A Dominant-Negative Strategy for Studying Roles of G Proteins in Vivo*

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G proteins play a critical role in transducing a large variety of signals into intracellular responses. Increasingly, there is evidence that G proteins may play other roles as well. Dominant-negative constructs of the α subunit of G proteins would be useful in studying the roles of G proteins in a variety of processes, but the currently available dominant-negative constructs, which target Mg2+-binding sites, are rather leaky. A variety of studies have implicated the carboxyl terminus of G protein α subunits in both mediating receptor-G protein interaction and in receptor selectivity. Thus we have made minigene plasmid constructs that encode oligonucleotide sequences corresponding to the carboxyl-terminal undecapeptide of Goα, Gaqα, or Gαs. To determine whether overexpression of the carboxyl-terminal peptide would block cellular responses, we used as a test system the activation of the M2 muscarinic receptor activated K+ channels in HEK 293 cells. The minigenes were transiently transfected along with G protein-regulated inwardly rectifying K+ channels (GIRK) into HEK 293 cells that stably express the M2 muscarinic receptor. The presence of the Goα carboxyl-terminal peptide results in specific inhibition of GIRK activity in response to agonist stimulation of the M2 muscarinic receptor. The Goα minigene construct completely blocks agonist-mediated M2 mACHR K+ channel response whereas the control minigene constructs (empty vector, pcDNA3.1, and the Ga carboxyl peptide in random order, pcDNA-GaR) had no effect on agonist-mediated M2 muscarinic receptor GIRK response. The inhibitory effects of the Goα minigene construct were specific because overexpression of peptides corresponding to the carboxyl terminus of Goα or Gaqα had no effect on M2 muscarinic receptor stimulation of the K+ channel.

Many biologically active molecules transduce their signals through heptahelical receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins).1 G proteins play important roles in determining the specificity and temporal characteristics of a variety of cellular responses. Upon activation, G protein-coupled receptors (GPCRs) interact with their cognate heterotrimeric G protein, inducing GDP release with subsequent GTP binding to the α subunit. The exchange of GDP for GTP leads to dissociation of the Gβγ dimer from the Ga subunit, and both initiate unique intracellular signaling responses (for review, see Refs. 1 and 2). Molecular cloning has resulted in the identification of 18 distinct Ga subunits that are commonly divided into four families based on their sequence similarity: Gαq, Gαi, Gαz, and Gα12. Similarly, multiple Gβ (5) and Gγ (11) subunits have been identified.

In all G proteins studied GTP is bound as a complex with Mg2+ and the GTP- and Mg2+-binding sites are tightly coupled. Dominant-negative constructs of the α subunit of G proteins have been made in which mutations are made in residues that contact the magnesium ion. Although this approach was quite successful with p21ras and other small G proteins (3, 4), dominant-negative Goα, Goqα, Gαs, and Gαi1 have been less effective (5–10). This is probably because of the degree to which Mg2+ is necessary to support GTP binding. p21ras forms a tight and nearly irreversible GDP-Mg2+ complex, whereas Goα subunits bind Mg2+ in the GDP-Mg2+ complex with lower affinity than in the GTP-Mg2+ complex (11–14).

Specific determinants of receptor-G protein interaction have been under investigation for many years. It is thought that there are multiple sites of contact between the activated receptor and the G protein. Studies using ADP-ribosylation by pertussis toxin, site-directed mutagenesis, peptide-specific antibodies, and chimeric proteins indicate that the carboxyl terminus of the Ga subunit is not only an essential region for receptor contact, but is also important for determining G protein receptor specificity (reviewed in Refs. 1 and 15). The crystal structures of various Ga subunits show that the last 4–7 amino acids of Go were not observed (16–22) indicating that the region is conformationally flexible in the absence of other interactions.

In vitro assays, as well as microinjection studies of intact cells, indicate Ga carboxyl-terminal peptides can competitively block G protein-coupled downstream events (23–26). A carboxyl-terminal peptide from Goα not only binds, but will also directly stabilize photoactivated rhodopsin (27, 28). Using a combinatorial peptide library Martin et al. (29) have shown that specific residues within the carboxyl terminus of Goα are critical for high affinity binding of the Goα peptide to rhodopsin. Similarly, a carboxyl-terminal peptide from Goα (384–394), but not corresponding peptides from Goq12, inhibits the ability of β2-adrenergic receptors to activate Goq and adenyl cyclase (30). In addition, a carboxyl-terminal undecapeptide from

1 The abbreviations used are: G proteins, guanine nucleotide-binding proteins; GPCR, G protein-coupled receptor; IP, inositol phosphate; HEK, human embryonic kidney; mACHR, muscarinic receptor; ACh, acetylcholine; IiLACH, inwardly rectifying K+ channel; GIRK, G protein-regulated inwardly rectifying K+ channel; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).
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Gαi1/2 can bind the adenosine A1 receptor, whereas the corresponding peptide from Goα, which differs by only 1 amino acid residue does not (31). Thus, the carboxyl terminus of G protein α subunits is critical in both mediating receptor-G protein interactions and in receptor selectivity (31–35).

“Minigene” plasmid vectors are constructs designed to express relatively short polypeptide sequences following their transfection into mammalian cells. Minigenes have been used by investigators to look at a variety of responses related to G proteins including (i) binding of pleckstrin homology (PH) domains to Gβγ (36), (ii) inhibiting GPCRs by expressing the carboxyl terminus of β2 adrenergic receptor kinase (37–39), and (iii) identifying intracellular domains of GPCRs critical for G protein coupling (40–44). Experiments using minigenes that express the last 55 amino acids of Goα to target the receptor-Gq interface to achieve class-specific inhibition were recently published by Akhter et al. (45). Transient transfection of COS-7 cells with α1B-adrenergic receptors or M1 muscarinic receptors and the Goq carboxyl-terminal minigene (residues 305–359) inhibits agonist stimulated inositol phosphate (IP) production, whereas co-expression with the Goq amino terminus (residues 1–54) has no effect. Inhibition by Goq (305–359) was apparent for Gq-coupled receptors because neither α2α- adrenergic receptor-mediated IP production (Gβγ-coupled), nor dopamine D1A receptor-mediated cAMP production (Gγ-coupled) were inhibited. In addition, transgenic mice made by targeting the Goq carboxyl-terminal minigene to the myocardium resulted in a marked inhibition of α1B-adrenergic receptor-mediated IP production and blockade of cardiac hypertrophy.

In this paper, we study the effects of several carboxyl termini Goα peptides using a minigene approach. To test whether minigene constructs encoding the carboxyl-terminal 11 amino acid residues from Goα subunits could effectively inhibit G protein-coupled receptor-mediated cellular responses, we chose a system in which 1) the importance of the carboxyl terminus and 2) the downstream effector system had been well established. Numerous studies (46–49) have shown that the M2 muscarinic receptor (mACHR) couples exclusively to the Gq/11 family. The M2 mACHR can efficiently couple to mutant Goαq in which the last 5 amino acids of Goαq are substituted with the corresponding residues from Goq or Goα (34), suggesting that this receptor contains domains that are specifically recognized by the carboxyl terminus of Goαq subunits. The effector system that we selected was the M2 mACHR-activated inwardly rectifying K+ channel (I_KACH). In cardiac cells, the I_KACH channel is formed as a heterotetramer of G protein-regulated inwardly rectifying K+ channels (GIK), with two GIK1 and two GIK4 subunits (50, 51). This channel is activated upon stimulation of M2 mACHR in a manner that is completely pertussis toxin-sensitive and is the prototype for a direct Gβγ-activated channel (52–54). Our experiments indicate that the Goαi carboxyl terminus minigene construct can completely block M2 mACHR-mediated K+ channel activation. The inhibition appears specific as constructs producing Goαi, Goαq, or a scrambled Goαi carboxyl-terminal peptide had no effect.

**MATERIALS AND METHODS**

**Construction of Go Carboxyl-terminal Minigenes**—The cDNA encoding the last 11 amino acids of human Goα1 subunits (Goα1/2, Goa1, Goa2) or the Goαq carboxyl terminus in random order (GoαR) were synthesized (Great American Gene Co.) with newly engineered 5′- and 3′-ends (Fig. 1). The 5′-end contained a BamHI site followed by the ribosome binding consensus sequence (5′-GCGGCCGACC-3′), a methionine (ATG) for translation initiation, and a glycine (GGA) to protect the ribosome binding site during translation and the nascent peptide against proteolytic degradation. A HindIII site was synthesized at the 3′-end immediately following the translational stop codon (TGA).

The DNA was brought up in sterile ddH2O (stock concentration 100 µM). Complimentary DNA was annealed in 1× NEBuffer 3 (50 mM Tris·HCl, 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol; New England Biolabs) at 85 °C for 10 min then allowed to cool slowly to room temperature. The annealed cDNA were ligated for 1 h at room temperature into plasmid vector (pDNA 3.1–+) (Promega) with BamHI and HindIII. After digestion with each restriction enzyme, the plasmid DNA was run on a 0.8% agarose gel, the appropriate band cut out, and the DNA purified (GeneClean II Kit, Bio101). For the ligation reaction the ratio of insert to vector was approximately 25 µg to 50 ng, respectively. Following ligation, the sample was heated to 65 °C for 5 min to deactivate the T4 DNA polymerase.

Ligation reaction (1 ml) was electroporated into 50-µl competent ARI814 cells (Bio-Rad) (Escherichia coli Pulsar; 29) and there were cells immediately plated into 1 ml of SOC (Life Technologies, Inc.) for 1 h at 37 °C, 100 µl was spread on LB/Amp plates and incubated at 37 °C for 12–16 h. To verify that insert was present, several colonies were grown overnight in LB/Amp and their plasmid DNA purified (Qiagen SpinKit). The plasmid DNA was digested with NcoI (New England Biolabs, Inc.) for 1 h at 37 °C and run on a 1.5% agarose gel. Vector alone produced 3 bands (3.4, 1.3, and 0.7 kilobases), whereas vector with insert resulted in 4 bands (3.4, 1.0, 0.7, and 0.3 kilobases). DNA with the correct pattern was sequenced (Northwestern University BioTechnology Center) to confirm the appropriate sequence. The Goαi minigenes used for transfection (45) were: pcDNA3.1-Goαq, pcDNA3.1-GoαR, pcDNA3.1-Goα, and pcDNA3.1 were purified from 500-ml cultures using endotoxin-free maxi-prep kits (Qiagen).

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells, stably expressing the M2 mACHR (~400 fmol receptor/mg protein) (55) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and G418 (500 µg/ml; Life Technologies, Inc.). Cells were grown under 10% CO2 at 37 °C. In all transfections for electrophysiological studies the CD8 reporter gene system was used to visualize transected cells (56). Dynabeads coated with anti-CD8-antibodies were purchased from Dynal. A standard calcium phosphate procedure was used for transient transfection of HEK cells. The following amounts of cDNA were used for transient transfections (unless otherwise indicated): M2 mACHR (human), 1 µg; pc11-GIRK1 (rat), 1 µg; pcDNA1-GIRK4 (rat), 1 µg; pcDNA3.1, pcDNA-Goα, pcDNA-Goα-R, pcDNA-Goα, or pcDNA-GoR, 4 µg. Typically the total amount of cDNA used for transfecting one 10-cm dish was 7 µg. All assays were performed 48–72 h posttransfection. The cDNAs for the GIRK1 and GIRK4 were gifts from F. Lesage and M. Lasiska (N掛け, France). pcDNA for CD8 was from G. Yellen (Harvard University, Boston, MA).

To determine minigene RNA expression, transiently transfected cells were washed twice with phosphate-buffered saline, lysed with 350 µl of RLT lysis buffer (Qiagen, Rneasy Mini Kit), homogenized using a QIAshredder column (Qiagen), and total RNA was processed according to the manufacturer’s protocol. Total RNA was eluted in diethyl pyrocarbonated water, quantitated, and stored at −20 °C. PCRs were made from total RNA using a reverse transcribed polymerase chain reaction (RT-PCR) (CLONTECH Advantage RT-for-PCR kit) according to the manufacturer’s protocol. To verify the presence of insert in cells transfected with pcDNA-Goα or pcDNA-Goα-R constructs, their cDNA was used as the template for PCR with forward and reverse primers that correspond to Go insert and vector, respectively (forward: 5′-ATTCCGCGGCCGACCATGGGGA; reverse: 5′-GGCAAGAGGACCGGGCGCT-A). The primers for the Goα minigenes amplify a 434-bp fragment only if the insert carboxyl termini oligonucleotides are present; no band is observed in cells transfected with empty vector (pcDNA3.1). As controls, PCR was also performed using T7 forward with the vector reverse primer, which amplified a 486-bp fragment in all cDNA tested or GSDPH primers (CLONTECH), which amplified a 953-bp fragment in all cDNA tested.

Additionally, transiently transfected cells were trypsinized, pelleted, washed twice with phosphate-buffered saline, and stored at −80 °C. Cellular extracts were prepared by homogenizing the cell pellets for 15 s (ESGE Bio-homogenizer M133/1281–0) in fractionation buffer (10 mM Tris·HCl, pH 7.5, 15.5 mM NaCl, 1 mM EDTA, 1 mM G75β, 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 3,000 × g for 20 min, and the supernatant centrifuged at 100,000 × g for 30 min. The cytosolic fraction from the resulting supernatant was collected; and the fractions stored at −80 °C until needed. For high pressure liquid chromatography analysis, 100 µl of cytosolic extract was loaded onto a C4 column (Vydac) equilibrated with 0.1% trifluoroacetic acid in ddH2O. Elution of the peptide was performed using 0.1% trifluo-
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orocetic acid in acetonitrile. The amount of acetonitrile was increased from 0 to 60% over 45 min. Peaks were collected, lyophilized, and analyzed using ion mass spray analysis (University of Illinois-Urbana Champaign).

Measurement of $I_{K_{ACh}}$ Currents—For the measurement of inwardly rectifying $K^+$ current, whole cell currents were recorded as described previously (57, 58). The extracellular solution contained 120 mM NaCl, 20 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM Hepes-NaOH, pH 7.4. The solution for filling the patch pipettes was composed of 100 mM potassium glutamate, 40 mM KCl, 5 mM MgATP, 10 mM Hepes-KOH, pH 7.4, 5 mM NaCl, 2 mM EGTA, 1 mM MgCl$_2$, and 0.01 mM GTP. All standard salts as well as acetylene were from Sigma.

To minimize variations caused by different transfections or culture conditions, control experiments (transfection with pcDNA-GaR) were done in parallel. Membrane currents were recorded under voltage clamp, using conventional whole cell patch techniques (59). Patch pipettes were fabricated from borosilicate glass capillaries, (GF-150–10, Warner Instrument Corp.) using a horizontal puller (P-85 Fleming & Poulsen) and were filled with the solutions listed above. The DC resistance of the filled pipettes ranged from 3 to 6 mega-ohms. Membrane currents were recorded using a patch-clamp amplifier (Axopatch 200, Axon Instruments). Signals were analog filtered using a low pass Bessel filter (1–3 kHz corner frequency). Data were digitally stored using an IBM compatible PC equipped with a hardware/software package (pClamp; Molecular Devices, Sunnyvale, CA). For voltage control, data acquisition, and data evaluation.

To measure $K^+$ currents in the inward direction, the potassium equilibrium potential was set to about −50 mV and the holding potential was −90 mV as described (57, 58). Agonist-induced currents were evoked by application of acetylcholine (ACh; 1 μM) using a solenoid-operated superfusion device, which allowed for solution exchange within 300 ms. Linear voltage ramps (from −120 mV to +60 mV within 500 ms) applied every 10 s. By subtracting nonagonist-dependent currents we were able to resolve the current voltage properties of the agonist-induced currents. For analysis of the data the maximal current density (peak amplitude) of ACh-induced inwardly rectifying $K^+$ currents were measured at −90 mV and compared.

Data Analysis—Data are presented as mean ± S.E. The statistical differences were determined using the Student’s $t$ test (GraphPad Prism; version 2.0).

RESULTS AND DISCUSSION

Dominant-negative constructs of the $α$ subunit of G proteins have been made in which mutations are made in regions that contact the magnesium ion. For the $α$ subunit of G proteins, this includes mutations of the Gly residue within the invariant sequence (G203T, G204A), as well as mutations of a Ser residue that include mutations of the Gly residue within the invariant sequence (G203T, G204A). As shown in Fig. 1, when insert is present there is a new NcoI site resulting in a shift in the band pattern, such that the digest pattern goes from three bands (3345, 1352, and 735 bp) to four bands (3345, 1011, 735, and 380 bp).

As our minigene approach depends on competitive inhibition, a key element for success is the expression of adequate amounts of peptides to block intracellular signaling pathways. To confirm the presence of the minigene constructs in transfected cells, total RNA was isolated 48 h posttransfection, cDNA made with RT-PCR, and PCR analysis was performed using the cDNA as template with primers specific for the Ga carboxyl-terminal peptide insert. Separation of the PCR products on 1.5% agarose gels (Fig. 2A) indicates the presence of the Ga carboxyl terminus peptide minigene RNA by a single 434-bp band. Control experiments were done using a T7 forward primer with the vector reverse primer to verify the presence of the pcDNA3.1 vector, and G3DPH primers (CLONTECH) to approximate the amount of total RNA (data not shown).

To verify that the peptide was being produced in the transfected cells, 48 h posttransfection, cells were lysed and homogenized. Cytosolic extracts were analyzed by high pressure liquid chromatography, and peaks (Fig. 2B) were analyzed by ion mass spray analysis. The mass spectrometer analysis for peak 1 from the pcDNA-GaR transfected cells, and peak 1 from cells transfected with a vector expressing the carboxyl terminus in random order (pcDNA-GaR) indicate that a 1450 molecular weight peptide was found in both cytosolic extracts. This is the expected molecular weight for both 13 amino acid peptide sequences. The fact that they were the major peptides found in the cytosol from cells transiently transfected with the pcDNA-GaR or pcDNA-GaR vectors strongly suggests that the vectors are producing the appropriate peptide sequences. Therefore, analysis of the transiently transfected HEK 293 cells indicates (1) minigene vectors are present, and (2) the corresponding peptides are being expressed.

We examined whether the presence of the Ga carboxyl-terminal peptide minigene would result in a significant inhibition of a downstream functional response following agonist stimulation of the transfected cells. G protein-regulated inwardly rectifying $K^+$ channels modulate electrical activity in many excitable cells (for review, see Refs. 60–62). Because the channel opens as a consequence of a direct interaction with $G_{αi}$, whole cell patch clamp recording of inwardly rectifying $K^+$ currents can be used as a readout of G protein activity in single intact cells. Thus, we tested whether the Ga carboxyl-terminal peptide minigenes could inhibit $M_1$ mACHR activation of inwardly rectifying $K^+$ currents. Superfusion of HEK 293 cells transiently transfected with GIRKI1/GIRK4 and either pcDNA-GaR or pcDNA-GaR DNA with 1 μM ACh re-
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Fig. 1. The eDNA minigene constructs. Insert DNA, all Go carboxyl-terminal peptide minigenes contain a BamHI restriction enzyme site at the 5’-end followed by a ribosomal binding site sequence, a methionine for translation initiation, a glycine for stabilization of the peptide, the peptide sequence, a stop codon, and a HindIII restriction enzyme site at the 3’-end. The GoR contains the GoR carboxyl peptide sequence in random order. Vector, following annealing, complimentary oligonucleotides were ligated into BamHI/HindIII cut pcDNA 3.1 plasmid vector, and the ligated insert/vector DNA was electroporated into competent cells. Neol digest, plasmid DNA was purified, digested with Neol, and separated on a 1.5% agarose gel to determine whether insert was present. Lane 1 is a 1-kilobase pair DNA ladder; lane 2 is pcDNA3.1; lane 3 is pcDNA-Go; lane 4 is pcDNA-GoR; and lane 5 is pcDNA-GoQ. When insert is present there is a new Neol site resulting in a shift in the band pattern, such that the digest pattern goes from three bands (3345, 1352, and 735 bp) to four bands (3345, 1011, 735, and 380 bp).

vealed that cells transfected with pcDNA-GoQ DNA have a dramatically impaired response to the M2 mACHR agonist (Fig. 3). Fig. 3, A and B shows representative recordings of whole cell membrane currents at −90 mV. Superfusion of the cells with ACh activates inward currents in cells transfected with pcDNA-GoQ-R (Fig. 3A) but not in cells transfected with pcDNA-GoQ (Fig. 3B). The inwardly rectifying IV-curve for the ACh-induced current from the experiment shown in Fig. 3A is illustrated in Fig. 3D. The strong inwardly rectifying properties of this current is characteristic of IKACh channels. Summarized data for the maximum amplitude of ACh-evoked currents are shown for three different transfection conditions as indicated by the black bars. The maximum current evoked by ACh was 3.7 ± 1.5 pA/pF (n = 14) in cells transfected with the pcDNA-GoQ compared with 24.1 ± 8.8 pA/pF (n = 11) in cells transfected with pcDNA-GoQ-R. As a control we transfected cells with empty vector (pcDNA3.1). The ACh responses in these cells (16.5 ± 7.7 pA/pF (n = 5)) was not significantly different from the responses measured in cells transfected with pcDNA-GoQ-R (Fig. 3C). Basal levels for all three conditions were equivalent (pcDNA 3.2 ± 1.8 pA/pF (n = 5); GoQ 6.1 ± 0.9 pA/pF (n = 14); GoR 5.6 ± 2.0 pA/pF (n = 10)). To exclude experiments in which we recorded currents from cells that may not have expressed the functional channel, only those cells that exhibited a basal nonagonist-dependent Ba2+ (200 μM) sensitive inwardly rectifying current were used for analysis. Thus, it appears that the GoQ minigene construct completely blocks the agonist-mediated M2 mACHR GIRK1/4 response, whereas the control minigene constructs (empty vector, pcDNA3.1, and the GoQ carboxyl peptide in random order, pcDNA-GoQ-R) had no effect on the agonist-mediated M2 mACHR GIRK1/4 response.

The cardiac IKACh channel is activated upon stimulation of M2 mACHR via G proteins of the Gq family. The carboxy-terminal region of Go has also been shown to be critical in determining the specificity of GPCR-G protein interactions (34, 63). Substitution of 3–5 carboxy-terminal amino acids from Go, with corresponding residues from Go allowed receptors that signal exclusively through Go subunits to activate the chimeric α subunits and stimulate the Go effector, phospholipase C-β. To determine whether carboxy-terminal peptides from other classes of G proteins could inhibit the agonist-mediated M2 mACHR GIRK1/4 response, we transiently transfected HEK 293 cells stably expressing the M2 mACHR with GIRK1/GIRK4 and with minigene constructs encoding Go carboxyl termini for GoQ or GoQ. ACh-stimulated IKACh currents from cells transfected with pcDNA-GoQ (Fig. 4B; 19.5 ± 5.5 pA/pF (n = 6)) or pcDNA-GoQ (Fig. 4C; 35.5 ± 9.7 pA/pF (n = 5)) were not significantly different from those of cells transfected with the control minigene vector, pcDNA-GoQ-R (23.7 ± 10.5 pA/pF (n = 6) and 26.0 ± 7.9 pA/pF (n = 5), respectively). This is very different from cells transfected with pcDNA-GoQ whose ACh-stimulated IKACh currents were significantly de-
creased as compared with cells transfected with pcDNA-GαiR (Fig. 4A; 3.7 ± 1.5 pA/pF (n = 14) versus 24.1 ± 8.8 pA/pF (n = 11)). These findings confirm the specificity of the inhibition of M2 mACHR-activated G protein-coupled I\textsubscript{K,ACH} responses by expression of the Gαi minigene, pcDNA-Gαi.

Recent experiments targeting receptor-G protein interaction (45) by constructing minigenes that encode the last 55 amino acids of Gαq also indicate that class-specific inhibition can be achieved. However, transient transfection experiments with this construct resulted in only a 27–48% inhibition of inositol

FIG. 2. Transient transfection of minigene vectors. HEK 293 cells that stably express M\textsubscript{2} mACHR were transiently transfected with DNA from GIRK1/4 and pcDNA3.1, pcDNA-Gαi, or pcDNA-GαiR. A, to confirm the presence of the minigenes in the transiently transfected HEK 293 cells, total RNA was isolated 48 h posttransfection (Qiagen RNeasy Kit with QIAshredder). The cDNA was made through RT-PCR (CLONTECH). The PCR analysis was completed using the cDNA as template with primers specific for the Go carboxyl-terminal peptide insert. Separation of the PCR products on 1.5% agarose gels indicates the presence of the Go carboxyl terminus peptide minigene RNA by a single 494-bp band. Lane 1 is a 1-kilobase pair DNA ladder; lane 2 is PCR products from cells transfected with pcDNA-GαiR; lane 3 is cells transfected with pcDNA-Gα; lane 4 is cells transfected with pcDNA3.1. B, to verify that the peptide was being produced in the transiently transfected cells, the cells were lysed 48 h posttransfection, homogenized, and cytosolic extracts analyzed by HPLC. Peaks from cells transfected with pcDNA3.1, pcDNA-Gαi, or pcDNA-GαiR were analyzed by ion mass spray analysis.

FIG. 3. Minigenes encoding carboxyl-terminal Go peptides inhibit M\textsubscript{2} mACHR activated I\textsubscript{K,ACH}. HEK 293 cells stably expressing the M\textsubscript{2} mACHR were transiently transfected with DNA from GIRK1/4 and pcDNA3.1, pcDNA-Go1, or pcDNA-GoαiR. A, a representative example of the activation of inwardly rectifying K\textsuperscript{+} currents upon superfusion of 1 μM ACh in a HEK 293 cell transiently transfected with GIRK1, GIRK4, and pcDNA-GαiR DNA. B, a representative example of the activation of inwardly rectifying K\textsuperscript{+} currents upon superfusion of 1 μM ACh in a HEK 293 cell transiently transfected with GIRK1, GIRK4, and pcDNA-Gαq DNA. C, the maximum current evoked by ACh (black bars) was 3.7 ± 1.5 pA/pF (n = 14) in cells transfected with the pcDNA-Gαq compared with 16.5 ± 7.7 pA/pF (n = 5) in cells transfected with empty vector (pcDNA3.1) or 24.1 ± 8.8 pA/pF (n = 11) in cells transfected with pcDNA-GαiR. The white bars represent the basal Ba\textsuperscript{2+}-sensitive currents. D, the current voltage relation of the ACh-induced current shows the characteristic inward rectification and a reversal potential near the potassium equilibrium potential typical for I\textsubscript{K,ACH}.
phosphate accumulation. Our Go1 minigene constructs that encode only the last 11 amino acids of the carboxyl terminus of Go1/2 resulted in an 85% inhibition of the I_{Kach} response. This difference may be caused by variations in the length of the expressed minigene (55 versus 11 residues). The longer peptide may fold in such a way that the critical carboxyl-terminal region is partly buried. We have shown that shorter peptides can effectively bind to receptors (27, 29–31). Because the extreme carboxyl terminus of Go subunits in their GDP-bound conformation is disordered in crystal structures (16, 20), the smaller peptide may be able to fit into its binding site more effectively. Alternatively, the difference may be in the amount of peptide being expressed because of differences in methods of transfection and cell type being studied.

Molecular determinants other than the carboxyl terminus are also involved in the recognition between heterotrimeric G proteins and their cognate receptors (27, 64). However, a variety of studies have shown that the carboxyl terminus of G protein α subunits is critical in both mediating receptor-G protein interaction and in receptor selectivity (31–35). Our results confirm that the carboxyl terminus of Go is able to block agonist-mediated responses completely and thus is important in receptor selectivity and specificity. Most importantly, this method appears to be a promising approach for completely turning off G protein-mediated responses in transfected cells and in vivo. Transfection of different Go carboxyl-terminal peptide should allow us to selectively block signal transduction through any G protein and thus provides a novel dominant-negative strategy. We have now made minigene constructs encoding Go carboxyl-terminal undcapeptide sequences for each of the Go subunits. These minigenes should provide an effective dominant-negative approach that will allow us to define new roles of G proteins in vivo. The approach may also allow us to explore the coupling mechanisms of receptors that interact with multiple G proteins and tease out the downstream responses mediated by each G protein.

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**Fig. 4.** Transfection of the Go α carboxyl-terminal minigene inhibits the M₃ mAChR activated I_{Kach} response, whereas Go₅ or Go₆ carboxyl-terminal minigenes do not. Stably m2 mAChR-expressing HEK 293 cells were transiently transfected with GIRK1, GIRK4 and pcDNA-Gq, pcDNA-Gq, pcDNA-Gq, or pcDNA-Gq. A, the maximum current evoked by 1 μM ACh was 3.7 ± 1.5 pA/pF (n = 14) in cells transfected with pcDNA-Gq compared with 24.1 ± 8.8 pA/pF (n = 11) in cells transfected with pcDNA-Gq. B, the maximum current evoked by ACh was 19.5 ± 5.6 pA/pF (n = 6) in cells transfected with the pcDNA-Gq compared with 23.8 ± 10.5 pA/pF (n = 6) in cells transfected with pcDNA-Gq, R. C, the maximum current evoked by ACh was 35.5 ± 9.7 pA/pF (n = 5) in cells transfected with pcDNA-Gq compared with 26.0 ± 8.0 pA/pF (n = 5) in cells transfected with pcDNA-Gq.
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