\alpha$ in pcDNA3.

2.3. Cell culture and transfection

COS-7 cells were maintained in DMEM containing 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 60-mm culture dishes and grown to 60–80% confluency (18–24 h) prior to transfection with pCDNA 3 con-

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Abbreviations: α_{2A}R-$G_{i1}\alpha$, fusion protein containing the porcine α_{2A}-adrenoceptor linked to the α subunit of G_{i1}; α_{2A}R-Cys^{351}Gly$G_{i1}\alpha$, fusion protein containing the porcine α_{2A}-adrenoceptor linked to the α subunit of G_{i1} in which cysteine^{351} was converted to glycine

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taining the relevant cDNA species using lipofectamine reagent (Life Technologies, Inc.) [10]. For transfection, 2.5–2.8 μg of DNA was mixed with 10 μl of lipofectamine in 0.2 ml of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 30 min prior to the addition of 1.8 ml of Opti-MEM. COS-7 cells were exposed to the DNA-lipofectamine mixture for 5 h. Two ml of 20% (v/v) foetal calf serum in DMEM was then added to the cells. Cells were harvested 48 h after transfection. In a number of experiments cells were treated for the final 24 h prior to cell harvest with pertussis toxin (between 25–200 ng/ml in different experiments).

2.4. Preparation of membranes

Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80°C following harvest as described previously [14].

2.5. [^{3}H]RS-79948-197 binding studies

Binding assays were initiated by the addition of 2–4 μg of protein to an assay buffer (10 mM Tris-HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5) containing [^{3}H]RS-79948-197 [15] (0–1 nM). Non-specific binding was determined in the presence of 100 μM idazoxan. Reactions were incubated at 30°C for 45 min, and bound ligand was separated from free by vacuum filtration through GF/C filters. The filters were washed with 3×5 ml of assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

2.6. High affinity GTPase assays

High affinity GTPase assays were performed as described in [10,12]. Non-specific GTPase was assessed by parallel assays containing 100 μM GTP.

All experiments were performed on at least three membrane preparations derived from different transient transfections. Because levels of expression of the fusion proteins varied between the individual transfections over a greater than 2-fold range the data from individual experiments was pooled only when presenting GTPase activity measurements as turnover number (see Table 1).

3. Results

Fusion proteins were generated between the porcine α_{2A}-adrenoceptor and wild type rat $G_{i1}\alpha$ to generate α_{2A}R-$G_{i1}\alpha$ or with a pertussis toxin-resistant, $Cys^{351}Gly$, mutant of $G_{i1}\alpha$ to generate α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ by attachment of the N-terminus of the G protein to the C-terminus of the receptor. These proteins were expressed transiently in COS-7 cells. Saturation specific binding studies using the α_2-adrenoceptor antagonist [^{3}H]RS-79948-197 were performed routinely on membranes from these cells to ascertain levels of expression of the constructs (Fig. 1). The alteration in sequence at the C-terminus of the fusion protein did not alter the affinity of binding of the [^{3}H]ligand, K_d at α_{2A}R-$G_{i1}\alpha = 0.30 \pm 0.05$ nM, $n = 4$, K_d at α_{2A}R-$Cys^{351}GlyG_{i1}\alpha = 0.39 \pm 0.12$ nM, $n = 10$, means ± S.D., $P = 0.17$.

Basal high affinity GTPase activity and its stimulation by a range of concentrations of adrenaline was then measured in membranes expressing α_{2A}R-$G_{i1}\alpha$ using 0.5 μM GTP as substrate. Adrenaline produced a concentration-dependent increase in high affinity GTPase activity with an EC_{50} of $1.6 \pm 0.2 \times 10^{-7}$ M (mean ± S.E.M., $n = 3$) (Fig. 2). Equivalent experiments on membranes expressing (α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$) also resulted in a concentration-dependent increase in high affinity GTPase activity but now the EC_{50} for adrenaline was higher ($1.7 \pm 0.2 \times 10^{-6}$ M, mean ± S.E.M., $n = 3$, $P = 0.0014$) (Fig. 2). Expression of the two fusion proteins to similar levels in COS-7 cell membranes (Fig. 1) resulted in a substantially higher maximal stimulation of high affinity GTPase activity by adrenaline at α_{2A}R-$G_{i1}\alpha$ compared to α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (Fig. 2). To examine this more extensively the fusion proteins were treated as agonist-activated enzymes and basal and adrenaline (100 μM)-stimulated high affinity GTPase activity measured at varying [GTP] (Fig. 3). Analysis of such data demonstrated the adrenaline-stimulated V_{max} of α_{2A}R-$G_{i1}\alpha$ to be substantially greater than α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (Fig. 3) whereas the K_m for GTP (α_{2A}R-$G_{i1}\alpha = 0.42 \pm 0.14$ μM, α_{2A}R-$Cys^{351}GlyG_{i1}\alpha = 0.43 \pm 0.16$ μM,

Table 1
Comparison of the adrenaline-stimulated turnover number of α_{2A}R-$G_{i1}\alpha$ and α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$

	Turnover number (min^{-1})
α_{2A}R-$G_{i1}\alpha$	8.0 ± 0.6
α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$	3.5 ± 0.7

Turnover numbers (means ± S.E.M., $n = 4$) for adrenaline-stimulated GTPase activity were calculated at V_{max} from experiments similar to those displayed in Figs. 3 and 5 and saturation ^{3}H-antagonist binding data performed on the same membrane preparations. The contribution of endogenous G proteins to the total high affinity GTPase signal was subtracted as defined in Section 3.

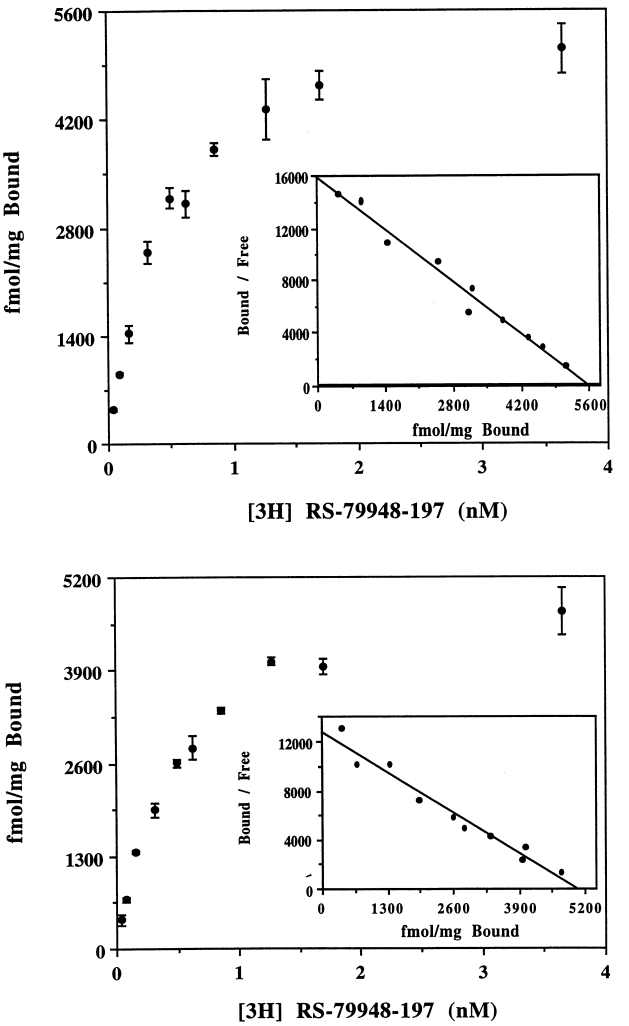


Fig. 1. Binding of [^{3}H]RS-79948-197 to α_{2A}R-$G_{i1}\alpha$ and α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$. The specific binding of [^{3}H]RS-79948-197 to membranes of COS-7 cells expressing either α_{2A}R-$G_{i1}\alpha$ (upper) or α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (lower) was assessed following transient transfection of their cDNAs. Insets represent Scatchard transformation of the data. Equivalent analyses were performed on all the individual transient transfections used in the study. Levels of expression varied over a greater than 2-fold range in the individual transfections.

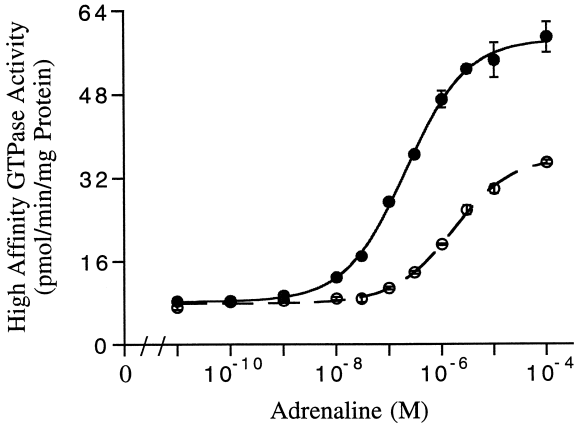


Fig. 2. Adrenaline stimulates the GTPase activity of α_{2A}R-$G_{i1}\alpha$ to a greater extent than α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$. Basal high affinity GTPase activity and its regulation by varying concentrations of adrenaline were measured using 0.5 μM GTP in membranes of COS-7 cells expressing either α_{2A}R-$G_{i1}\alpha$ (filled symbols) or α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (open symbols). Data is derived from the same set of membranes displayed in Fig. 1 and is representative of 4 separate experiments performed on membranes from different sets of transfections.

mean ± S.D., $n = 4$ in each case) was not different for the two constructs ($P = 0.94$).

Prior pertussis toxin (25 ng/ml, 24 h) treatment of COS-7 cells is sufficient to cause complete ADP-ribosylation of endogenously expressed G_i-like G proteins and to fully attenuate agonist stimulation of high affinity GTPase activity by a transiently expressed α_{2A}-adrenoceptor [16]. Such pretreatment of COS-7 cells expressing α_{2A}R-$G_{i1}\alpha$ resulted in a substantial (67.7 ± 2.4%, mean ± S.E.M., $n = 3$), but not complete, reduction in the capacity of adrenaline to stimulate high affinity GTPase activity which was accompanied by a small (5.1-fold) but statistically significant ($P = 0.04$) increase in the EC_{50} for adrenaline (Fig. 4, upper panel). Although such treatment did not abolish adrenaline stimulation of high affinity GTPase activity, pretreatment of the cells with higher levels of pertussis toxin (up to 200 ng/ml, 24 h) produced no further reduction in the response to adrenaline (data not shown). As such, the remaining agonist-stimulated GTPase activity is unlikely to simply reflect a requirement for higher levels of the toxin to modify wild-type $G_{i1}\alpha$ within the fusion protein.

Equivalent pertussis toxin pretreatment of cells expressing α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ did not alter agonist-stimulated GTPase activity substantively (Fig. 4, lower panel) and did not alter the EC_{50} for adrenaline ($2.0 \pm 0.5 \times 10^{-6}$ M, mean ± S.E.M., $n = 3$, $P = 0.64$).

Adrenaline-stimulated GTPase activity vs. [GTP] experiments equivalent to those detailed in Fig. 3 confirmed both that pertussis toxin pretreatment had little apparent effect on the agonist-stimulated V_{max} of α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (Fig. 5, upper panel). In contrast such treatment substantially reduced, but did not abolish, agonist-stimulated GTPase activity of α_{2A}R-$G_{i1}\alpha$ when measured at V_{max} (Fig. 5, lower panel) again without altering the K_m for GTP (following pertussis

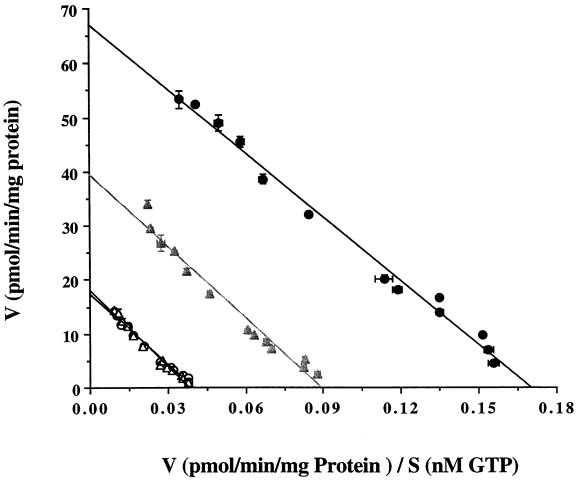
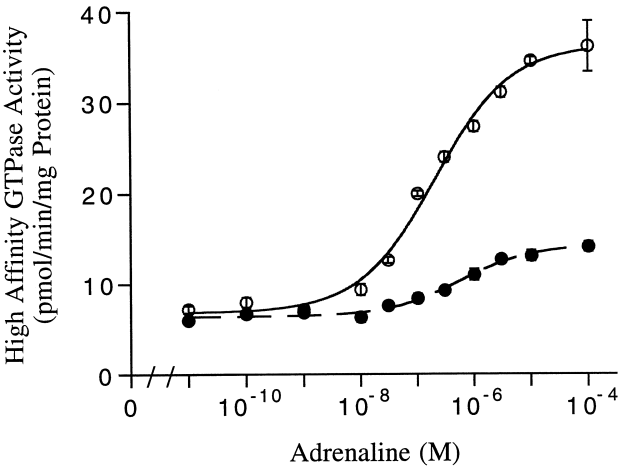


Fig. 3. Adrenaline increases the V_{max} of the GTPase activity of α_{2A}R-$G_{i1}\alpha$ to a greater extent than α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ although their K_m for GTP is not different. Basal high affinity GTPase activity (open symbols) and its stimulation by adrenaline (100 μM) was measured at a range of concentrations of GTP in membranes of COS-7 cells expressing either α_{2A}R-$G_{i1}\alpha$ (circles) or α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (triangles). The data are presented as an Eadie-Hofstee transformation.



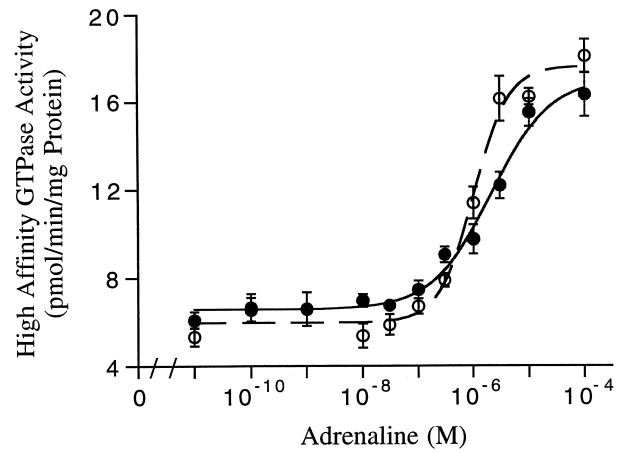


Fig. 4. The effect of pertussis toxin on adrenaline stimulation of the GTPase activity of α_{2A}R-$G_{i1}\alpha$ and α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$. Basal high affinity GTPase activity and its regulation by varying concentrations of adrenaline was measured using 0.5 μM GTP in membranes from either untreated (open symbols) or pertussis toxin pretreated (25 ng/ml, 24 h) COS-7 cells transiently transfected to express α_{2A}R-$G_{i1}\alpha$ (upper panel) or α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (lower panel).

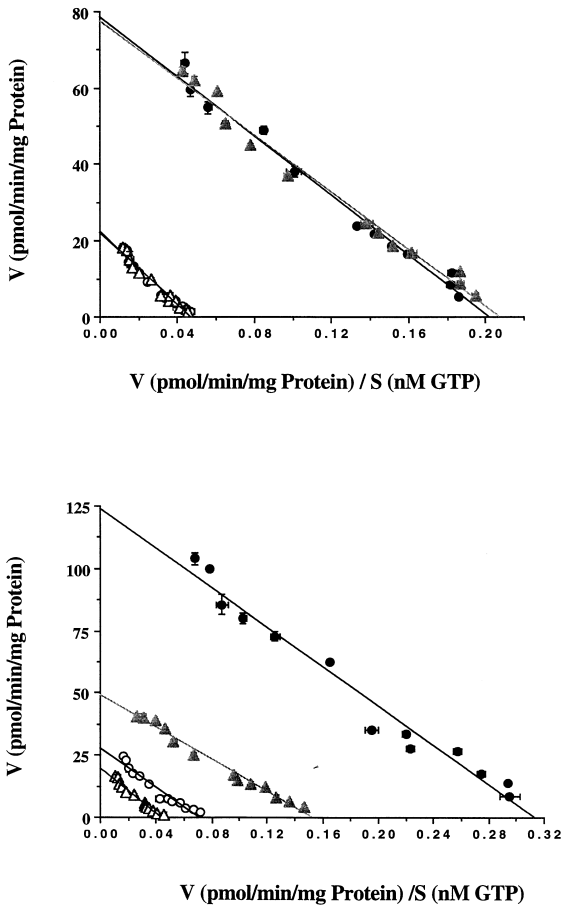


Fig. 5. The effect of pertussis toxin on the V_{max} of the GTPase activity of α_{2A}R-$G_{i1}\alpha$ and α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$. Basal high affinity GTPase activity (open symbols) and its stimulation by adrenaline (100 μM) (filled symbols) were measured at a range of concentrations of GTP in membranes of COS-7 cells expressing either α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ (upper) or α_{2A}R-$G_{i1}\alpha$ (lower) which were untreated (circles) or pretreated with pertussis toxin (25 ng/ml, 24 h) (triangles). The data are presented as Eadie-Hofstee transformations.

toxin treatment α_{2A}R-$G_{i1}\alpha = 0.39 \pm 0.11$ μM, mean ± S.D., $P = 0.1$).

Levels of expression of the receptor-G protein fusion proteins varied between individual transfections and thus translated into differences in absolute levels of agonist-stimulated GTPase activity in different membrane preparations (note the variation in absolute GTPase activity numbers in the different experiments displayed). Therefore, data from experiments such as those in Figs. 3 and 5 were converted to adrenaline-induced GTPase turnover numbers (GTPase activity at V_{max} (pmol GTP hydrolysed/min/mg membrane protein)/fusion protein expression levels (pmol/mg membrane protein) (Table 1). This demonstrated unequivocally the greater capacity of adrenaline to stimulate the GTPase activity of α_{2A}R-$G_{i1}\alpha$ compared to α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$. However, when presented in this manner, although the measured GTPase turnover number for α_{2A}R-$G_{i1}\alpha$ was reduced from 9.7 ± 0.6 min^{-1} to 2.9 ± 0.3 min^{-1} by pertussis toxin treatment there was also a reduction from 5.6 ± 0.5 min^{-1} to 4.4 ± 0.5 min^{-1} in the adrenaline-stimulated turnover number of α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ following pertussis toxin treatment (means ± S.E.M., $n = 4$ in each case). As α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ is resistant to pertussis toxin treatment this contribution to the overall GTPase signal must reflect activation of the endogenously expressed pertussis-sensitive G proteins (see Section 4 for details). This contribution was thus subtracted to allow direct comparison of turnover number of α_{2A}R-$G_{i1}\alpha$ and α_{2A}R-$Cys^{351}GlyG_{i1}\alpha$ in response to adrenaline and thus a direct measurement of the maximal capacity (44%) of the adrenaline-occupied α_{2A}-adrenoceptor to active $Gly^{351}G_{i1}\alpha$ compared to wild type $(Cys^{351})G_{i1}\alpha$ (Table 1).

4. Discussion

Minor mutations in both receptors and G proteins are known which interfere with functional contacts between these proteins. A good example of this is the *unc* mutation of $G_s\alpha$ in which alteration of Arg^{389} to Pro, six amino acids from the C-terminal of the protein, prevents functional activation of the G protein by an agonist-occupied β-adrenoceptor. The deleterious effects of such a mutation can easily be considered when it is appreciated that the C-terminal region of G protein α subunits represents a key interaction domain for receptors and this specific mutation is likely to produce a marked structural deformation in this region. Pertussis toxin, elucidated by *Bordetella pertussis*, attenuates functional contacts between receptors and the G_i-family of G proteins because it functions as an ADP-ribosyltransferase, the acceptor for which is a conserved cysteine residue 4 amino acids from the C-terminus of these G proteins [1]. Because of this capacity of pertussis toxin and the routine co-expression of a number of pertussis toxin-sensitive G-proteins by most cells, a number of groups have generated mutants of the G_i-proteins which have been used to examine potential selectivity of interactions between receptors and individual G_i-G proteins. Such studies have used alterations of the pertussis toxin-sensitive Cys to either Ser or Gly, presumably as these have been considered conservative mutations which might not interfere dramatically with function. However, in studies on the interaction of rhodopsin and transducin, a combinatorial library of peptides derived from the C-terminal region of transducin has suggested the absolute importance of the presence of the pertussis toxin-sensitive Cys for high affinity interactions [17] whereas direct mutational alterations of the protein have suggested greater flexibility, with a Cys-Ala mutation having little effect [18] and a Cys-Tyr mutant resulting in complete loss of function [19]. The rhodopsin-transducin system has a range of specific features which make such assays relatively easy to perform, including a capacity to measure the stabilisation of meta-rhodopsin II spectrophotometrically. However, despite the many features of commonality between rhodopsin-transducin and the interactions of other receptor and G protein pairs, there is no a priori reason to assume that such mutational data can be directly compared.

Available studies utilising co-expression of a receptor with mutationally modified G_i-like G proteins which have rendered them insensitive to pertussis toxin [2–8] have not been designed to provide quantitative analysis of the effects of the mutation. Therefore, we have taken the strategy of utilising fusion proteins [9–12] in which a receptor is linked to each of the wild-type and mutated G protein to provide such quantitation. The basis for this approach is three-fold. (i) The nature of the fusion proteins defined that the stoichiometry of receptor and G protein is fixed as 1:1. This would be difficult to achieve in most other systems and eliminates concerns that the

mutation in the G protein might significantly alter its state level of expression. This strategy also allowed absolute quantitation of expression of the proteins from saturation ^{3}H-antagonist ligand binding studies. (ii) The nature of the fusion proteins defines that the receptor and G protein must be in proximity following expression. Again there is no inherent reason to assume this is the case in co-transfection studies. Mutational alteration of the G protein may alter this as we and others have demonstrated for acylation mutations of G proteins [20–23]. On this basis we have previously used a fusion protein strategy to ascertain whether acylation is integral to signal transduction from receptor to G protein or simply required to target the G protein appropriately to the membrane [11]. (iii) Using the fusion proteins, agonist-activated GTPase activity can be converted to enzyme catalytic centre activity and thus provide a direct measure of activation.

Using this approach we have demonstrated herein that the adrenaline-stimulated turnover number of α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ is only 44% of that of α_{2A}R-G$_{i1}\alpha$ when measured at V_{max} using a maximally effective concentration of adrenaline. There was no difference, however, in the K_m for GTP (Fig. 3). These are unique sets of measurements and could not have been calculated without development of the fusion protein strategy.

A number of elements of the study deserve further comment. Pertussis toxin treatment of α_{2A}R-G$_{i1}\alpha$ expressing COS-7 cells did not result in complete ablation of adrenaline-stimulated GTPase activity, even when using 10-fold higher concentrations of the toxin than we have previously demonstrated to be required to fully attenuate GTPase stimulation in membranes of these cells by the expressed, separated, α_{2A}-adrenoceptor [16]. Indeed, at V_{max} some 30% of the total adrenaline-stimulated function was resistant to pertussis toxin treatment. Although we have recently shown the capacity of α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ to activate both endogenously expressed G$_i$ and the fusion protein partner G protein following stable expression of this construct in Rat 1 fibroblasts [24], the pertussis toxin resistant fraction of activity cannot result from interaction with endogenous G$_i$ as this was fully ADP-ribosylated by the toxin treatment (data not shown). The most obvious explanation for this data is that addition of ADP-ribose to Cys^{351} of G$_{i1}\alpha$ within the confines of the fusion protein is simply insufficient to fully prevent adrenaline-mediated GDP-GTP exchange and subsequent hydrolysis, even though this is generally held to be true for interactions between the separated receptor and G protein [1]. A second possibility is that the conformation of the receptor-G protein fusion protein is such that a fraction of the fusion protein population was shielded from ADP-ribosylation catalysed by pertussis toxin. Although this possibility cannot be firmly excluded, the lack of further reduction of the effects of adrenaline following treatment with between 25–200 ng/ml pertussis toxin does not favour a concept of simple variation in toxin efficiency. Residual agonist-activated GTPase activity is not uncommon in situations in which all of the measurable G$_i$-G protein has become ADP-ribosylated. Such observations are often taken to imply interaction of the receptor with further, pertussis toxin-insensitive, G proteins but this view is rarely tested directly. Certainly, expression of the isolated porcine α_{2A}-adrenoceptor in COS-7 cells at substantially higher levels than used herein for the fusion proteins does not result in noticeable stimulation of GTPase activity following pertussis toxin treatment [16]. Such results suggest that any interactions with other classes of G proteins could not have been detected with this assay.

Although the data of Figs. 4 and 5 seem to indicate that pertussis toxin treatment had no measurable effect on adrenaline-stimulated GTPase activity on COS-7 cells membranes expressing α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ this is not strictly true. Although no more than a 20% effect, there were consistently somewhat higher steady-state levels of α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ in the samples treated with pertussis toxin compared to vehicle treated controls. Therefore, in calculations of adrenaline-induced turnover number (Table 1), this was lower in pertussis toxin treated COS-7 cells expressing α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ (see Section 3) even though this construct cannot be modified by the toxin. The obvious conclusion is that in this system the pertussis toxin-sensitive contribution to the total adrenaline-stimulated GTPase must be provided by interactions between the α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$ fusion protein and endogenous G$_i$. As such, these values were subtracted, as were equivalent values from the data with α_{2A}R-G$_{i1}\alpha$, to allow absolute turnover numbers to be calculated for the two fusion proteins. It is noteworthy that the α_{2A}R-G$_{i1}\alpha$ protein also maintained a 10-fold lower EC$_{50}$ for adrenaline than α_{2A}R-Cys^{351}GlyG$_{i1}\alpha$, a feature we have also noted following individual co-expression of the α_{2A}-adrenoceptor and the wild-type and Cys^{351}Gly forms of G$_{i1}\alpha$ [16].

Many assay systems for agonist ligands at G protein-coupled receptors utilise G protein activation assays based on either agonist-enhancement of GTPase activity or of the binding of ligands such as [^{35}S]GTPγS [25–29]. The current studies provide an entirely novel approach to examine the quantitative details of alterations in receptor-G protein interactions which should be equally amenable to mutations in both receptor or G protein.

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