Comparative Analysis of the Efficacy of A_1 Adenosine Receptor Activation of $G_{i/o}\alpha$ G Proteins following Coexpression of Receptor and G Protein and Expression of A_1 Adenosine Receptor−$G_{i/o}\alpha$ Fusion Proteins†

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ABSTRACT: HEK293T cells were transiently transfected to express either the human A_1 adenosine receptor together with pertussis toxin-resistant cysteine-to-glycine forms of the α subunits of G_{i1} (C351G), G_{i2} (C352G), and G_{i3} (C351G) and wild-type $G_{o1}\alpha$ or fusion proteins comprising the A_1 adenosine receptor and these $G_{i/o}$ G proteins to compare A_1 adenosine receptor agonist-mediated activation of these G_i family G proteins upon coexpression of individual $G_{i/o}$ G proteins and receptor versus expression as receptor−G protein fusion proteins. Addition of the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) to membranes of pertussis toxin-treated cells resulted in a concentration-dependent stimulation of [^{35}S]-GTPγS binding with comparable amounts of NECA required to produce half-maximal stimulation following transfection of A_1 adenosine receptor and $G_{i/o}$ G proteins either as fusion proteins or as separate polypeptides. However, the magnitude of agonist-mediated activation of GTPγS binding was greatly enhanced by expressing the A_1 adenosine receptor and G_i family G proteins from chimaeric open reading frames. This observation was consistent following the study of more than 40 agonists. No preferential activation of any G protein was observed with more than 40 A_1 receptor agonists following cotransfection of receptor with G protein or transfection of receptor−G protein fusion proteins. These studies demonstrate the utility of using fusion proteins to study receptor−G protein interaction, show that the A_1 adenosine receptor couples equally well to the $G_{i/o}$ G proteins $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, and $G_{o1}\alpha$, and demonstrate that for a range of agonists there is no selectivity for activation of any particular A_1 adenosine receptor−$G_{i/o}$ G protein combination.

Heterotrimeric G proteins of the $G_i\alpha$ family play a pivotal role in the transduction and amplification of extracellularly derived signals serving as membrane-bound mediators between many seven transmembrane-spanning G protein-coupled receptors (GPCRs)[1] and intracellular effectors such as adenylyl cyclase and MAP kinase (*1*). Six members of the G_i family of G proteins have been characterized to date: $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{o1}\alpha$, $G_{o2}\alpha$, and $G_z\alpha$. These G proteins, with the exception of $G_z\alpha$, can be modified by pertussis toxin-catalyzed transfer of an ADP−ribose moiety onto a cysteine residue four amino acids from their C-termini to result in the attenuation of receptor coupling to the G protein (*2*). To examine the specificity of coupling between a receptor and a particular G_i G protein, we (*2*) and others (*3*, *4*) have generated modified versions of these G protein α subunits in which this cysteine residue has been mutated to serine (*3*) or glycine (*2*, *4*). Such modification renders the G protein resistant to pertussis toxin exposure. Pertussis toxin treatment of cells expressing a particular pertussis toxin-resistant G protein precludes coupling between GPCRs and endogenous G_i G proteins to allow isolated study of receptor coupling to the modified $G_i\alpha$.

Agonist stimulation of the A_1 adenosine receptor leads to modulation of numerous intracellular signaling events such as pertussis toxin-sensitive inhibition of adenylate cyclase, stimulation of phosphoinositidase C, activation of inwardly rectifying K^+ channels, and inhibition of neuronal calcium channels as a consequence of coupling to multiple pertussis toxin-sensitive $G_i\alpha$ family G proteins (*5*−*7*). Indeed, a number of studies have demonstrated that the A_1 adenosine receptor is capable of signaling to various G_i family G proteins, coupling preferentially to particular $G_i\alpha$ subunits (*8*−*10*). For example, following affinity chromatography of

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[1] Abbreviations: GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; ORF, open reading frame; DMEM, Dulbecco's modified Eagle's medium; [^{35}S]GTPγS, guanosine 5′-[γ-^{35}S]triphosphate; ECL, enhanced chemiluminescence; SPA, scintillation proximity assay; Cys→Gly, mutated G protein in which a C-terminal cysteine residue is replaced with glycine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; GR79236X, *N*-[(1*S*)-*trans*-2-hydroxycyclopentyl]adenosine; CCI4019, N^6-phenyladenosine; GR56071, 2-chloro-N^6-cyclopentyl-5′-deoxy-5′-(ethylamino)-5′-oxoadenosine; GR56072, N^6-cyclopentyl-5′-deoxy-5′-(ethylamino)-5′-oxoadenosine; GR66683, N^6-cyclopentyl-2-methyladenosine; R-PIA, (*R*)-N^6-phenylisopropyladenosine; S-PIA, (*S*)-N^6-phenylisopropyladenosine; CHA, N^6-cyclohexyladenosine; A_1AR, A_1 adenosine receptor.

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bovine brain, A_1 adenosine receptors copurified with $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ (*8*). In another study, A_1 adenosine receptors purified from bovine brain have been shown to interact with similarly purified $G_i\alpha$ or $G_o\alpha$, or with recombinantly expressed $G_i\alpha$ G proteins from *Escherichia coli*, to reconstitute high-affinity agonist binding (*9*). In this study, species differences were observed in the G protein selectivity of the human and bovine A_1 adenosine receptors since $G_{i3}\alpha$ was found to be more potent at reconstituting high-affinity agonist binding to bovine membranes than other G_i family G proteins, whereas $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ were equipotent in human membranes. More recently, recombinant bovine A_1 adenosine receptors expressed in Sf9 cell membranes were shown to reconstitute with recombinant $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, and $G_o\alpha$ and various $\beta\gamma$ combinations purified from baculovirus-infected Sf9 cells (*10*). These workers demonstrated that the bovine A_1 adenosine receptor coupled equally well to $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, and $G_o\alpha$ in combination with $\beta\gamma$ subunits containing $\beta1$ and $\beta2$ and $\gamma1$ and $\gamma2$. Since the A_1 adenosine receptor has been demonstrated to interact with $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$, we were interested in comparing the ability of this receptor to activate each of these G_i family G proteins following either expression as constrained receptor−G protein fusion proteins or as separate signaling molecules. To address this issue, we have both coexpressed the A_1 adenosine receptor with the various pertussis toxin-resistant forms of $G_i\alpha$ and expressed fusion proteins comprising the A_1 adenosine receptor and these G_i G proteins in HEK293T cells and studied receptor−G protein interactions following pertussis toxin treatment. Furthermore, to investigate the possibility that different agonist ligands may be able to selectively activate different $G_i\alpha$ G proteins following receptor activation, we have examined the ability of more than 40 agonist ligands to activate each receptor−$G_i\alpha$ G protein combination. We demonstrate that each ligand is able to activate each receptor G protein combination with similar concentration dependence and with similar maximal stimulation. This study further demonstrates the utility of receptor−G protein fusion proteins in studying receptor−G protein interaction and indicates that there is no selectivity for activation of any particular A_1 adenosine receptor−$G_{i/o}$ G protein combination.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]GTPγS (1175 Ci/mmol) was purchased from Amersham, and [^{3}H]DPCPX (120 Ci/mmol) and [^{3}H]-CCPA (30 Ci/mmol) were from New England Nuclear. Lipofectamine was obtained from Life Technologies Inc., and all other cell culture reagents and pertussis toxin (50 μg/mL) were purchased from Sigma. All other chemicals were from Fisher Scientific or Sigma and were of the highest purity commercially available.

Construction of A_1 Adenosine Receptor−Pertussis Toxin-Insensitive $G_i\alpha$ Fusion Proteins. Construction of pertussis toxin-insensitive Cys→Gly variants of rat $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ is described in ref *2*. All $G_i\alpha$ constructs were subsequently cloned into the *Eco*RI site of pCDNA3 such that they were 5′ to a unique *Xho*I site in the multiple cloning region of the vector. Initially, the human A_1 adenosine receptor was fused to Cys→Gly$G_{i2}\alpha$ using a PCR-based approach. The open reading frame (ORF) of the receptor DNA was amplified using oligonucleotides 5′-AAGAATTCGCCGCCATGCCGCCCTCCATCTCAGCTTTC-3′(sense) and 5′-AAGAATTCCATGGCATCAGGCCTCTCTTCTGGGAGATC-3′ (antisense) (restriction sites for *Eco*RI and *Nco*I underlined), and the product was ligated to PCR-Script (Stratagene) according to the manufacturer's instructions with a unique vector *Xho*I site 3′ to the receptor insert. Introduction of the *Nco*I site at the 3′ end of the ORF resulted in the C-terminal amino acid of the receptor being altered from aspartic acid to alanine and the removal of the stop codon. Rat Cys→Gly$G_{i2}\alpha$ possesses three *Nco*I sites: one straddling the ATG start codon and the other two 260 and 402 bp downstream from this. An *Nco*I fragment of Cys→Gly$G_{i2}\alpha$ comprising the initial 260 bp of the ORF was cloned into the *Nco*I site at the 3′ end of the A_1 adenosine receptor in PCR-Script, thus forming an in-frame fusion whereby the 3′ end of the A_1 adenosine receptor ORF and the 5′ end of Cys→Gly$G_{i2}\alpha$ were exactly adjacent. Cys→Gly$G_{i2}\alpha$ also harbors a unique *Eco*47III site 43 bp downstream from the start codon. Hence, *Eco*47III was used in concert with *Xho*I to generate a fragment consisting of the final 1022 bp of the ORF of Cys→Gly$G_{i2}\alpha$ which was subsequently cloned into the existing A_1 adenosine−Cys→Gly$G_{i2}\alpha$ construct to form a full in-frame fusion. Cys→Gly$G_{i1}\alpha$, Cys→Gly$G_{i3}\alpha$, and rat $G_{o1}\alpha$ possess *Nco*I sites straddling their ATG start codons and also an additional site about 260 bp downstream from this. Hence, fusions between the A_1 adenosine receptor and Cys→Gly$G_{i1}\alpha$, Cys→Gly$G_{i3}\alpha$, and wild-type $G_{o1}\alpha$ were simply formed by excising the Cys→Gly$G_{i2}\alpha$ ORF from the A_1 adenosine receptor−Cys→Gly$G_{i2}\alpha$ fusion by digestion with *Nco*I−*Xho*I and replacement with equivalently digested fragments of Cys→Gly$G_{i1}\alpha$, Cys→Gly$G_{i3}\alpha$, and $G_{o1}\alpha$. Full fusion constructs were excised from pCR-Script with *Eco*RI and *Xho*I and ligated into the eukaryotic expression vector pCDNA3.

Cell Culture and Transfection. HEK293T cells (HEK293 cells stably expressing the SV40 large T-antigen) were maintained in DMEM containing 10% (v/v) newborn calf serum and 2 mM glutamine. Cells were seeded in 60 mm culture dishes and grown to 60−80% confluency (18−24 h) prior to transfection with pCDNA3 containing the relevant DNA species using Lipofectamine reagent. For transfection, 3 μg of DNA was mixed with 10 μL of Lipofectamine in 0.2 mL of Opti-MEM (Life Technologies Inc.) and the mixture incubated at room temperature for 30 min prior to the addition of 1.6 mL of Opti-MEM. For cotransfection experiments, 1.5 μg of each cDNA species was used. Cells were exposed to the Lipofectamine/DNA mixture for 5 h, and 2 mL of 20% (v/v) newborn calf serum in DMEM was then added. Following transfection, growth medium was supplemented with adenosine deaminase (2 units/mL). Cells were harvested 48 h after transfection. Where relevant, cells were exposed to pertussis toxin (50 ng/mL) for 24 h prior to harvest to occasion ADP ribosylation of endogenous G_i family G proteins (*2*) and thus preclude potential interactions between these and the fusion proteins.

Membrane Preparations. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80 °C following harvest, as described previously (*2*).

Radioligand Binding Studies. Radioligand saturation binding experiments were carried out in 96-well plates using 50 mM HEPES buffer (pH 7.4) containing 2 units/mL adenosine

deaminase and, for the agonist radioligand only, 10 mM $MgCl_2$. The radioligands used were [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 120 Ci/mmol, New England Nuclear), an antagonist ligand used to measure the total receptor population, and [^{3}H]-2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA, 30 Ci/mmol, New England Nuclear), an agonist ligand used to measure the population of receptor in a high-affinity (presumably G protein-coupled) state. Nonspecific binding was defined using 10 μM NECA. Plates were incubated at room temperature for 1 h and filtered through Whatman GF/B filters using a Brandel harvester. Data were analyzed by nonlinear regression to determine B_{max} and K_D values.

Immunological Studies. Antisera SG1 (*11*) and I3C (*12*) were produced in New Zealand white rabbits, using conjugates of synthetic peptides and keyhole limpet haemocyanin (Calbiochem) as antigens. Antisera SG1 and I3C were raised against peptides corresponding to the C-terminal decapeptides of $G_{i1/2}\alpha$ and $G_{i3}\alpha$, respectively. Membrane samples were resolved by SDS−PAGE using 10% (w/v) acrylamide gels. Proteins were subsequently transferred to Hybond ECL nitrocellulose membrane (Amersham), probed with the relevant antiserum, and visualized using ECL according to the manufacturer's instructions.

High-Affinity [^{35}S]GTPγS Binding. Assays were performed in the 96-well format using a method modified from that described in ref *13*. Membranes (5 μg per point) were diluted to 0.083 mg/mL in assay buffer [20 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$ (pH 7.4)] supplemented with saponin (10 mg/L) and preincubated with 40 μM GDP. Agonist and [^{35}S]GTPγS (1170 Ci/mmol, Amersham) at 0.3 nM was added (total volume of 100 μL), and binding was allowed to proceed at room temperature for 30 min. Nonspecific binding was determined by the inclusion of 0.6 mM GTP. Wheatgerm agglutinin SPA beads (Amersham) (0.5 mg) in 25 μL of assay buffer were added, and the whole mixture was incubated at room temperature for 30 min while it was agitated. Plates were centrifuged at 1500*g* for 5 min, and the amount of [^{35}S]GTPγS bound was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

RESULTS

Construction of fusion proteins of the human A_1 adenosine receptor and previously described (*2*) C-terminal Cys→Gly pertussis toxin-resistant mutant forms of rat $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ is depicted in Figure 1.

The A_1 adenosine receptor was expressed in HEK293T cells as fusion proteins with Cys→Gly versions of $G_{i1-3}\alpha$ (Figure 2). Expression of the individual A_1 adenosine receptor−$G_i\alpha$ fusion proteins as polypeptides of approximately 80 kDa was detected by immunoblotting membrane fractions of transfected cells with antisera specific for $G_{i1}\alpha$ or $G_{i2}\alpha$ (SG1) and $G_{i3}\alpha$ (I3C) (Figure 2). Both mock- and fusion protein-transfected cells expressed detectable levels of endogenous $G_{i1/2}\alpha$ and $G_{i3}\alpha$, which migrated as 40−42 kDa polypeptides; however, only those expressing fusion proteins yielded immunoreactivity at 80 kDa.

The level of expression of the A_1 adenosine receptor was assessed by saturation analysis of specific binding of the A_1 adenosine receptor antagonist [^{3}H]DPCPX (Table 1). Similar

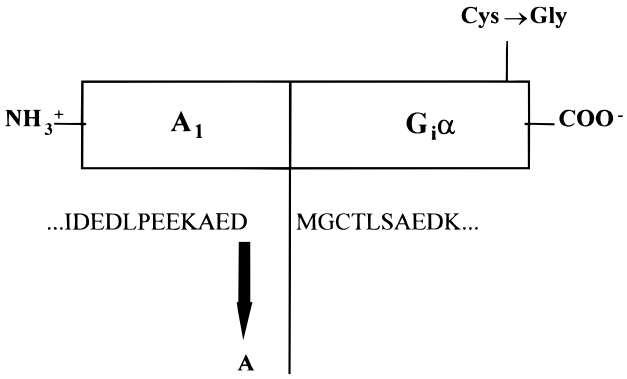

FIGURE 1: Construction of A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion proteins. Fusion of the N-terminus of pertussis toxin-resistant forms (Cys→Gly) of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ to the C-terminus of the A_1 adenosine receptor resulted in the receptor C-terminal amino acid aspartate (D) being converted into alanine (A) and maintenance of the methionine (M), which normally functions as the initiator in $G_i\alpha$.

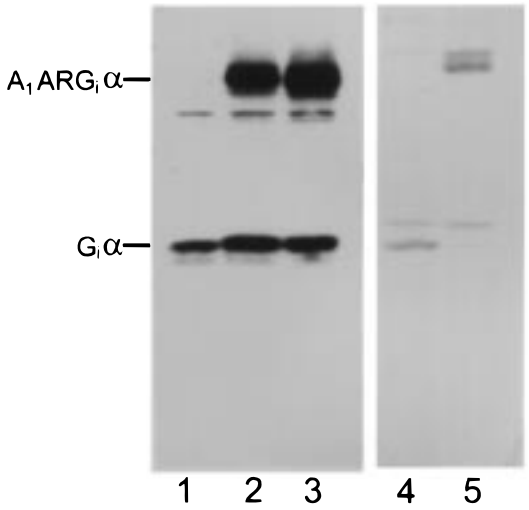

FIGURE 2: Expression of A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion proteins. HEK293T cells were either mock-transfected (lanes 1 and 4) or transfected with A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion protein cDNAs harboring $G_{i1}\alpha$ (lane 2), $G_{i2}\alpha$ (lane 3), and $G_{i3}\alpha$ (lane 5) in pCDNA3. P2 particulate fractions were prepared as described in Experimental Procedures, and 50 μg of protein was resolved by SDS−PAGE and immunoblotted: lanes 1−3, immunoblotted with $G_{i1}\alpha$- and $G_{i2}\alpha$-specific antiserum, SG1; and lanes 4 and 5, immunoblotted with $G_{i3}\alpha$-specific antiserum (I3C). In the P2 particulate fractions of cells expressing each fusion protein, a polypeptide of approximately 80 kDa was detected, which was not present in mock-transfected cells.

levels of receptor expression were achieved following cotransfection of HEK293T cells with the A_1 adenosine receptor and the various pertussis toxin-resistant forms of $G_{i1-3}\alpha$ or following transfection with A_1 adenosine receptor−$G_i\alpha$ fusion proteins (5−12 pmol/mg). K_d values for the interaction between [^{3}H]DPCPX and the A_1 adenosine receptor were of high affinity and were also comparable following either coexpression of receptor with individual G_i G proteins or the expression of receptor−G protein fusion proteins (Table 1). No specific binding of [^{3}H]DPCPX was demonstrated in membranes from cells transfected with empty vector.

In all experiments involving expression of Cys→Gly forms of $G_i\alpha$, cells were exposed to pertussis toxin (50 ng/mL) for 24 h prior to harvest to preclude interaction between the

Table 1: Expression Levels of A_1 Adenosine Receptor with Cys→Gly$G_i\alpha$ G Proteins and as Fusion Proteins with Cys→Gly Variants of $G_i\alpha$[a]

transfection	antagonist binding (pmol/mg)	dissociation constant K_d of antagonist binding (nM)	agonist binding (pmol/mg)
mock	0.3 ± 0.2	−	0
A_1AR and G_{i1}[b]	5.8 ± 0.3	0.68 ± 0.07	1.1 ± 0.4
A_1AR and G_{i2}[b]	6.3 ± 0.7	0.78 ± 0.18	1.8 ± 0.4
A_1AR and G_{i3}[b]	6.1 ± 0.3	0.64 ± 0.10	1.0 ± 0.6
A_1AR−G_{i1}[b]	9.6 ± 3.7	1.40 ± 0.55	3.2 ± 1.3
A_1AR−G_{i2}[b]	7.9 ± 2.8	0.90 ± 0.53	1.4 ± 1.0
A_1AR−G_{i3}[b]	7.9 ± 1.2	0.96 ± 0.26	1.6 ± 1.0

[a] HEK293T cells were transfected with A_1 adenosine receptor with either Cys→Gly variants of $G_i\alpha$ or as fusions with such G proteins. P2 particulate membrane fractions were prepared and subjected to saturation binding studies using the antagonist [^{3}H]DPCPX and the agonist [^{3}H]CCPA as described in Experimental Procedures. The values shown are the means ± SD of a representative experiment of three that were performed. [b] A_1AR and G_{ix} denote A_1 adenosine receptor and Cys→Gly variants of $G_i\alpha$, respectively.

introduced A_1 adenosine receptor and endogenous G_i family G proteins (*2*). Agonist binding studies demonstrated specific, high-affinity binding of the A_1 adenosine receptor agonist [^{3}H]CCPA in pertussis toxin-treated cells expressing the A_1 adenosine receptor both with the various Cys→Gly$G_i\alpha$ G proteins and in similarly treated cells expressing the receptor−G protein fusions (Table 1). Such data suggested coupling between expressed receptor and pertussis toxin-resistant G_i G protein since levels of agonist binding in cells transfected with receptor alone were significantly reduced following pertussis toxin exposure (3.1 ± 0.6 pmol mg^{-1} in untreated cells and 0.3 ± 0.1 pmol mg^{-1} following pertussis toxin exposure). Furthermore, similar levels of [^{3}H]CCPA binding were observed on membranes from cells transfected to express the A_1 adenosine receptor both with the various Cys→Gly$G_i\alpha$ G proteins and as receptor−G protein fusions (Table 1) which suggested that the physical linkage of receptor and G protein within the constraint of a fusion protein did not appear to influence the level of precoupling compared to the expression of receptor and G protein as separate entities.

Addition of the adenosine receptor agonist NECA to membranes of pertussis toxin-treated HEK293T cells transfected to express the A_1 adenosine receptor did not result in activation of endogenous G_i G proteins as measured by the extent of [^{35}S]GTPγS binding (Figure 3). However, cotransfection of the A_1 adenosine receptor with each of the Cys→Gly mutants of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ produced a robust pertussis toxin-resistant NECA-mediated stimulation of [^{35}S]-GTPγS binding (Figure 3), demonstrating receptor interaction with each of these G protein subtypes. Moreover, transfection of the individual A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion cDNAs into HEK293T cells led to significantly greater pertussis toxin-resistant NECA-induced elevation of [^{35}S]-GTPγS binding (Figure 3). Similar results were also demonstrated in membranes of untreated cells transfected to express the A_1 adenosine receptor and wild-type $G_{o1}\alpha$ either as fusion proteins or as separate polypeptides (Figure 3).

To study the relative affinities of interaction between the A_1 adenosine receptor and the Cys→Gly forms of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$, each of these polypeptides was coexpressed with the receptor and the ability of various concentrations of

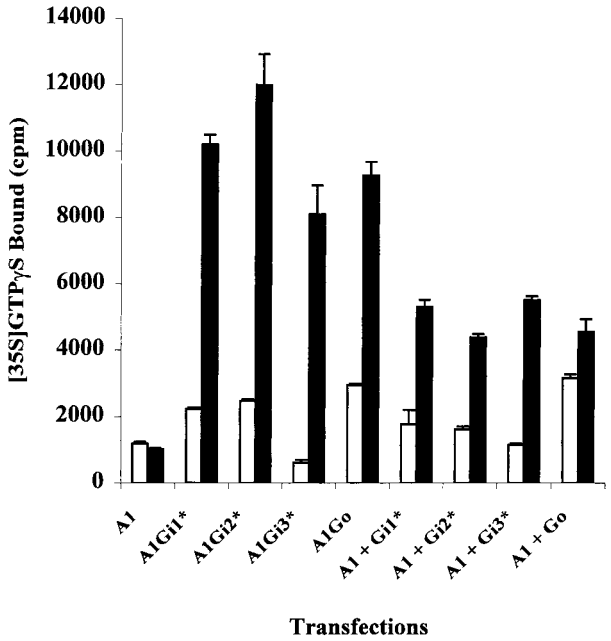

FIGURE 3: Agonist-mediated stimulation of [^{35}S]GTPγS binding to A_1 adenosine receptor−Cys→Gly$G_i\alpha$ and wild-type $G_{o1}\alpha$ fusion proteins and to coexpressed A_1 adenosine receptor and Cys→Gly$G_i\alpha$ and wild-type $G_{o1}\alpha$ G proteins. HEK293T cells were transfected with A_1 adenosine receptor alone, together with Cys→Gly variants of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$, or as fusion proteins with each of these G_i family G proteins. Cells were treated with pertussis toxin (50 ng/mL) 16 h prior to being harvested. Cells transfected to express the A_1 adenosine receptor with $G_{o1}\alpha$ either as separate proteins or as a fusion protein were not exposed to pertussis toxin prior to being harvested. [^{35}S]GTPγS binding was assessed on P2 particulate fractions without (white bars) or with (black bars) exposure to NECA (10 μM) as described in Experimental Procedures. Asterisks denote a Cys→Gly variant of $G_i\alpha$. The data shown are the means ± SD of triplicate measurements and are representative of three independent experiments.

NECA to elevate [^{35}S]GTPγS binding was measured in pertussis toxin-treated cells. This was found to be concentration-dependent with EC_{50} values for coupling of the A_1 adenosine receptor to the pertussis toxin-insensitive G proteins of (5.6 ± 1.7) × 10^{-8} M (Cys→Gly$G_{i1}\alpha$), (4.2 ± 0.6) × 10^{-8} M (Cys→Gly$G_{i2}\alpha$), and (4.9 ± 0.9) × 10^{-8} M (Cys→Gly$G_{i3}\alpha$) (means ± SEM, $n = 3$) (Figure 4B). Similar data were also obtained following coexpression of the A_1 adenosine receptor with $G_{o1}\alpha$ [EC_{50} = (4.5 ± 1.5) × 10^{-8} M, $n = 3$] (Figure 4B). We then proceeded to examine the affinity of interaction between the A_1 adenosine receptor and the Cys→Gly forms of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ when the receptor−G protein interaction is constrained within the fusion protein. Individual A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion protein constructs were transfected into HEK293T cells, and [^{35}S]GTPγS binding was assessed following exposure to increasing concentrations of NECA (Figure 4A). This was again found to be concentration-dependent with similar EC_{50} values of (12.8 ± 1.2) × 10^{-8} M (Cys→Gly$G_{i1}\alpha$), (14.6 ± 1.0) × 10^{-8} M (Cys→Gly$G_{i2}\alpha$), and (16.0 ± 1.5) × 10^{-8} M (Cys→Gly$G_{i3}\alpha$). EC_{50} values for NECA stimulation of the receptor−G protein fusions were approximately 3-fold less potent compared to those measured upon stimulation of the A_1 adenosine receptor when coexpressed with individual Cys→GlyG_i G proteins. Expression of an A_1 adenosine receptor−$G_{o1}\alpha$ fusion protein also yielded an EC_{50} value for NECA stimulation [(17.1 ± 3.2) × 10^{-8} M, n =

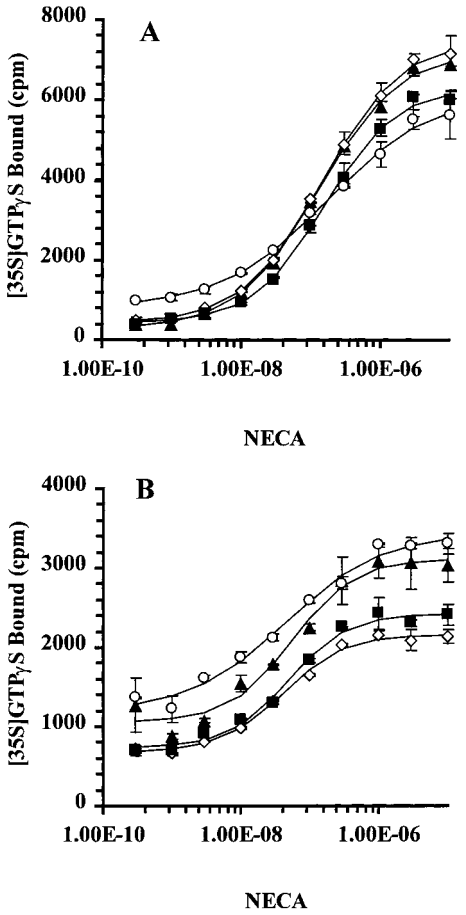

FIGURE 4: Dose-dependent agonist-mediated stimulation of [^{35}S]-GTPγS binding to A_1 adenosine receptor−Cys→Gly$G_i\alpha$ and −wild-type $G_{o1}\alpha$ fusion proteins and to coexpressed A_1 adenosine receptor and Cys→Gly$G_i\alpha$ and wild-type $G_{o1}\alpha$ G proteins. The ability of varying concentrations of NECA to stimulate the binding of [^{35}S]-GTPγS in membranes of (A) pertussis toxin-treated HEK293T cells transfected to express A_1 adenosine receptor−Cys→Gly$G_i\alpha$ fusion proteins containing $G_{i1}\alpha$ (▲), $G_{i2}\alpha$ (◇), and $G_{i3}\alpha$ (■) or in membranes of untreated HEK293T cells transfected to express an A_1 adenosine receptor−$G_{o1}\alpha$ fusion protein (○) and (B) coexpressed A_1 adenosine receptor and Cys→Gly variants of $G_{i1}\alpha$ (▲), $G_{i2}\alpha$ (◇), $G_{i3}\alpha$ (■), and wild-type $G_{o1}\alpha$ (○) was measured as described in detail in Experimental Procedures. Results are means from three separate experiments.

3] comparable to those observed with the other fusion proteins.

To expand these studies and to attempt to identify A_1 adenosine receptor agonists capable of preferential activation of particular receptor−G protein combinations, a range of 40 adenosine receptor and A_1 adenosine receptor-specific agonists were compared to NECA for their ability to promote elevation of [^{35}S]GTPγS binding in cells transfected with each of the A_1 adenosine receptor−Cys→Gly$G_i\alpha$ and -$G_{o1}\alpha$ fusion proteins. No significant differences in either potency or efficacy for any ligand were observed between cells expressing fusions composed of Cys→Gly forms of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ and wild-type $G_{o1}\alpha$ (Table 2 shows representative data for 10 such compounds). For comparison, the A_1 adenosine receptor was cotransfected into HEK293T cells with each of the Cys→Gly forms of $G_i\alpha$, and the potency and efficacy of the same series of agonists were assessed following pertussis toxin treatment. Similar studies were conducted on untreated cells coexpressing the A_1 adenosine receptor and $G_{o1}\alpha$. All of the agonists tested promoted nonselective activation of all three Cys→Gly forms of $G_i\alpha$ and wild-type $G_{o1}\alpha$ via the A_1 adenosine receptor (Table 2).

Table 2: Potency (A) and Efficacy (B) Measurements of Ligands at the A_1 Adenosine Receptor When Coexpressed with Cys→Gly Variants of $G_i\alpha$ and Wild-Type $G_{o1}\alpha$ (Top Half of Each Section) or as Fusion Proteins with These G Proteins (Bottom Half of Each Section)[a]

A

	potency (EC_{50} in nM)			
ligand	A_1AR and G_{i1}[b]	A_1AR and G_{i2}[b]	A_1AR and G_{i3}[b]	A_1AR and G_o
NECA	56.4 ± 17	42.0 ± 6	49.2 ± 9	45.6 ± 14.9
CPA	5.3 ± 1.2	14.5 ± 5.5	7.8 ± 2.8	17.0 ± 7.4
GR79236X	19.0 ± 4.5	22.0 ± 6.3	21.0 ± 6.2	18.2 ± 0.6
CCI4019	46.6 ± 3.6	63.7 ± 6.1	66 ± 7.5	55.5 ± 11.9
GR56071	7.7 ± 0.9	11.8 ± 2.7	10.8 ± 1.3	6.5 ± 2.6
GR56072	3.5 ± 1.1	3.5 ± 1.6	4.5 ± 2.5	6.9 ± 2.4
GR66683	33.2 ± 5.4	52.1 ± 13.4	46.0 ± 6.4	52.6 ± 6.4
S-PIA	300 ± 39	357 ± 80	320 ± 41	224 ± 31.3
R-PIA	8.5 ± 1.2	9.3 ± 2.3	10.9 ± 1.7	5.7 ± 1.5
CHA	11.9 ± 3.1	23.4 ± 3.1	10.3 ± 1.8	6.6 ± 4.1

	potency (EC_{50} in nM)			
ligand	A_1AR−G_{i1}[b]	A_1AR−G_{i2}[b]	A_1AR−G_{i3}[b]	A_1AR−G_o
NECA	128 ± 11.7	145.8 ± 10.1	159.8 ± 15.3	171 ± 32.2
CPA	31.6 ± 1.6	41.3 ± 3.5	39.7 ± 2.0	22.1 ± 2.4
GR79236X	96.6 ± 5.6	129 ± 10.0	125 ± 9.6	87.4 ± 6.8
CCI4019	317 ± 38	340 ± 18	312 ± 10.9	175 ± 23.2
GR56071	46 ± 2.8	56 ± 1.6	65 ± 2.8	64.7 ± 5.9
GR56072	20.7 ± 2.8	25.2 ± 1.2	25.5 ± 4.0	22.2 ± 4.4
GR66683	214 ± 18.6	247 ± 13.8	241 ± 15.0	166 ± 24.0
S-PIA	1850 ± 300	1330 ± 167	1470 ± 147	685 ± 81.2
R-PIA	53.6 ± 4.1	64.3 ± 3.3	73.3 ± 3.7	42.9 ± 4.5
CHA	64.2 ± 6.0	90.2 ± 8.6	98 ± 6.7	73.4 ± 3.5

B

	efficacy to activate (% of NECA)			
ligand	A_1AR and G_{i1}[b]	A_1AR and G_{i2}[b]	A_1AR and G_{i3}[b]	A_1AR and G_o
CPA	90 ± 29	113 ± 10	96 ± 10	107 ± 11
GR79236X	121 ± 7	119 ± 8	125 ± 8	127 ± 15
CCI4019	114 ± 2	119 ± 3	112 ± 3	98 ± 9
GR56071	115 ± 4	125 ± 7	107 ± 4	97 ± 5
GR56072	125 ± 10	126 ± 15	105 ± 19	109 ± 6
GR66683	111 ± 5	116 ± 3	107 ± 4	83 ± 10
S−PIA	136 ± 2	141 ± 7	117 ± 4	96 ± 3
R−PIA	118 ± 4	156 ± 6	123 ± 6	100 ± 4
CHA	110 ± 8	129 ± 4	129 ± 5	98 ± 8

	efficacy to activate (% of NECA)			
ligand	A_1AR−G_{i1}[b]	A_1AR−G_{i2}[b]	A_1AR−G_{i3}[b]	A_1AR−G_o
CPA	98 ± 1	102 ± 2	103 ± 1	110 ± 3
GR79236X	100 ± 2	105 ± 2	97 ± 2	100 ± 2
CCI4019	96 ± 3	100 ± 2	95 ± 1	101 ± 4
GR56071	101 ± 2	104 ± 1	103 ± 1	116 ± 3
GR56072	103 ± 4	106 ± 1	99 ± 3	113 ± 5
GR66683	103 ± 2	101 ± 2	96 ± 2	107 ± 4
S-PIA	102 ± 9	85 ± 4	103 ± 1	95 ± 4
R-PIA	95 ± 2	103 ± 1	103 ± 1	108 ± 3
CHA	96 ± 3	111 ± 3	104 ± 2	115 ± 1

[a] Measurements were made using agonist-mediated stimulation of [^{35}S]GTPγS binding to membranes from transfected HEK293T cells. Efficacy data are presented as the percentage of the stimulation produced by 10 μM NECA. Results are means from two independent experiments. [b] A_1AR and G_{ix} denote A_1 adenosine receptor and Cys→Gly variants of $G_i\alpha$, respectively.

DISCUSSION

The pertussis toxin-sensitive G proteins $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ are often coexpressed in cells. Hence, to study the

specificity of interaction between a receptor and individual members of this G protein family, we (*2*) and others (*3*, *4*) have mutationally altered these proteins such that they are refractory to pertussis toxin-catalyzed ADP ribosylation. These pertussis toxin-insensitive Cys→Gly mutants of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ can then be introduced into cells with a receptor of interest. Specificity of interaction between expressed receptor and Cys→Gly$G_i\alpha$ can be studied in isolation following exposure of cells to pertussis toxin to eliminate any potential coupling to endogenous G_i family G proteins. More recently, we (*14*−*16*) and others (*17*−*19*) have adopted an alternative approach to studying the mechanisms and specificities governing receptor−G protein interaction in which the two signaling entities are introduced into cells as a constrained fusion protein. Initially, we generated a fusion protein between the α_{2A}-adrenoceptor and Cys→Gly$G_{i1}\alpha$ by simply fusing the N-terminus of the G protein to the C-terminus of the receptor (*14*). This construct has proved to be a useful tool for studying the enzymic capacity of $G_{i1}\alpha$ (*14*) and also as a means of measuring ligand efficacy at defined GPCR-G protein tandems since the two signaling polypeptides are expressed in a fixed stoichiometric ratio and are in close proximity to one another (*15*). Seifert et al. (*19*) used fusions between the β_2-adrenoceptor and the long and short splice variants of $G_s\alpha$ to demonstrate subtle differences in their coupling mechanisms. They concluded that coupling of the β_2-adrenoceptor to $G_s\alpha$L but not to $G_s\alpha$S renders the receptor constitutively active.

Our primary aim in this study was to examine the possibility that agonist compounds may exhibit altered efficacy at receptor−G protein fusions versus coexpressed receptor and G protein combinations. We also wanted to study the specificity of interaction of the A_1 adenosine receptor with individual $G_{i/o}\alpha$ G proteins following activation by a variety of agonists since the A_1 adenosine receptor couples to the G_i family of G proteins to produce a wide variety of second messenger-derived responses. Hence, in this work, we have examined the ability of more than 40 A_1 adenosine receptor agonists to activate Cys→Gly pertussis toxin-insensitive variants of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ and wild-type $G_{o1}\alpha$ upon coexpression with the A_1 adenosine receptor and in comparison to their expression as A_1 adenosine receptor−$G_{i/o}\alpha$ fusion proteins following transient transfection in HEK293T cells.

In all experiments, receptor interaction with coexpressed Cys→Gly G_i G protein was measured as a stimulation of high-affinity [^{35}S]GTPγS binding following agonist exposure of membranes from pertussis toxin-treated transfected cells. In such studies, all three G_i G proteins were able to interact functionally with the A_1 adenosine receptor (Figure 3) following coexpression as separate entities. This was not surprising since a number of reports have demonstrated interactions of this receptor with multiple G_i family G proteins (*8*−*10*). The affinity of receptor interaction with each coexpressed $G_i\alpha$ was also very similar (Figure 4). We also observed such a phenomenon when examining interactions between the α_{2A}-adrenoceptor and $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ (*2*). In addition, $G_{o1}\alpha$ was also found to interact functionally with the A_1 adenosine receptor with an affinity similar to that observed with the other G_i G proteins (Figure 4).

To further explore the specificity of interaction between the A_1 adenosine receptor and various G_i G proteins, fusion proteins were constructed between the receptor and each Cys→Gly variant of $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ and wild-type $G_{o1}\alpha$. Minimal disruption was caused to each polypeptide following manufacturing of the fusion with only the C-terminal amino acid of the receptor being altered from aspartic acid to alanine and the initiator methionine of the G protein (which would normally be removed) remaining in the new protein (Figure 1). Introduction of each fusion protein into HEK293T cells led to robust agonist-mediated stimulation of [^{35}S]GTPγS binding activity which exhibited a magnitude significantly greater than that observed when receptor and G protein were coexpressed (Figure 3). This is probably due to more efficient coupling between receptor and G protein when they are constrained within a fusion protein as opposed to being overexpressed in a cell as separate entities. Certainly, such enhanced G protein activation cannot be attributed to altered levels of receptor expression since saturation ^{3}H antagonist binding studies revealed similar quantities of A_1 adenosine receptor in membranes from cells transfected with cDNAs encoding receptor−G protein fusions and also cells transfected with receptor together with the individual G_i family G proteins (Table 1). Moreover, ^{3}H agonist binding studies provided no evidence of altered levels of receptor−G protein precoupling upon expression of the A_1 adenosine receptor together with the G_i family G proteins or constrained as receptor−G protein fusions (Table 1).

Recently, evidence has arisen implying that particular agonists can interact with GPCRs in a defined manner to harness activation of specific G proteins (see refs *20* and *21* for review). For example, Negishi et al. (*22*) observed that the prostaglandin E receptor EP3D displayed agonist-dependent G protein coupling specificity. Furthermore, it was recently reported that the relative efficacies of agonists acting at 5-HT_{2A} and 5-HT_{2C} receptors differed depending on the effector pathway that was activated (*23*). Thus, taken together these data provide evidence for the existence of multiple active receptor states. We thought it appropriate to study this phenomenon of "agonist trafficking" since the A_1 adenosine receptor causes activation of a variety of intracellular signaling pathways via interaction with G_i family G proteins and in this study was shown to couple to all four G_i G protein subtypes tested using the adenosine receptor agonist NECA. Hence, a range of A_1 adenosine receptor agonists were characterized for their ability to promote activation of a particular G_i family G protein following expression of the individual fusions in HEK293T cells. No preferential activation of any expressed $G_{i/o}$ G protein via the A_1 adenosine receptor was observed with any of the compounds tested (representative data for 10 compounds out of 40 studied are shown in Table 2). We were aware that using the fusion approach to investigate agonist trafficking at the A_1 adenosine receptor may prove to be misleading since the physical proximity of receptor to G protein may promote their interaction and also that the linkage between them may constrain the capacity of agonist ligands to promote particular conformations within the GPCR requisite for specific G protein activation. Therefore, we also recorded efficacy and potency of the same series of ligands following coexpression of the A_1 adenosine receptor with each Cys→Gly$G_i\alpha$ and wild-type $G_{o1}\alpha$. Table 2 demonstrates that all agonists tested produced similar activation of each G_i family G protein,

although the magnitude of each response was much lower than observed using the fusion protein approach (data not shown). The data presented in Table 2 also demonstrate that the rank order of efficacy was unchanged between fusion proteins and when expressing separated receptor and G protein. This was not surprising as we had previously shown similar findings using a fusion approach to study agonist efficacy at the α_{2A}-adrenoreceptor when expressed as a fusion with Cys→Gly$G_{i1}\alpha$ (*15*). Hence, we were unable to demonstrate agonist-induced "channelling" of the human A_1 adenosine receptor that caused activation of distinct members of the G_i family of G proteins. A more exhaustive study in which a wider bank of agonist ligands was employed may provide evidence for such a phenomenon at the A_1 adenosine receptor. Alternatively, to investigate agonist trafficking, it may be more pertinent to select GPCRs which are known to couple to less closely related G proteins such as the α_{2A}-adrenoceptor which has been shown to interact with both G_i and G_s subtypes (*24*).

In conclusion, these studies further demonstrate the application of mutant G_i-like G proteins, in which the C-terminal cysteine residue which serves as the substrate for pertussis toxin-catalyzed ADP ribosylation has been replaced, as useful tools for the study of receptor−G protein interaction. In addition, they also illustrate the advantages of fixing receptor and G protein stoichiometry and proximity in a system through the use of receptor−G protein fusions. We expect that the use of receptor−G protein fusion proteins will improve the understanding of the complexities of GPCR-mediated signaling and may provide a novel means of identifying regulatory molecules interacting with specific receptor−G protein fusions.

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