A Cysteine-3 to Serine Mutation of the G-Protein $G_{i1}\alpha$ Abrogates Functional Activation by the α_{2A}-Adrenoceptor but Not Interactions with the $\beta\gamma$ Complex

Alan Wise,[‡] Morag A. Grassie,[‡] Marco Parenti,[§] Melanie Lee,[∥] Stephen Rees,[∥] 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., Receptor Systems Unit, Glaxo−Wellcome Research and Development, Gunnels Wood Road, Stevenage SG1 2NY Hertfordshire, England, U.K., and Dipartimento di Farmacologia, Universita di Milano, Via Vanvitelli 32, 20129 Milano, Italy

Received February 10, 1997; Revised Manuscript Received June 9, 1997[⊗]

ABSTRACT: Pertussis toxin-resistant (C351G) and also palmitoylation-negative (C3S/C351G), myristoylation-negative (G2A/C351G) and combined acylation-negative (G2A/C3S/C351G) forms of the G-protein $G_{i1}\alpha$ were expressed in COS-7 cells along with the porcine α_{2A}-adrenoceptor. G2A/C3S/C351G $G_{i1}\alpha$ and G2A/C351G $G_{i1}\alpha$ were largely cytosolic and failed to interact with the agonist-occupied α_{2A}-adrenoceptor in membrane preparations. In contrast, C351G $G_{i1}\alpha$ was almost entirely particulate and the α_2-adrenoceptor agonist UK14304 caused a marked stimulation of its GTPase activity and binding of [^{35}S]GTPγS which was not prevented by pertussis toxin treatment of the cells. C3S/C351G $G_{i1}\alpha$ was present in both the particulate and cytosolic fractions but the GTPase activity of the membrane bound fraction was only slightly activated by the α_{2A}-adrenoceptor. Coexpression of C3S/C351G $G_{i1}\alpha$ and the α_{2A}-adrenoceptor along with β_1 and γ_2 subunits increased the P2 membrane complement of the α subunit and increased substantially the ratio of membrane bound to cytosolic protein. However, this also failed to allow marked stimulation of high-affinity GTPase activity by the α_{2A}-adrenoceptor despite the increased proportion of G-protein in the P2 membrane fraction. Despite the low fractional activation of C3S/C351G $G_{i1}\alpha$ by the α_{2A}-adrenoceptor compared to C351G $G_{i1}\alpha$, the palmitoylation-resistant G-protein caused a marked reduction in pertussis toxin-resistant, agonist (UK14304)-mediated stimulation of adenylyl cyclase activity. UK14304 caused the same degree of effect on adenylyl cyclase activity in pertussis toxin-treated cells following transfection of the same amounts of C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$, as both appear to act to sequester $\beta\gamma$ subunits. By contrast, neither G2A/C351G $G_{i1}\alpha$ nor G2A/C3S/C351G $G_{i1}\alpha$ resulted in effective regulation of adenylyl cyclase activity.

The α subunits of heterotrimeric guanine nucleotide binding proteins (G-proteins)[1] are modified by the addition of fatty acyl chains (Casey, 1995; Milligan et al., 1995; Wedegaertner et al., 1995). In the subfamily of the G_i-like pertussis toxin-sensitive G-proteins all of the widely expressed members have a glycine at codon 2 that acts as the acceptor site for cotranslational addition of the 14-carbon saturated fatty acid myristate (Casey, 1995; Milligan et al., 1995; Wedegaertner et al., 1995). Both in these G-proteins and in the others that are not cotranslationally modified by the addition of myristate, cysteine residues within the first 10 positions act as substrates for posttranslational addition of the 16-carbon saturated fatty acid palmitate (Casey, 1995; Milligan et al., 1995; Wedegaertner et al., 1995). Considerable interest has centered on the role of this palmitate as this modification is dynamic (Milligan et al., 1995) and at least in the case of $G_s\alpha$ has been indicated to be regulated by activation of the G-protein (Degtyarev et al., 1993a; Wedegaertner & Bourne, 1994; Mumby et al., 1994). Furthermore, in these studies depalmitoylation of the G-protein was correlated with a movement of this polypeptide away from the membrane to the cytoplasm (Wedegaertner & Bourne, 1994). Such studies indicate that one role of palmitoylation may be to regulate interaction of the G-protein with suitable receptors, either in a rather direct manner or by defining spatial opportunities for interactions between the proteins.

In the G_i family the site of palmitoylation has been defined by site-directed mutagenesis as cysteine 3 (Parenti et al., 1993; Degtyarev et al., 1993b; Galbiati et al., 1994). Mutation of this amino acid does not prevent cotranslational attachment of myristate in either $G_{i1}\alpha$ or $G_{o1}\alpha$ and thus such mutated forms of the polypeptide can be used to examine potential roles for palmitoylation. We have recently generated mutations in the α subunits of the G_i-like G-proteins that render them insensitive to the actions of pertussis toxin (Wise et al., 1997). To do so, the cysteine residue four amino acids from the C-terminus, which functions as the acceptor site for ADP-ribose donated via the toxin, was converted to glycine. These polypeptides couple effectively to the α_{2A}-adrenoceptor following transient coexpression in COS-7 cells, and following pertussis toxin treatment of the cells, α_{2A}-adrenoceptor interaction with these proteins could be studied in isolation (Wise et al., 1997). In the current study we examine the ability of a double mutant of $G_{i1}\alpha$ that is insensitive to pertussis toxin treatment and cannot be

[†] This work was supported by grants from the MRC and BBSRC (to G.M.) and from the Wellcome Trust (to G.M. and M.P.).

[‡] University of Glasgow.

[∥] Glaxo−Wellcome Research and Development.

[§] Universita di Milano.

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.

[1] Abbreviations: G-protein, guanine nucleotide binding protein; G2A, glycine at codon 2 converted to alanine; C3S, cysteine at codon 3 converted to serine; C351G, cysteine at codon 351 converted to glycine.

S0006-2960(97)00299-7 CCC: $14.00

palmitoylated (C3S/C351G $G_{i1}\alpha$) to interact functionally with the α_{2A}-adrenoceptor and with the $\beta\gamma$ complex and to regulate adenylyl cyclase activity.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, Scotland). [^{3}H]RS-79948−197 (90 Ci/mmol) and [^{3}H]adenine (21 Ci/mmol) were purchased from Amersham International. [γ-^{32}P]GTP (30 Ci/mmol) and [^{35}S]GTPγS (1175 Ci/mmol) were 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 synthesized on a Millipore Expedite nucleic acids synthesis system.

Methods

Construction of $G_{i1}\alpha$ Subunit Mutations. A pertussis toxin-resistant form of rat $G_{i1}\alpha$ was generated (Wise et al., 1997) following excision of this on an *Eco*RI restriction fragment from the vector pGEM2 and insertion into the *Eco*RI site of pBluescript KS− (Stratagene), creating pBS/$G_{i1}\alpha$. To generate pBS/$G_{i1}\alpha$(C351G), the 3′ 44 base pairs (bp) of the $G_{i1}\alpha$ open reading frame (ORF) and the 3′ flanking sequence were excised from pBS/$G_{i1}\alpha$ upon digestion with restriction enzymes *Aat*II and *Hin*dIII, and replaced with the synthetic oligonucleotide linker created following annealing of the oligonucleotides 5′-CATCATAAAGAATAACCTAAAAGAC**GGT**GGTCTCTTCTAAGAATTCA-3′ and 5′-AGCTTGAATTCTTAGAAGAGACC**ACC**GTCTTTTAGGTTATTCTTTATGATGACGT-3′ (C351G in boldface type; restriction sites for *Eco*RI, *Hin*dIII, and *Aat*II sites underlined) to recreate the 3′ end of $G_{i1}\alpha$. The vector was sequenced following cloning to demonstrate the presence of the cysteine to glycine mutation and the authenticity of the 3′ end of the $G_{i1}\alpha$ open reading frame. C3S, G2A, and G2A/C3S mutations of $G_{i1}\alpha$ were produced as previously described (Galbiati et al., 1994) and were then subcloned into pcDNA3 (Invitrogen). C3S, G2A, and G2A/C3S forms of C351G $G_{i1}\alpha$ were generated by excision of the 5′ 575 bp of the C3S, G2A or G2A/C3S $G_{i1}\alpha$ ORF by digestion with *Bgl*II and ligation of these fragments to the 3′ 487 bp of the C351G $G_{i1}\alpha$ ORF digested with the same restriction enzyme.

Cell Culture and Transfection. COS-7 cells were maintained in DMEM containing 10% (v/v) fetal calf serum (FCS), 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 pcDNA3 containing either the porcine α_{2A}-adrenoceptor or the various forms of $G_{i1}\alpha$ using lipofectamine reagent (Life Technologies, Inc.) (Wise et al., 1997). 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 milliliters of 20% (v/v) FCS in DMEM was then added to the cells. Cells were harvested 48 h after transfection.

Preparation of Membranes. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80 °C following harvest. Cell pellets were resuspended in 0.5 mL of 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 (buffer A), and rupture of the cells was achieved with 50 strokes of a hand-held Teflon on-glass homogenizer followed by passage (10 times) through a 25-gauge needle. Cell lysates were centrifuged at 1000*g* for 10 min in a Beckman TJ-6 centrifuge to pellet the nuclei and unbroken cells, and P2 particulate fractions were then recovered by centrifugation of the supernatant at 200000*g* for 30 min in a Beckman TL 100 bench-top ultracentrifuge using a Beckman TLA 100.2 rotor. P2 particulate fractions were resuspended in buffer A and stored at −80 °C until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure (Smith et al., 1985) using BSA as standard.

[^{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 (Gillard et al., 1996) (1 nM). Nonspecific 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.

Previous studies (Wise et al., 1997) have established that transfection of COS-7 cells with amounts of α_{2A}-adrenoceptor cDNA above 0.7 μg results in similar levels of expression of this receptor, in the region of 15 pmol/mg of membrane protein.

Immunological Studies. Both antiserum I1C and BN3 (Green et al., 1990) were produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide and keyhole limpet hemocyanin (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 this antiserum for $G_{i1}\alpha$ has been demonstrated previously. 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 containing 6 M urea overnight at 100 V. Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell), probed with relevant antiserum, and visualized as described (McKenzie et al., 1990).

High-affinity GTPase assays were performed essentially as described in Koski and Klee (1981) using [γ-^{32}P]GTP (0.5 μM, 60 000 cpm) and UK14304 (10 μM). Nonspecific GTPase was assessed by parallel assays containing 100 μM GTP.

[^{35}S]GTPγS binding studies were performed as in Thomas et al. (1995). Briefly, membranes (5 μg) were incubated at 30 °C for 30 min in a final assay volume of 100 μL in a reaction mix comprising 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM ascorbate, 10 μM GDP, and 0.3−0.5 nM [^{35}S]GTPγS (50 nCi) in the presence or absence of agonist as described in the text. The incubation was terminated by the addition of 2.5 mL of ice-cold washing buffer B (20 mM HEPES, pH 7.4, and 3 mM $MgCl_2$) and rapid filtration through Whatman GF/C filters followed by three washes (5 mL) with ice-cold buffer B. Filters were

maintained overnight in 5 mL of Ultima-Flo AF scintillant before liquid scintillation counting.

Solubilization of $G_{i1}\alpha$ from P2 Particulate Fractions. P2 particulate fractions (100 μg) from COS-7 cells transfected to express either C3S/C351G $G_{i1}\alpha$ or C351G $G_{i1}\alpha$ were resuspended in buffer A to a final concentration of 1 mg/mL. Aliquots (100 μL) were extracted using either 1 M NaCl, 4 M urea, 0.1% (w/v) sodium cholate, and 1% (w/v) and sodium carbonate. In the case of sodium carbonate extractions, original P2 particulate fractions (100 μg) were adjusted to 200 μL with 0.2 M Na_2CO_3 and then diluted to 500 μL with 0.1 M Na_2CO_3. All extractions were performed at 4 °C for 45 min prior to recovery of nonextractable material by centrifugation at 14000*g* for 30 min. Extracted and particulate fractions were dissolved in SDS−PAGE sample buffer and analyzed by SDS−10% PAGE.

Trypsinization Studies. P2 particulate fractions (100 μg) from rat brain and from COS-7 cells transfected to express either C3S/C351G $G_{i1}\alpha$ or C351G $G_{i1}\alpha$ and cytosolic fractions (80 μg) from C3S/C351G 24 h $G_{i1}\alpha$-expressing cells were incubated in the presence or absence of 100 μM GTPγS for 15 min at 37 °C in buffer containing 20 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 2 mM DTT, and 0.1 mM EDTA. Samples were then treated with or without L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (0.1 mg/mL) for 15 min at 37 °C (Eide et al., 1987). Proteolysis was terminated by the addition of soybean trypsin inhibitor to a final concentration of 0.5 mg/ml. Samples were then dissolved in SDS−PAGE sample buffer and analyzed by SDS−10% PAGE.

Adenylyl cyclase activity measurements were performed essentially as described by Wong (1994). COS-7 cells were transfected with combinations of the α_{2A}-adrenoceptor and C351G or C3S/C351G $G_{i1}\alpha$ or the other N-terminal mutations as described above. Twenty-four hours after transfection, each 60 mm dish of cells was split into six wells of a 12-well plate and the cells were allowed to reattach. Cells were then incubated in medium containing [^{3}H]adenine (1.5 μCi/well) and pertussis toxin (50 ng/mL) for 16−24 h. The generation of [^{3}H]cAMP in response to treatment of the cells with UK14304 (10 μM) was then assessed.

RESULTS

The acylation and pertussis toxin-resistant $G_{i1}\alpha$ proteins were constructed as follows. C3S $G_{i1}\alpha$ and C351G $G_{i1}\alpha$ in pcDNA3 were digested with the enzyme *Bgl*II, which has a single restriction site within codon 192 of the open reading frame of the $G_{i1}\alpha$ polypeptide. The 5′ fragment of C3S $G_{i1}\alpha$ and the 3′ fragment of C351G $G_{i1}\alpha$ were recovered and ligated to generate C3S/C351G $G_{i1}\alpha$ pcDNA3. By similar cloning strategies G2A/C351G and G2A/C3S/C351G forms of $G_{i1}\alpha$ were also generated in pcDNA3.

Transient expression of C3S/C351G $G_{i1}\alpha$ pcDNA3 in COS-7 cells led to expression of a polypeptide recognized by the specific anti-$G_{i1}\alpha$ antiserum I1C (Green et al., 1990), raised against a synthetic peptide corresponding to amino acids 160−169 of $G_{i1}\alpha$. Equivalent expression of C351G $G_{i1}\alpha$ pcDNA3 also resulted in expression of an I1C-reactive polypeptide. As expected, both of these polypeptides were resistant to the action of pertussis toxin. SDS−PAGE analysis of the P2 membrane fraction of cells expressing these constructs resulted in no alteration in mobility of the C351G $G_{i1}\alpha$ polypeptides following treatment with pertussis toxin, whereas in equivalent experiments with wild-type $G_{i1}\alpha$ the mobility of the polypeptide was reduced by this treatment (Figure 1).

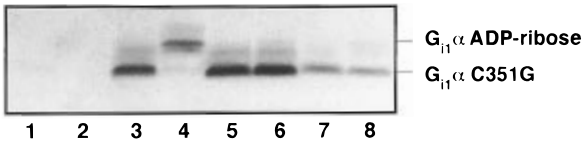

FIGURE 1: C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ are not modified by pertussis toxin-catalyzed ADP-ribosylation. COS-7 cells were mock-transfected (lanes 1 and 2) or transfected to express wild-type $G_{i1}\alpha$ (lanes 3 and 4), C351G $G_{i1}\alpha$ (lanes 5 and 6), or C3S/C351G $G_{i1}\alpha$ (lanes 7 and 8). Twenty-four hours prior to harvest, the cells were either treated with vehicle (lanes 1, 3, 5, and 7) or with pertussis toxin (50 ng/mL) (lanes 2, 4, 6, and 8). P2 membrane fractions were then resolved by SDS−PAGE that incorporated 6 M urea, and the samples were transferred to nitrocellulose and immunoblotted using the $G_{i1}\alpha$-specific antiserum I1C. Successful ADP-ribosylation of $G_{i1}\alpha$ is recorded by a reduced mobility of the protein in SDS−PAGE (see lane 4).

Cellular distribution studies demonstrated that expressed C351G $G_{i1}\alpha$ was almost entirely particulate whereas C3S/C351G $G_{i1}\alpha$ showed a pattern of distribution that was partially particulate and partially cytosolic (Figure 2A). Expression of G2A/C351G and G2A/C3S/C351G forms of $G_{i1}\alpha$ also resulted in the expression of I1C-reactive polypeptides (Figure 2A). However, these were found almost entirely in the cytosolic rather than the particulate fraction and it was noticeable that the myristoylation-deficient G2A/C351G and G2A/C3S/C351G forms of $G_{i1}\alpha$ migrated rather more slowly in SDS−PAGE than C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$, which remain substrates for myristoylation (Figure 2A). This observation is in agreement with Mumby and Linder (1994).

The pertussis toxin-resistant forms of $G_{i1}\alpha$ were transiently coexpressed in COS-7 cells along with the porcine α_{2A}-adrenoceptor. The cells were treated with pertussis toxin (50 ng/mL) for the last 24 h prior to cell harvest and the P2 particulate membrane fractions were examined for the ability of the α_2-adrenoceptor agonist UK14304 to stimulate high-affinity GTPase activity. In cells transfected with the α_{2A}-adrenoceptor alone, UK14304 (10 μM) was unable to stimulate this activity above basal levels. In contrast, coexpression of the α_{2A}-adrenoceptor with C351G $G_{i1}\alpha$ allowed a robust stimulation of high-affinity GTPase activity by the agonist (Figure 2B). No stimulation by UK14304 could be observed in equivalent experiments following coexpression of the receptor with myristoylation- or palmitoylation-deficient G2A/C351G $G_{i1}\alpha$, G2A/C3S/C351G $G_{i1}\alpha$, and C3S/C351G $G_{i1}\alpha$ (Figure 2B). While both G2A/C351G $G_{i1}\alpha$ and G2A/C3S/C351G $G_{i1}\alpha$ might not be expected to have the capacity to couple to the α_{2A}-adrenoceptor in assays performed on membrane preparations due to their cytosolic distribution, detectable levels of C3S/C351G $G_{i1}\alpha$ are seen in the P2 particulate fraction (Figure 2A). To examine if guanine nucleotide exchange and hydrolysis by C3S/C351G $G_{i1}\alpha$ could be stimulated by the α_{2A}-adrenoceptor, we attempted to generate levels of expression in the P2 particulate membrane fraction equivalent to those seen in transfections with C351G $G_{i1}\alpha$. We thus transfected COS-7 cells with the α_{2A}-adrenoceptor cDNA and a single amount of C351G $G_{i1}\alpha$ or varying amounts of C3S/C351G $G_{i1}\alpha$ cDNA. Immunoblots of P2 membranes with antiserum I1C

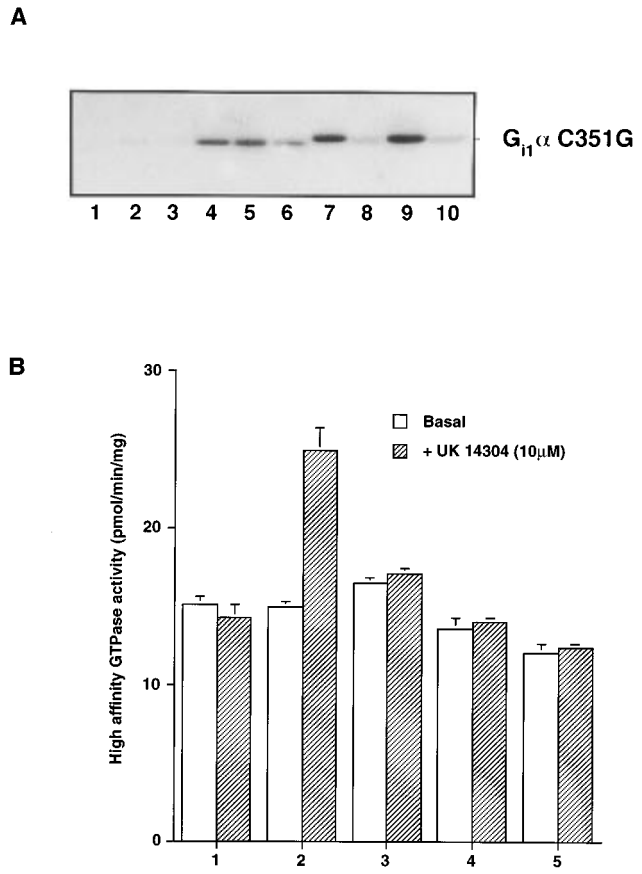

FIGURE 2: Cellular distribution and receptor interactions of N-terminal mutants of $G_{i1}\alpha$. (A) Cellular distribution of C351G $G_{i1}\alpha$ and various N-terminal modifications of this protein following expression in COS-7 cells. P2 membrane fractions (lanes 2, 4, 6, 8, and 10) and cytosol (lanes 1, 3, 5, 7, and 9) were derived from COS-7 cells that were either mock-transfected (lanes 1 and 2) or transfected to express C351G $G_{i1}\alpha$ (lanes 3 and 4), C3S/C351G $G_{i1}\alpha$ (lanes 5 and 6), G2A/C351G $G_{i1}\alpha$ (lanes 7 and 8), or G2A/C3S/C351G $G_{i1}\alpha$ (lanes 9 and 10). In all cases the cells were also transfected to express the porcine α_{2A}-adrenoceptor, and 24 h prior to harvest the cells were treated with pertussis toxin (50 ng/mL). These samples were resolved by SDS−PAGE [10% (w/v) acrylamide], transferred to nitrocellulose, and immunoblotted using antiserum I1C as primary reagent. (B) Of these G-proteins only membrane-associated C351G $G_{i1}\alpha$ interacts effectively with the α_{2A}-adrenoceptor to cause stimulation of high-affinity GTPase activity. High-affinity GTPase measurements were performed in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM) on the P2 particulate fractions described in panel A derived from COS-7 cells transfected with vector alone (1) or with 0.7 μg of porcine α_{2A}-adrenoceptor in combination with 1.8 μg of either C351G $G_{i1}\alpha$ (2), C3S/C351G $G_{i1}\alpha$ (3), G2A/C351G $G_{i1}\alpha$ (4), or G2A/C3S/C351G $G_{i1}\alpha$ (5).

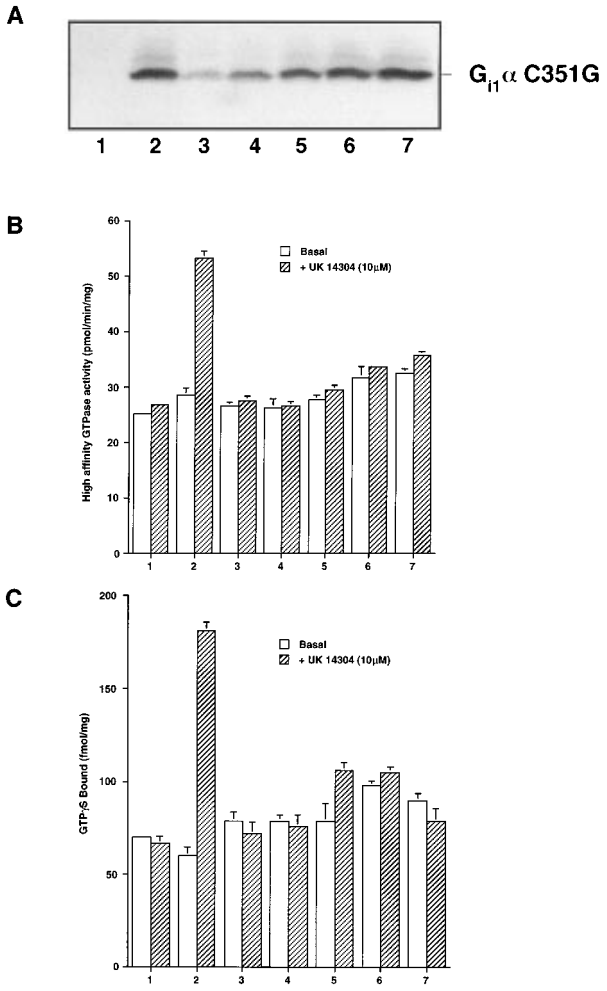

FIGURE 3: Higher levels of expression of C3S/C351G $G_{i1}\alpha$ do not result in effective coupling of C3S/C351G $G_{i1}\alpha$ to the α_{2A}-adrenoceptor. (A) Immunoblot of P2 membrane fractions. COS-7 cells were either mock-transfected (lane 1) or transfected with 0.9 μg of C351G $G_{i1}\alpha$ pcDNA3 (lane 2) or with 0.5 (lane 3), 0.9 (lane 4), 1.2 (lane 5), 1.5 (lane 6), and 1.8 μg (lane 7) of C3S/C351G $G_{i1}\alpha$ pcDNA3. All samples were also transfected with 0.7 μg of porcine α_{2A}-adrenoceptor pcDNA3, and 24 h prior to harvest the cells were treated with pertussis toxin (50 ng/mL). P2 membrane fractions were resolved by SDS−PAGE, and the samples were transferred to nitrocellulose and immunoblotted using antiserum I1C.(B) UK14304-mediated regulation of high-affinity GTPase activity. High-affinity GTPase measurements were performed in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM) on the P2 particulate fractions described in panel A. (C) UK14304-mediated stimulation of [^{35}S]GTPγS binding. The specific binding of [^{35}S]GTPγS was measured in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM) on the P2 particulate fractions described in panel A.

demonstrated equivalent expression of C3S/C351G $G_{i1}\alpha$ and C351G $G_{i1}\alpha$ in cells transfected with either 1.2−1.5 μg of C3S/C351G $G_{i1}\alpha$ plasmid or with 0.9 μg of C351G $G_{i1}\alpha$ expression vector (Figure 3A). Despite the equivalent level of expression in the P2 fraction, virtually no stimulation of the GTPase activity of C3S/C351G $G_{i1}\alpha$ by UK14304 was observed in cells transfected with up to 1.8 μg of C3S/C351G $G_{i1}\alpha$ cDNA (Figure 3B). Similar results were obtained in assays that measured UK14304 stimulation of the binding of [^{35}S]GTPγS (Figure 3C).

It was clearly possible that C3S/C351G $G_{i1}\alpha$ was unable to exchange guanine nucleotide. To assess this we expressed either C351G $G_{i1}\alpha$ or C3S/C351G $G_{i1}\alpha$ in COS-7 cells, generated P2 membrane and cytosolic fractions, and examined whether the addition of GTPγS could protect partially clipped forms of these variants of $G_{i1}\alpha$ from extensive tryptic digestion. A positive control was provided by performing parallel experiments on membranes from rat frontal cortex, which express high levels of $G_{i1}\alpha$. In the presence of both trypsin and GTPγS, two I1C-immunoreactive polypeptides were produced from the rat brain membranes that were smaller than native $G_{i1}\alpha$ (Figure 4). In the absence of GTPγS only low levels of a single, relatively small, digested I1C-reactive polypeptide was observed following treatment with trypsin (Figure 4). An identical pattern was observed when the incubations were performed with P2 membrane fractions of COS-7 cells transfected to express either C351G $G_{i1}\alpha$ or C3S/C351G $G_{i1}\alpha$ (Figure 4). Treatment of cytosolic

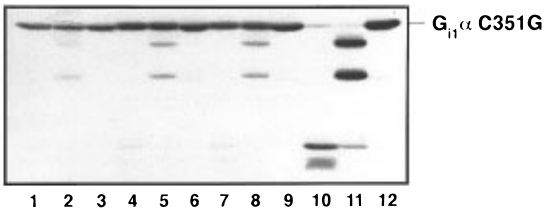

FIGURE 4: Identical GTPγS protection of C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ from tryptic digestion. P2 membrane fractions derived from either rat brain frontal cortex (lanes 1−3) and COS-7 cells transfected to express either C351G $G_{i1}\alpha$ (lanes 4−6) or C3S/C351G $G_{i1}\alpha$ (lanes 7−9) or cytosol derived from COS-7 cells transfected to express C3S/C351G $G_{i1}\alpha$ (lanes 10−12) were either untreated (lanes 3, 6, 9, and 12) or subjected to limited tryptic proteolysis performed in the absence (lanes 1, 4, 7, and 10) or presence (lanes 2, 5, 8, and 11) of GTPγS (100 μM) for 15 min. The reaction was stopped by the addition of soybean trypsin inhibitor and the samples were resolved by SDS−PAGE, transferred to nitrocellulose, and immunoblotted using antiserum I1C.

fractions of COS-7 cells transfected to express C3S/C351G $G_{i1}\alpha$ with trypsin plus GTPγS also resulted in the production of I1C-reactive polypeptides of identical size to those produced from the particulate fraction. However, a much greater fraction of the total C3S/C351G $G_{i1}\alpha$ population was degraded upon treatment of the cytosolic samples with trypsin and GTPγS than was achieved with identical treatment of the P2 membrane fractions. Again, in the absence of GTPγS a low molecular mass I1C-reactive fragment was observed and the high degree of overall trypsinization allowed detection of further, smaller, I1C-reactive fragments (Figure 4). Such results clearly demonstrate that particulate and cytosolic C3S/C351G $G_{i1}\alpha$ have a similar ability to exchange guanine nucleotide and bind GTPγS as both C351G $G_{i1}\alpha$ and native wild-type $G_{i1}\alpha$.

We next examined whether coexpression of excess $\beta\gamma$ subunit would allow greater amounts of the G-protein α subunits to become associated with the P2 particulate fraction and whether this would influence the capacity of the α_{2A}-adrenoceptor to activate these G-proteins. The α_{2A}-adrenoceptor was expressed alone or together with either C351G $G_{i1}\alpha$ or C3S/C351G $G_{i1}\alpha$, in the presence and absence of β_1 and γ_2. The presence of excess β_1 subunit in the P2 membrane fractions following transfection with this cDNA was confirmed in immunoblots using antiserum BN3 (Green et al., 1990) (Figure 5A). This polypeptide was entirely particulate (data not shown). Parallel immunoblots with antiserum I1C demonstrated that coexpression of $\beta_1\gamma_2$ resulted in higher immunologically detected levels of C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ in the P2 particulate fractions than when the G-protein α subunits were expressed in the absence of excess $\beta\gamma$ (Figure 5B). Although, as noted earlier, essentially all of the C351G $G_{i1}\alpha$ was present in P2 particulate fraction following its expression both with and without excess $\beta_1\gamma_2$ (Figure 5B), coexpression of $\beta_1\gamma_2$ resulted in a considerably smaller fraction of C3S/C351G $G_{i1}\alpha$ being present in the cytosol and more in the P2 particulate fraction. The higher levels of P2-associated $G_{i1}\alpha$ subunits following coexpression of $\beta_1\gamma_2$ reflected, to some degree, greater total cellular levels of expression of the α subunits. Immunoblotting of whole-cell lysates of COS-7 cells transfected with either C3S/C351G $G_{i1}\alpha$ or C351G $G_{i1}\alpha$ with or without coexpression of $\beta_1\gamma_2$ showed higher levels of total cell I1C immunoreactivity in the presence of $\beta_1\gamma_2$ (data not shown). This may reflect a protection of the

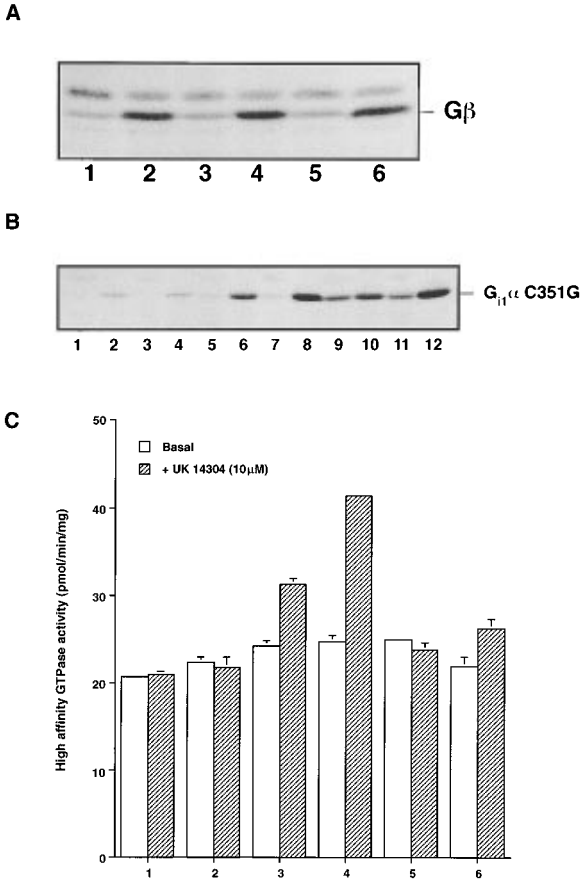

FIGURE 5: Coexpression of $\beta_1\gamma_2$ increases P2 membrane-associated levels of both C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ but fails to allow α_{2A}-adrenoceptor activation of C3S/C351G $G_{i1}\alpha$. (A) Coexpression of $\beta_1\gamma_2$ increases P2 membrane levels of β-subunit. COS-7 cells were transfected with 0.7 μg of the porcine α_{2A}-adrenoceptor in conjunction with no other plasmid (lane 1) or with 0.7 μg of C351G $G_{i1}\alpha$ (lanes 3 and 4) or 0.7 μg of C3S C351G $G_{i1}\alpha$ (lanes 5 and 6), and with (lanes 2, 4, and 6) or without (lanes 3 and 5) a combination of $\beta_1$and γ_2 (0.7 μg each). P2 membrane fractions from these cells were then immunoblotted for β-subunit with antiserum BN3. (B) Coexpression of $\beta_1\gamma_2$ increases P2 membrane levels of both C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$. COS-7 cells were transfected with the porcine α_{2A}-adrenoceptor (0.7 μg) in conjunction with no other plasmid (lanes 1 and 2) or with 0.7 μg of C351G $G_{i1}\alpha$ (lanes 5−8) or 0.7 μg of C3S C351G $G_{i1}\alpha$ (lanes 9−12) with (lanes 3, 4, 7, 8, 11, and 12) or without (lanes 5, 6, 9, and 10) a combination of $\beta_1$and γ_2 (0.7 μg each). The cells were treated with pertussis toxin for the last 24 h before cell harvest. P2 membrane (lanes 2, 4, 6, 8, 10, and 12) and cytosol (lanes 1, 3, 5, 7, 9, and 11) fractions were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted for forms of $G_{i1}\alpha$ with antiserum I1C. (C) Higher P2 membrane levels of C351G $G_{i1}\alpha$ produced by coexpression of $\beta_1\gamma_2$ results in greater α_{2A}-adrenoceptor-stimulated GTPase activity. This is not true for C3S/C351G $G_{i1}\alpha$. Basal (open bars) high-affinity GTPase activity and its regulation by UK14304 (hatched bars) was performed on the P2 membrane fractions described in panel A derived from COS-7 cells transfected with the porcine α_{2A}-adrenoceptor (0.7 μg) alone (1 and 2) or in conjunction with 0.7 μg of C351G $G_{i1}\alpha$ (3 and 4) or 0.7 μg of C3S/C351G $G_{i1}\alpha$ (5 and 6) with (2, 4, and 6) or without (1, 3, and 5) a combination of β_1 and γ_2 (0.7 μg each).

expressed α subunit from proteolytic degradation in the presence of excess $\beta_1\gamma_2$. Coexpression of $\beta_1\gamma_2$ along with C351G $G_{i1}\alpha$ and the α_{2A} adrenoceptor resulted in a greater stimulation of high-affinity GTPase activity by UK14034 in pertussis toxin-treated COS-7 cells (Figure 5C) than when the transfections were performed without β_1 and γ_2. However, even when it was coexpressed with excess β_1 and γ_2,

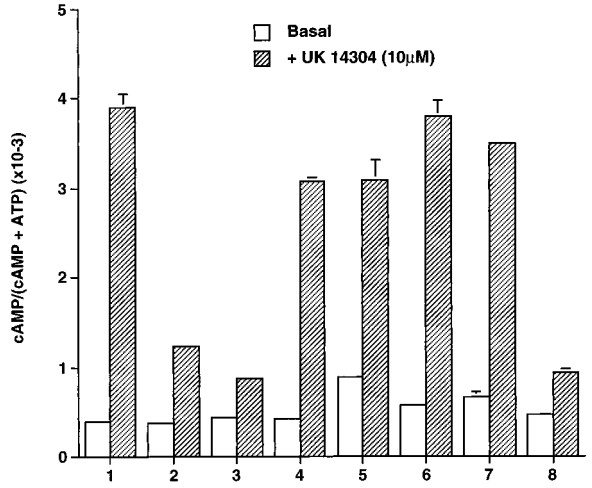

FIGURE 6: Expression of C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ but not G2A/C351G $G_{i1}\alpha$, or G2A/C3S/C351G $G_{i1}\alpha$ prevents α_{2A}-adrenoceptor-mediated stimulation of adenylyl cyclase. COS-7 cells were transfected to express 0.7 μg of the α_{2A}-adrenoceptor alone (1) or in combination with 0.9 and 1.8 μg of either C351G $G_{i1}\alpha$ (2 and 3), G2A/C351G $G_{i1}\alpha$ (4 and 5) or G2A/C3S/C351G $G_{i1}\alpha$ (6 and 7), respectively, or with 1.8 μg of C3S/C351G $G_{i1}\alpha$ (8). Twenty-four hours prior to harvest the cells were treated with pertussis toxin (50 ng/ml) and labeled with [^{3}H]adenine (1.5 μCi/mL. Adenylyl cyclase activity was determined as described under Materials and Methods in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM).

C3S/C351G $G_{i1}\alpha$ failed to display any significant increase of GTPase activity in response to UK14304 (Figure 5C), despite the increased P2 membrane localization of this G-protein (Figure 5B).

We have previously observed that expression of the α_{2A}-adrenoceptor alone in pertussis toxin-treated COS-7 cells results in a UK14304-mediated stimulation of whole cell adenylyl cyclase activity measured following labeling of the ATP pool with [^{3}H]adenine (Wise et al., 1997) (Figure 6). This is likely to be a reflection of the ability of this receptor to interact with and activate $G_s\alpha$ as well as with forms of $G_i\alpha$ as reported by Eason and colleagues (Eason et al., 1992; Eason & Liggett, 1995) and/or the capacity of $\beta\gamma$ released from activated G-proteins to synergistically stimulate an adenylyl cyclase of the type II/type IV class (Federman et al., 1992; Taussig & Gilman, 1995). Cotransfection of the α_{2A}-adrenoceptor with C351G $G_{i1}\alpha$ resulted in a large reduction of the stimulation of adenylyl cyclase activity measured in the presence of UK14304 and with pertussis toxin pretreatment (Figure 6). Perhaps surprisingly, given the lack of observed agonist-enhanced guanine nucleotide exchange and hydrolysis (Figure 3), this effect was also observed on coexpression of the α_{2A}-adrenoceptor with C3S/C351G $G_{i1}\alpha$ (Figure 6). By contrast, expression of G2A/C351G $G_{i1}\alpha$ resulted in a very limited reduction in agonist-mediated stimulation of adenylyl cyclase, and expression of G2A/C3S/C351G $G_{i1}\alpha$ failed to reduce the level of adenylyl cyclase activity in response to UK14034 from that observed in the absence of expressed G-protein α subunit (Figure 6). Expression of a range of amounts of either C351G $G_{i1}\alpha$ or C3S/C351$G_{i1}\alpha$ along with the α_{2A}-adrenoceptor resulted in very similar levels of reduction in agonist-mediated stimulation of adenylyl cyclase in response to a maximally effective concentration of UK14304 at each amount of either C351G $G_{i1}\alpha$ or C3S/C351$G_{i1}\alpha$ (Figure 7). To examine whether the overexpressed $G_{i1}\alpha$ might be sequestering the $\beta\gamma$ complex

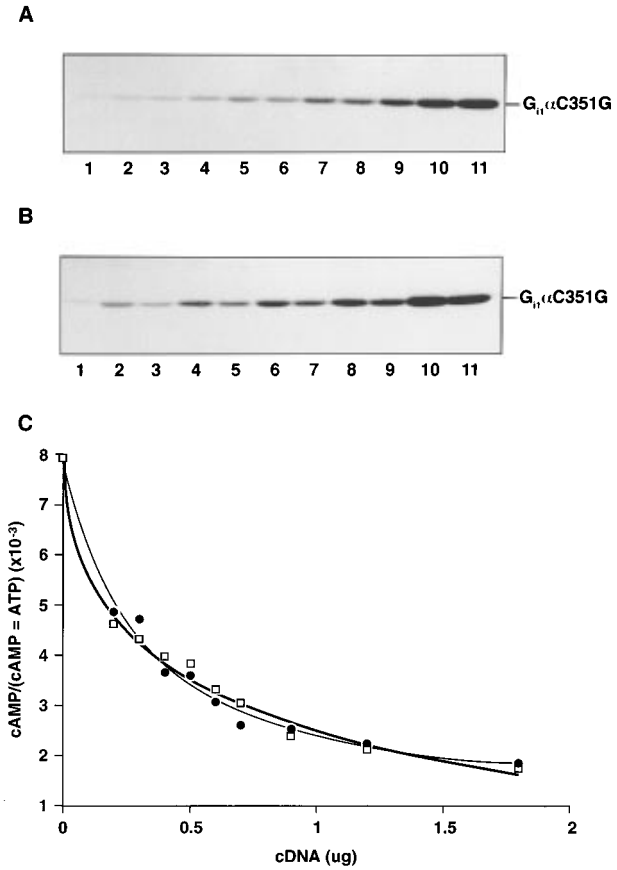

FIGURE 7: Expression of C351G $G_{i1}\alpha$ and C3S C351G $G_{i1}\alpha$ results in equivalent reductions in α_{2A}-adrenoceptor-mediated stimulation of adenylyl cyclase. (A) Immunoblot of whole cell lysates. (B) Immunoblot of P2 membrane fractions. COS-7 cells were either mock-transfected (lane 1) or transfected with 0.2 μg (lanes 2 and 3), 0.4 μg (lanes 4 and 5), 0.6 μg (lanes 6 and 7), 0.9 μg (lanes 8 and 9), and 1.8 μg (lanes 10 and 11) of C351G $G_{i1}\alpha$ pcDNA3 (lanes 2, 4, 6, 8, and 10) or C3S/C351G $G_{i1}\alpha$ pcDNA3 (lanes 3, 5, 7, 9, and 11). All samples were also transfected with 0.7 μg of porcine α_{2A}-adrenoceptor pcDNA3, and 24 h prior to harvest the cells were treated with pertussis toxin (50 ng/mL). Whole cell lysates (A) and P2 membrane fractions (B) were resolved by SDS−PAGE, and the samples were transferred to nitrocellulose and immunoblotted using antiserum I1C. (C) Whole-cell [^{3}H]adenine labeling. Differing amounts of either C351G $G_{i1}\alpha$ (●) or C3S/C351G $G_{i1}\alpha$ (□) cDNA were transfected into COS-7 cells along with the α_{2A}-adrenoceptor. Twenty-four hours prior to harvest the cells were treated with pertussis toxin (50 ng/mL) and labeled with [^{3}H]adenine (1.5 μCi/mL). Adenylyl cyclase activity in the presence of UK14034 (10 μM) was determined as described under Materials and Methods.

to inhibit $\beta\gamma$-mediated stimulation of adenylyl cyclase type II/type IV, we coexpressed additional β_1 and γ_2 along with these forms of $G_{i1}\alpha$ and the α_{2A}-adrenoceptor. Overexpression of $\beta_1\gamma_2$ in these experiments resulted in a greater degree of stimulation of adenylyl cyclase produced by UK14304 than in the absence of $\beta_1\gamma_2$ (Figure 8) and was able to partially reverse the effects of both C351G $G_{i1}\alpha$ and C3S/C351$G_{i1}\alpha$ (Figure 8). To examine this effect further, we proceeded to coexpress wild-type transducin α along with the α_{2A}-adrenoceptor. Transducin α is a pertussis toxin-sensitive member of the G_i-like G-protein family that is expressed endogenously only in photoreceptor-expressing cells and that has been demonstrated to sequester and inhibit $\beta\gamma$-mediated signaling in a number of systems. This G-protein functions to regulate a cGMP phosphodiesterase and does not mediate direct inhibition of adenylyl cyclase. Following pertussis toxin treatment of the cells to prevent

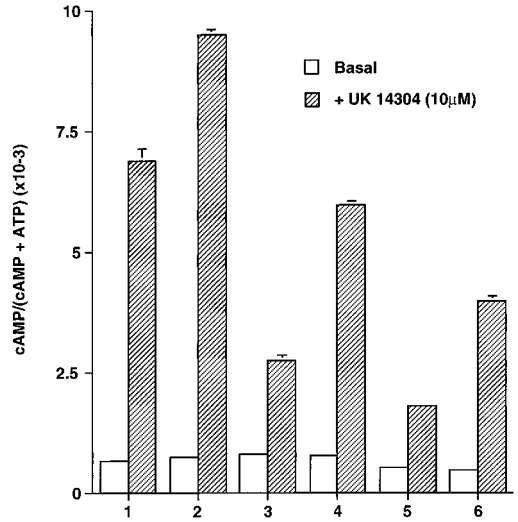

FIGURE 8: Concurrent expression of $\beta_1\gamma_2$ partially reverses the reduction of stimulation of adenylyl cyclase activity produced by coexpression of the α_{2A}-adrenoceptor and the forms of $G_{i1}\alpha$. COS-7 cells were transfected to express 0.7 μg of the α_{2A}-adrenoceptor alone (1 and 2) or in conjunction with 0.7 μg of each of C351G $G_{i1}\alpha$ (3 and 4) or C3S/C351G $G_{i1}\alpha$ (5 and 6) with (2, 4, and 6) or without (1, 3, and 5) a combination of β_1 and γ_2 (0.7 μg each). Twenty-four hours prior to harvest the cells were treated with pertussis toxin (50 ng/mL) and labeled with [^{3}H]adenine (1.5 μCi/ml). Adenylyl cyclase activity was determined as described under Materials and Methods in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM).

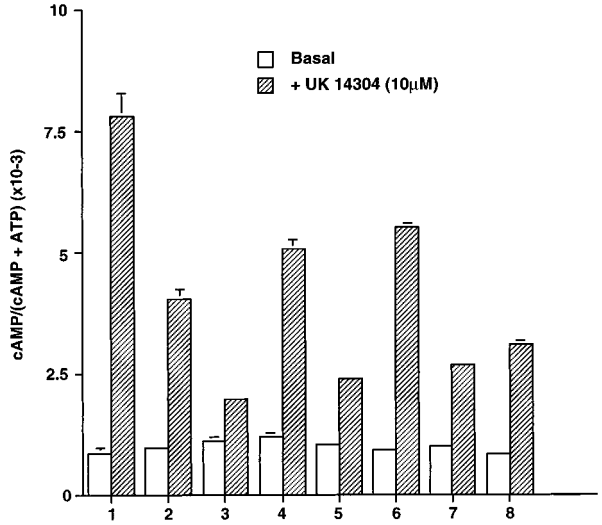

FIGURE 9: Inhibitory effects of transducin α on α_{2A}-adrenoceptor-mediated stimulation of adenylyl cyclase activity. COS-7 cells were transfected to express 0.7 μg of the α_{2A}-adrenoceptor alone (1) or in combination with 0.4 and 1.8 μg of either C351G $G_{i1}\alpha$ (2 and 3) or C3S/C351G $G_{i1}\alpha$ (4 and 5), respectively, with 0.4 μg of transducin α (6), with 0.4 μg each of both C351G $G_{i1}\alpha$ and transducin α (7) or with 0.4 μg each of both C3S/C351G $G_{i1}\alpha$ and transducin α (8). Twenty-four hours prior to harvest the cells were treated with pertussis toxin (50 ng/mL) and labeled with [^{3}H]adenine (1.5 μCi/ml). Adenylyl cyclase activity was determined as described under Materials and Methods in the presence (hatched bars) or absence (open bars) of UK14304 (10 μM).

potential interactions between transducin α and the co-expressed α_{2A}-adrenoceptor, this also resulted in a substantially lower stimulation of adenylyl cyclase activity by UK14304 than that observed when the α_{2A}-adrenoceptor was expressed alone (Figure 9). Furthermore, the effects of submaximal amounts of transducin α and either C351G $G_{i1}\alpha$ or C3S/C351G $G_{i1}\alpha$ were essentially additive (Figure 9). These data, in conjuction with those of Figures 7 and 8, also provide evidence for a similar capability of C3S/C351G $G_{i1}\alpha$ and C351G $G_{i1}\alpha$ to interact with $\beta_1\gamma_2$.

DISCUSSION

The role of the fatty acyl chains that can be shown to be attached to G-protein α subunits has resisted easy analysis. Mutational replacement of the glycine residue at codon 2 in the G_i-like G-proteins prevents myristoylation of these proteins (Jones et al., 1990; Mumby et al., 1990), is associated with a reduction in affinity of interaction with $\beta\gamma$ subunits (Linder et al., 1991), and causes a loss of membrane association (Jones et al., 1990; Mumby et al., 1990). Such studies have indicated that acylation plays a key role in the correct targeting and localization of these polypeptides. Subsequently, it was determined that cysteine 3 of these proteins was the site of posttranslational attachment of other fatty acids (predominantly palmitate) (Parenti et al., 1993; Degtyarev et al., 1993b), that mutation of glycine 2 often prevents subsequent palmitoylation of cysteine 3 (Galbiati et al., 1994; Hallak et al., 1994), an effect that may relate to the cellular location of the posttranslational modification (Degtayarev et al., 1994), and that the half-life of association of [^{3}H]palmitate with the G-protein α subunit was very much less than the half-life of the protein (Degtyarev et al., 1993a; Wedegaertner & Bourne, 1994; Mumby et al., 1994). Such studies suggested a key role for attachment of palmitate to this site in G-protein localization and function and its dynamic nature suggested it to be a process that might be regulated (Degtyarev et al., 1993a; Wedegaertner & Bourne, 1994; Mumby et al., 1994). Although demonstration of dynamic regulation of the palmitoylation status of the G_i-like proteins has yet to be produced, this has been forthcoming for G_s, where both activation of the G-protein via agonist occupancy of relevant receptors and direct activation by treatment of cells with cholera toxin results in accelerated depalmitoylation of this protein (Degtyarev et al., 1993a; Wedegaertner & Bourne, 1994; Mumby et al., 1994). As the G_i-like G-proteins are dually acylated at their N-terminus by both myristate and palmitate, resolution of the contributions of each of these to membrane association and signaling functions have been difficult to assess. However, mutation of cysteine 3 does not prevent myristoylation of the expressed protein at glycine 2, allowing a means to examine the activity of the polypeptide containing only the myristate moiety.

In the present study we have assessed the contribution of the palmitoylation of cysteine 3 of $G_{i1}\alpha$ to the functional interactions of this G-protein with the porcine α_{2A}-adrenoceptor (Wise et al., 1997). Expression of this receptor normally allows signal transduction to occur via interaction with G_i-like G-proteins expressed endogenously by COS-7 cells. To eliminate these interactions we have routinely treated the cells with pertussis toxin. To prevent the expressed forms of $G_{i1}\alpha$ from also acting as substrates for this toxin we have used versions of $G_{i1}\alpha$ in which we have converted cysteine 351 to glycine to generate proteins resistant to pertussis toxin-catalyzed ADP-ribosylation (Wise et al., 1997). A convenient means to ascertain whether a G_i-like protein has been modified by pertussis toxin-catalyzed ADP-ribosylation is that this modification reduces their mobility through SDS−PAGE. Particularly large differences

in mobility can be recorded when the resolving gel contains 6 M urea (Wise et al., 1997). We confirmed the inability of C351G and C3S/C351G $G_{i1}\alpha$ to be modified by pertussis toxin, as the mobility of these proteins on such gels was not reduced by toxin treatment (Figure 1). A concern with the use of such mutants is that pertussis toxin treatment of wild-type G_i-like G-proteins prevents them from being activated by receptors as the C-terminal region of the G-protein, which encompasses this cysteine residue, is a key element in receptor/G-protein interactions (Wise et al., 1997). Although others have reported variable results with the use of such C-terminally mutated G_i-like proteins (Senogles, 1994; Hunt et al., 1994) we have shown that, in transient assays, the porcine α_{2A}-adrenoceptor is able to interact with C → G mutations of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ (Wise et al., 1997). The affinity of UK14304 to stimulate the GTPase activity of each of these proteins was, however, reduced some 10−15-fold compared to parallel experiments with the wild-type G-proteins (Wise et al., 1997). As such, in the current experiments we have restricted studies to use of a single high concentration of the agonist that we have previously shown to be able to cause maximal activation of C351G $G_{i1}\alpha$ (Wise et al., 1997).

We (McCallum et al., 1995) and others (Edgerton et al., 1994) have previously noted that fractions of "particulate", transiently expressed mutated G-protein α subunits can be resistant to solubilization by sodium cholate and other detergents and thus do not display the anticipated characteristics of properly folded and located G-proteins. This, to a large extent, reflects protein that is not at the plasma membrane. Hence, in the current studies we restricted analyses to P2 membrane fractions and eliminated the crude P1 particulate fractions, which contain much of this detergent-insoluble G-protein.

Because we have previously noted that C3S forms of both $G_{i1}\alpha$ and $G_{o1}\alpha$ partition between the membrane and cytosolic compartments of cells when expressed both transiently and stably [see Grassie et al. (1995) for example] we used a range of amounts of C3S/C351G $G_{i1}\alpha$ cDNA in these studies to ensure that the P2 membrane fractions used for the functional assays contained similar levels of C3S/C351G $G_{i1}\alpha$ and C351G $G_{i1}\alpha$ polypeptides. This was assessed immunologically using an antiserum targeted against a central segment of $G_{i1}\alpha$ in which the epitope identified is remote from the sites of either mutational alteration (Green et al., 1990). Despite such controls we were unable to record significant activation of [^{35}S]GTPγS binding or of GTP hydrolysis by the palmitoylation-resistant, pertussis toxin-insensitive C3S/C351G $G_{i1}\alpha$ in response to the α_2-adrenoceptor agonist UK14304, whereas C351G $G_{i1}\alpha$ displayed marked regulation (Figure 3).

We were concerned to eliminate two potential explanations for the observed lack of functional coupling observed with the C3S/C351G $G_{i1}\alpha$. The first was that this protein simply failed to exchange guanine nucleotides. The ability of analogues of GTP to protect trypsin-clipped forms of G-protein α subunits from further degradation is a well-established assay used to assess this question (Eide et al., 1987). The capacity of GTPγS to function in this manner was identical for P2 membrane-associated C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ (Figure 4). Furthermore, the pattern of GTPγS-protected tryptic fragments from these mutants was the same as that produced in parallel experiments with rat brain, which expresses high levels of native $G_{i1}\alpha$. Second, it was possible that differences in $\beta\gamma$ interactions occur between C3S $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ because the N-terminal region of G-protein α subunits is a key element in interaction with the $\beta\gamma$ complex (Wall et al., 1995; Lambright et al., 1996) and functional interaction with $\beta\gamma$ is required to allow effective interactions between receptors and G-proteins. Coexpression of both C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ along with a combination of β_1 and γ_2 cDNAs resulted in higher levels of P2 membrane-associated forms of the G-protein α subunits (Figure 5). In the case of C351G $G_{i1}\alpha$ this resulted in greater stimulation of high-affinity GTPase activity by the coexpressed α_{2A}-adrenoceptor in membranes of pertussis toxin-treated cells. We have previously established a strong correlation between the maximal stimulation of high-affinity GTPase activity by the α_{2A}-adrenoceptor and the levels of P2 membrane-associated C351G $G_{i1}\alpha$ so this was not an unexpected observation (Wise et al., 1997). In contrast, the higher levels of P2-associated C3S/C351G $G_{i1}\alpha$ observed following coexpression of $\beta_1\gamma_2$ still failed to allow significant α_{2A}-adrenoceptor activation of the GTPase activity of this G-protein mutant (Figure 5) despite a marked increase in the P2 membrane versus cytosolic distribution ratio of C3S/C351G $G_{i1}\alpha$, an observation that indicates that C3S/C351G $G_{i1}\alpha$ can interact with $\beta_1\gamma_2$ to facilitate transport to the membrane.

The only previous study of the ability of a palmitoylation-resistant mutant form of a $G_i\alpha$-subfamily protein to interact with receptors and effectors indicated that an epitope-tagged form of C3A $G_z\alpha$ could be activated in CHO-K1 cells by a coexpressed D_2 dopamine receptor to mediate inhibition of amplified adenylyl cyclase (Wilson & Bourne, 1995). However, this effect was relatively small, possibly because C3A $G_z\alpha$ appeared to have a strong constitutive capacity to inhibit adenylyl cyclase; indeed, more so than wild-type $G_z\alpha$ (Wilson & Bourne, 1995). In our studies we observed no obvious constitutive activity of C3S/C351G $G_{i1}\alpha$ to inhibit adenylyl cyclase compared to C351G $G_{i1}\alpha$. Following pertussis toxin treatment of COS-7 cells transiently transfected with the α_{2A}-adrenoceptor alone, we have observed an agonist-stimulated activation of adenylyl cyclase (Wise et al., 1997), an effect believed to be mediated through activation of $G_s\alpha$ (Eason et al., 1992, 1994). In the experiments reported here we observed that UK14304 produced much less effective stimulation of adenylyl cyclase activity following expression of either C3S/C351G $G_{i1}\alpha$ or C351G $G_{i1}\alpha$ together with the α_{2A}-adrenoceptor. Given that COS-7 cells are known to express an adenylyl cyclase isoform that can be stimulated by production of the $\beta\gamma$ complex (Federman et al., 1992), then an obvious interpretation of these results is that both C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ can act as effective scavengers of receptor-generated $\beta\gamma$, released following the activation of G_s, to attenuate the activation of adenylyl cyclase. Furthermore, as expression of equal amounts of the two forms of $G_{i1}\alpha$ resulted in the same degree of reduction of UK14304-mediated regulation of adenylyl cyclase activity (Figure 7), we conclude that the alteration of cysteine 3 to serine does not substantially alter the affinity of $\beta\gamma$ interaction. The fact that expression of transducin α, a reagent often used to examine roles of the $\beta\gamma$ complex in signaling, resulted in a very similar reduction in adenylyl cyclase stimulation by UK14304 (Figure 8) and that coexpression of transducin α and either C3S/C351G $G_{i1}\alpha$

or C351G $G_{i1}\alpha$ (Figure 9) acted additively to reduce UK14304-mediated stimulation of adenylyl cyclase provides yet further evidence that each of these G-proteins are capable of interacting with and sequestering $\beta\gamma$. By contrast, following expression of either G2A/C351G $G_{i1}\alpha$ or G2A/C3S/C351G $G_{i1}\alpha$ along with the receptor we could record only a very limited (G2A/C351G $G_{i1}\alpha$) or no (G2A/C3S/C351G $G_{i1}\alpha$) ability to modify UK14034-mediated stimulation of adenylyl cyclase (Figure 6). This is consistent with our observation that these polypeptides were almost entirely soluble and with the previously reported fact that lack of myristoylation reduces the affinity of $\beta\gamma$ interaction (Linder et al., 1991).

The α_{2A}-adrenoceptor is also modified by palmitoylation, at Cys442. However, studies by Kennedy and Limbird (1993) have indicated that the interactions between this receptor and G-proteins are not perturbed by mutation at this site. Although evidence has been presented to indicate a role for receptor palmitoylation in regulation of signal transduction for both the β_2-adrenoceptor (O'Dowd et al., 1989; Moffet et al., 1993) and rhodopsin (Morrison et al., 1991), a palmitoylation-resistant form of the α_{2A}-adrenoceptor appears to differ from the wild-type protein only in its capacity to be internalized and downregulated (Eason et al., 1994). Expression of this palmitoylation-resistant form of the α_{2A}-adrenoceptor along with C351G $G_{i1}\alpha$ in the current studies produced neither an alteration in maximal GTPase activity in response to UK14304 nor an alteration in the concentration of this agonist required to elicit half-maximal stimulation (I. C. Carr, A. Wise, and G. Milligan, unpublished observations).

There has been considerable recent interest in the concept that dual acylation by myristate and palmitate may be involved in targeting expressed proteins to distinct regions of the plasma membrane of cells termed caveolae, based on the presence of the marker protein caveolin in these regions (Lisanti et al., 1994a). It has further been proposed that these may act as areas in which to concentrate polypeptides involved in signal transduction to increase the local concentrations and thus the efficiency of interactions between proteins (Sargiacomo et al., 1993; Lisanti et al., 1994b; Chang et al., 1994). A recent study has demonstrated the potential for direct interactions between caveolin and certain G-protein α subunits and furthermore that this may be regulated by the activation status of the G-protein, with inactive, GDP-bound forms interacting preferentially with caveolin (Li et al., 1995). It will be intriguing to examine whether C351G $G_{i1}\alpha$ and C3S/C351G $G_{i1}\alpha$ localize differentially to caveolae in transfected COS-7 cells and whether this might contribute to the lack of effective coupling to the α_{2A}-adrenoceptor by C3S/C351G $G_{i1}\alpha$.

In conclusion, we have demonstrated that mutation of cysteine 3 to serine in $G_{i1}\alpha$ generates a protein, containing a single myristate moiety, that can be partially localized in the P2 particulate fraction but is incapable of interacting with an agonist-occupied α_{2A}-adrenoceptor in a functional manner as measured by enhanced guanine nucleotide binding and hydrolysis. However, when overexpressed it appears to have the capacity to sequester $\beta\gamma$ subunits, resulting in attenuation of the α_{2A}-adrenoceptor-mediated stimulation of adenylyl cyclase observed following pertussis toxin treatment.

ACKNOWLEDGMENT

We thank Dr. L. Limbird, Vanderbilt University, Nashville, TN, for provision of the porcine α_{2A}-adrenoceptor cDNA and Dr. H. Hamm, University of Illinois, Chicago, IL, for the transducin α cDNA.

REFERENCES

Casey, P. J., (1995) *Science 268*, 221−225.
Chang, W. J., Ying, Y., Rothberg, K., Hooper, N., Turner, A., Gambliel, H., Dr-Gunzburg, J., Mumby, S., Gilman, A., & Anderson, R. G. W. (1994) *J. Cell. Biol. 126*, 127−138.
Degtyarev, M. Y., Spiegel, A. M., & Jones, T. L. Z. (1993a) *J. Biol. Chem. 268*, 23769−23772.
Degtyarev, M. Y, Spiegel, A. M., & Jones, T. L. Z. (1993b) *Biochemistry 32*, 8057−8061.
Degtyarev, M. Y., Spiegel, A. M., & Jones, T. L. Z. (1994) *J. Biol. Chem. 269*, 30898−30903.
Eason, M. G., & Liggett, S. B. (1995) *J. Biol. Chem. 270*, 24753−24760.
Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., & Liggett, S. B. (1992) *J. Biol. Chem. 267*, 15795−15801.
Eason, M. G., Jacinto, M. T., Theiss, C. T., & Liggett, S. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11178−11182.
Edgerton, M. D., Chabert, C., Chollet, A., & Arkinstall, S. (1994) *FEBS Lett. 354*, 195−199.
Eide, B., Gierschik, P., Milligan, G., Mullaney, I., Unson, C. G., Goldsmith, P., & Spiegel, A. (1987) *Biochem. Biophys. Res. Commun. 148*, 1398−1405.
Federman, A. D., Conklin, B. R., Schrader, K. A., Reed. R. R., & Bourne, H. R. (1992) *Nature 356*, 159−161.
Galbiati, F., Guzzi, F., Magee, A. I., Milligan, G., & Parenti, M. (1994) *Biochem. J. 303*, 697−700.
Gillard, N. P., Linton, C. J., Milligan, G., Carr, I. C., Patmore, L., & Brown, C. M. (1996) *Br. J. Pharmacol. 117*, 298P.
Grassie, M. A., McCallum, J. F., Magee, A. I., Milligan, G., & Parenti, M. (1995) *Biochem. J. 302*, 913−920.
Green, A., Johnson, J. L., & Milligan, G. (1990) *J. Biol. Chem. 265*, 5206−5210.
Hallak, H., Brass, L. F., & Manning, D. R. (1994) *J. Biol. Chem. 269*, 4571−4576.
Helper, J. R., Biddlecome, G. H., Kleuss, C., Camp, L. A., Hofmann, S. L., Ross, E. M., & Gilman, A. G. (1996) *J. Biol. Chem. 271*, 496−504.
Hunt, T. W., Carroll, R. C., & Peralta, E. G. (1994) *J. Biol. Chem. 269*, 29565−29570.
Jones, T. L. Z., Simonds, W. F. Jr., Merendino, J. J., Brann, M. R., & Spiegel, A. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 568−572.
Kennedy, M. E., & Limbird, L. E. (1993) *J. Biol. Chem. 268*, 8003−8011.
Koski, G., & Klee, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4185−4189.
Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., & Sigler, P. B. (1996) *Nature 379*, 311−319.
Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., & Lisanti, M. P. (1995) *J. Biol. Chem. 270*, 15693−15701.
Linder, M. E., Pang, I.-H., Duronio, R. J., Gordon, J. I., Sternweis, P. C., & Gilman, A. G. (1991) *J. Biol. Chem. 266*, 4654−4659.
Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994a) *Trends Cell Biol. 4*, 231−235.
Lisanti, M. P, Scherer, P. E., Vidugiriene, J., Tang, Z.-L., Hermanoski-Vosatka, A., Tu, Y.-H., Cook, R. F., & Sargiacomo, M. (1994b) *J. Cell Biol. 126*, 111−126.
McCallum, J. F., Wise, A., Grassie, M. A., Magee, A. I., Guzzi, F., Parenti, M., & Milligan, G. (1995) *Biochem. J. 310*, 1021−1027.
McKenzie, F. R., & Milligan, G. (1990) *Biochem. J. 267*, 391−398.
Milligan, G.; Streaty, R. A., Gierschik, P., Spiegel, A. M., & Klee, W. A. (1987) *J. Biol. Chem. 262*, 8626−8630.
Milligan, G., Parenti, M., & Magee, A. I. (1995) *Trends Biochem. Sci. 20*, 181−186.

Moffet, S., Mouillac, B., Bonin, H., & Bouvier, M. (1993) *EMBO J. 12*. 349−356.
Morrison, D. F., O'Brien, P. J., & Pepperberg, D. R. (1991) *J. Biol. Chem. 266*, 20118−20123.
Mumby, S. M., & Linder, M. E. (1994) *Methods Enzymol. 237*, 254−268.
Mumby, S. M., Heukeroth, R. O., Gordon, J. I., & Gilman, A. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 728−732.
Mumby, S. M., Kleuss, C., & Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2800−2804.
O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., & Bouvier, M. (1989) *J. Biol. Chem. 264*, 7564−7569.
Parenti, M., Vigano, M. A., Newman, C. M. H., Milligan, G., & Magee, A. I. (1993) *Biochem. J. 291*, 349−353.
Sargiacomo, M., Sudol, M., Tang, Z.-L., & Lisanti, M. P. (1993) *J. Cell Biol. 122*, 789−807.
Senogles, S. E. (1994) *J. Biol. Chem. 269*, 23120−23127.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
Taussig, R., & Gilman, A. G. (1995) *J. Biol. Chem. 270*, 1−4.
Thomas, D. R., Faruq, S. A., Balcarek, J. M., & Brown, A. M. (1995) *J. Rec. Sig. Trans. 15*, 199−211.
Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A.G., & Sprang, S. R. (1995) *Cell 83*, 1047−1058.
Wedegaertner, P. B., & Bourne, H. R. (1994) *Cell 77*, 1063−1070.
Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., & Bourne, H. R. (1993) *J. Biol. Chem. 268*, 25001−25008.
Wedegaertner, P. B., Wilson, P. T., & Bourne, H. R. (1995) *J. Biol. Chem. 270*, 503−506.
Wilson, P. T., & Bourne, H. R. (1995) *J. Biol. Chem. 270*, 9667−9675.
Wise, A., Watson-Koken, M.-A., Rees, S., Lee, M., & Milligan, G. (1997) *Biochem. J. 321*, 721−728.
Wong, Y. H. (1994) *Methods Enzymol. 238*, 81−94.

BI9702997