RESEARCH COMMUNICATION

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

Alan WISE*, I. Craig CARR and Graeme MILLIGAN†

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

A fusion protein was generated between the porcine α_{2A}-adrenoceptor and a pertussis-toxin-insensitive ($Cys^{351} \rightarrow Gly$) variant of the α subunit of $G_{i1}\alpha$ by direct in-frame fusion of the N-terminus of the G-protein to the C-terminus of the receptor. The fusion protein could be transiently expressed to high levels in COS-7 cells. Addition of the α_2-adrenoceptor agonist 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14304) to membranes of pertussis-toxin-treated transfected cells resulted in a concentration-dependent stimulation of high-affinity GTPase activity. V_{max} estimations for the GTPase activity demonstrated an induced catalytic-centre activity of $2.0 \pm 0.2\ min^{-1}$ for $G_{i1}\alpha$ when the α_{2A}-adrenoceptor was maximally stimulated by UK14304 with a K_m for GTP of $0.37 \pm 0.07\ \mu M$. Co-expression of excess $\beta_1\gamma_2$ along with the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein resulted in greater maximal UK14304-induced stimulation of high-affinity GTPase activity (2.1 ± 0.2-fold) without alteration in agonist EC_{50}. These studies demonstrate the functionality of the fusion construct, its capacity to interact with $\beta\gamma$ complex and its utility in measuring agonist regulation of the catalytic-centre activity of GTP by a receptor-associated G-protein.

INTRODUCTION

Signal transduction via heterotrimeric G-proteins requires the capacity of agonist-occupied receptors to increase the rate of exchange of GTP for GDP in the nucleotide-binding pocket of the G-protein α subunit [1–3]. The GTP-liganded α subunit can then dissociate from both receptor and from the G-protein $\beta\gamma$ complex allowing regulation of effectors [1–3]. Deactivation of the G-protein α subunit is produced by the intrinsic capacity of the α subunit to act as a GTPase, resulting once again in the presence of GDP in the binding site [1–3]. Measurements of the GTPase activity of isolated G-proteins have routinely resulted in estimates which are too low to account for the rapid kinetics of ligand-induced activation and deactivation of signal transduction cascades (see [1] for review of early data; [4,5]). These values were increased in experiments which have reconstituted G-proteins with receptors in lipid vesicles [4] or detergent was removed [6], and also by reports showing that a number of effector enzymes [7,8] and so-called regulators of G-protein signalling 'RGS' [9–11] can act as GTPase activating proteins 'GAPs' for certain G-protein α subunits. Despite these studies, direct estimations of catalytic-centre activity based on agonist-induced enhancement of GTPase activity, whether performed in membrane preparations or in reconstituted systems, have been difficult to achieve because, either the absolute levels of expression of the receptor and G-protein are unknown or it has been unclear whether the agonist-occupied receptor has the capacity to activate all of the G-protein provided.

In the present work we have adopted a markedly different strategy in which we have generated a fusion protein between the α_{2A}-adrenoceptor and the G-protein $G_{i1}\alpha$. Only one report of the generation of such a receptor–G-protein fusion construct has been published [12], using the β_2-adrenoceptor and the α subunit of its cognate G-protein G_s to demonstrate the capacity of the fusion protein to activate adenylate cyclase.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies Inc. The α_2-adrenoreceptor antagonist [^{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, and were of the highest purity available. Oligonucleotides were purchased from Genosys (Cambridge, U.K.).

Construction of Cys^{351}-Gly $G_{i1}\alpha$

A pertussis-toxin-resistant form of rat $G_{i1}\alpha$ was generated as in [13] following excision of the wild-type sequence on an *Eco*RI restriction fragment from the vector pGEM2 and insertion into the *Eco*RI site of pBluescript KS (Stratagene), creating pBS/$G_{i1}\alpha$.

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

The porcine α_{2A}-adrenoceptor [14] was obtained from Dr. L. E. Limbird, Vanderbilt University, Nashville, TN, U.S.A. The pertussis-toxin-resistant $Cys^{351} \rightarrow Gly$ form of rat $G_{i1}\alpha$ was linked to the α_{2A}-adrenoceptor. To do so, the open reading frame (ORF) of the α_{2A}-adrenoceptor DNA was amplified by PCR using the oligonucleotides: sense, 5′-TTGGTACCATGTATCCTTACGACGTTC-3′; antisense, 5′-AAGAATTCCATGGCGATCCGTTTCCTGTCCCCACGGC-3′. The restriction sites

Abbreviations used: UK14304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; ORF, open reading frame.

† To whom correspondence should be addressed.

* Present address: Receptor Systems Unit, GlaxoWellcome Research and Development, Stevenage SG1 2NY, Herts., U.K.

for *Kpn*1, *Eco*R1 and *Nco*1 are underlined. The PCR-amplifed fragment was digested with *Kpn*1 and *Eco*R1 and ligated to pBluescript (pPS) (Stratagene) through these restriction sites. Introduction of the *Nco*1 site at the 3′ end of the ORF resulted in the C-terminal amino acid of the receptor being altered from valine to alanine and to removal of the stop codon. The rat $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ cDNA contains two *Nco*1 sites, one straddling the ATG start codon and the other 268 bp downstream from this. This 268 bp fragment was removed from $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ in pBS by digestion with *Nco*1 and the remaining $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ pBS cDNA was re-ligated. The shortened cDNA was excised from pBS with *Eco*R1 and cloned into the *Eco*R1 site of the α_{2A}-adrenoceptor in pBS, adjacent to the 3′ end of the receptor ORF. The 268 bp fragment was then inserted between the *Nco*1 sites at the 3′ end of the α_{2A}-adrenoceptor ORF and at the 5′ end of the $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ ORF. This resulted in production of an in-frame construct, whereby the 3′ end of the α_{2A}-adrenoceptor ORF was exactly adjacent to the 5′ end of the $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ ORF. The full fusion construct was then excised from pBS with *Kpn*1 and *Eco*R1 and ligated into the eukaryotic expression vector pCDNA3.

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium 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) before transfection with pCDNA3 containing 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 was incubated at room temperature for 30 min before the addition of 1.8 ml of Opti-MEM. COS-7 cells were exposed to the DNA/lipofectamine mixture for 5 h and 2 ml of 20% (v/v) foetal-calf serum in Dulbecco's modified Eagle's medium was then added. The cells were harvested 48 h after transfection. In the bulk of experiments, cells were treated with pertussis toxin (25 ng/ml) for the final 16 h before harvesting in order to bring about ADP-ribosylation of the endogenous G_i-family G-proteins [13], and thus prevent potential interactions between these and the fusion protein.

Preparation of membranes

Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80 °C after harvesting, as described previously [13].

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

Binding assays were initiated by the addition of 2–4 μg of membrane 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. The samples were incubated at 30 °C for 45 min, and bound was separated from free ligand by vacuum filtration through GF/C filters. The filters were washed with 3 × 5 ml of assay buffer, and the radioactivity contained in bound ligand was estimated by liquid scintillation spectrometry.

Immunological studies

Both antisera I1C and BN3 [16] were produced in New Zealand White rabbits, using conjugates of synthetic peptides and keyhole-limpet haemocyanin (Calbiochem) as antigen. Antiserum I1C was raised against a synthetic peptide corresponding to amino acids 160–169 of the $G_{i1}\alpha$ subunit. The specificity of the antiserum for $G_{i1}\alpha$ has been reported previously [16]. Antiserum BN3 was raised against a peptide corresponding to the N-terminal decapeptide of the β1 subunit. Membrane samples were resolved by SDS/PAGE using 10% (w/v) acrylamide gels. Proteins were subsequently transferred to nitrocellulose membranes (Schleicher and Schuell), probed with relevant antiserum and revealed as described previously [17].

High-affinity GTPase assays

The assays were performed essentially as described previously [18]. Non-specific GTPase was assessed by parallel assays containing 100 μM GTP.

RESULTS

Expression in COS-7 cells of a fusion protein consisting of the porcine α_{2A}-adrenoceptor and a form of the α subunit of $G_{i1}\alpha$ which is resistant to treatment with pertussis toxin due to $Cys^{351} \rightarrow Gly$ substitution (Figure 1), resulted in a high level of

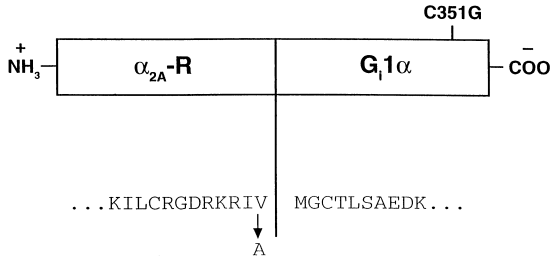

Figure 1 Construction of α_{2A}-adrenoceptor–$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$

Fusion of the N-terminus of a pertussis-toxin-resistant form ($Cys^{351} \rightarrow Gly$) of $G_{i1}\alpha$ to the C-terminus of the porcine α_{2A}-adrenoceptor resulted in the receptor C-terminal amino acid valine (V) being converted into alanine (A) and maintainance of the methionine (M), which normally functions as initiator in $G_{i1}\alpha$.

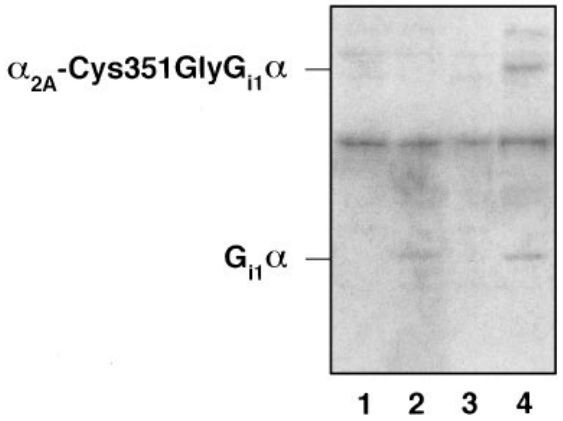

Figure 2 Expression of α_{2A}-adrenoceptor–$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$

COS-7 cells were either mock-transfected (lanes 1 and 2) or transfected with the α_{2A}-adrenoceptor–$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ fusion protein (α_{2A}-Cys351Gly$G_{i1}\alpha$) cDNA (lanes 3 and 4) in pCDNA3. Cell homogenates were separated into cytosolic (lanes 1 and 3) and P2 particulate (lanes 2 and 4) fractions and portions containing 25 μg of protein were resolved by SDS/PAGE and immunoblotted to detect the presence of $G_{i1}\alpha$. In the P2 particulate fraction of cells expressing the fusion protein a polypeptide of approx. 100 kDa was detected, which was not present in the mock-transfected cells.

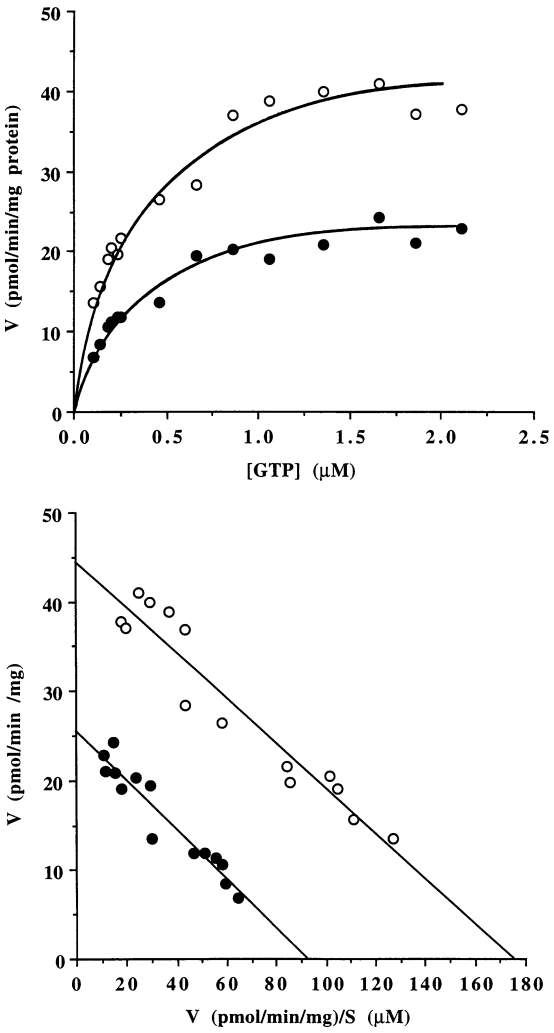

Figure 3 UK14304 stimulates high-affinity GTPase activity of the α_{2A}-adrenoceptor–Cys^{351} → Gly $G_{i1}\alpha$ fusion protein

P2 particulate membrane fractions from pertussis-toxin-treated COS-7 cells transfected to express the α_{2A}-adrenoceptor–Cys^{351} → Gly $G_{i1}\alpha$ fusion protein were used to measure high-affinity GTPase activity. Upper panel; high-affinity GTPase activity was measured over a range of GTP concentrations in the absence (●) or presence (○) of UK14304 (10 μM). Lower panel; the data are presented as an Eadie–Hofstee transformation. In the example shown, the increase in V_{max} produced by UK14304 was 18.8 pmol/min per mg of membrane protein. As these membranes expressed the fusion protein at 6.2 pmol per mg of protein, then UK14304 stimulation of turnover of GTP by the fusion-protein-associated G-protein could be calculated as 3.0 min^{-1}.

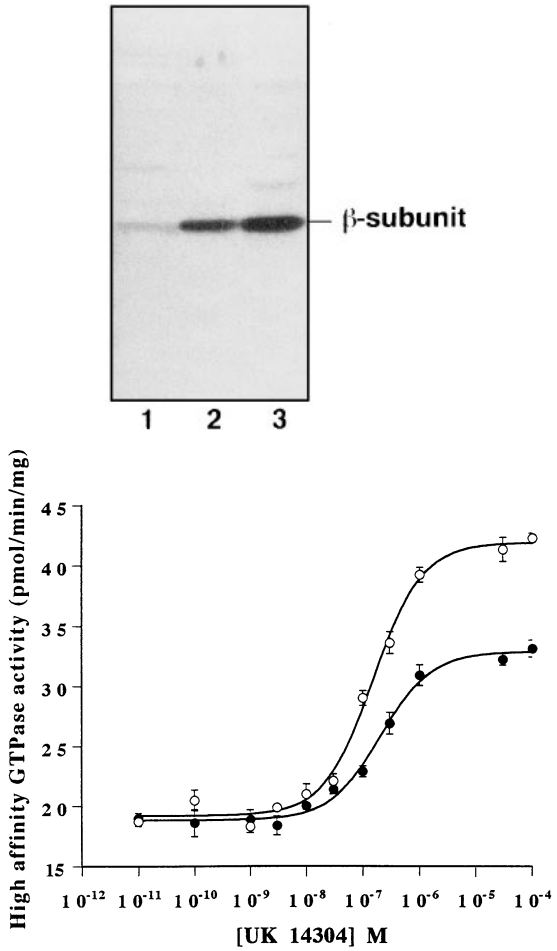

Figure 4 Co-expression of $\beta_1\gamma_2$ increases the GTPase capacity of the α_{2A}-adrenoceptor–Cys^{351} → Gly $G_{i1}\alpha$ fusion protein

COS-7 cells were transfected to express either the α_{2A}-adrenoceptor–Cys^{351} → Gly $G_{i1}\alpha$ fusion protein alone (1 μg cDNA) or in combination with β_1 and γ_2 (each at 1 μg cDNA). Upper panel; P2 particulate fractions (25 μg) from: (lane 1) COS-7 cells containing fusion protein alone, (lane 2) COS-7 cells containing fusion protein plus $\beta_1\gamma_2$, or (lane 3) rat brain (positive control), were immunoblotted with antiserum BN3 [15] to detect the β-subunit. Lower panel; P2 particulate fractions were exposed to various concentrations of UK14304 and high-affinity GTPase activity was measured using 0.5 μM GTP. In the example shown, the EC_{50} for UK14304 was 2.1×10^{-7} M in the absence of $\beta_1\gamma_2$ (●) and 1.5×10^{-7} M in the presence of excess $\beta_1\gamma_2$ (○). Three further experiments produced similar data.

expression (6–15 pmol/mg of membrane protein in individual transfections) in P2 particulate membrane fractions of this construct when measured by saturation analysis of the specific binding of the highly selective α_2-adrenoceptor antagonist [^{3}H]RS-79948-197 (results not shown). The K_d for this interaction was 0.35 ± 0.02 nM (mean $\pm$ S.E.M., $n = 5$), a value similar to that obtained following individual co-expression of the α_{2A}-adrenoceptor and Cys^{351} → Gly $G_{i1}\alpha$ in COS-7 cells (results not shown). Expression of the fusion protein as an entirely particulate polypeptide of approx. 100 kDa was also detected by immunoblotting membrane and cytosolic fractions of transfected cells with the $G_{i1}\alpha$-specific antiserum I1C [16] (Figure 2). Both fusion-protein-transfected and mock-transfected cells expressed detectable levels of authentic endogenous $G_{i1}\alpha$ which migrated as a 41 kDa polypeptide (Figure 2) but the mock-transfected cells did not express the 100 kDa polypeptide.

Addition of the α_2-adrenoceptor agonist UK14304 (10^{-5} M) to membranes of pertussis-toxin-treated COS-7 cells expressing the α_{2A}-adrenoceptor-Cys^{351} → Gly $G_{i1}\alpha$ fusion protein resulted in stimulation of high-affinity GTPase activity when measured using 0.5μM GTP as substrate, a standard condition for assays of this type [18]. The enzymic properties of the fusion protein were then measured following incubation with various concentrations of GTP and either vehicle or 10^{-5} M UK14304. In the results of the experiment shown in Figure 3, the V_{max} above basal high-affinity GTPase activity induced by the agonist was 18.8 pmol/min per mg of membrane protein. As the fusion protein was expressed to 6.2 pmol per mg of membrane protein in this transfection, the UK14304-induced turnover number was calculated to be 3.0 min^{-1}. Data from independent transfections resulted in a measured catalytic-centre activity of 2.0 ± 0.2

(mean ± S.E.M., $n = 5$) for the receptor-attached $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$. The measured K_m for GTP of the fusion protein was $0.37 \pm 0.07\ \mu M$ (mean ± S.E.M., $n = 5$), which was similar to that of basal high-affinity GTPase activity ($0.32 \pm 0.02\ \mu M$, mean ± S.E.M., $n = 5$).

UK14304 stimulated the high-affinity GTPase activity of the α_{2A}-adrenoceptor-$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ fusion protein in a concentration-dependent manner (Figure 4, lower panel) with an $EC_{50} = 2.3 \pm 0.3 \times 10^{-7}$ M ($n = 3$). Co-expression of the α_{2A}-adrenoceptor-$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ fusion protein with excess $\beta\gamma$ complex (Figure 4, upper panel), provided as the β_1 and γ_2 subunits, resulted in greater maximal (2.1 ± 0.2-fold, mean ± S.E.M., $n = 4$) UK14304 stimulation of high-affinity GTPase activity (Figure 4, lower panel). This did not reflect either higher steady-state levels of expression of the fusion protein under these conditions (results not shown) or a substantial alteration in the EC_{50} for UK14304 ($EC_{50} = 1.5 \pm 0.2 \times 10^{-7}$M, $n = 3$) (Figure 4, lower panel). Unlike UK14304, addition of the α_2-adrenoceptor antagonist yohimbine ($10^{-5}\ \mu M$) did not modify the GTPase activity of membranes expressing the fusion protein (results not shown). However, yohimbine interacted with the fusion protein as co-addition of yohimbine and UK14304 blocked the effect of the agonist (results not shown).

DISCUSSION

In the present study we have constructed a fusion protein between the α_{2A}-adrenoceptor and the α subunit of the G-protein G_{i1} as a means to examine agonist regulation of G-protein activation. The fusion protein was generated by the apparently simplistic expedient of linking the N-terminus of the G-protein directly to the C-terminal tail of the receptor. The strategy used resulted in minimal alteration to the sequence of the protein in the region of fusion. Indeed, only the C-terminal amino acid of the receptor was altered (valine to alanine) and the initiator methionine of the G-protein, which would normally be removed, remained in the sequence of the new protein (see Figure 1). As an aid to subsequent analysis we generated the fusion protein using a form of $G_{i1}\alpha$ in which Cys^{351}, which is the normal target for pertussis-toxin-catalysed ADP-ribosylation, was replaced by glycine. We have demonstrated previously that $Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ is not a substrate for pertussis-toxin-catalysed ADP-ribosylation [13]. It can, however, be activated by the α_{2A}-adrenoceptor, the only clear difference, compared with the wild-type G-protein, was that approx. 10–15-fold higher concentrations of agonist were required to produce the same degree of stimulation [13]. This is presumably a reflection of the alteration in conformation of the C-terminus of the G-protein, an element known to be a key receptor contact site [19]. This mutation, however, allows receptor regulation of the mutated G-protein to be studied in isolation following pertussis-toxin treatment of cells to eliminate potential functional contacts between the receptor and the endogenously expressed G_i-family G-proteins [13,20–21].

Expression of the α_{2A}-adrenoceptor-$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ fusion protein in COS-7 cells was monitored by two separate strategies. The first of these involved immunodetection of the fusion protein with an antiserum which identifies $G_{i1}\alpha$. Following transfection, an approx. 100 kDa polypeptide was detected with the antiserum, which was not present in mock-transfected cells (Figure 2). The second strategy utilized saturation binding studies with the α_2-adrenoceptor antagonist [^{3}H]RS-79948-197 (results not shown). As the fusion protein defines a stoichiometry of expression of 1 : 1 for the receptor and its associated G-protein and, furthermore, defines that they will be in proximity following expression, it provided an interesting model with which to examine the enzymic properties of a receptor-activated G-protein. Addition of the agonist UK14304 caused marked stimulation of high-affinity GTPase activity in pertussis-toxin-treated COS-7 cells transfected to express the fusion protein. Measurement of the V_{max} of this reaction and the simple estimation of expression levels of the fusion protein based on ^{3}H-labelled ligand-binding studies allowed the first direct estimations of catalytic-centre activity for a receptor activated G-protein.

Given the nature of the physical linkage between the receptor and G-protein, the knowledge that the N-terminal of the G-protein α subunit plays a central role in interaction with the $\beta\gamma$ complex [22,23] and the concept that the $\beta\gamma$ complex may play a key role in receptor interactions with the α subunit [24–26], it was of considerable interest to observe that co-expression of excess $\beta_1\gamma_2$ along with the α_{2A}-adrenoceptor-$Cys^{351} \rightarrow Gly$ $G_{i1}\alpha$ fusion protein resulted in greater maximal UK14304 stimulation of GTPase activity without alteration in the measured EC_{50} for the agonist. Under these conditions, the measured catalytic-centre activity for the receptor-associated G-protein increased by some 50 %. A trivial explanation for this observation, based on higher steady-state levels of expression of the fusion protein in the presence of excess $\beta\gamma$, was eliminated by performing ^{3}H-labelled antagonist binding studies (results not shown). These results imply interaction of the fusion protein with the $\beta\gamma$ complex and that formation of a receptor–$\alpha\beta\gamma$ complex is required to allow the most effective signal transduction between receptor and G-protein. It is of interest in this regard to note that Taylor et al. [27] have previously indicated a role for $\beta\gamma$ in receptor stimulation of the GTPase activity of the α_{2A}-adrenoceptor and that Kisselev and Gautam [28] have provided evidence for selectivity of γ subunits in receptor interactions in as much as rhodopsin has the capacity to interact with a G-protein containing γ_1 but not γ_2 or γ_3 subunits.

These studies provide the first direct measurements of the enzymic capacity of a G-protein when constrained within a receptor–G-protein fusion protein and demonstrate the utility of this fusion protein to examine the functional interactions between receptors and G-proteins. The availability of the fusion protein should also offer a novel means to examine the capacity of other regulators to modify this rate and to further understand why physiological deactivation processes appear to outstrip the intrinsic GTPase capacity of G-proteins.

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