Selective Inhibition of Heterotrimeric Gs Signaling

TARGETING THE RECEPTOR-G PROTEIN INTERFACE USING A PEPTIDE MINIGENE ENCODING THE GaS CARBOXYL TERMINUS*

The blockade of heptahelical receptor coupling to heterotrimeric G proteins by the expression of peptides derived from G protein Ga s 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 Ga s (GsCT). In membranes prepared from GsCT-expressing cells, the peptide blocked high affinity agonist binding to β2 adrenergic receptors (AR) and inhibited β2AR-induced [35S]GTPγS loading of Ga s. GsCT expression inhibited β2AR- and dopamine D1A receptor-mediated cAMP production, without affecting the cellular response to cholera toxin or forskolin, indicating that the peptide inhibited receptor-Gs coupling without impairing G protein or adenyl cyclase function. [35S]GTPγS loading of Gαq/11 by α2ARs and Gαi by α2AARs and Gαq/11- or G i-mediated phosphatidylinositol hydrolysis was unaffected, indicating that the inhibitory effects of GsCT were selective for Gs. We next employed the GsCT construct to examine the complex role of Gs 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 β2AR in HEK-293 cells, where PKA activity is required for ERK activation, expression of GsCT caused a net inhibition of ERK activation. In contrast, α2AAR-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 Gαq/11-coupled α1BAR 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.

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The abbreviations used are: G protein, heterotrimeric GTP-binding protein; AR, adrenergic receptor; D1, D2, dopamine receptor third intracellular domain; ERK, extracellular signal-regulated kinase; PBS, fetal bovine serum; Gαi, a subunit of the heterotrimeric G i protein; Gαq/11, a subunit of the heterotrimeric Gαq/11 protein; Gαs, a subunit of the heterotrimeric G s protein; GsCT, the carboxyl-terminal residues 313–395 of bovine Gα 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-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 Gαq11-coupled α1a adrenergic receptor (AR) and M1 muscarinic acetylcholine receptor, the Gq-coupled α2aAR and M2 acetylcholine receptor, and the Gβγ-coupled D1A dopamine receptor have been shown to inhibit Gαq11, Gγ, and Gβγ-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 Gαi, containing three point mutations that impair its function strongly inhibits Gq-dependent stimulation of adenylyl cyclase in cultured cells (12). Modified xanthine nucleotide-binding mutants of Gαs, (13–14) and Gα16 (15) inhibit signaling by Gq-coupled receptors when expressed in COS-7 cells, whereas xanthine nucleotide-binding mutants of Gα11 and Gα16 (15) inhibit Gi-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 Gαq inhibits Gq/11-coupled receptor signaling (18). Minigene plasmids encoding oligopeptides representing the carboxyl termini of Gαs, Gαi1/2, and Gα13 have recently been employed to determine the contribution of different G protein pools to signaling by M2 muscarinic and thrombin receptors (19, 20).

To determine whether an expressible peptide could be identified that interrupts signaling at the receptor-Gq interface, we have prepared a series of minigene constructs encoding varying length polypeptides derived from the carboxyl terminus of Gαq. In this paper, we characterize the mechanism of action and consequences of expression of an 83-amino acid Gαq carboxyl-terminal polypeptide (GqCT). We find that the GqCT peptide selectively inhibits receptor-Gq coupling in isolated plasma membranes and second messenger production in intact cells, without affecting Gαq11 or Gi signaling. When employed to examine the role of Gqα in regulation of the ERK MAP kinase cascade, we find that GqCT expression reveals both stimulatory and inhibitory effects of Gqα in response to activation of Gi, Giq, and Giq11-coupled adrenergic receptors. These data indicate that expression of peptides derived from the carboxyl terminus of Gαα can induce G protein-specific blockade of Gq-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), genetin (G418), and penicillin/streptomycin were from Invitrogen. FuGENE 6 was from Roche Molecular Biochemicals. 3',5'-[3H]AMP was from Amersham Biosciences. myo-3H]inositol and [35S]GTPγS were from PerkinElmer Life Sciences. Monoclonal anti-Gαs, anti-Gα3q11, and anti-Gαq11 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, H989, forskolin, isoproterenol, and 6-chloro-2B-PB-hydrobromide were from Sigma. Bordetella pertussis toxin was from List Biological. The cDNAs encoding the hamster α1aAR and the human α2aAR were provided by R. J. Leffkowitz. The cDNA encoding the human D1A dopamine receptor was from M. G. Caron. The cDNA encoding the bovine Gαq subunit was provided by A. G. Gilman.

**Construction of Minigenes Encoding the Gαq Carboxyl Terminus**—The construction of the Gαq peptide minigenes is depicted schematically in Fig. 1A. The PCR was employed to amplify cDNA encoding amino acids 286–395, 313–395, or 337–395 of bovine Gαq and the translation stop codon. Restriction sites at the 5′ and 3′ ends of the Gαq-derived sequence were incorporated into the oligonucleotides primers used for DNA amplification. For the construct encoding a Gαq-derived peptide capable of post-translational prenylation, the DNA sequence TGGCTCCTCTTCT, encoding the peptide sequence CVL5, was incorporated into the end of the cDNA minigene prior to the initiation of the hemagglutinin (HA) epitope-tagged constructs. The Gαq, carboxyl-terminal sequences were subcloned as EcoRI to SalI 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, Gαq, carboxyl-terminal sequences were subcloned into the pEgg vector, which encodes a carboxyl-terminal GST epitope. The minigene plasmid encoding the third intracellular domain of the human D1A dopamine receptor (D1a3) 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 minigene Gαq11 (1315–395), 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 Gαq carboxyl terminus. All assays on transiently transfected cells were performed after 48–72 h. Prior to assay, transfected cells were split into multwell 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, [35S]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 βAR competition binding analyses, membranes were resuspended in binding buffer (10 mM Tris-HCl, pH 7.4, 4 mM MgCl2 at a concentration of 0.5 µg of protein/ml. Membrane aliquots (20 µg of protein) were incubated with [3H]-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 GαqCT effects were performed under conditions of equal receptor expression, the level of α1aAR, α2aAR, βAR, and D1A dopamine receptor expression in HEK-293 and COS-7 cells was determined by saturation binding analysis, as described previously (11).

**[35S]GTPγS Loading of Gα Subunits**—Assays of [35S]GTPγS loading of endogenous Gαs, Gαq11, and Gαq12 subunits were performed on cell membranes prepared from HEK-293 cells. Membrane pellets were resuspended in TMF buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4) containing 2 mM EDTA and 4.8 mM MgCl2 for Gq loading assays or 100 mM EDTA and 120 mM MgCl2 for Gαq11 and Gαq12 loading assays. [35S]GTPγS loading was performed by incubating 25 µg of membrane protein in TME buffer, 1 µM GDP, and 30 mM [35S]GTPγS, plus agonist or vehicle, for 5 min at 30 °C in a total volume of 100 µl. Reactions were terminated by subheling the membranes for 30 min at 4 °C in IP/Stop buffer (150 mM NaCl, 5% Nonidet P-40, 20 mM MgCl2, 100 µM GDP, 100 µM GTP, 1% aprotinin, 50 mM Tris-Cl, pH 7.5). Specific G protein subunits were isolated by immunoprecipitation for 1 h at 4 °C using monoclonal antibodies specific for Gαs, Gαq11, or Gαq12, collected on protein A-Sepharose. Immune complexes on Sepharose were washed three times with IP/Stop buffer, and [35S]GTPγS bound to the immunoprecipitated Gα subunits was determined by liquid scintillation counting. To determine the presence of [35S]GTPγS binding, membranes were 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 Bio-trak [3H]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 nystatin (500 U/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 KHCO₃. Total inositol phosphates were isolated by anion exchange chromatography on Dowex AG 1-X8 columns and quantified by liquid scintillation spectrometry, 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 Multimag. 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 Ga₃ Inhibits Gs-coupled Receptor Signaling by Blocking Receptor-Gs Protein Coupling—** To create a peptide inhibitor of receptor-Gs coupling, we initially prepared a series of minigene constructs encoding 59, 83, and 110 amino acid polypeptides derived from the carboxyl terminus of bovine Ga₃. These polypeptides contain the major region of Ga₃ thought to mediate contact with the intracellular domains of GPCRs but lack the sequences that contact adenyl cyclases. As shown in Fig. 1A, each minigene was composed of a minimal Kozak sequence, followed by the Ga₃-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 Ga₃-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. 1B 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-Ga₃(313–395) (lane 2), HA-Ga₃(313–395) and a modified HA-Ga₃(313–395)-CVLS bearing the protein prenylation sequence CVLS at the carboxyl terminus (lane 4). As shown in Fig. 1C, transient transfection of COS-7 cells with increasing amounts of the HA-Ga₃(313–395) plasmid produced a progressive increase in peptide expression that reached levels significantly in excess of the expression of endogenous Ga₃ isoforms. As shown, minigene expression had no significant effect on the level of endogenous Ga₃ expression.

To determine whether expression of Ga₃-derived peptides affected signaling by a Gs-coupled GPCR, we measured basal and agonist-stimulated cAMP production in COS-7 cells transiently expressing the D₁A dopamine receptor and either the GST-Ga₃(313–395), HA-Ga₃(313–395), or HA-Ga₃(313–395)-CVLS minigenes. As shown in Fig. 1D, the GST-Ga₃(313–395) peptide had no significant effect on D₁A 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-Ga₃(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 Ga₃(313–395) (GstCT) for further characterization.

The basic unit of heptahelical receptor signaling consists of receptor, heterotrimeric G protein, and effector. As depicted schematically for the βAR-Gs-adenyl cyclase module in Fig.
Selective Peptide Inhibitor of Receptor-\(G_s\) Coupling

To elucidate further the mechanism of the inhibition produced by GsCT expression, we assayed the effect of the peptide on the affinity of \(\beta_2\)AR for agonist binding. As shown in Fig. 3A, the GsCT peptide, like the endogenous Go\(_s\), protein, was present almost exclusively in the plasma membrane fraction following cell fractionation. Neither agonist exposure nor coexpression of FLAG epitope-tagged \(\beta_2\)AR increased the amount of GsCT in the membrane fraction, suggesting that the peptide inherently partitions into the membrane. As shown in Fig. 3B, the GsCT peptide specifically immunoprecipitates with the FLAG-\(\beta_2\)AR 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 GTP\(_{\gamma}\)S, which causes irreversible dissociation of \(G\) protein subunits, only the low affinity state of the receptor is present. Fig. 3C compares competition binding curves generated for the displacement of the \(\beta_2\)AR antagonist \([^{125}\text{I}]-\text{cyanopindolol}\) by isoproterenol in COS-7 cells membranes in the presence of either GTP\(_{\gamma}\)S or GsCT. In control membranes, the competition binding curve fits a two-site model with the high affinity site containing 22% of the total specific \([^{125}\text{I}]-\text{cyanopindolol}\)-binding sites. In the presence of GTP\(_{\gamma}\)S, 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 \(\beta_2\)AR 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 \([^{35}\text{S}]\text{GTP}\gamma S\) loading of endogenous \(G\) proteins in membranes isolated from parental and GsCT-expressing HEK-293 cells. For these assays, endogenous \(\beta_2\)ARs or transiently expressed \(\alpha_{1B}\)ARs and \(\alpha_{2A}\)ARs were employed to stimulate the endogenous pools of Go\(_s\), Go\(_{\gamma/11}\), and Go\(_i\), respectively. As shown in Fig. 4A, membranes from GsCT-expressing cells showed a 72% decrement in the isoproterenol-induced increase in \([^{35}\text{S}]\text{GTP}\gamma S\) loading of Go\(_s\), compared with membranes from parental cells, with no significant effect on basal Go\(_s\) loading. As shown in Fig. 4, B and C, no significant differences in basal or agonist-stimulated \(G\) protein loading were observed when \(\alpha_{2A}\)AR-mediated Go\(_{\gamma1}\) and \(\alpha_{2A}\)AR-mediated Go\(_i\) loading in GsCT-expressing and parental cells were compared. Thus, GsCT expression led to Go\(_s\)-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 Gs, one would expect it to inhibit the generation of Gs-dependent, but not Go\(_{\gamma1}\)- or Gi-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. 5A depicts the effects of increasing GsCT expression on cAMP production in response to stimulation of coexpressed Go\(_{i}\)-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. 5B depicts the dose-response relationship for D1A 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 D1A3i. 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. 5C, similar, apparently non-competitive inhibition of β2AR-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 D1AR in COS-7 cells, and to a lesser
extent the endogenous β2AR 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-Gs coupling or to an additional receptor-independent effect of GsCT, we compared the effect of GsCT expression with that of the β2AR inverse agonist ICI118551 (23, 24). In these assays we employed a constitutively activated point mutant of the β2AR (β2ARonco). COS-7 cells were transfected with plasmid encoding the β2ARonco (2 μg/100-mm dish) plus either empty vector (control) or pcDNA3.1-HA-GsCT, pcDNA3.1-HA-GsCT (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. 

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 Gs 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 D1A receptor-mediated cAMP production did vary with the level of receptor expression.

The α2AR stimulates phosphatidylinositol (PI) hydrolysis primarily by Gq/11-dependent activation of the phospholipase C (PL-C) β1 isoform (25). As shown in Fig. 6A, α2AAR-mediated PI hydrolysis is unaffected by coexpression of increasing amounts of the GsCT polypeptide. The α2AAR weakly stimulates PI hydrolysis by Gq-dependent activation of the PL-C β2 and β3 isoforms (26). As shown in Fig. 6B, α2AAR-mediated PI hydrolysis is unaffected by coexpression of increasing amounts of the GsCT polypeptide. The α2AAR weakly stimulates PI hydrolysis by Gq-dependent activation of the PL-C β2 and β3 isoforms (26). As shown in Fig. 6B, α2AAR-mediated PI hydrolysis is unaffected by coexpression of increasing amounts of the GsCT polypeptide. The α2AAR weakly stimulates PI hydrolysis by Gq-dependent activation of the PL-C β2 and β3 isoforms (26).
Presented in arbitrary units, such that the basal amount of [3H]inositol/11006 of 1. In each panel, the data shown represent the mean phosphate detected in cells not expressing GsCT was assigned a value of 1. myocytes (28), and pancreatic acinar cells (29), and inhibition of ERK activity. In HEK-293 cells (27), cardiac agonist, UK14304 (10−6), was determined as described. Data are presented in arbitrary units, such that the basal amount of [3H]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.

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 Gα-coupled receptors, in that β2AR and D1A dopamine receptor cAMP production are similarly affected, but are specific for signals mediated by Gs, in that Gα11- and Gα-dependent PI hydrolysis is unimpaired.

**Use of GsCT to Examine the Contribution of Gs to ERK Activation by β2 Adrenergic, α1B Adrenergic, and α2A Adrenergic Receptors**—The role of Gs 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 Gs-coupled receptors can produce both stimulation and inhibition of ERK activity. In HEK-293 cells (27), cardiac myocytes (28), and pancreatic acinar cells (29), β2AR-mediated ERK activation involves both PKA and activation of pertussis toxin-sensitive G proteins. It has been proposed that phosphorylation of the β2AR by PKA switches receptor coupling from Gs to Gα, allowing the receptor to mediate pertussis toxin-sensitive ERK1/2 activation through a Gβγ 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 Gs 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 Gs-mediated signaling, we employed the construct to examine the contribution of Gs to ERK activation by β2, α2A, and α1B adrenergic receptors. For the β2AR, 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. 8A, β2AR-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 Gs 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. 8B, are consistent with the proposed requirement for Gs activation in β2AR signaling to ERK.

The α2AAR couples to Gαs family G proteins and in COS-7 cells mediates ERK activation through a pertussis toxin-sensitive pathway that is blocked by expression of a Gβγ subunit sequestor polypeptide derived from the carboxyl terminus of G protein-coupled receptor kinase 2 (34). However, the α2AAR

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**Fig. 6.** Effect of GsCT expression on agonist-stimulated PI hydrolysis by the Gsα11-coupled α1B and Gα2α2A adrenergic receptors. A, effect of increasing GsCT expression on agonist-stimulated PI hydrolysis in COS-7 cells transiently expressing α1BAR. Cells in 100-mm dishes were cotransfected the pRK5–α1BAR plasmid, along with the indicated amounts of pcDNA3.1-HA-GsCT (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 α2AAR. Cells in 100-mm dishes were cotransfected the pRK5–α2AAR plasmid, along with the indicated amounts of pcDNA3.1-HA-GsCT (313–395). PI hydrolysis in response to 1 h of exposure to the α2AAR agonist, UK14304 (10 μM), was determined as described. Data are presented in arbitrary units, such that the basal amount of [3H]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 β2AR. Activation of the Gs-adenyl cyclase (AC)-PKA pathway results in PKA-mediated phosphorylation of the β2AR. PKA phosphorylation increases receptor coupling to pertussis toxin-sensitive Gi proteins, resulting in Gβγ 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.
also couples to \( G_s \), particularly at high levels of receptor expression (35). In contrast to the \( \beta_2AR \), \( \alpha_2AAR \) 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 \( G_s \), 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. 9A, \( \beta_2AR \)s transiently expressed in COS-7 cells, like \( \beta_2AR \)s in HEK-293 cells, activate ERK1/2 via pertussis toxin-sensitive G proteins. However, in contrast to the \( \beta_2AR \) system, treatment with H89 enhances, rather than inhibits, \( \alpha_2AAR \)-mediated ERK activation. This presumably reflects relief of the inhibitory effect of PKA on ERK activation that results from phosphorylation of c-RafI. As shown in Fig. 9B, transfection of COS-7 cells with increasing amounts of the GsCT plasmid, like H89 treatment, caused a progressive enhancement of \( \alpha_2AAR \)-mediated ERK phosphorylation.

**FIG. 8.** Effect of GsCT expression on \( \beta_2AR \)-mediated ERK1/2 phosphorylation. A, effect of the PKA inhibitor H89 and pertussis toxin on \( \beta_2AR \)-stimulated ERK1/2 activation in HEK-293 cells. Cells in 6-well plates were preincubated with H89 (10 \textmu M) for 30 min or with pertussis toxin (100 ng/ml, PTX) for 16 h, prior to 5 min of stimulation with isoproterenol (Iso) (10 \textmu M). 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 \( \beta_2AR \)-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 \textmu M) 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 \pm S.E. for three separate experiments. * p < 0.05.

**FIG. 9.** Effect of GsCT expression on agonist-stimulated PI hydrolysis by the \( G_{q11} \)-coupled \( \alpha_1BAR \) and \( G_i \)-coupled \( \alpha_2AAR \). A, effect of the PKA inhibitor H89 and pertussis toxin on \( \alpha_2AAR \)-stimulated ERK1/2 phosphorylation in COS-7 cells. Cells in 6-well plates were preincubated with H89 (10 \textmu M) for 30 min or with pertussis toxin (100 ng/ml, PTX) for 16 h, prior to 5 min of stimulation with UK14304 (10 \textmu M). 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 \( \alpha_2AAR \)-mediated ERK1/2 activation in COS-7 cells. Cells in 100-mm dishes were cotransfected the pRK5-\( \alpha_2AAR \) plasmid, along with the indicated amounts of pcDNA3.1-HA-G\( \alpha_2 \)-(313–395), prior to passage into 6-well plates. Phospho-ERK1/2 levels after 5 min of stimulation with UK14304 (10 \textmu M) were determined as described. C, effect of increasing GsCT expression on \( \alpha_1BAR \)-stimulated ERK1/2 activation in COS-7 cells. Cells in 100-mm dishes were cotransfected the pRK5-\( \alpha_1BAR \) plasmid, along with the indicated amounts of pcDNA3.1-HA-G\( \alpha_1 \)-(313–395), prior to passage into 6-well plates. Phospho-ERK1/2 levels after 5 min of stimulation with phenylephrine (1 \textmu M) were determined as described. B and C, the radiograph depicts a representative immunoblot. Data shown represent the mean \pm S.E. for three separate experiments. *, greater than control, p < 0.05.
Selective Peptide Inhibitor of Receptor-\(G_s\) Coupling

In COS-7 cells, stimulation of transiently expressed \(\alpha_{12}\)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 \(G_o\) (18). As shown in Fig. 9C, transfection of COS-7 cells with increasing amounts of the GsCT plasmid had no effect on \(\alpha_{12}\)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 \(G_o\) has indicated that receptor coupling specificity is likely determined by a surface formed by the continuous carboxyl-terminal \(\alpha\)-helix between Asp-368 and Leu-394, and the loop between the \(\alpha5\)-helix and \(\beta6\)-strand (6). Contact between the \(G_o\) subunit and the second and third intracellular domains of heptahelial receptors determines the efficiency and specificity of the receptor-G protein interaction (3, 37, 38). The \(G_o\) subunit carboxyl-terminal helix may insert into a cavity between the third and sixth receptor transmembrane domains of the heptahelial receptor bundle that forms as a consequence of agonist-induced conformational changes (39). NMR studies have demonstrated that short polypeptides derived from the \(G_o\) carboxyl terminus form stable \(\alpha\)-helices in solution. In isolated plasma membranes, 11-amino acid peptides representing the carboxyl termini of \(G_{o1/2}\) or \(G_o\) 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 \(G_o\) inhibit high affinity agonist binding to the adenosine \(A_{1A}\) receptors, and impair \(A_{2A}\) receptor-mediated adenylyl cyclase activation (17). These data suggest that the isolated carboxyl-terminal \(\alpha\)-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 \(G_o\) in intact cells. Expression of the GaCT peptide impaired adenylyl cyclase activation by \(G_{o}\)-coupled \(\beta_2\) adrenergic and \(D_{1A}\) dopamine receptors, without affecting the response to cholina 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 \(G_o\) pool for access to ligand-occupied receptors. Furthermore, the inhibition was apparently specific for \(G-o\) because PI hydrolysis induced by stimulation of the \(G_{q11}\)-coupled \(\alpha_{12}\)AR and \(G_{i}\)-coupled \(\alpha_{2A}\)AR was unaffected by GsCT expression.

A significant aspect of the approach of using receptor- or G protein-derived peptides to inhibit heptahelial 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 Ga 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 \(G_o\), \(G_{o2}\), \(G_{o3}\), and \(G_o4\) 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 GaCt expression to examine the role of \(G_o\) in a complex process, activation of the ERK MAP kinase cascade in fibroblasts, where \(G_o\) activation has been reported previously to produce both stimulation and inhibition of ERK activity. Consistent with previous reports, we found that \(\beta_o\)-mediated ERK activation, which is blocked by PKA inhibition, is inhibited by GaCT, whereas \(\alpha_{2A}\)-mediated ERK activation, which is accentuated by PKA inhibition, is enhanced in cells expressing GaCT (27–33). These data support a dual role for PKA in ERK activation by the \(\beta_o\)AR, where PKA phosphorylation of the receptor promotes receptor-G coupling and pertussis toxin-sensitive ERK activation (27), but where PKA phosphorylation of Ra1 attenuates ERK activation downstream of Ras (30–33). In the case of the constitutively \(G_o/G_i\)-coupled \(\alpha_{2A}\)AR, only the downstream inhibitory effect of \(G_o\) activation, which is relieved by GaCT 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 \(G_o\) 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 \(G_{q11}\) proteins in this process. Recombinant adenosviruses-mediated expression of a \(G_{\beta\gamma}\) subunit sequestering 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 \(G_o\) signaling, such as the GaCt mimige, may provide the opportunity to obtain similar insights into the complex roles of \(G_o\) in control of cellular hypertrophy and proliferation in a variety of tissues.

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