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Selective Inhibition of Heterotrimeric G_s Signaling

TARGETING THE RECEPTOR-G PROTEIN INTERFACE USING A PEPTIDE MINIGENE ENCODING THE $G\alpha_s$ CARBOXYL TERMINUS*

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The blockade of heptahelical receptor coupling to heterotrimeric G proteins by the expression of peptides derived from G protein $G\alpha$ subunits represents a novel means of simultaneously inhibiting signals arising from multiple receptors that share a common G protein pool. Here we examined the mechanism of action and functional consequences of expression of an 83-amino acid polypeptide derived from the carboxyl terminus of $G\alpha_s$ (GsCT). In membranes prepared from GsCT-expressing cells, the peptide blocked high affinity agonist binding to β_2 adrenergic receptors (AR) and inhibited β_2AR-induced [^{35}S]GTPγS loading of $G\alpha_s$. GsCT expression inhibited β_2AR- and dopamine D_{1A} receptor-mediated cAMP production, without affecting the cellular response to cholera toxin or forskolin, indicating that the peptide inhibited receptor-G_s coupling without impairing G protein or adenylyl cyclase function. [^{35}S]GTPγS loading of $G\alpha_{q/11}$ by α_{1B}ARs and $G\alpha_i$ by α_{2A}ARs and $G_{q/11}$- or G_i-mediated phosphatidylinositol hydrolysis was unaffected, indicating that the inhibitory effects of GsCT were selective for G_s. We next employed the GsCT construct to examine the complex role of G_s in regulation of the ERK mitogen-activated protein kinase cascade, where activation of the cAMP-dependent protein kinase (PKA) pathway reportedly produces both stimulatory and inhibitory effects on heptahelical receptor-mediated ERK activation. For the β_2AR in HEK-293 cells, where PKA activity is required for ERK activation, expression of GsCT caused a net inhibition of ERK activation. In contrast, α_{2A}AR-mediated ERK activation in COS-7 cells was enhanced by GsCT expression, consistent with the relief of a downstream inhibitory effect of PKA. ERK activation by the $G_{q/11}$-coupled α_{1B}AR was unaffected by GsCT. These findings suggest that peptide G protein inhibitors can provide insights into the complex interplay between G protein pools in cellular regulation.

Heptahelical, or G protein-coupled, receptors represent the single most diverse class of cell surface receptors, both evolutionarily and within the human genome. The basic unit of G protein-coupled receptor signaling is composed of three parts as follows: a heptahelical receptor, a heterotrimeric G protein,[1] and an effector, such as a G protein-regulated enzyme or ion channel. The binding of an extracellular agonist ligand to the receptor changes its conformation so as to permit productive coupling with the G protein, thereby catalyzing the exchange of GTP for GDP on the $G\alpha$ subunit, and dissociation of $G\alpha$-GTP from $G\beta\gamma$ subunits. Regulation of effectors is achieved through their interaction with free GTP-bound $G\alpha$ or $G\beta\gamma$ subunits. Based upon data from crystallographic, biochemical, and mutagenesis studies, physical coupling of receptor and G protein is thought to involve primarily the second and third intracellular domains of the receptor, which make physical contact with the carboxyl terminus of the $G\alpha$ subunit (1–6). In particular, the last ~50 amino acids of the $G\alpha$ subunit are important for discriminating between different receptor subtypes and between different functional states of the receptor (3, 4, 6–9).

Pharmacologic agents that act as agonists or antagonists of heptahelical receptors represent the most common type of drug in clinical use today. Irrespective of chemical composition, these agents share a common mechanism of action in that they act extracellularly either to mimic, or to preclude, agonist binding at its receptor. By interacting with the molecular determinants of ligand binding in the extracellular or transmembrane domains of the receptor, often remarkable receptor subtype-specific agonist or antagonist effects can be obtained.

An alternative approach to antagonism of heptahelical receptor signaling is to target the receptor-G protein interface with agents that block coupling between the receptor and G protein intracellularly. Such an approach differs fundamentally from classical heptahelical receptor pharmacology in that the blockade of receptor-G protein coupling might be expected to produce G protein-specific, rather than receptor-specific, antagonism. Several successful applications of this strategy, using polypeptides derived from the putative contact surfaces on

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[1] The abbreviations used are: G protein, heterotrimeric GTP-binding protein; AR, adrenergic receptor; D_{1A}R3i, D_{1A} dopamine receptor third intracellular domain; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; $G\alpha_i$, α subunit of the heterotrimeric G_i protein; $G\alpha_{q/11}$, α subunit of the heterotrimeric $G_{q/11}$ protein; $G\alpha_s$, α subunit of the heterotrimeric G_s protein; GsCT, the carboxyl-terminal residues 313–395 of bovine $G\alpha_s$; GST, glutathione *S*-transferase; HA, influenza virus hemagglutinin; MAP, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PKA, protein kinase A; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GPCR, G protein-coupled receptor.

the receptor, or the G protein Gα subunit, have been reported. For example, cellular expression of peptides derived from the third intracellular domains of the $G_{q/11}$-coupled α_{1B} adrenergic receptor (AR) and M_1 muscarinic acetylcholine receptor, the G_i-coupled α_{2A}AR and M_2 acetylcholine receptor, and the G_s-coupled D_{1A} dopamine receptor have been shown to inhibit $G_{q/11}$-, G_i-, and G_s-coupled receptor signaling, respectively (10, 11).

Analogous strategies have been applied using modified Gα subunits or Gα subunit-derived peptides. Cellular expression of a mutant Gα_s containing three point mutations that impair its function strongly inhibits G_s-dependent stimulation of adenylyl cyclase in cultured cells (12). Modified xanthine nucleotide-binding mutants of Gα_o (13–14) and Gα_{16} (15) inhibit signaling by G_i-coupled receptors when expressed in COS-7 cells, whereas xanthine nucleotide-binding mutants of Gα_{11} and Gα_{16} (15) inhibit G_q-coupled receptor signaling. Smaller peptides, derived from the carboxyl terminus of Gα subunits, have been shown to produce similar inhibitory effects in membrane preparations and in intact cells (16–20). Cellular expression of a minigene encoding the last 55 amino acids of Gα_q inhibits $G_{q/11}$-coupled receptor signaling (18). Minigene plasmids encoding oligopeptides representing the carboxyl termini of Gα_i, Gα_q, Gα_{12}, and Gα_{13} have recently been employed to determine the contribution of different G protein pools to signaling by M_2 muscarinic and thrombin receptors (19, 20).

To determine whether an expressible peptide could be identified that interrupts signaling at the receptor-G_s interface, we have prepared a series of minigene constructs encoding varying length polypeptides derived from the carboxyl terminus of Gα_s. In this paper, we characterize the mechanism of action and consequences of expression of an 83-amino acid Gα_s carboxyl-terminal polypeptide (GsCT). We find that the GsCT peptide selectively inhibits receptor-G_s coupling in isolated plasma membranes and second messenger production in intact cells, without affecting $G_{q/11}$ or G_i signaling. When employed to examine the role of G_s in regulation of the ERK MAP kinase cascade, we find that GsCT expression reveals both stimulatory and inhibitory effects of G_s in response to activation of G_s-, G_i-, and $G_{q/11}$-coupled adrenergic receptors. These data indicate that expression of peptides derived from the carboxyl terminus of Gα_s can induce G protein-specific blockade of G_s-coupled receptor signaling. By selectively blocking a single G protein pool, this approach can potentially provide insights into the contribution of different G protein pools to complex signaling processes.

EXPERIMENTAL PROCEDURES

Materials—HEK-293 and COS-7 cells were from the American Type Culture Collection. Tissue culture media, fetal bovine serum (FBS), geneticin (G418), and penicillin/streptomycin were from Invitrogen. FuGENE 6 was from Roche Molecular Biochemicals. 3′,5′-[^{3}H]cAMP was from Amersham Biosciences. *myo*-[^{3}H]Inositol and [^{35}S]GTPγS were from PerkinElmer Life Sciences. Monoclonal anti-Gα_s, anti-G$\alpha_{i1/2}$, and anti-G$\alpha_{q/11}$ IgG were from Calbiochem and PerkinElmer Life Sciences. Polyclonal anti-FLAG and anti-HA were from Santa Cruz Biotechnology. Anti-FLAG M2 affinity-agarose was from Sigma. Polyclonal anti-ERK1/2 and anti-phospho-ERK1/2 IgG were from Cell Signaling Technology. Horseradish peroxidase-conjugated donkey anti-mouse IgG was from Amersham Biosciences. Cholera toxin, H89, forskolin, isoproterenol, and 6-chloro-PB hydrobromide were from Sigma. *Bordetella pertussis* toxin was from List Biological. The cDNAs encoding the hamster α_{1B}AR and the human α_{2A}AR were provided by R. J. Lefkowitz. The cDNA encoding the human D_{1A} dopamine receptor was from M. G. Caron. The cDNA encoding the bovine Gα_s subunit was provided by A. G. Gilman.

Construction of Minigenes Encoding the Gα_s Carboxyl Terminus—The construction of the Gα_s peptide minigenes is depicted schematically in Fig. 1*A*. The PCR was employed to amplify cDNA encoding amino acids 286–395, 313–395, or 337–395 of bovine Gα_s and the translation stop codon. Restriction sites at the 5′ and 3′ ends of the Gα_s-derived sequence were incorporated into the oligonucleotide primers used for DNA amplification. For the construct encoding a G_s-derived peptide capable of post-translational prenylation, the DNA sequence TGCGTC-CTCTCTT, encoding the peptide sequence CVLS, was incorporated into the 3′ end of the cDNA sequence prior to the stop codon. For the hemagglutinin (HA) epitope-tagged constructs, the Gα_s carboxyl-terminal sequences were subcloned as *Eco*RI to *Sal*I fragments into a modified pcDNA3.1 that contained a Kozak sequence and an amino-terminal HA epitope upstream of the cloning sites. For the glutathione *S*-transferase (GST)-tagged constructs, Gα_s carboxyl-terminal sequences were subcloned into the pEBG vector, which encodes an amino-terminal GST epitope. The minigene plasmid encoding the third intracellular domain of the human D_{1A} dopamine receptor (D_{1A}3i) in pRK5 was prepared as described previously (10).

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 50 μg/ml penicillin/streptomycin. HEK-293 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum and 50 μg/ml penicillin/streptomycin. Transient transfection of 40–50% confluent cultures of COS-7 or HEK-293 cells in 100-mm dishes was performed using a ratio of 3 μl of FuGENE 6 per μg of plasmid DNA, according to the manufacturer's directions. Empty pcDNA3.1 vector DNA was added to each transfection as needed to keep the mass of DNA constant. A stable HEK-293 cell line expressing the GsCT minigene, Gα_s-(313–395), was prepared by calcium phosphate transfection using 5 μg/ml G418 for selection, as described previously (21). Minigene expression following transient or stable transfection was detected by protein immunoblotting using antisera directed against the Gα_s carboxyl terminus. All assays on transiently transfected cells were performed after 48–72 h. Prior to assay, transfected cells were split into multiwell plates, as appropriate, and incubated overnight in growth medium supplemented with 0.5% FBS and 10 mM HEPES, pH 7.4.

Competition and Saturation Binding Assays—Plasma membrane preparations for use in binding assays, [^{35}S]GTPγS loading of Gα subunits, and immunoblotting were prepared by differential centrifugation. Monolayers of appropriately transfected COS-7 or HEK-293 cells were scraped into 4 °C lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA) and subjected to Dounce homogenization. Membranes were isolated by sequential centrifugation at 300 × *g* for 3 min to remove cell nuclei and unbroken cells, and 40,000 × *g* for 30 min to collect plasma membranes. The supernatant from the second centrifugation represented the cytosolic fraction. For β_2AR competition binding analyses, membranes were resuspended in binding buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) at a concentration of 0.5 mg of protein/ml. Membrane aliquots (20 μg of protein) were incubated with [^{125}I]-cyanopindolol for 30 min at 37 °C in the presence of varying concentrations of isoproterenol (0–10^{-5} M) and then filtered over Whatman GF-C filters and washed to separate unbound ligand. Nonspecific binding was determined in the presence of 25 μM alprenolol. To confirm that assays of GsCT effects were performed under conditions of equal receptor expression, the level of α_{1B}AR, α_{2A}AR, β_2ARonco, and D_{1A} dopamine receptor expression in HEK-293 and COS-7 cells was determined by saturation binding analysis, as described previously (11).

[^{35}S]GTPγS Loading of Gα Subunits—Assays of [^{35}S]GTPγS loading of endogenous Gα_s, G$\alpha_{q/11}$, and G$\alpha_{i1/2}$ subunits were performed on cell membranes prepared from HEK-293 cells. Membrane pellets were resuspended in TME buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 2 mM EDTA and 4.8 mM $MgCl_2$ for G_s loading assays or 100 μM EDTA and 120 μM $MgCl_2$ for $G_{q/11}$ and G_i loading assays. [^{35}S]GTPγS loading was performed by incubating 25 μg of membrane protein in TME buffer, 1 μM GDP, and 30 nM [^{35}S]GTPγS, plus agonist or vehicle, for 5 min at 30 °C in a total volume of 100 μl. Reactions were terminated by solubilizing the membranes for 30 min at 4 °C in IP/Stop buffer (150 mM NaCl, 0.5% Nonidet P-40, 20 mM $MgCl_2$, 100 μM GDP, 100 μM GTP, 1% aprotinin, 50 mM Tris-HCl, pH 7.5). Specific G protein subunits were isolated by immunoprecipitation for 1 h at 4 °C using monoclonal antisera specific for Gα_s, G$\alpha_{q/11}$, or G$\alpha_{i1/2}$, collected on protein A-Sepharose. Immune complexes on Sepharose were washed three times with IP/Stop buffer, and [^{35}S]GTPγS bound to the immunoprecipitated Gα subunits was determined by liquid scintillation counting. [^{35}S]GTPγS binding in the presence of 25 mM $MgCl_2$ was used as a positive control. Immunoprecipitations performed in the absence of primary antibody were used to determine nonspecific background.

cAMP Production—Appropriately transfected HEK-293 or COS-7 cells were split into 6-well plates and serum-starved overnight. Monolayers were preincubated with 1 μM 3-isobutyl-1-methylxanthine for 15 min at 37 °C, prior to stimulation with agonist for 6–10 min as de-

scribed in the figure legends. Reactions were terminated by aspirating medium and adding 250 μl/well of cAMP buffer (4 mM EDTA, 50 mM Tris-HCl, pH 7.5) on ice. Monolayers were collected by scraping into Eppendorf tubes, boiled for 10 min, and clarified by microcentrifugation at 14,000 rpm for 15 min. The cAMP content of the supernatants was determined according to the manufacturer's instructions using the Biotrack [^{3}H]cAMP Assay System from Amersham Biosciences (22). Data were normalized to protein content as determined by Bradford assay of the cell lysates and expressed as pmol of cAMP/mg cell protein.

Phosphatidylinositol Hydrolysis—Appropriately transfected HEK-293 or COS-7 cells were split into 6-well plates and incubated for 18–24 h with *myo*-[^{3}H]inositol at 4 μCi/ml in low serum growth medium. After labeling, cells were washed once with phosphate-buffered saline (PBS) and preincubated for 1 h in PBS at 37 °C followed by fresh PBS containing 20 mM LiCl for 20 min. Cells were then stimulated for 1 h with agonist. Reactions were terminated by the addition of 1.0 ml of 0.4 M perchloric acid and neutralized with 0.4 ml of 0.72 M KOH and 0.6 M $KHCO_3$. Total inositol phosphates were isolated by anion exchange chromatography on Dowex AG1-X8 columns and quantified by liquid scintillation spectroscopy, as described (11).

Phosphorylation of ERK1/2—Appropriately transfected COS-7 cells were split to 6-well plates and incubated for 18–24 h in low serum growth medium in the presence or absence of inhibitors, as indicated. Agonist stimulation was carried out for 5 min, after which monolayers were washed once in 4 °C PBS and lysed in 200 μl of Laemmli sample buffer. For the determination of total cellular ERK1/2 and phospho-ERK1/2, aliquots containing ~20 μg of cell protein were resolved by SDS-PAGE. ERK1/2 and phospho-ERK1/2 were detected by protein immunoblotting using polyclonal anti-ERK1/2 and anti-phospho-ERK1/2 antisera, respectively, with horseradish peroxidase-conjugated polyclonal donkey anti-rabbit IgG used as secondary antibody. Immune complexes were visualized by enzyme-linked chemiluminescence and quantified using a Fluor-S MultiImager. In each experiment, equal loading of ERK1/2 protein was confirmed by probing parallel immunoblots using anti-ERK1/2 antisera.

RESULTS

Cellular Expression of a Polypeptide Derived from the Carboxyl Terminus of $G\alpha_s$ Inhibits G_s-coupled Receptor Signaling by Blocking Receptor-G Protein Coupling—To create a peptide inhibitor of receptor-G_s coupling, we initially prepared a series of minigene constructs encoding 59, 83, and 110 amino acid polypeptides derived from the carboxyl terminus of bovine $G\alpha_s$. These polypeptides contain the major region of $G\alpha_s$ thought to mediate contact with the intracellular domains of GPCRs but lack the sequences that contact adenylyl cyclases. As shown schematically in Fig. 1*A*, each minigene was composed of a minimal Kozak sequence, followed by the $G\alpha_s$-derived cDNA and a 3′-untranslated region. To facilitate detection of the expressed polypeptides, HA or GST epitopes were incorporated into the amino termini of each construct. Transient expression studies in COS-7 cells revealed robust expression of the 83- and 110-amino acid $G\alpha_s$-derived polypeptides. We were unable to detect expression of the 59-amino acid construct, suggesting that the polypeptide product was subject to rapid intracellular degradation. Fig. 1*B* shows an immunoblot of whole cell lysates from COS-7 cells transiently transfected with three versions of the 83-amino acid peptide as follows: GST-$G\alpha_s$-(313–395) (*lane 2*), HA-$G\alpha_s$-(313–395) (*lane 3*), and a modified HA-$G\alpha_s$-(313–395)-CVLS bearing the protein prenylation sequence CVLS at the carboxyl terminus (*lane 4*). As shown in Fig. 1*C*, transient transfection of COS-7 cells with increasing amounts of the HA-$G\alpha_s$-(313–395) plasmid produced a progressive increase in peptide expression that reached levels significantly in excess of the expression of endogenous $G\alpha_s$ isoforms. As shown, minigene expression had no significant effect on the level of endogenous $G\alpha_s$ expression.

To determine whether expression of $G\alpha_s$-derived peptides affected signaling by a G_s-coupled GPCR, we measured basal and agonist-stimulated cAMP production in COS-7 cells transiently expressing the D_{1A} dopamine receptor and either the GST-$G\alpha_s$-(313–395), HA-$G\alpha_s$-(313–395), or HA-$G\alpha_s$-(313–395)-CVLS minigenes. As shown in Fig. 1*D*, the GST-$G\alpha_s$-(313–395) peptide had no significant effect on D_{1A} receptor-mediated cAMP production, despite robust levels of expression. In contrast, expression of either HA epitope-tagged version of the construct led to a marked reduction in the cAMP response. Interestingly, addition of the prenylation sequence CVLS to the carboxyl terminus of the HA-$G\alpha_s$-(313–395) peptide, which might be expected to enhance membrane localization of the peptide, did not significantly increase its effectiveness. Based upon these data, we selected the unmodified HA epitope-tagged version of the 83-amino acid polypeptide $G\alpha_s$-(313–395) (GsCT) for further characterization.

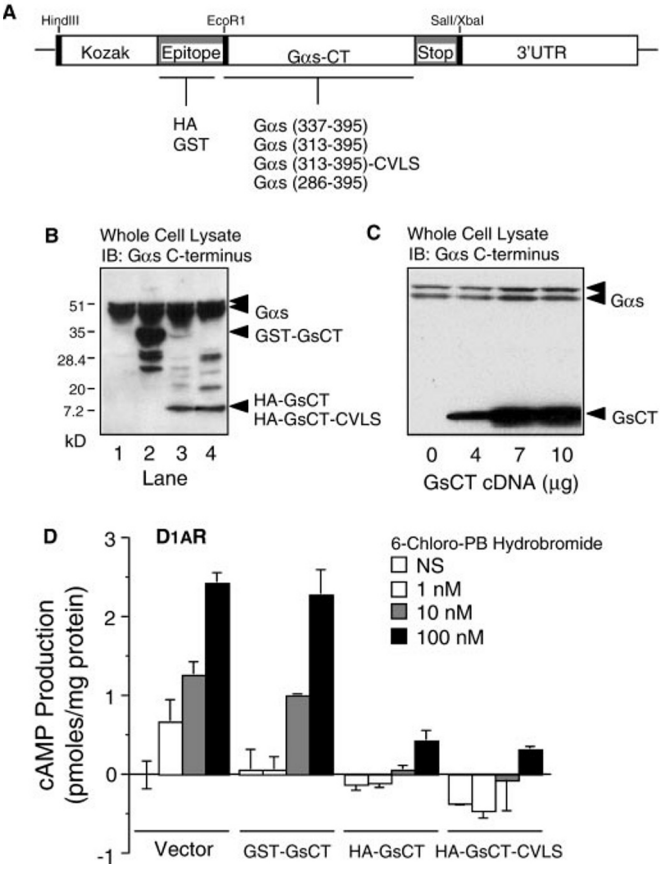

FIG. 1. **Construction of minigenes for expression of polypeptides derived from the $G\alpha_s$ carboxyl terminus.** *A*, schematic representation of minigene constructs encoding the carboxyl-terminal 59, 83, and 110 amino acids of bovine $G\alpha_s$. *B*, protein immunoblot (*IB*) of total cell lysates from COS-7 cells transiently transfected with empty vector, pEBG-GST-$G\alpha_s$-(313–395), pcDNA3.1-HA-$G\alpha_s$-(313–395), and pcDNA3.1-HA-$G\alpha_s$-(313–395)-CVLS (5 μg/100-mm dish), performed using antisera directed against the carboxyl terminus of $G\alpha_s$. *C*, representative immunoblot of COS-7 cell lysates transiently transfected with increasing amounts of the pcDNA3.1-HA-$G\alpha_s$-(313–395) plasmid (0–10 μg/100-mm dish). *B* and *C*, the position of the endogenous p45 and p52 isoforms of $G\alpha_s$, as well as the GsCT minigene products, are as indicated. *D*, effect of GST-$G\alpha_s$-(313–395), HA-$G\alpha_s$-(313–395), and HA-$G\alpha_s$-(313–395)-CVLS expression on D_{1A} dopamine receptor-mediated cAMP production. COS-7 cells were transiently transfected with the pRK5-D_{1A}R (2 μg/100-mm dish), plus either empty vector, pEBG-GST-$G\alpha_s$-(313–395), pcDNA3.1-HA-$G\alpha_s$-(313–395) or pcDNA3.1-HA-$G\alpha_s$-(313–395)-CVLS (8 μg/100-mm dish), and basal and 6-chloro-PB hydrobromide-stimulated cAMP production was determined as described under "Experimental Procedures." Data were normalized to the basal cAMP level measured cells transfected with D_{1A}R plus empty vector (1.25 pmol/mg protein). Data shown represent the mean ± S.D. for triplicate determinations in one of five separate experiments. *UTR,* untranslated region.

The basic unit of heptahelical receptor signaling consists of receptor, heterotrimeric G protein, and effector. As depicted schematically for the β_2AR-G_s-adenylyl cyclase module in Fig.

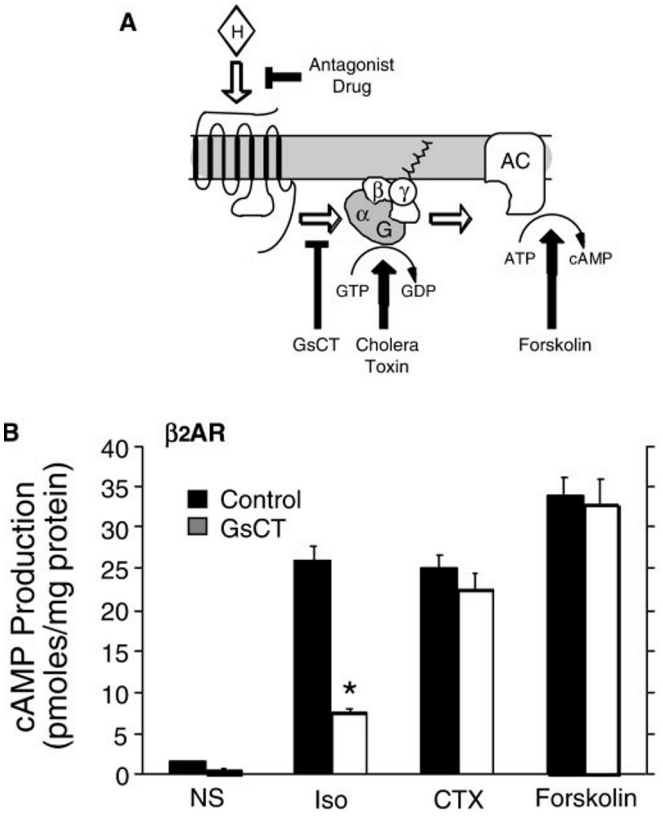

FIG. 2. **Inhibition of β_2AR coupling to G_s by stable expression of the GsCT minigene in HEK-293 cells.** *A*, diagram of the heptahelical receptor-G protein-adenylyl cyclase unit, showing the site of action of agonist hormone (*H*), cholera toxin, and forskolin, all of which stimulate cAMP production. The target of antagonist drugs, which block hormone binding to the receptor, and the putative locus of GsCT action, at the receptor-G protein interface, are shown. *B*, effect of isoproterenol (*Iso*), cholera toxin (*CTX*), and forskolin on cAMP production in parental HEK-293 cells and HEK-293 cells stably expressing the GsCT minigene. Cells were treated with vehicle, isoproterenol (10 μM) for 6 min, cholera toxin (100 ng/ml) for 12 h, or forskolin (5×10^{-4} M) for 6 min, prior to the determination of cAMP content as described. Data shown represent the mean ± S.E. values from four separate experiments. *, less than control, $p < 0.01$. *NS*, not stimulated.

2*A*, it is possible to stimulate cAMP production in cells either by applying agonist, by activating G_s directly using cholera toxin, or by activating adenylyl cyclase directly using forskolin. To determine the effect of the GsCT polypeptide on adenylyl cyclase activation by β_2ARs, we employed a stable GsCT-expressing HEK-293 cell line. Fig. 2*B* compares the cAMP response of parental and GsCT-expressing HEK-293 cells to stimulation with the β_2AR agonist, isoproterenol, cholera toxin, or forskolin. In the presence of GsCT, isoproterenol-stimulated cAMP production stimulation was attenuated by ~68% compared with parental HEK-293 cells. In contrast, cAMP production occurring in response to receptor-independent activation of G_s with cholera toxin, or of adenylyl cyclase with forskolin, was indistinguishable between the two cell lines. These data suggest that the GsCT peptide inhibits receptor-G protein coupling without directly impairing G protein or adenylyl cyclase function.

To elucidate further the mechanism of the inhibition produced by GsCT expression, we assayed the effect of the peptide on the affinity of β_2AR for agonist binding. As shown in Fig. 3*A*, the GsCT peptide, like the endogenous $G\alpha_s$ protein, was present almost exclusively in the plasma membrane fraction following cell fractionation. Neither agonist exposure nor coexpression of FLAG epitope-tagged β_2AR increased the amount of GsCT in the membrane fraction, suggesting that the peptide inherently partitions into the membrane. As shown in Fig. 3*B*, the GsCT peptide specifically immunoprecipitates with the FLAG-β_2AR from cotransfected cells, suggesting that once associated with the membrane, the peptide is capable of binding to the receptor.

In the absence of exogenous guanine nucleotide, many GPCRs exhibit characteristic high and low affinity states for agonist binding. The high affinity state is thought to represent pre-coupling of the GPCR to GDP-bound heterotrimeric G protein, whereas the low affinity state represents free GPCR. In the presence of a nonhydrolyzable GTP analogue, such as GTPγS, which causes irreversible dissociation of G protein subunits, only the low affinity state of the receptor is present. Fig. 3*C* compares competition binding curves generated for the displacement of the β_2AR antagonist [^{125}I]-cyanopindolol by isoproterenol in COS-7 cells membranes in the presence of either GTPγS or GsCT. In control membranes, the competition binding curve fits a two-site model with the high affinity site composing 22% of the total specific [^{125}I]-cyanopindolol-binding sites. In the presence of GTPγS, the curve was shifted to the right, with only a single low affinity site present. In membranes from cells expressing the GsCT, the curve was similarly right-shifted, such that the high affinity site composed only 7% of the total. No significant differences were detected in the EC_{50} values for the high and low affinity sites between control and GsCT-containing membranes. These data strongly support the hypothesis that GsCT binding to the β_2AR precludes receptor-G protein coupling.

Because GsCT expression appeared to target the receptor-G protein interface, we sought to determine whether the effect of GsCT expression was specific for G_s by assaying receptor-stimulated [^{35}S]GTPγS loading of endogenous G proteins in membranes isolated from parental and GsCT-expressing HEK-293 cells. For these assays, endogenous β_2ARs or transiently expressed α_{1B}ARs and α_{2A}ARs were employed to stimulate the endogenous pools of G_s, $G_{q/11}$, and G_i, respectively. As shown in Fig. 4*A*, membranes from GsCT-expressing cells showed a 72% decrement in the isoproterenol-induced increase in [^{35}S]GTPγS loading of $G\alpha_s$ compared with membranes from parental cells, with no significant effect on basal $G\alpha_s$ loading. As shown in Fig. 4, *B* and *C*, no significant differences in basal or agonist-stimulated G protein loading were observed when α_{1B}AR-mediated $G\alpha_{q/11}$ and α_{2A}AR-mediated $G\alpha_i$ loading in GsCT expressing and parental cells were compared. Thus, GsCT expression led to G_s-specific inhibition of receptor-G protein coupling.

GsCT Expression Results in G Protein-specific Inhibition of Heptahelical Receptor Signaling—If expression of the GsCT polypeptide selectively uncouples heptahelical receptors from G_s, one would expect it to inhibit the generation of G_s-dependent, but not $G_{q/11}$- or G_i-dependent second messengers after stimulation of receptors coupled to these G protein pools. To test this hypothesis, we employed a transfected COS-7 cell system in which various heptahelical receptors were transiently expressed in the presence or absence of GsCT. Fig. 5*A* depicts the effects of increasing GsCT expression on cAMP production in response to stimulation of coexpressed G_s-coupled D_{1A} dopamine receptors in COS-7 cells. At the highest levels of expression, GsCT inhibited D_{1A} receptor-mediated cAMP production to an extent comparable with that obtained by expression of a 59-amino acid polypeptide derived from the third intracellular domain of the D_{1A} receptor (D_{1A}R3i). We have shown previously that the D_{1A}R3i peptide, which represents the receptor side of the putative receptor-G protein interface, inhibits D_{1A} receptor-mediated cAMP production when expressed in HEK-293 and COS-7 cells (10, 11).

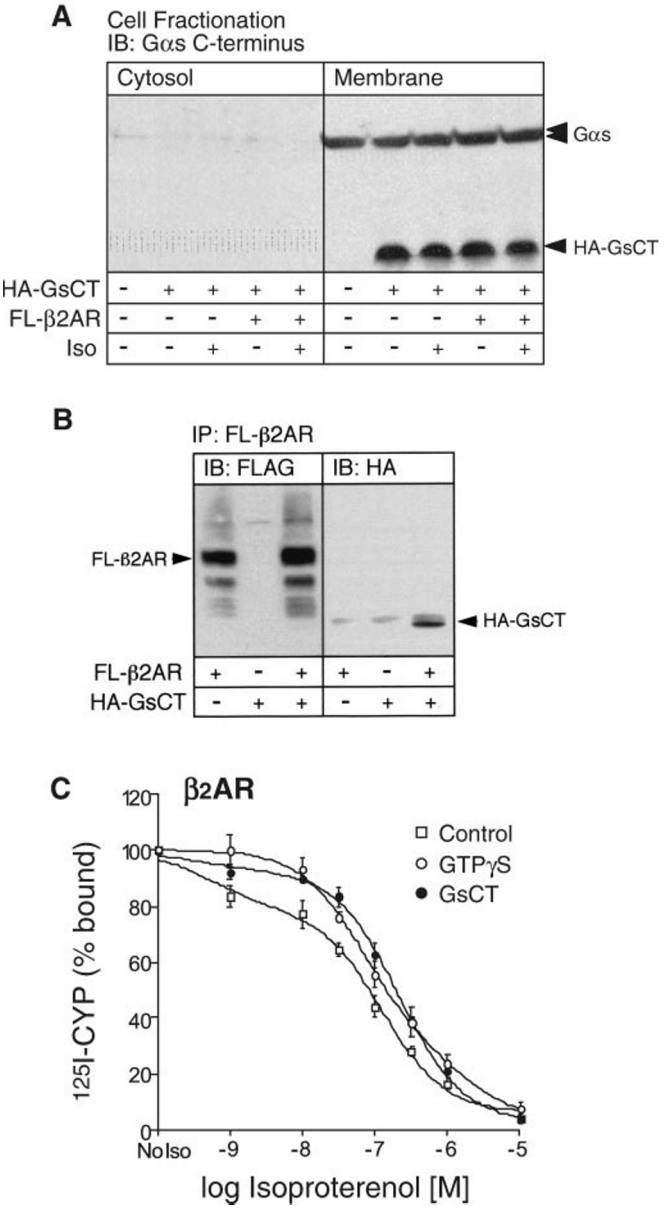

FIG. 3. **Effect of GsCT on high affinity agonist binding to the β_2AR.** *A*, partitioning of GsCT between cytosol and membrane fractions. COS-7 cells were transfected with the HA-GsCT minigene (8 μg/100-mm dish) in the presence or absence of FLAG-β_2AR (2 μg/dish) and treated for 5 min with isoproterenol (10 μM) or vehicle prior to the preparation plasma membrane and cytosolic fractions. Immunoblots of 2% of the protein from each fraction were performed using antisera directed against the carboxyl terminus of $G\alpha_s$. The position of the endogenous p45 and p52 isoforms of $G\alpha_s$, as well as the GsCT minigene product, are as indicated. *B*, coprecipitation of HA-GsCT with FLAG-β_2AR. COS-7 cells were transfected with the HA-GsCT minigene (8 μg/100-mm dish) and FLAG-β_2AR (2 μg/dish), alone or in combination, as indicated. FLAG immunoprecipitates (*IP*) were subjected to immunoblotting (*IB*) using polyclonal anti-FLAG (*left panel*) and polyclonal anti-HA antisera (*right panel*) to detect the FLAG-β_2AR and HA-GsCT peptide, respectively. The positions of FLAG-β_2AR and coprecipitated HA-GsCT peptide are as indicated. *C*, agonist displacement curves for endogenous β_2AR in COS-7 cell membranes in the presence or absence of GTPγS or HA-GsCT. Isoproterenol (*Iso*) displacement of [^{125}I]cyanopindolol (*^{125}I-CYP*) was performed using plasma membranes prepared from untransfected COS-7 cells or cells transfected with GsCT (20 μg/150-mm dish). Agonist affinities and abundance were calculated by nonlinear regression analysis with one- and two-site models. Calculated values were as follows: control membranes, high affinity site 5.4×10^{-10} M (22%) and low affinity site 1.25×10^{-7} M (78%); control membranes plus GTPγS, low affinity site 1.08×10^{-7} M (100%); GsCT membranes, high affinity site 3.1×10^{-10} M (7%) and low affinity site 2.1×10^{-7} M (93%). Data shown represent the mean ± S.E. for duplicate determinations in two to three separate experiments.

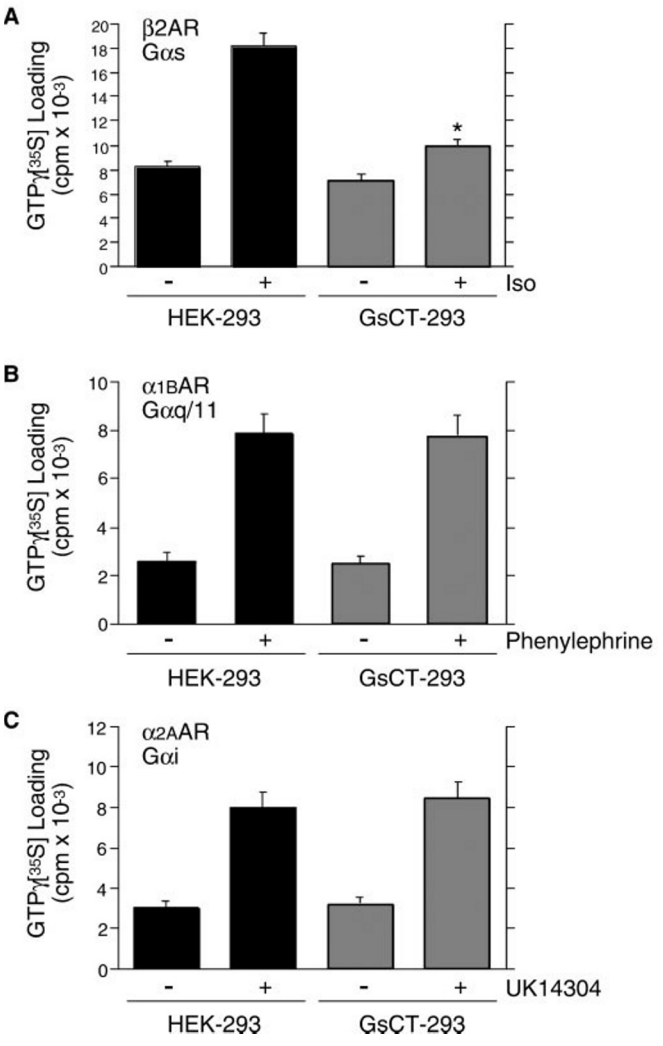

FIG. 4. **Effect of GsCT expression on GTPγS loading of endogenous $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_i$ pools.** *A*, comparison of basal and isoproterenol-stimulated binding of [^{35}S]GTPγS to endogenous $G\alpha_s$ in membranes isolated from parental (HEK-293) and HA-GsCT-expressing HEK-293 (GsCT-293) cells. Membrane fractions were incubated with [^{35}S]GTPγS in the presence and absence of isoproterenol (10 μM) for 10 min, prior to detergent solubilization and immunoprecipitation of $G\alpha_s$ for the determination of [^{35}S]GTPγS binding as described. *B*, comparison of basal and phenylephrine (1 μM) stimulated binding of [^{35}S]GTPγS to endogenous $G\alpha_{q/11}$ in membranes isolated from parental and GsCT-expressing HEK-293 cells transiently expressing hamster α_{1B}AR. *C*, comparison of basal and UK14304 (10 μM)-stimulated binding of [^{35}S]GTPγS to endogenous $G\alpha_{i1/2}$ in membranes isolated from parental and GsCT-expressing HEK-293 cells transiently expressing human α_{2A}AR. In each panel, data shown represent the mean ± S.E. for four separate experiments. * less than control, $p < 0.01$.

Fig. 5*B* depicts the dose-response relationship for D_{1A} receptor-stimulated cAMP production in COS-7 cells expressing a comparable level of receptor (0.9–1.15 pmol/mg membrane protein) in the presence or absence of coexpressed GsCT or D_{1A}3i. In the presence of either polypeptide, 6-chloro-PB hydrobromide-stimulated cAMP production was inhibited by at least 70% at each agonist concentration tested. The observed inhibition was not surmountable by even supersaturating concentrations of agonist. As shown in Fig. 5*C*, similar, apparently noncompetitive inhibition of β_2AR-mediated cAMP production was observed in the stable GsCT-expressing HEK-293 cell line. In the GsCT-expressing cells, isoproterenol-stimulated cAMP production was attenuated by at least 66% at each agonist concentration.

For the overexpressed D_{1A}R in COS-7 cells, and to a lesser

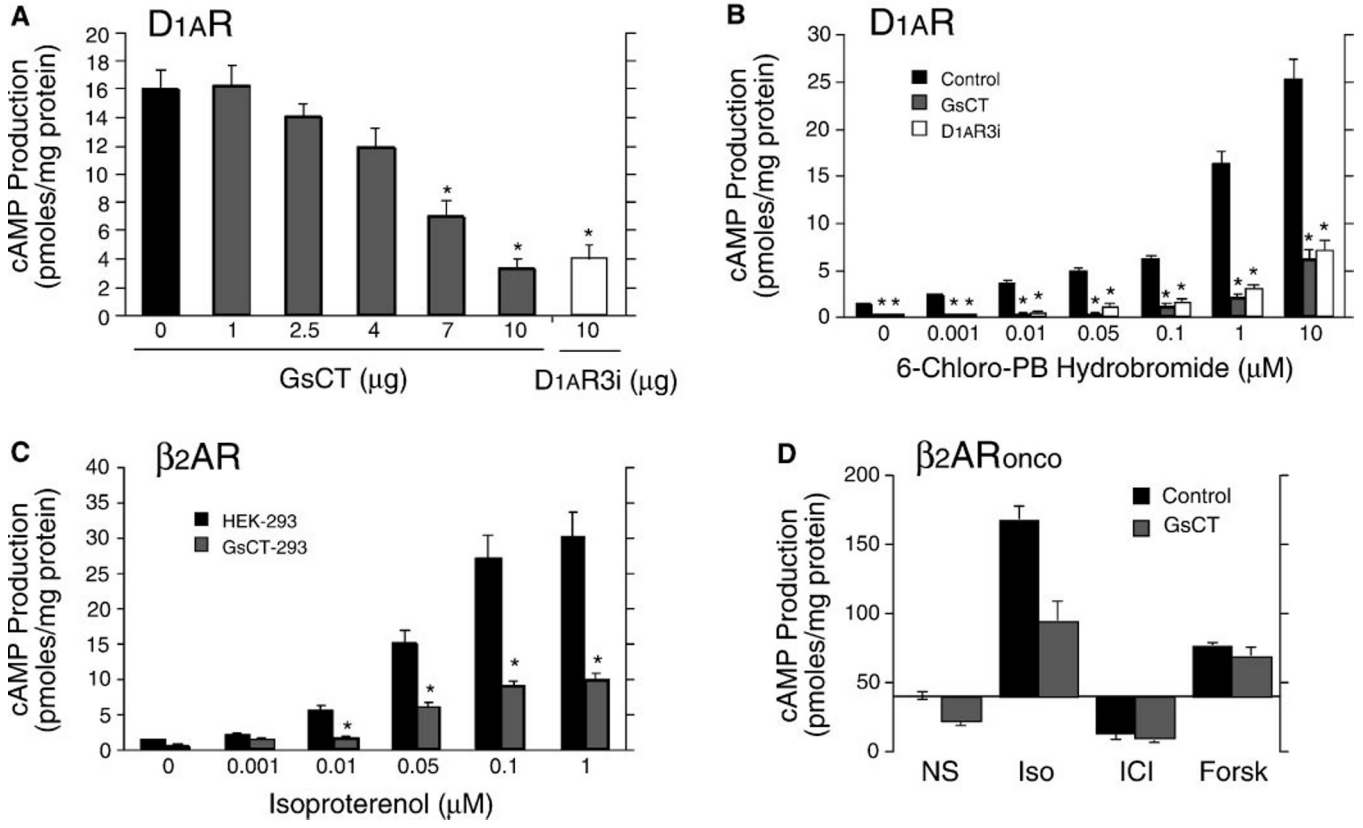

FIG. 5. **Effect of GsCT expression on agonist-stimulated cAMP production by the G_s-coupled D_{1A} dopamine and β_2 adrenergic receptors.** *A*, effect of increasing GsCT expression on agonist-stimulated cAMP production in COS-7 cells transiently expressing D_{1A} dopamine receptors (D_{1A}R). Cells in 100-mm dishes were cotransfected the pRK5-D_{1A}R plasmid, along with the indicated amounts of either pcDNA3.1-HA-Gα_s-(313–395) or pRK5-D_{1A}R3i. The production of cAMP in response to 6 min of exposure to the dopamine receptor agonist 6-chloro-PB hydrobromide (10 μM) was determined as described. *B*, dose-response curves for 6-chloro-PB hydrobromide-stimulated cAMP production in COS-7 cells in the presence and absence of coexpressed HA-GsCT or D_{1A}3i peptides. Cells were cotransfected the pRK5-D_{1A} receptor plasmid along with either the pcDNA3.1-HA-Gα_s-(313–395) or pRK5-D_{1A}3i plasmid (10 μg/100-mm dish). The production of cAMP in response to 6 min of exposure to the indicated concentration of 6-chloro-PB hydrobromide was determined as described. *C*, dose-response curves for isoproterenol-stimulated cAMP production in parental HEK-293 cells and HEK-293 cells stably expressing HA-GsCT (GsCT-293). The production of cAMP in response to 6 min of exposure to the indicated concentration of isoproterenol was determined as described. *A–C*, data shown represent the mean ± S.E. for four separate experiments. *, less than control, $p < 0.05$. *D*, effect of HA-GsCT expression on cAMP production in COS-7 cells transiently expressing a constitutively active mutant of the β_2AR (β_2ARonco). COS-7 cells were transfected with plasmid encoding the β_2ARonco (2 μg/100-mm dish) plus either empty vector (control) or pcDNA3.1-HA-Gα_s-(313–395). Determinations of cAMP production were made under basal conditions and following treatment for 10 min with isoproterenol (1 μM) or forskolin (1 μM) or for 30 min with the inverse agonist ICI118551 (10 μM). Data shown represent the mean ± S.D. values of triplicate determinations in one of three identical experiments.

extent the endogenous β_2AR in HEK-293 cells, GsCT expression reduced basal as well as agonist-stimulated cAMP levels. To determine whether this effect was due to inhibition of basal receptor-G_s coupling or to an additional receptor-independent effect of GsCT, we compared the effect of GsCT expression with that of the β_2AR inverse agonist ICI118551 (23, 24). In these assays we employed a constitutively activated point mutant of the β_2AR, β_2ARonco (23), because the higher basal levels of cAMP generated by the mutated receptor facilitated measurement of the effects of GsCT and ICI118551 on basal cAMP. As shown in Fig. 5*D*, basal cAMP production in COS-7 cells expressing the β_2ARonco was increased 4-fold in the presence of agonist and inhibited by 66% in the presence of maximally effective concentrations of ICI118551. As with the wild type β_2AR, both basal and agonist-stimulated cAMP production were attenuated in cells expressing GsCT. Treatment with ICI118551 had little additional effect on cAMP levels in the GsCT-expressing cells. The cAMP response to a submaximal dose of forskolin was equivalent between the two cell populations. The lack of additivity of the effects of GsCT and ICI118551 on β_2ARonco signaling suggests that the predominant effect of the GsCT is mediated through its effects on receptor-G protein coupling.

The apparently noncompetitive pattern of inhibition we observed is consistent with expression of an inhibitor that competes with the endogenous G protein pool for access to ligand-bound receptor. Increasing agonist concentration would have no effect on the ratio of GsCT to functional G_s heterotrimer and would thus not be expected to surmount the inhibitory effect of the polypeptide. Consistent with this, we found that the maximal extent of GsCT-induced inhibition of D_{1A} receptor-mediated cAMP production did vary with the level of receptor expression. At a D_{1A} receptor density of <0.75 pmol/mg, the cAMP response to saturating concentrations of agonist was almost completely blocked by high levels of GsCT expression. Increasing D_{1A} receptor expression to levels >1.5 pmol/mg partially overcame the inhibition (data not shown). In the presence of a saturating concentration of agonist, increasing receptor expression might increase the likelihood of an activated receptor encountering a functional G_s heterotrimer, leading to less inhibition of second messenger generation.

The α_{1B}AR stimulates phosphatidylinositol (PI) hydrolysis primarily by $G_{q/11}$-dependent activation of the phospholipase C (PL-C) β1 isoform (25). As shown in Fig. 6*A*, α_{1B}AR-mediated PI hydrolysis is unaffected by coexpression of increasing amounts of the GsCT polypeptide. The α_{2A}AR weakly stimulates PI hydrolysis by G_i-dependent activation of the PL-C β_2 and β_3 isoforms (26). As shown in Fig. 6*B*, α_{2A}AR-mediated PI

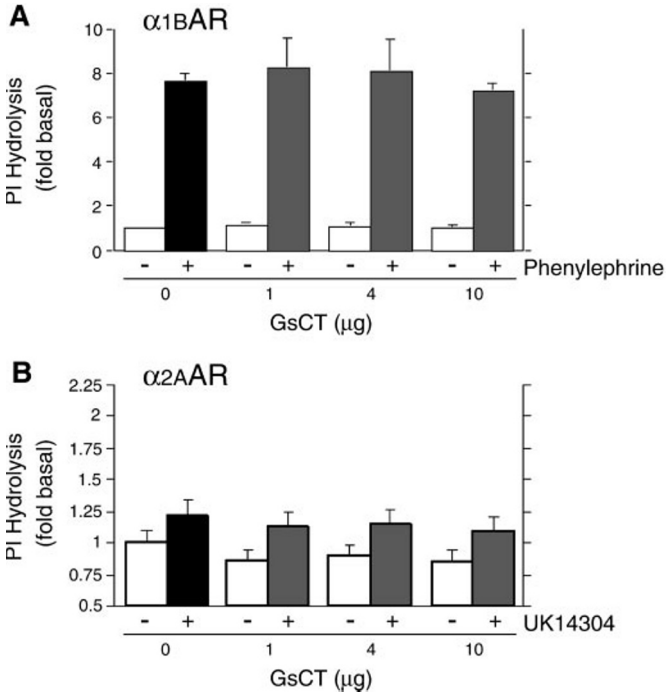

FIG. 6. **Effect of GsCT expression on agonist-stimulated PI hydrolysis by the $G_{q/11}$-coupled α_{1B} and G_i-coupled α_{2A} adrenergic receptors.** *A*, effect of increasing GsCT expression on agonist-stimulated PI hydrolysis in COS-7 cells transiently expressing α_{1B}AR. Cells in 100-mm dishes were cotransfected the pRK5-α_{1B}AR plasmid, along with the indicated amounts of pcDNA3.1-HA-Gα_s-(313–395). PI hydrolysis in response to 1 h of exposure to phenylephrine (1 μM) was determined as described. *B*, effect of increasing GsCT expression on agonist-stimulated PI hydrolysis in COS-7 cells transiently expressing α_{2A}AR. Cells in 100-mm dishes were cotransfected the pRK5-α_{2A}AR plasmid, along with the indicated amounts of pcDNA3.1-HA-Gα_s-(313–395). PI hydrolysis in response to 1 h of exposure to the α_{2A}AR agonist, UK14304 (10 μM), was determined as described. Data are presented in arbitrary units, such that the basal amount of [^{3}H]inositol phosphate detected in cells not expressing GsCT was assigned a value of 1. In each panel, the data shown represent the mean ± S.E. of triplicate determinations in four separate experiments.

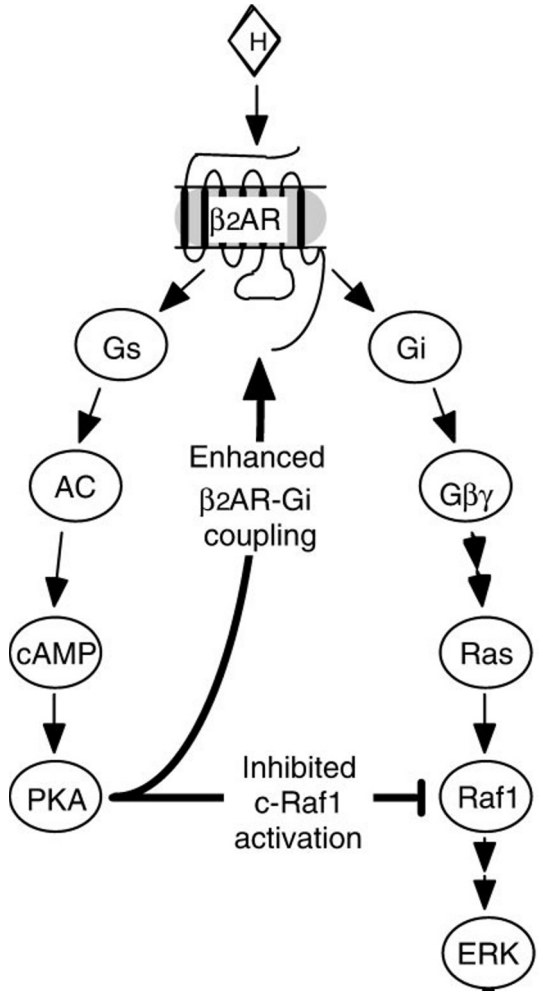

FIG. 7. **Putative stimulatory and inhibitory effects of PKA phosphorylation on activation of the ERK1/2 MAP kinase cascade by β_2ARs.** Activation of the G_s-adenylyl cyclase (*AC*)-PKA pathway results in PKA-mediated phosphorylation of the β_2AR. PKA phosphorylation increases receptor coupling to pertussis toxin-sensitive G_i proteins, resulting in G$\beta\gamma$ subunit and Ras-dependent activation of the ERK1/2 pathway. At the same time, PKA activation exerts an inhibitory effect on ERK1/2 activation by phosphorylating the MAP kinase kinase kinase, Raf1.

hydrolysis was likewise unaffected by GsCT expression. Collectively, these data suggest that expression of the GsCT polypeptide produces G protein-specific inhibition of heptahelical receptor-G protein coupling. Its effects are generalizable to multiple G_s-coupled receptors, in that β_2AR and D_{1A} dopamine receptor cAMP production are similarly affected, but are specific for signals mediated by G_s, in that $G_{q/11}$- and G_i-dependent PI hydrolysis is unimpaired.

Use of GsCT to Examine the Contribution of G_s to ERK Activation by β_2 Adrenergic, α_{1B} Adrenergic, and α_{2A} Adrenergic Receptors—The role of G_s proteins in GPCR-mediated ERK activation is complex. As depicted schematically in Fig. 7, previous studies have indicated that activation of protein kinase A (PKA) by G_s-coupled receptors can produce both stimulation and inhibition of ERK activity. In HEK-293 cells (27), cardiac myocytes (28), and pancreatic acinar cells (29), β_2AR-mediated ERK activation involves both PKA and activation of pertussis toxin-sensitive G proteins. It has been proposed that phosphorylation of the β_2AR by PKA switches receptor coupling from G_s to G_i, allowing the receptor to mediate pertussis toxin-sensitive ERK1/2 activation through a G$\beta\gamma$ subunit-dependent pathway (27). On the other hand, PKA-mediated phosphorylation of Raf-1 has been shown to attenuate growth factor-stimulated ERK activation in several cell types (30–33). Thus, the net effect of G_s stimulation on GPCR-mediated ERK activation likely reflects a balance between two opposing mechanisms of regulation.

Having determined that expression of the GsCT polypeptide leads to selective inhibition of G_s-mediated signaling, we employed the construct to examine the contribution of Gα_s to ERK activation by β_2, α_{2A}, and α_{1B} adrenergic receptors. For the β_2AR, which is endogenously expressed, we compared isoproterenol-stimulated ERK1/2 phosphorylation in parental HEK-293 cells with that in stable GsCT-expressing HEK-293 cells. As shown in Fig. 8*A*, β_2AR-mediated ERK phosphorylation in HEK-293 cells was inhibited by pretreatment with either the PKA inhibitor, H89, or with pertussis toxin, consistent with the previously described roles of PKA and G_i in the pathway (27). When isoproterenol-stimulated ERK1/2 phosphorylation was compared in parental and GsCT-expressing HEK-293 cells, a significant reduction was observed in the cells expressing the GsCT peptide. These data, shown in Fig. 8*B*, are consistent with the proposed requirement for G_s activation in β_2AR signaling to ERK.

The α_{2A}AR couples to $G_{i/o}$ family G proteins and in COS-7 cells mediates ERK activation through a pertussis toxin-sensitive pathway that is blocked by expression of a G$\beta\gamma$ subunit sequestrant polypeptide derived from the carboxyl terminus of G protein-coupled receptor kinase 2 (34). However, the α_{2A}AR

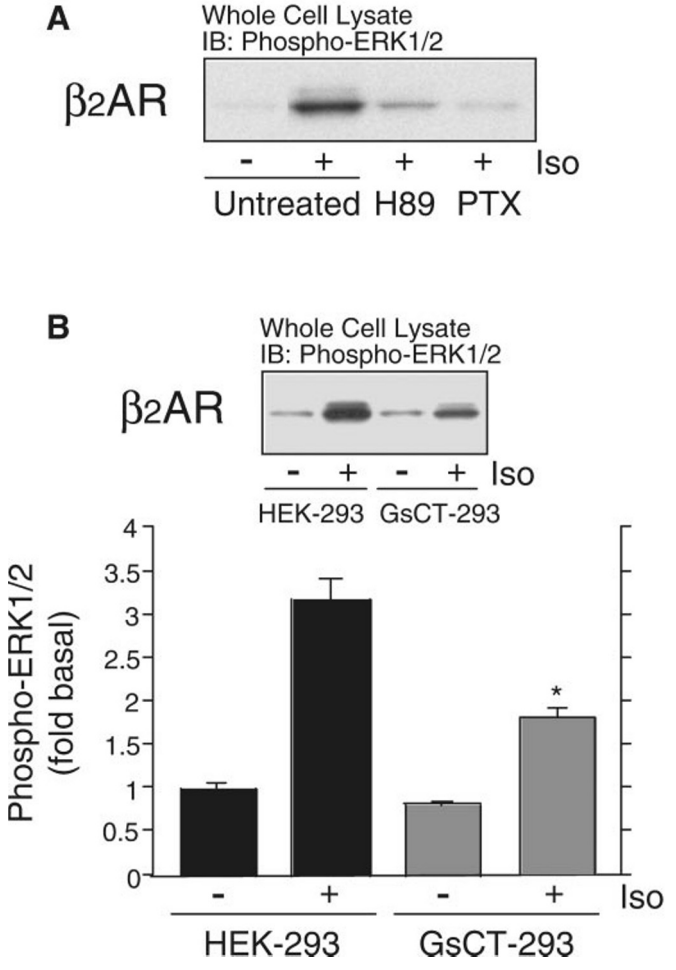

FIG. 8. **Effect of GsCT expression on β_2AR-mediated ERK1/2 phosphorylation.** *A,* effect of the PKA inhibitor H89 and pertussis toxin on β_2AR-stimulated ERK1/2 activation in HEK-293 cells. Cells in 6-well plates were preincubated with H89 (10 μM) for 30 min or with pertussis toxin (100 ng/nl, *PTX*) for 16 h, prior to 5 min of stimulation with isoproterenol (*Iso*) (10 μM). Phospho-ERK1/2 levels in whole cell lysates were determined by immunoblotting (*IB*) as described. The immunoblot shown is representative of at least three separate experiments. *B*, effect of GsCT on β_2AR-stimulated ERK1/2 activation in parental HEK-293 and GsCT-expressing HEK-293 cells (GsCT-293). Serum-starved cells in 6-well plates were stimulated for 5 min of stimulation with isoproterenol (10 μM) prior to determination of phospho-ERK1/2 levels as described. The *upper panel* depicts a representative immunoblot. Data shown in the *lower panel* represent the mean ± S.E. for three separate experiments. *, less than control, $p < 0.05$.

also couples to G_s, particularly at high levels of receptor expression (35). In contrast to the β_2AR, α_{2A}AR coupling to both G_i and G_s is a constitutive property of the receptor, not one that is modulated by PKA phosphorylation. Furthermore, G_s activation apparently antagonizes GPCR-stimulated ERK activation in COS-7 cells, because expression of an activated mutant of $G\alpha_s$, or treatment with the cell-permeant cAMP analog, 8-bromo-cAMP, attenuates ERK activation in response to either isoproterenol or epidermal growth factor in this system (33). As shown in Fig. 9*A*, α_{2A}ARs transiently expressed in COS-7 cells, like β_2ARs in HEK-293 cells, activate ERK1/2 via pertussis toxin-sensitive G proteins. However, in contrast to the β_2AR system, treatment with H89 enhances, rather than inhibits, α_{2A}AR-mediated ERK activation. This presumably reflects relief of the inhibitory effect of PKA on ERK activation that results from phosphorylation of c-Raf1. As shown in Fig. 9*B*, transfection of COS-7 cells with increasing amounts of the GsCT plasmid, like H89 treatment, caused a progressive enhancement of α_{2A}AR-mediated ERK phosphorylation.

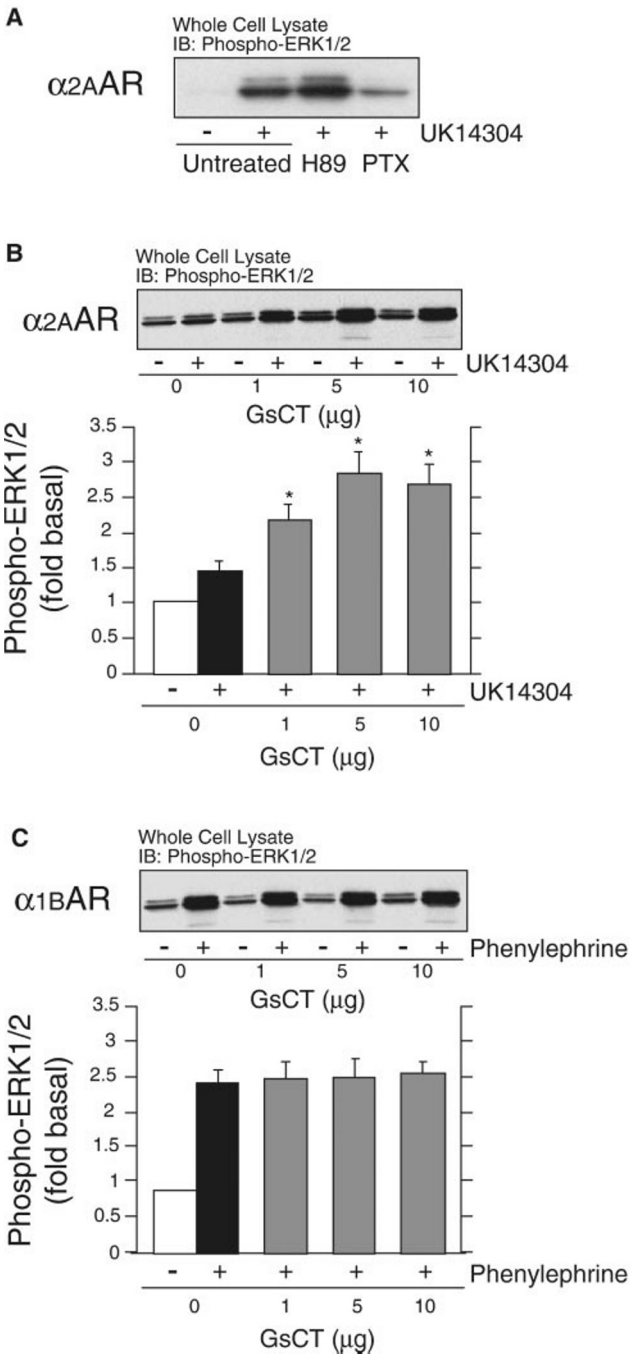

FIG. 9. **Effect of GsCT expression on agonist-stimulated PI hydrolysis by the $G_{q/11}$-coupled α_{1B}AR and G_i-coupled α_{2A}AR.** *A,* effect of the PKA inhibitor H89 and pertussis toxin on α_{2A}AR-stimulated ERK1/2 phosphorylation in COS-7 cells. Cells in 6-well plates were preincubated with H89 (10 μM) for 30 min or with pertussis toxin (100 ng/nl, *PTX*) for 16 h, prior to 5 min of stimulation with UK14304 (10 μM). Phospho-ERK1/2 levels in whole cell lysates were determined by immunoblotting (*IB*) as described. The immunoblot shown is representative of at least three separate experiments. *B,* effect of increasing GsCT expression on α_{2A}AR-mediated ERK1/2 activation in COS-7 cells. Cells in 100-mm dishes were cotransfected the pRK5-α_{2A}AR plasmid, along with the indicated amounts of pcDNA3.1-HA-Gα_s-(313–395), prior to passage into 6-well plates. Phospho-ERK1/2 levels after 5 min of stimulation with UK14304 (10 μM) were determined as described. *C*, effect of increasing GsCT expression on α_{1B}AR-stimulated ERK1/2 activation in COS-7 cells. Cells in 100-mm dishes were cotransfected the pRK5-α_{1B}AR plasmid, along with the indicated amounts of pcDNA3.1-HA-Gα_s-(313–395), prior to passage into 6-well plates. Phospho-ERK1/2 levels after 5 min of stimulation with phenylephrine (1 μM) were determined as described. *B* and *C,* the radiograph depicts a representative immunoblot. Data shown represent the mean ± S.E. for three separate experiments. *, greater than control, $p < 0.05$.

In COS-7 cells, stimulation of transiently expressed α_{1B}ARs leads to ERK activation that is pertussis toxin-insensitive (36) but blocked by expression of a polypeptide derived from the carboxyl-terminal 55 amino acids of $G\alpha_q$ (18). As shown in Fig. 9*C*, transfection of COS-7 cells with increasing amounts of the GsCT plasmid had no effect on α_{1B}AR-mediated ERK phosphorylation. Thus, ERK1/2 activation by a receptor that does not activate G_s was unaffected by GsCT expression.

DISCUSSION

The precise structural determinants underlying activation of heterotrimeric G proteins by heptahelical receptors are incompletely understood. Crystallographic analysis of the structure of $G\alpha_s$ has indicated that receptor coupling specificity is likely determined by a surface formed by the continuous carboxyl-terminal α-helix between Asp-368 and Leu-394, and the loop between the α5-helix and β6-strand (6). Contact between the $G\alpha$ subunit and the second and third intracellular domains of heptahelical receptors determines the efficiency and specificity of the receptor-G protein interaction (3, 37, 38). The $G\alpha$ subunit carboxyl-terminal helix may insert into a cavity between the third and sixth receptor transmembrane domains of the heptahelical receptor bundle that forms as a consequence of agonist-induced conformational changes (39). NMR studies have demonstrated that short polypeptides derived from the $G\alpha_s$ carboxyl terminus form stable α-helices in solution. In isolated plasma membranes, 11-amino acid peptides representing the carboxyl termini of $G\alpha_{i1/2}$ or $G\alpha_o$ modulate ligand binding to the adenosine A_1 receptor by disrupting the high affinity receptor-G protein complex (16). Similarly, modified 16–21-amino acid peptides derived from the carboxyl terminus of $G\alpha_s$ inhibit high affinity agonist binding to the adenosine A_{2A} receptors, and impair A_{2A} receptor-mediated adenylyl cyclase activation (17). These data suggest that the isolated carboxyl-terminal α-helix can interact with a receptor in a manner that precludes productive receptor-G protein coupling.

We have examined the mechanism of action and functional consequences of expression of an 83-amino acid polypeptide derived from the carboxyl terminus of $G\alpha_s$ in intact cells. Expression of the GsCT peptide impaired adenylyl cyclase activation by G_s-coupled β_2 adrenergic and D_{1A} dopamine receptors, without affecting the response to cholera toxin or forskolin, suggesting that the peptide specifically impairs receptor-G protein coupling. At a constant level of receptor expression, the inhibition was not surmountable by increasing agonist concentration. The magnitude of the effect was partially reversed by increasing receptor density, consistent with the hypothesis that the peptide competes with the endogenous G_s pool for access to ligand-occupied receptors. Furthermore, the inhibition was apparently specific for G_s, because PI hydrolysis induced by stimulation of the $G_{q/11}$-coupled α_{1B}AR and G_i-coupled α_{2A}AR was unaffected by GsCT expression.

A significant aspect of the approach of using receptor- or G protein-derived peptides to inhibit heptahelical receptor signaling is that the resulting antagonism affects a class of G protein, rather than a specific receptor. Such reagents differ from pharmacologic antagonists of ligand binding in that expression of a single polypeptide should be able to uncouple multiple receptors from a single G protein pool. In this regard, their function is more like *B. pertussis* toxin, which uncouples all $G_{i/o}$ family proteins from their cognate receptors by catalyzing the ADP-ribosylation of a carboxyl-terminal cysteine residue on the $G\alpha$ subunit.

Because of their ability to selectively uncouple specific G proteins from multiple receptors, peptide inhibitors of receptor-G protein coupling may be useful for determining the contribution of a given G protein pool to signaling by a receptor that couples to multiple G proteins. Minigene constructs encoding the carboxyl termini of $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ have recently been employed to examine the contribution of different G protein pools to second messenger generation by the thrombin receptor in endothelial cells (20). We have employed GsCT expression to examine the role of $G\alpha_s$ in a complex process, activation of the ERK MAP kinase cascade in fibroblasts, where G_s activation has been reported previously to produce both stimulation and inhibition of ERK activity. Consistent with previous reports, we found that β_2AR-mediated ERK activation, which is blocked by PKA inhibition, is inhibited by GsCT, whereas α_{2A}AR-mediated ERK activation, which is accentuated by PKA inhibition, is enhanced in cells expressing GsCT (27–33). These data support a dual role for PKA in ERK activation by the β_2AR, where PKA phosphorylation of the receptor promotes receptor-G_i coupling and pertussis toxin-sensitive ERK activation (27), but where PKA phosphorylation of Raf1 attenuates ERK activation downstream of Ras (30–33). In the case of the constitutively G_i/G_s-coupled α_{2A}AR, only the downstream inhibitory effect of G_s activation, which is relieved by GsCT expression, is discernible.

Tissue-specific expression of peptide G protein inhibitors has already provided valuable information about the roles of individual G proteins in complex physiologic responses *in vivo*. Cardiomyocyte-specific expression of a 55-amino acid peptide derived from the carboxyl terminus of $G\alpha_q$ reduces cardiac hypertrophy (18) and inhibits activation of the ERK and c-Jun amino-terminal kinase MAP kinase cascades (40), in response to surgically induced pressure overload in a transgenic murine model, underscoring the important role of $G_{q/11}$ proteins in this process. Recombinant adenovirus-mediated expression of a $G\beta\gamma$ subunit sequestrant polypeptide derived from the carboxyl terminus of G protein-coupled receptor kinase 2 (41), which results in generic inhibition of $G\beta\gamma$ subunit-mediated signaling events (34, 42), blocks ERK activation and vascular smooth muscle hypertrophy in a rat carotid artery model of vascular restenosis (43). The availability of specific polypeptide inhibitors of $G\alpha_s$ signaling, such as the GsCT minigene, may provide the opportunity to obtain similar insights into the complex roles of G_s in control of cellular hypertrophy and proliferation in a variety of tissues.

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