Interactions of the α_{2A}-adrenoceptor with multiple G_i-family G-proteins: studies with pertussis toxin-resistant G-protein mutants

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The α_{2A}-adrenoceptor is the prototypic example of the family of G-protein-coupled receptors which function by activation of 'G_i-like' pertussis toxin-sensitive G-proteins. A number of members of this subfamily of G-proteins are often co-expressed in a single cell type. To examine the interaction of this receptor with individual G_i-family G-proteins the porcine α_{2A}-adrenoceptor was transiently transfected into COS-7 cells either alone or with each of wild-type $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ or mutations of each of these G-proteins in which the cysteine residue which is the target for pertussis toxin-catalysed ADP-ribosylation was exchanged for a glycine residue. The α_2-adrenoceptor agonist UK14304 stimulated both high-affinity GTPase activity and the binding of guanosine 5′-[γ-^{35}thio]-triphosphate (GTP[^{35}S]), when expressed without any additional G-protein. These effects were greatly reduced by pretreatment of the cells with pertussis toxin. Co-expression of each of the wild-type G_i-like G-protein α-subunits resulted in enhanced agonist activation of the cellular G-protein population which was fully prevented by pretreatment with pertussis toxin. Co-expression of the receptor along with the cysteine-to-glycine mutations of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ resulted in agonist stimulation of these G-proteins, which was as great as that of the wild type proteins, but now the agonist stimulation produced over that due to the activation of endogenously expressed G_i-like G-proteins was resistant to pertussis toxin treatment. The Cys → Gly mutations of $G_i1\alpha$, $G_i2\alpha$ and $G_{i3}\alpha$ were each also able to limit agonist-mediated stimulation of adenylate cyclase activity. The degree of agonist-mediated activation of the pertussis toxin-resistant mutant of G_i1a was correlated highly both with the level of expression of this G-protein and with the level of expression of the α_{2A}-adrenoceptor. Half-maximal stimulation of high-affinity GTPase activity of the Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ required 10–15-fold higher concentrations of agonist than did stimulation of their wild-type counterparts, consistent with a model in which the affinity of functional interactions of the α_{2A}-adrenoceptor with the wild-type G-protein is greater than with the pertussis toxin-resistant mutant G-protein.

INTRODUCTION

The α_2-adrenoceptor subtypes represent the prototypic examples of G-protein-coupled receptors (GPCRs) which mediate inhibition of adenylate cyclase activity via interaction with members of the G_i-subfamily of heterotrimeric G-proteins [1]. With the exception of $G_z\alpha$ [2,3], the α-subunits of the G_i-like G-proteins all possess a conserved cysteine residue four amino acids away from the C-terminus which can act as the acceptor for ADP-ribosylation catalysed by pertussis toxin [4]. As other G-protein α-subunits do not have this feature and pertussis toxin-catalysed ADP-ribosylation prevents functional contacts between GPCRs and the G_i-like G-proteins [4], then a simple way of defining that a cellular response to an agonist at a GPCR is transduced via G-proteins of this subfamily is simply to record attenuation of function following pertussis toxin treatment of cells or tissue [4]. However, as products of the three distinct G_i-like genes, $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$, which encode pertussis toxin-sensitive G-proteins can be co-expressed, then definition of the specificity of interactions of a receptor with individual G-proteins from this family cannot be attempted using such a limited approach. Strategies to overcome this problem have included: the use of antibodies which selectively identify individual G_i-like G-proteins to either interfere with receptor–G-protein interactions [5–7], co-immunoprecipitation of a G-protein/receptor complex [8–10], and immunoprecipitation of a G-protein following covalent modification in a GPCR agonist-dependent manner [11–14]. All of these strategies have contributed greatly to an understanding of the selectivity, or otherwise, of interactions between GPCRs and the G_i-like G-proteins. An alternative scheme which has recently been employed by Senogles [15] and by Hunt et al. [16] has been to replace the cysteine residue which acts as the target for pertussis toxin-catalysed ADP-ribosylation. In this way, treatment of a cell expressing this pertussis toxin-resistant form of a G_i-like G-protein with pertussis toxin results in attenuation of the coupling of a GPCR to the endogenous wild-type G_i-like G-proteins, thus allowing examination of the interaction of the GPCR with the mutationally modified G-protein in isolation. There are inherent concerns with this strategy as the site for pertussis toxin-catalysed ADP-ribosylation is within a key contact site between GPCRs and G-proteins, and because an approach, based upon combinatorial chemistry, in which the sequence of the peptide which comprises the C-terminus of the G_i-like G-protein transducin was altered suggested that the presence of the cysteine residue is vital for high-affinity interactions of these peptides with rhodopsin [17]. In contrast with these findings, however, direct mutational strategies have indicated that alteration of the cysteine → alanine (Cys347 → Ala) residue in transducin does not alter its interaction with rhodopsin [18], whereas a cysteine → tyrosine mutation prevented this interaction [19].

In the present report we examine the use of such mutationally

Abbreviations used: GTP[^{35}S], guanosine 5′-[γ-^{35}thio]-triphosphate; Cys → Gly, mutated G-protein in which a C-terminal cysteine residue is replaced with glycine; GPCR, G-protein-coupled receptor.

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modified forms of pertussis toxin-insensitive $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ to define interactions between these polypeptides and the α_{2A}-adrenoceptor and compare the interactions with those produced by the wild-type G-proteins.

MATERIALS AND METHODS

All materials for tissue culture were supplied by Life Technologies. [^{3}H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham International. [γ-^{32}P]GTP (30 Ci/mmol) and guanosine 5′-[γ-^{35}thio]-triphosphate (GTP[^{35}S]) (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 and were of the highest purity available. Oligonucleotides were synthesized on a Millipore Expedite nucleic acids synthesis system.

Construction of pertussis toxin-resistant $G_i\alpha$-subunits

To generate pertussis toxin-resistant $G_i\alpha$-subunits, rat $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ were excised on an *Eco*RI restriction fragment from the vector pGEM2 and inserted into the *Eco*RI site of pBluescript KS- (Stratagene) creating pBS/$G_i1\alpha$, pBS/$G_i2\alpha$ and pBS/$G_i3\alpha$. To generate pBS/$G_i1\alpha$(C351G) the 3′-44 bp of the $G_i1\alpha$ open reading frame and the 3′ flanking sequence was 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**GGTCTC TTCTAAGAATTCA-3′ and 5′-AGCTTGAATTCTTAGAA-GAGACC**ACC**GTCTTTTAGGTTATTCTTTATGAT GACGT-3′ (Cys351 → Gly in bold; restriction sites for *Eco*RI, *Hin*dIII and *Aat*II sites underlined) to recreate the 3′ end of $G_i1\alpha$. Similarly, to generate pBS/$G_i2\alpha$(C352G) the plasmid pBS/$G_i2\alpha$ was digested with *Aat*II and *Not*I to remove the 3′-44 bp and the 3′ untranslated sequence of $G_i2\alpha$ which was replaced with the synthetic oligonucleotide linker created following annealing of the oligonucleotides 5′-CATCATCAAGAACAACCTGAA-GGAC**GGT**G GCCTCTTCTGAGAATTCGC -3′ and 5′-GGCCGCGAATTCTCAGAAGAGGCC**ACC**GTCCTTCA-GGTTGTTCTTGATGATGACGT -3′ (C352G, in bold; restriction sites for *Eco*RI, *Not*I and *Aat*II, underlined) to recreate the 3′ end of $G_i2\alpha$. Finally, to generate pBS/$G_i3\alpha$(Cys351 → Gly) the plasmid pBS/$G_i3\alpha$ was digested with *Bgl*II and *Sal*I to release a 770 bp fragment containing the 3′-112 bp and the 3′ untranslated sequence of $G_i3\alpha$. The 3′ end of $G_i3\alpha$ was then amplified from pBS/$G_i3\alpha$ using a PCR with Pfu DNA Polymerase (Stratagene) and the synthetic oligonucleotide primers 5-GTTTGAAGATCTGAACCGAAGAAAGGACAC-3′ and 5-GCTGATGTCGACGAATTCTCAGTAAAGCCC**ACC**TTC CTT-3′ (Cys351 → Gly, in bold; *Bgl*II, *Sal*I and *Eco*RI sites, underlined). The 136 bp fragment obtained following the PCR was digested with restriction enzymes *Bgl*II and *Sal*I and ligated between the *Bgl*II and *Sal*I sites of pBS/$G_i3\alpha$ to generate pBS/$G_i3\alpha$(Cys351 → Gly). All vectors were sequenced after cloning to verify the presence of the Cys → Gly mutation and the authenticity of the 3′ end of each $G_i\alpha$ open reading frame.

Each of these mutants and the wild-type forms of the G-proteins were subcloned into pcDNA3 (Invitrogen) for the transfection studies.

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 penicillin/ml and 100 μg streptomycin/ml.

Table 1 Generation and specificity of antisera

The antisera were generated against synthetic peptides predicted to represent the sequences noted. See Materials and methods section and the listed references for further details. TD, transducin.

Antiserum	Sequence	Identification	Reference
SG1	TDα (341–350)	TDα, $G_i1\alpha$, $G_i2\alpha$	[25]
LE3	$G_i2\alpha$ (160–169)	$G_i2\alpha$	[26]
I1C	$G_i1\alpha$ (159–168)	$G_i1\alpha$	[25]
I3C	$G_i3\alpha$ (345-354)	$G_i3\alpha$	[30]

Cells were seeded in 60-mm culture dishes and grown to 60–80 % confluency (18–24 h) before transfection with pcDNA3 containing either the porcine α_{2A}-adrenoceptor, wild-type or pertussis toxin-insensitive forms of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$ using lipofectamine reagent (Life Technologies). For transfection, 2.0 μg of DNA was mixed with 10 μl of lipofectamine in 0.2 ml of Opti-MEM (Life Technologies) and incubated at room temperature for 30 min before 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 to the cells. The 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 after harvesting. Cell pellets were resuspended in 0.5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and rupture of the cells was achieved with 50 strokes of a hand-held Teflon/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 200 000 ***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 with the bicinchoninic acid (BCA) procedure [20] 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 [21] (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 and free ligands were separated 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.

Immunological studies

The generation and specificities of the various antisera used in this study are defined in Table 1. Each antiserum was produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide and keyhole limpet haemocyanin (Calbiochem) as antigen. Membrane samples were resolved by SDS/PAGE overnight at 100 V, using 10 % (w/v) acrylamide gels containing 6 M urea.

Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell), probed with the relevant antiserum and visualized as described [6].

High-affinity GTPase assays

The GTPase assays were performed essentially as described in [22] using [γ-^{32}P]GTP (0.5 μM, 60000 c.p.m.) and UK14304 (up to 1×10^{-5} M). Non-specific GTPase was assessed by parallel assays containing 100 μM GTP.

GTP[^{35}S] binding studies

The binding studies were performed as in [23]. Briefly, membranes (5 μg) were incubated at 30 °C for 30 min in a final volume of 100 μl of reaction mixture comprising 20 mM Hepes (pH 7.4)/3 mM $MgCl_2$/100 mM NaCl/0.2 mM ascorbate and 10 μM GDP, 0.3–0.5 nM GTP[^{35}S] (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 7.4/3 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 Ultima-Flo AF scintillant before counting by liquid scintillation spectroscopy.

Regulation of adenylate cyclase activity

This was performed essentially as described by Wong [24]. COS-7 cells transfected with combinations of the α_{2A}-adrenoceptor and the pertussis toxin-resistant Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$ were labelled with [^{3}H]adenine and pertussis toxin-treated as described above. The generation of [^{3}H]cAMP in response to treatment of the cells with combinations of UK14304 and/or forskolin was then assessed.

RESULTS

Cys → Gly mutations of the cysteine residue located 4 amino acids from the C-terminus of the α-subunits of rat G_i1, G_i2 and G_i3 were generated (see Materials and methods section). This cysteine residue is the target amino acid for pertussis toxin-catalysed ADP-ribosylation of each of these G-protein polypeptides. After subcloning of both wild-type and Cys → Gly mutants into the plasmid pcDNA3, these proteins were transiently expressed in COS-7 cells. Plasma membrane-containing P2 particulate fractions from COS-7 cells were analysed by SDS/10% (w/v) PAGE containing 6 M urea and subsequent immunoblotting. These gel conditions allowed excellent resolution of the G_i-like G-proteins. Immunoblotting of mock-transfected cells with antiserum SG1 [25], which identifies $G_i1\alpha$ and $G_i2\alpha$ equally as it is directed against the C-terminal decapeptide that is absolutely conserved in these two G-proteins, indicated that both of these G-proteins are expressed endogenously by COS-7 cells (Figure 1; upper panel, lane 1). Examination of gels after SDS/PAGE in 6 M urea showed that the entire population of endogenous $G_i1\alpha$ and $G_i2\alpha$ proteins was ADP-ribosylated following pretreatment of the mock-transfected cells with pertussis toxin (50 ng/ml for 16 h), as migration through the gel was retarded (Figure 1; upper panel, lane 2). Transfection with wild-type $G_i2\alpha$ resulted in satisfactory expression of this polypeptide (Figure 1; upper panel, lane 3) and pretreatment of the cells with pertussis toxin brought about ADP-ribosylation of the full complement of the expressed polypeptide, as assessed by its reduced mobility after toxin treatment (Figure 1; upper panel, lane 4). Expression of the Cys → Gly mutant of $G_i2\alpha$ also resulted in ample levels of this polypeptide but, as predicted, the

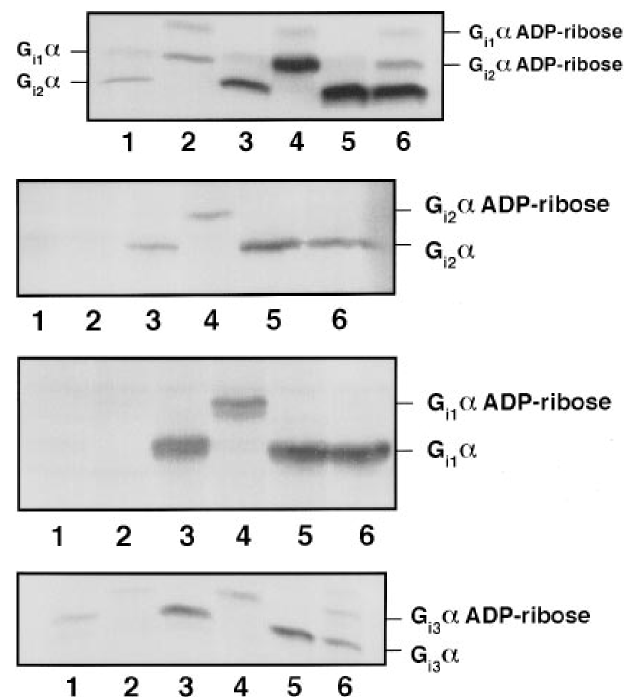

Figure 1 Expression and pertussis toxin-sensitivity of wild-type and Cys → Gly mutants of G_i-like G-proteins

COS-7 cells were either mock transfected (lanes 1 and 2) or transfected with wild-type G_i-like G-protein α-subunits (lanes 3 and 4) or pertussis toxin-resistant Cys → Gly mutations of these G-proteins (lanes 5 and 6) as described in the Materials and methods section. Samples in lanes 2, 4 and 6 were infected with pertussis toxin (50 ng/ml) 16 h before harvesting. Cell-membrane preparations were resolved by SDS/PAGE in gels containing 6 M urea and were immunoblotted. Figure 1 (upper panel), transfections with wild-type or Cys → Gly $G_i2\alpha$. Antiserum SG1 was used for the immunoblots, allowing identification of both $G_i1\alpha$ and $G_i2\alpha$. Figure 1 (second panel), transfections with wild-type or Cys → Gly $G_i2\alpha$. Immunoblots were with the $G_i2\alpha$-specific antiserum, LE3. Figure 1 (third panel), transfections with wild-type or Cys → Gly $G_i1\alpha$. Immunoblots were with the $G_i1\alpha$-specific antiserum, I1C. Figure 1 (fourth panel), transfections with wild-type or Cys → Gly $G_i3\alpha$. Immunoblots were with the $G_i3\alpha$-specific antiserum, I3C. The results shown are from representative experiments which were performed at least three times.

polypeptide was not a substrate for ADP-ribosylation by pertussis toxin and mobility through urea-containing SDS-polyacrylamide gels was similar to that of the polypeptide from untreated cells (Figure 1; upper panel, lanes 5 and 6). In these experiments, however, the endogenously expressed G_i-like G-proteins were modified by the addition of ADP-ribose as the mobility of these polypeptides was reduced in the resolving gel (Figure 1; upper panel, lane 6). When immunoblots were performed with the $G_i2\alpha$ specific antiserum LE3 [26] (Figure 1, second panel), identical conclusions as to the expression and pertussis toxin sensitivity of wild-type and Cys → Gly $G_i2\alpha$ were obtained. Expression and complete pertussis toxin sensitivity of wild-type $G_i1\alpha$ (Figure 1, third panel) and $G_i3\alpha$ (Figure 1, fourth panel) were recorded after transfection of COS-7 cells with the appropriate construct and immunoblotting of P2 particulate fractions of these cells with specific antisera (see Table 1 for details). The Cys → Gly mutants of both $G_i1\alpha$ and $G_i3\alpha$ were also well expressed and in each case were shown to be resistant to modification by pertussis toxin treatment of the cells (Figure 1, third panel and fourth panels).

Transfection of COS-7 cells with the porcine α_{2A}-adrenoceptor resulted in high-level expression of this receptor, assessed by the

Table 2 Expression of the α_{2A}-adrenoceptor is not compromised by co-expression of G-protein α-subunits or by treatment with pertussis toxin

COS-7 cells were transfected with the α_{2A}-adrenoceptor (α_{2A}-AR) in pcDNA3 (1 μg) with either wild-type (WT) or Cys → Gly variants of G-proteins (1 μg) as described in the Materials and methods section. Cells were harvested after 48 h, membranes were prepared and the specific binding of [^{3}H]RS-79948-197 was measured. In some cases the cells were treated with pertussis toxin (50 ng/ml for 16 h) before harvesting. The values shown are the means ± S.D. of a representative experiment of three performed.

Transfections	[^{3}H]RS-79948-197 binding (pmol/mg of protein) Without pertussis toxin	With pertussis toxin
$\alpha_{2\alpha}$-AR	15.9 ± 0.3	16.0 ± 0.8
$\alpha_{2\alpha}$-AR + $G_i2\alpha$ WT	15.7 ± 1.1	17.3 ± 0.2
$\alpha_{2\alpha}$-AR + $G_i3\alpha$ WT	18.3 ± 1.3	16.6 ± 0.8
$\alpha_{2\alpha}$-AR + $G_i2\alpha$ Cys352 → Gly	15.3 ± 1.3	14.3 ± 0.4
$\alpha_{2\alpha}$-AR + $G_i3\alpha$ Cys351 → Gly	18.6 ± 0.6	20.9 ± 0.7

Table 3 Agonist stimulation of high affinity GTPase following co-expression of the α_{2A}-adrenoceptor and wild-type G_i-like G proteins

COS-7 cells were transfected with either the α_{2A}-adrenoceptor (α_{2A}-AR) alone, or with wild-type $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$. Cells were treated either with pertussis toxin (50 ng/ml) or with vehicle 32 h after transfection and were harvested 16 h later. Cell membranes were prepared and treated with UK14304 (10 μM) (basal values were from membranes not treated with UK 14304) and the high-affinity GTPase activity was measured as described in the Materials and methods section. The values are the means ± S.D. of triplicate measurements. A second, independent experiment gave qualitatively similar data.

	High-affinity GTPase activity (pmol/min per mg of protein)			
	With pertussis toxin		Without pertussis toxin	
Transfections	Basal	+ UK14304 (10 μM)	Basal	+ UK14304 (10 μM)
α_{2A}-AR	13.6 ± 0.7	20.4 ± 0.4	12.1 ± 0.6	11.3 ± 0.4
α_{2A}-AR + $G_i1\alpha$WT	14.8 ± 1.1	36.7 ± 0.7	12.7 ± 2.8	13.9 ± 0.4
α_{2A}-AR + $G_i2\alpha$WT	15.4 ± 1.0	30.0 ± 0.8	11.3 ± 0.7	12.3 ± 0.1
α_{2A}-AR + $G_i3\alpha$WT	15.1 ± 1.4	31.1 ± 1.0	13 ± 0.6	13.1 ± 0.8

Table 4 Agonist stimulation of high-affinity GTPase following co-expression of the α_{2A}-adrenoceptor and Cys → Gly mutant G_i-like G proteins

COS-7 cells were transfected with either the α_{2A}-adrenoceptor alone (α_{2A}-AR) or in combination with Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$. Cells were treated either with pertussis toxin (50 ng/ml) or with vehicle 32 h after transfection and were harvested 16 h later. Cell membranes were prepared and treated with UK14304 (10 μM) (basal values were from membranes not treated with UK14304) and the high-affinity GTPase activity was measured as described in the Materials and methods section. The values are the means ± S.D. of triplicate measurements and are representative of two independent experiments.

	High-affinity GTPase activity (pmol/min per mg of protein)			
	With pertussis toxin		Without pertussis toxin	
Transfections	Basal	+ UK14304 (10 μM)	Basal	+ UK14304 (10 μM)
α_{2A}-AR	13.6 ± 0.7	20.4 ± 0.4	12.1 ± 0.6	11.3 ± 0.4
α_{2A}-AR + $G_i1\alpha$ Cys351 → Gly	12.6 ± 1.0	33.0 ± 1.7	13.0 ± 0.1	30.0 ± 0.1
α_{2A}-AR + $G_i2\alpha$ Cys352 → Gly	12.1 ± 0.3	25.9 ± 1.8	10.9 ± 1.1	20.1 ± 0.1
α_{2A}-AR + $G_i3\alpha$ Cys351 → Gly	13.3 ± 0.6	32.0 ± 1.3	12.8 ± 0.4	27.6 ± 1.3

Table 5 Agonist stimulation of GTP[^{35}S] following co-expression of the α_{2A}-adrenoceptor and Cys → Gly mutant G_i-like G proteins

COS-7 cells were transfected with either the α_{2A}-adrenoceptor alone (α_{2A}-AR) or in combination with Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$. Cells were treated with either pertussis toxin (50 ng/ml) or vehicle 32 h after transfection and were harvested 16 h later. Cell membranes were prepared and treated with UK 14304 (10 μM) (basal values were from membranes not treated with UK 14304) and the specific binding of GTP[^{35}S] was measured as described in the Materials and methods section. The values are the means ± S.D. of triplicate measurements. A second independent experiment gave qualitatively similar results.

	Specific GTP[^{35}S] binding (fmol/mg of protein)			
	With pertussis toxin		Without pertussis toxin	
Transfections	Basal	+ UK14304 (10 μM)	Basal	+ UK14304 (10 μM)
α_{2A}-AR	44.1 ± 3.9	87.2 ± 8.9	27.2 ± 1.0	37.0 ± 5.5
α_{2A}-AR + $G_i1\alpha$ Cys351 → Gly	47.0 ± 5.0	186.0 ± 18.9	40.0 ± 11.6	111.0 ± 15.2
α_{2A}-AR + $G_i2\alpha$ Cys352 → Gly	47.7 ± 2.1	130.0 ± 5.6	43.9 ± 4.1	86.0 ± 9.9
α_{2A}-AR + $G_i3\alpha$ Cys351 → Gly	50.4 ± 7.3	170.0 ± 11.6	35.0 ± 0.8	107.8 ± 13.0

specific binding of the high-affinity and highly α_2-adrenoceptor-selective ligand [^{3}H]RS-79948-197 (Table 2). The levels of expression of the receptor were unaffected by co-expression with any of either the wild-type or mutant, pertussis toxin-resistant, Cys → Gly G_i-like G-proteins (Table 2).

Addition of the α_2-adrenoceptor agonist UK14304 (1×10^{-5} M) to membranes of COS-7 cells transfected to express the α_{2A}-adrenoceptor resulted in activation of the endogenous G-protein population as measured by a stimulation of high-affinity GTPase activity (Table 3). Pretreatment of the cells with pertussis toxin completely prevented this effect (Table 3). Co-transfection of COS-7 cells to express the α_{2A}-adrenoceptor along with wild-type forms of each of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ resulted in significantly greater activation of high-affinity GTPase activity in the presence of UK14304 than when the receptor was expressed in isolation, thus confirming the capacity of the agonist-occupied receptor to interact with and stimulate each of these G-proteins (Table 3). As anticipated from the results shown in Figure 1, pertussis toxin pretreatment of the cells fully prevented UK14304 stimulation of high-affinity GTPase activity after expression of wild-type $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ (Table 3). Co-transfection of the α_{2A}-adrenoceptor and the Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ also resulted in greater activation of high-affinity GTPase activity in the presence of UK14304 than after expression of the receptor in the absence of additional G-protein (Table 4). Pretreatment of these cells with pertussis toxin did not prevent stimulation of high-affinity GTPase activity by UK14304 (Table 4). Although pertussis toxin treatment reduced the maximal effect of UK14304 after expression of each of the mutant Cys → Gly $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ proteins, the reduction was similar in magnitude to the degree of stimulation of high-affinity GTPase activity produced by UK14304 after expression of the α_{2A}-adrenoceptor in isolation, and thus presumably reflects the ability of pertussis toxin to eliminate contact between the receptor and the endogenously expressed G_i-like G-proteins. Very similar data to those obtained with the high-affinity GTPase assays were recorded when the ability of UK14304 to stimulate binding of GTP[^{35}S] to membranes of COS-7 cells transfected to express the Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ was examined (Table 5, and results not shown).

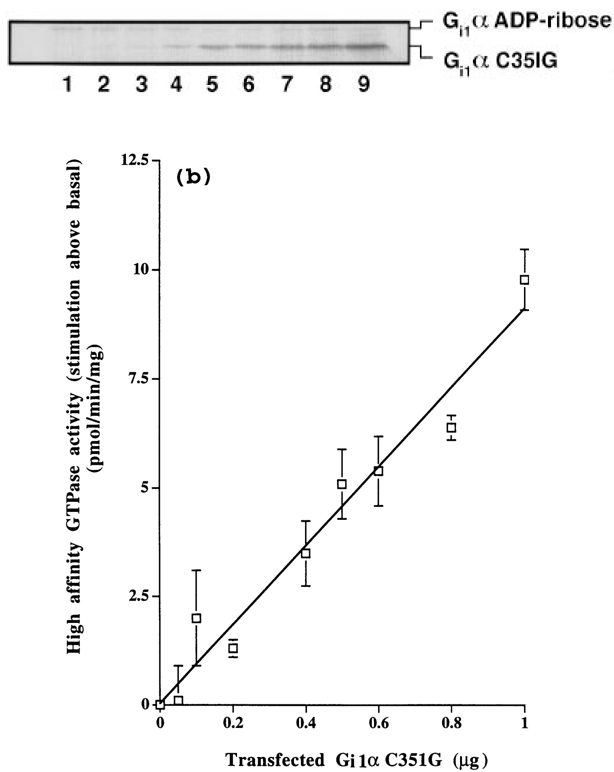

Figure 2 Agonist stimulation of the high-affinity GTPase activity of Cys → Gly $G_i1\alpha$ depends on the level of expression of the G-protein

COS-7 cells were transfected with the α_{2A}-adrenoceptor in pcDNA3 (1 μg) together with various amounts (0–1 μg) of Cys → Gly $G_i1\alpha$ in pcDNA3. The cells were treated with pertussis toxin (50 ng/ml) 32 h after transfection and were harvested 16 h later. The cell membranes were immunoblotted with antiserum I1C to allow the relative levels of expression of Cys → Gly $G_i1\alpha$ to be assessed (Figure 2, upper panel). Lanes 1–9 show increasing levels of Cys → Gly $G_i1\alpha$ transfected. Endogenous $G_i1\alpha$, which was modified by the addition of ADP-ribose during the pertussis-toxin treatment, was detected with this antiserum as a constant, low level of a more slowly migrating I1C-reactive polypeptide (compare with Figure 1, upper panel). The results shown are from a representative experiment. (**b**) Agonist stimulation of the high-affinity GTPase activity measured in transfected Cys → Gly $G_i1\alpha$ ($G_i1\alpha$ C351G) cell membranes in the presence or absence of UK14304 (10 μM). The results shown are the means ± S.E.M. of three experiments.

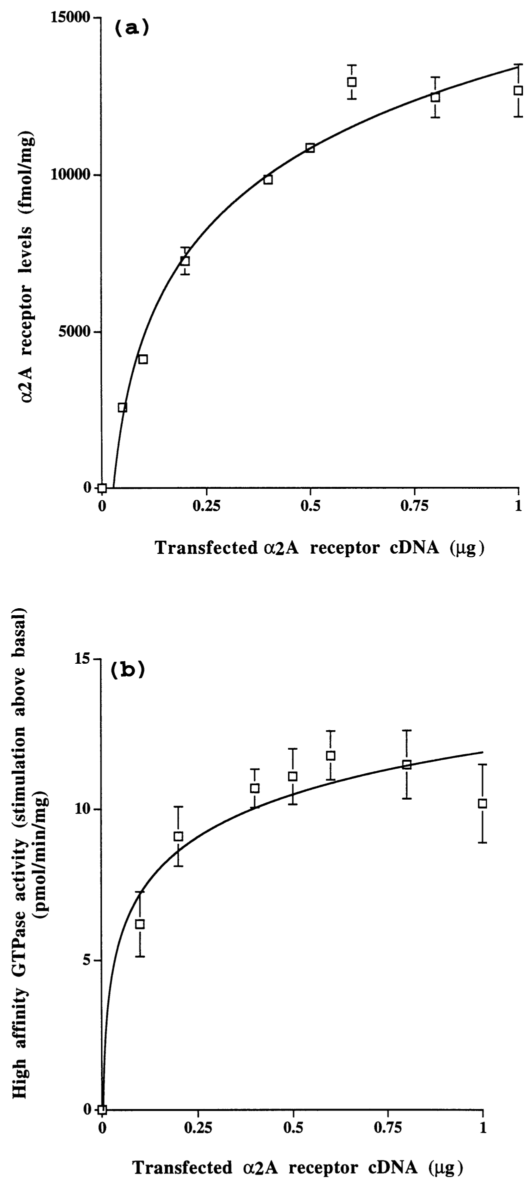

Figure 3 Agonist stimulation of the high-affinity GTPase activity of Cys → Gly $G_i1\alpha$ depends on the level of expression of the receptor

COS-7 cells were transfected with various amounts of the α_{2A}-adrenoceptor in pcDNA3 (0–1 μg) together with 1 μg of Cys → Gly $G_i1\alpha$ in pcDNA3. The cells were treated with pertussis toxin (50 ng/ml) 32 h after transfection and were harvested 16 h later. Cell membranes were immunoblotted to detect the relative levels of expression of Cys → Gly $G_i1\alpha$, which did not vary between the transfections (results not shown), and were used to measure the specific binding of [^{3}H]RS-79948-197 (**a**) or high-affinity GTPase activity, measured in the presence or absence of UK14304 (10 μM) (**b**). Means ± S.E.M., $n = 3$.

Transfection of increasing amounts of the Cys → Gly $G_i1\alpha$ plasmid (up to 1 μg DNA) into COS-7 cells along with a constant amount (1 μg) of the α_{2A}-adrenoceptor plasmid resulted in increasing levels of expression of Cys → Gly $G_i1\alpha$ (Figure 2, upper panel). After pertussis toxin pretreatment of the cells, UK14304 (1×10^{-5} M) caused stimulation of high-affinity GTPase activity above basal levels, which was linearly related to the amount of G-protein cDNA used and protein expressed (Figure 2b). Transfection of varying levels of the α_{2A}-adrenoceptor plasmid along with a constant level of Cys → Gly $G_i1\alpha$ resulted in a plateau in the expression of the receptor at levels above 0.25 μg DNA. The increase in high-affinity GTPase activity in response to 10 μM UK14304 again reflected the levels of expression of the receptor (Figure 3).

To investigate the relative affinities of interaction of the α_{2A}-adrenoceptor with wild-type $G_i1\alpha$ versus Cys → Gly $G_i1\alpha$, each of these polypeptides was co-expressed with the receptor and the ability of various concentrations of UK14304 to stimulate high-affinity GTPase activity was measured in untreated and pertussis toxin-pretreated cells. As anticipated, stimulation of high-affinity GTPase was not observed after pertussis toxin treatment of cells expressing wild-type $G_i1\alpha$. As noted earlier, a similar maximal stimulation was achieved using either form of $G_i1\alpha$. However, the EC_{50} (mean ± S.E.M., $n = 3$) for UK14304 stimulation of the high-affinity GTPase activity of wild-type $G_i1\alpha$ [$(6.1 \pm 0.2) \times 10^{-9}$ M] was approx. 10-fold lower than that of Cys → Gly $G_i1\alpha$ [$(5.4 \pm 0.2) \times 10^{-8}$ M in untreated cells and $(6.4 \pm 0.7) \times 10^{-8}$ M in pertussis toxin-treated cells] (Figure 4a). Equivalent studies after co-transfection of the α_{2A}-adrenoceptor with wild-type or Cys → Gly $G_i2\alpha$ (Figure 4b) [the EC_{50} (mean ± S.E.M., $n = 3$) for UK14304 stimulation of

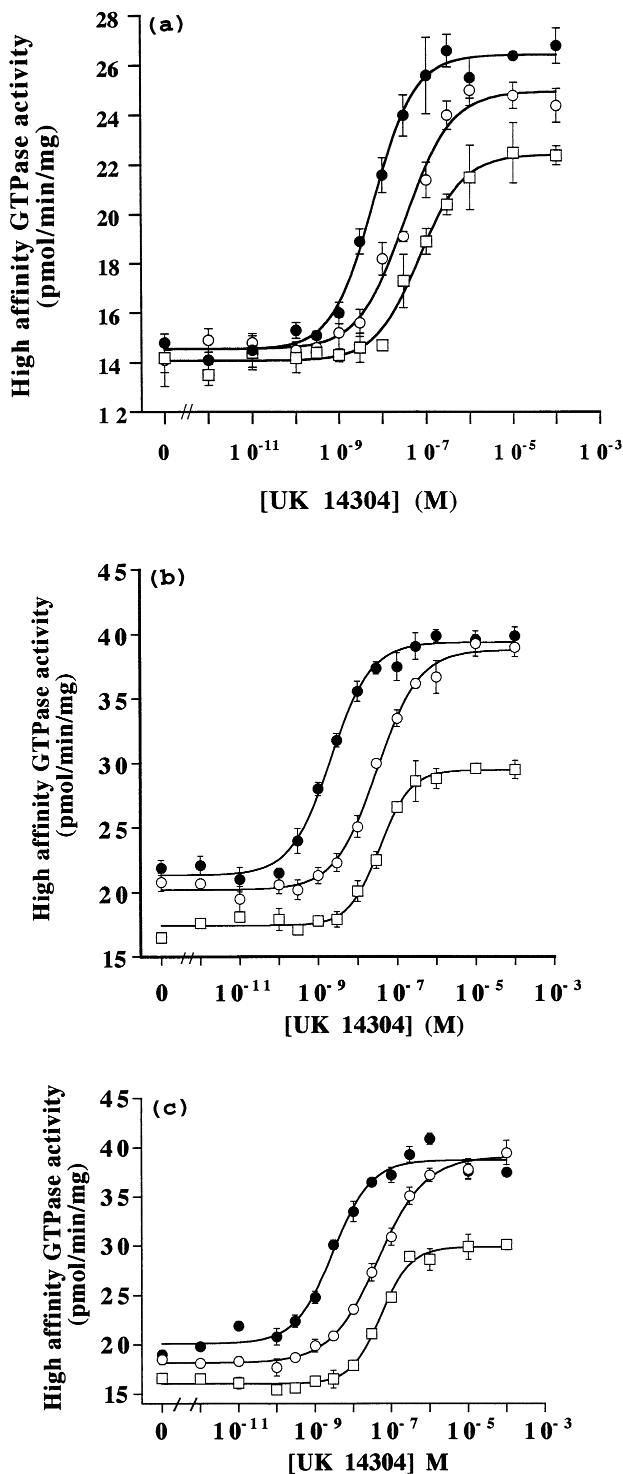

Figure 4 Less agonist is required to stimulate the high-affinity GTPase activity of wild-type G_i -proteins than that of the pertussis toxin-resistant Cys → Gly mutants

COS-7 cells were co-transfected with the α_{2A}-adrenoceptor in pcDNA3 (1 μg) and with either wild-type or mutated G-proteins in pcDNA3. The cells were treated with pertussis toxin (50 ng/ml) (□) or with vehicle (controls) (○,●) 32 h after transfection and the cells were harvested 16 h later. Cell membranes were used to measure high-affinity GTPase activity in the presence of UK14304 (0–10^{-5} M). (**a**) Wild-type $G_i1\alpha$ (●), Cys → Gly $G_i1\alpha$ (○), pertussis toxin-treated Cys → Gly $G_i1\alpha$ (□); (**b**) wild-type $G_i2\alpha$ (●), Cys → Gly $G_i2\alpha$ (○) pertussis toxin-treated Cys → Gly $G_i2\alpha$ (□); (**c**) wild-type $G_i3\alpha$ (●), Cys → Gly $G_i3\alpha$ (○), pertussis toxin-treated Cys → Gly $G_i3\alpha$ (□). Means ± S.E.M., $n = 3$.

Table 6 Pertussis toxin-resistant Cys → Gly mutants of G_i-like G-proteins can limit α_{2A}-adrenoceptor-mediated stimulation of adenylate cyclase activity

COS-7 cells were transfected with either the α_{2A}-adrenoceptor alone (α_{2A}-AR) or in combination with Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$. Cells were treated with pertussis toxin (50 ng/ml) 32 h after transfection and labelled with [^{3}H]adenine (1.5 μCi/ml). The cells were treated with UK14304 (10 μM) in the presence or absence of forskolin (50 μM) 20 h later (basal values were from cells not treated with either UK14304 or forskolin). The generation of [^{3}H]cAMP was then measured as described in [24]. The values shown are the ratio ([^{3}H]cAMP/total [^{3}H]adenine nucleotides) × 1000 and are the means ± S.D. of triplicate measurements of a single experiment. A second, independent experiment produced similar results.

	cAMP		cAMP	
Transfections	Basal	+ UK14304 (10 μM)	Forskolin	Forskolin + UK14304 (10 μM)
α_{2A}-AR	0.62 ± 0.01	6.8 ± 0.2	20.2 ± 0.81	44.7 ± 2.0
α_{2A}-AR + $G_i1\alpha$ Cys351 → Gly	0.77 ± 0.05	1.3 ± 0.17	21.1 ± 1.3	26.7 ± 3.8
α_{2A}-AR + $G_i2\alpha$ Cys352 → Gly	0.64 ± 0.04	1.3 ± 0.05	23.0 ± 0.73	21.8 ± 1.4
α_{2A}-AR + $G_i3\alpha$ Cys351 → Gly	0.73 ± 0.05	1.1 ± 0.12	21.6 ± 2.0	20.1 ± 0.52

the high-affinity GTPase activity of wild-type $G_i2\alpha$ was $(2.2 \pm 0.3) \times 10^{-9}$ M, and for Cys → Gly $G_i2\alpha$ it was $(3.2 \pm 0.4) \times 10^{-8}$ M in untreated cells and $(3.7 \pm 0.4) \times 10^{-8}$ M in pertussis toxin-treated cells] and wild-type or Cys → Gly $G_i3\alpha$ (Figure 4c) [the EC_{50} (means ± S.E.M., $n = 3$) for UK14304 stimulation of the high-affinity GTPase activity of wild-type $G_i3\alpha$ was $(3.0 \pm 0.7) \times 10^{-9}$M, and for Cys → Gly $G_i3\alpha$ it was $(4.5 \pm 0.5) \times 10^{-8}$ M in untreated cells and $(5.3 \pm 0.6) \times 10^{-8}$ M in pertussis toxin-treated cells] produced very similar results and the EC_{50} for UK14304 stimulation of high-affinity GTPase activity required 10–15-fold higher concentrations when using the pertussis toxin-resistant forms of these G-proteins.

To assess whether the pertussis toxin resistant Cys → Gly forms of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ were capable of transducing a signal to an effector enzyme, transfected COS-7 cells were labelled with [^{3}H]adenine and adenylate cyclase activity was subsequently measured. In cells transfected to express only the α_{2A}-adrenoceptor and then treated with pertussis toxin to eliminate interactions with the endogenous G_i population, both basal and forskolin (5×10^{-5} M)-amplified adenylate cyclase was further stimulated by the addition of UK14304 (1×10^{-5} M) (Table 6). This effect was not unexpected as Eason et al. [27] and Eason and Liggett [28] have noted previously that high-level expression of the α_{2A}-adrenoceptor can result in activation of both G_s and G_i and after pertussis toxin treatment only potential interactions with G_s would be expected. After co-transfection of the α_{2A}-adrenoceptor and the pertussis toxin-resistant Cys → Gly mutations of each of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ the observed stimulatory effect of UK14304 on adenylate cyclase activity in pertussis toxin-treated cells in the absence of the mutant G_i proteins was abolished (Table 6). These results demonstrated clearly that each of these G-proteins was capable of preventing stimulation of adenylate cyclase by the α_{2A}-adrenoceptor.

DISCUSSION

COS-7 cells express each of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ endogenously (Figure 1) and transient expression of the porcine α_{2A}-adrenoceptor allows interaction with some combinations of these G-proteins as measured by the ability of the agonist UK14304 to stimulate high-affinity GTPase activity and the binding of

GTP[^{35}S] in a way which was completely attenuated by pretreatment of the cells with pertussis toxin. In all of the experiments presented herein we have taken steps to determine that pertussis toxin treatment of the transfected cells was effective and that it modified the entire pool of the relevant G-proteins. To do so we have utilized the fact, known for some time, that after addition of ADP-ribose to the G_i-like α-subunits they migrate more slowly when subjected to SDS/PAGE [26]. This may reflect the combined effects of the addition of approx. 500 molecular-mass units and the negatively charged nature of ADP-ribose. In many SDS-PAGE systems the change in mobility is small, but the inclusion of 6 M urea in the resolving gel produced excellent retardation of the ADP-ribosylated polypeptide when compared with the non-modified G-protein (Figure 1). Treatment of the cells with pertussis toxin produced ADP-ribosylation of the entire pool of the wild-type G_i-like proteins, which are substrates for the toxin, even when high concentrations of these were transiently expressed (Figure 1). Use of this system showed also that the Cys → Gly mutants of the G_i-like G-proteins used in this study were indeed fully resistant to pertussis toxin treatment, as changes in mobility were not observed after treatment of the cells, and that after their expression it was only the endogenous G_i-like G-proteins which were modified by the toxin (Figure 1).

Receptor interaction with overexpressed wild-type $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$ was recorded as an increase in maximal stimulation of both high-affinity GTPase activity and GTP[^{35}S] binding produced in response to UK14304 after expression of these polypeptides. All of these G-proteins were shown to have the capacity to interact functionally with the α_{2A}-adrenoceptor (Table 3). This was not unexpected as we have previously shown direct interactions of this receptor with each of $G_i2\alpha$, $G_i3\alpha$ and $G_{o1\alpha}$ in various cells lines transfected to stably express this receptor [11,29,30].

Both Senogles [15] and Hunt et al. [16] have previously made use of a strategy in which site-directed mutation of the cysteine residue, which acts as the acceptor for pertussis toxin-catalysed ADP-ribosylation, resulted in the production of a pertussis toxin-insensitive G-protein, and they then used these proteins after expression in either GH4C1 [15] or CHO [16] cells, to examine the signalling functions of a variety of receptors. The results obtained in these studies, although interesting, were rather variable. Hunt et al. [16] examined the interactions of the M_2-muscarinic acetylcholine receptor with a cysteine → serine mutant of $G_i3\alpha$ but were unable to detect functional coupling of this receptor to an equivalent cysteine → serine $G_i2\alpha$ mutant. Furthermore, they were unable to provide evidence for an interaction between this receptor and the endogenously expressed $G_i2\alpha$- or $G_i3\alpha$-proteins and did not examine potential interactions after overexpression of a wild-type $G_i3\alpha$-protein. Senogles [15] examined potential interactions of each of $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$ with either the long or short isoform of the dopamine D_2-receptor. A rather complex pattern of interactions was observed, in which a Cys → Gly mutant of $G_i1\alpha$ was unable to interact with either isoform, the short isoform interacted functionally and efficiently with Cys → Gly $G_i2\alpha$ although the long isoform did not, and the interaction of the short isoform with Cys → Gly $G_i3\alpha$ was poor but the long isoform interacted efficiently with this variant G-protein. Parallel experiments with the wild-type proteins were not performed.

To investigate if the pertussis toxin-insensitive mutants could be usefully employed to provide information on coupling to the wild-type proteins or if, at best, they are merely academic curiosities, in the present study we used Cys → Gly mutations of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ to study interactions with the porcine α_{2A}-adrenoceptor. The choice of receptor was based on our previous studies, which had showed that it interacted with stably expressed wild-type forms of each of these pertussis toxin-sensitive G-proteins. As controls for these studies, we examined the interaction of this receptor with the equivalent wild-type G-proteins. A receptor-saturating concentration of the agonist UK14304 resulted in stimulation of high-affinity GTPase activity in membranes of COS-7 cells expressing $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ together with the α_{2A}-adrenoceptor, which was significantly greater than that produced by receptor activation of the endogenous G-protein population (Table 3). Equivalent experiments with the Cys → Gly mutants of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ resulted in similar levels of expression when compared with the wild-type proteins and also similar degrees of stimulation of high-affinity GTPase activity in response to a maximally effective concentration of UK14304 (Table 4). Pertussis-toxin treatment of cells transfected to express either the α_{2A}-adrenoceptor alone or the receptor and each of the wild-type G-proteins, resulted in complete attenuation of UK14304 stimulation of high-affinity GTPase activity. By contrast, pertussis-toxin treatment of cells expressing the receptor together with the Cys → Gly mutants of these G-proteins resulted in partial inhibition of UK14304 stimulation. The degree of inhibition was similar to the stimulation achieved in the absence of expression of the mutant G-proteins, and thus represents impairment of interaction with the endogenous G_i-like G-proteins but not with the Cys → Gly mutant G-proteins. Interestingly, however, when we compared the affinity of functional interactions between the α_{2A}-adrenoceptor and either wild-type or Cys → Gly $G_i1\alpha$, measured by concentration-effect curves to UK14304, the mutant protein required approx. 10-fold higher concentrations of the agonist than the wild-type G-protein in order to be half-maximally activated, even though at maximally effective concentrations of UK14304 similar stimulations were achieved (Figure 4a). Similar results were seen when the concentration-effect curves to UK14304 for stimulation of wild-type versus the Cys → Gly mutants of $G_i2\alpha$ and $G_i3\alpha$ were compared (Figures 4b and 4c) These data indicate that the mutation does not prevent interaction of the G-protein with the α_{2A}-adrenoceptor but that the affinity of these interactions are reduced. This is not inherently surprising as the extreme C-terminal region of G-protein α-subunits represents a key site for interaction with receptors. At least, in the case of interactions between rhodopsin and mutated forms of transducin, a Cys → Gly mutation equivalent to those used here has been reported not to interfere with receptor interactions [18], while a less conservative cysteine → tyrosine mutation has been reported to result in a substantial loss of function [19]. An alternative approach, which involved the use of combinatorial chemistry to generate peptides related to the C-terminal region of transducin followed by selection of peptides displaying high affinity for rhodopsin, has indicated that the presence of the cysteine residue was an important feature in maintaining high-affinity interactions [17]. Interestingly, this approach also identified peptide sequences which interacted with rhodopsin with substantially higher affinity than the wild-type sequence [17]. Information such as this will, in time, be used to design mutations in the G_i-like G-proteins to permit investigation of the interactions of various receptors with the modified G-proteins.

The pertussis-toxin resistant forms of the G_i-like G-proteins also were able to prevent stimulation of adenylate cyclase in response to occupancy of the a_{2A}-adrenoceptor with UK14304. This was not surprising as the effector-recognition domains of these G-proteins lie further upstream from the C-terminus, and thus are unlikely to be affected by the Cys → Gly mutation. As reported previously by Eason et al. [27] and Eason and Liggett [28], we also observed a marked stimulation of adenylate cyclase

activity in response to UK14304 in pertussis-toxin treated cells which were transfected to express only the α_{2A}-adrenoceptor. Eason and co-workers [27,28] have identified elements within the primary structure of this receptor capable of activating G_s, and thus α_{2A}-adrenoceptor regulation of adenylate cyclase may be either stimulatory or inhibitory in individual cells depending on the relative levels of expression of G_s and G_i. Although the Cys → Gly mutation of each of the G_i proteins resulted in inhibition of both basal and forskolin-amplified adenylate cyclase activity (Table 6), the degree of inhibition produced by UK14304 in the presence of forskolin was similar to the level of activity produced by addition of forskolin to cells transfected to express the α_{2A}-adrenoceptor alone. This probably reflects the adenylate cyclase isoform profile of COS-7 cells. Federman et al. [31] have shown that after α_{2A}-receptor expression in HEK 293 cells, clear inhibition of adenylate cyclase activity is observed in response to UK14034, whereas, as found in the present study, in COS-7 cells stimulation of activity was observed. This effect is due to the expression of a $\beta\gamma$-sensitive isoform of adenylate cyclase in these cells, which can result in a complex pattern of regulation of cAMP generation.

In conclusion, these studies demonstrate that mutant G_i-like G-proteins, in which the cysteine residue which is the target for pertussis toxin-catalysed ADP-ribosylation has been replaced, can be usefully employed to examine receptor–G-protein interactions despite the fact that this residue lies within one of the key contact sites between these proteins. However, information obtained using the modified G-proteins should be treated with caution, and limiting parameters should be established empirically for each receptor under study. Without supporting data, the results of experiments in which the mutant proteins are used should not be interpreted as providing evidence for the lack of interaction of a receptor with the wild-type G-protein.

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