RGS3 and RGS4 Differentially Associate with G Protein-coupled Receptor-Kir3 Channel Signaling Complexes Revealing Two Modes of RGS Modulation

PRECOUPLING AND COLLISION COUPLING

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RGS3 and RGS4 are GTPase-activating proteins expressed in the brain and heart that accelerate the termination of Gi/o- and Gq-mediated signaling. We report here the determinants mediating selective association of RGS4 with several G protein-coupled receptors (GPCRs) that form macromolecular complexes with neuronal G protein-gated inwardly rectifying potassium (Kir3 or GIRK) channels. Kir3 channels are instrumental in regulating neuronal firing in the central and peripheral nervous system and pacemaker activity in the heart. By using an epitope-tagged degradation-resistant RGS4 mutant, RGS4(C2V), immunoprecipitation of several hemagglutinin-tagged Gi/o-coupled and Gq-coupled receptors expressed in Chinese hamster ovary (CHO-K1) cells readily co-precipitated both Kir3.1/Kir3.2a channels and RGS4(C2V). In contrast to RGS4(C2V), the closely related and functionally active RGS3 “short” isoform (RGS3S) did not interact with any of the GPCR-Kir3 channel complexes examined. Deletion and chimeric RGS constructs indicate both the N-terminal domain and the RGS domain of RGS4(C2V) are necessary for association with m2 receptor-Kir3.1/Kir3.2a channel complexes, where the GPCR was found to be the major target for RGS4(C2V) interaction. The functional impact of RGS4(C2V) “precoupling” to the GPCR-Kir3 channel complex versus RGS3S “collision coupling” was a 100-fold greater potency in the acceleration of G protein-dependent Kir3 channel-gating kinetics with no attenuation in current amplitude. These findings demonstrate that RGS4, a highly regulated modulator and susceptibility gene for schizophrenia, can directly associate with multiple GPCR-Kir3 channel complexes and may affect a wide range of neurotransmitter-mediated inhibitory and excitatory events in the nervous and cardiovascular systems.

RGS4, a member of the “Regulator of G protein Signaling” gene family (1, 2), is abundantly expressed in the mammalian brain and in peripheral tissues, including the heart (3–5). Functionally, RGS4 augments the GTPase activity of Gi/o and Gq/11 proteins and accelerates the termination of G protein-coupled receptor (GPCR)2 signaling (6–9). Genetic linkage and association analysis have identified the human RGS4 gene as a major susceptibility locus (chromosome 1q21-q22) for schizophrenia (10, 11), where gene profiling studies have shown RGS4 expression to be significantly reduced in the prefrontal cortex of schizophrenic subjects (12). These findings, together with the potential role of RGS4 in regulating several neurotransmitter systems known to affect the symptoms of schizophrenia (hallucinations, delusions, and depression), have implicated RGS4 in the etiology of this disorder (13). Decreased RGS4 levels are also reported to correlate with the reduced cholinergic signaling found in Alzheimer disease (14).

Aside from its potential role in neurological disorders, RGS4 is a highly regulated modulator that provides adaptive capabilities during various levels of cell signaling (15). At the transcriptional level, brain RGS4 mRNA levels are dynamically regulated by neurotransmitter activation of different GPCRs (16–18), several drugs of abuse (cocaine, morphine, and amphetamines) (19–21), stress and glucocorticoids (22), and electroconvulsive seizures (23). At the post-translational level, RGS4 protein is rapidly degraded via the ubiquitin-dependent N-end rule pathway, a process initiated by arginylation of Cys-2 by arginyltransferases and tightly coupled to the oxidative environment (24–26). Together these findings illustrate multiple levels of regulation that ultimately determine the RGS4 protein concentration affecting Gi/o and Gq/11 signaling in the brain and cardiovascular system.

One of the key effectors for Gi/o- and Gq/11-coupled receptors that modulate neuronal excitability and cardiac pacemaker activity is the G protein-gated inwardly rectifying K+ (Kir3/GIRK) channel (27, 28). Kir3 channels in neurons are predominantly localized to dendritic spines, dendrites, and the cell soma (29, 30) and are thus well positioned for suppressing excitation following activation by pertussis toxin (PTX)-sensitive Gi/o-coupled receptors, as evidenced in seizure-prone Kir3.2 knock-out mice (31, 32). In contrast to activation by PTX-sen-
Selective RGS Coupling to GPCR-Kir3 Channel Signaling Complexes

N-terminal Tagged GPCRs—Complementary DNAs encoding the human muscarinic m2 receptor (GenBank accession number NM_000739), human serotonin 1A receptor (GenBank accession number NM_000524), and mouse lysophosphatidic acid (LPA1/edg2) receptor (GenBank accession number NM_010336) were “tagged” at their N termini with the HA sequence (YPYDVPDYA). The HA tag was preceded by a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) for efficient membrane targeting (45). The signal sequence and HA tag sequence were introduced by annealing two complementary oligonucleotide primers (Sigma-Genosys) that contained a 5’ HindIII restriction site followed by a Kozak translation initiation sequence (GGCCGCACCC), the 16-amino acid signal sequence, the 9-amino acid HA sequence, and finally a 3’ XbaI restriction site. The annealed duplex was then cut with HindIII and XbaI and cloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen). The complete coding region of the human muscarinic m2 receptor, human serotonin 1A receptor, and mouse LPA receptor were then amplified by PCR and cloned in-frame at the XbaI site of the pcDNA3.1(+) vector. The HA tag was preceded by a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) for efficient membrane targeting (45). The signal sequence and HA tag sequence were introduced by annealing two complementary oligonucleotide primers (Sigma-Genosys) that contained a 5’ HindIII restriction site followed by a Kozak translation initiation sequence (GGCCGCACCC), the 16-amino acid signal sequence, the 9-amino acid HA sequence, and finally a 3’ XbaI restriction site. The annealed duplex was then cut with HindIII and XbaI and cloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen). The complete coding region of the human muscarinic m2 receptor, human serotonin 1A receptor, and mouse LPA receptor were then amplified by PCR and cloned in-frame at the XbaI site of the pcDNA3.1(+) vector. The cloning process resulted in two additional amino acids (SR) between the HA tag and starting methionine of the native GPCR sequence because of the XbaI sequence. The human adenylyl cyclase A1 receptor (GenBank accession number AY136746), human dopamine D2L receptor (GenBank accession number NM_000795), and human muscarinic m1 receptor (GenBank accession number AF498915) were obtained from the University of Missouri, Rolla cDNA Resource Center, and contained N-terminal triple (3×) HA tags, and were also cloned into the pcDNA3.1(+) vector.

C-terminal Tagged Kir3 Channels—The rat Kir3.1 channel subunit (GenBank accession number NM_031610) was tagged at the C terminus with the MYC epitope (EQKLISEEDL) by PCR and cloned into the pBudCE4.1 vector (Invitrogen). The pBudCE4.1 vector is a dual expression vector where Kir3.1-MYC expression was driven by the cytomegalo virus promoter. Mouse Kir3.2a (GenBank accession number NM_010606) was cloned into the second cloning site with expression driven by the EF-1a promoter. The Kir3.2a subunit was not modified by epitope tagging. The resulting Kir3.1-MYC/Kir3.2a-pBudCE4.1 vector yielded expression of both Kir3 channel subunits from a single DNA plasmid.

C-terminal Tagged RGS Proteins—Rat RGS4 (GenBank accession number NM_017214) and mouse RGS3s (GenBank accession number NM_134257) were tagged at their C termini with the FLAG epitope (DYKDDDDK) by PCR using primers that incorporated the FLAG sequence. The RGS-FLAG constructs were cloned into the pBudCE4.1 vector with expression driven by the cytomegalo virus promoter. Enhanced green fluorescent protein, GFP(S65T) (pGreenlantern-1; Invitrogen), was cloned into the second site with expression driven by the EF-1a promoter. The resulting RGS-FLAG/GFP-pBudCE4.1 plasmids provided expression of the RGS-FLAG protein and the GFP reporter protein from a single DNA plasmid. A pBudCE4.1 plasmid containing only GFP(S65T) was also generated for negative control (RGS−) experiments. All point mutations, deletion mutations, and chimeras of RGS3s-FLAG and RGS4-FLAG were constructed by PCR and also cloned into the cytomegalo virus promoter-driven site of the GFP-pBudCE4.1 vector.

The sequences of all epitope-tagged full-length cDNA constructs were confirmed by automated DNA sequencing (Molecular Biology Core Facility, Moffitt Cancer Center and Research Institute, Tampa, FL).

CHO-K1 Culture and DNA Transfection

CHO-K1 cells (American Type Culture Collection, Manassas, VA) were cultured in α-modified Eagle’s medium containing 5% fetal bovine serum and 0.1 mg/ml streptomycin and maintained in a humidified 5% CO2 incubator at 37 °C. For electrophysiological experiments, cells were plated at low density on 35-mm culture dishes. For biochemical experiments, cells were plated at a similar density on 100-mm culture dishes.

Cells were transfected using Lipofectamine (Invitrogen) and a mixture of 3–4 expression vectors. The total DNA (µg) to Lipofectamine (µg) ratio was kept constant at 1:5 when pre-forming the DNA-liposome complexes. The amount of each DNA vector in the mixture for each 35-mm dish was as follows: HA-GPCR-pcDNA3.1 (0.2 µg), Kir3.1-MYC/Kir3.2a-pBudCE4.1 (0.2 µg), and either RGS-FLAG/GFP-pBudCE4.1 or GFP-pBudCE4.1 (negative control) (1.0 µg). For transfection of cells plated in 100-mm dishes, the amounts were scaled eight times. Transfected CHO-K1 cells were incubated 24–36 h in serum-free Opti-MEM media (Invitrogen). For some experiments, mammalian expression vectors containing different Gα subunit cDNAs (Gαq(C352G), Gαs(C351G), or Gαq) were included (1.6 µg for 100-mm dish).
Selective RGS Coupling to GPCR-Kir3 Channel Signaling Complexes

Transfected CHO-K1 cells (100-mm dishes) were first washed with ice-cold Tris-buffered saline (TBS, pH 7.2). Three 100-mm plates were combined for each experimental condition. Cells were lysed and collected by cell scraping in 800 μl of extraction buffer at 4 °C. The extraction buffer was composed of 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% n-dodecyl-β-D-maltoside (MP Biomedicals), and a protease inhibitor mixture (Complete Mini EDTA-free; Roche Applied Science). The crude cell lysate was then left rotating end-over-end at 4 °C for 30 min to further solubilize cell membranes. Afterward, the sample was spun for 10 min at 14,000 × g to remove cellular debris. The protein concentrations of the final supernatants (cell lysates) were determined using a BCA assay (Pierce).

Immunoprecipitations were performed using anti-HA or anti-MYC antibodies conjugated to agarose beads (Profound IP/Co-IP kits; Pierce). Briefly, cell lysates (~750 μl or ~600 μg) were transferred to spin columns and either anti-HA or anti-MYC-agarose beads added (10 μg) followed by end-over-end rotation for 4 h at 4 °C. The columns were then spun to remove the cell lysate, and the beads then washed three times with extraction buffer (500 μl each). The immunoprecipitated proteins bound to the agarose beads were then eluted three times (10 μl each) with pH 2.8 elution buffer (Pierce). The acidic protein sample was then immediately neutralized with 1.5 μl of 1 M Tris, pH 9.5.

Western Blot Analysis

Western blotting was performed using standard methodology. The eluted protein samples (~30 μl) were added to 7.5 μl of a 5 × SDS loading buffer (0.3 M Tris-Cl, pH 6.8, 5% SDS, 50% glycerol, and a lane tracking dye) that also contained β-mercaptoethanol (~10%). The samples were heated for 5 min at 95 °C. A portion of the denatured protein sample (~20 μl) was then separated by gel electrophoresis using 4–15% or 8–16% Tris-HCl glycin polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore).

PVDF membranes were first incubated for 1 h in blocking buffer (5% nonfat dry milk powder in TBS with 0.05% Tween 20) and then incubated overnight at 4 °C with the appropriate primary antibody as follows: (1:1000) HRP-conjugated anti-HA 12CA5 antibody (Roche Applied Science); (1:1000) HRP-conjugated anti-MYC 9E10 antibody (Roche Applied Science); (1:1000) HRP-conjugated anti-FLAG M2 antibody, or 5–10 μg/ml anti-FLAG M2 antibody (F-3165; Sigma). For anti-FLAG immunodetection using the non-HRP-conjugated antibody (F-3165), membranes were washed in blocking buffer (five times) and subsequently incubated for 1 h with an HRP-conjugated goat anti-mouse secondary antibody diluted 1:10,000 in blocking buffer (sc-2318; Santa Cruz Biotechnology). Following all antibody incubations, PVDF membranes were washed four times (15 min each) with TBS containing 0.05% Tween 20, followed by two times (20 min each) with TBS. HRP-immunoreactive protein bands were then resolved by enhanced chemiluminescence (Luminol; Santa Cruz Biotechnology) and detected by exposure to blue-sensitive autoradiography film (Midwest Scientific). For some PVDF membranes, antibodies were stripped and re-probed with a different antibody.

Electrophysiological Recordings from CHO-K1 Cells

Electrophysiological recordings from CHO-K1 cells were performed using the whole-cell configuration of the patch clamp technique as described previously (42, 46). GFP-positive cells were identified by epifluorescence microscopy. Whole-cell recordings were performed with a patch clamp amplifier (Axopatch-1D; Axon Instruments) using patch pipettes having tip resistances of 3–5 megohms. Cells were voltage-clamped at a holding potential of ~100 mV, with voltage ramps from ~100 to +50 mV (0.5 s in duration) applied before and during ACh application to monitor inward rectification of the ACh-evoked Kir3 currents (I_{K,ACH}). All experiments were performed at room temperature (21–23 °C).

During whole-cell recording, cells were superfused with a high K + external solution to resolve the kinetics of inward I_{K,ACH}. The composition of the external solution was (in mM) as follows: NaCl 125, KCl 25, CaCl2 2, MgCl2 1, glucose 10, HEPES 5, pH 7.4. Rapid application and washout of different ACh concentrations was performed using a multibarrel perfusion system (SF-77B; Warner Instruments) that had a time constant for solution exchange of ~200 ms (46). The composition of the internal pipette solution was (in mM) as follows: KCl 120, NaCl 10, MgCl2 5, EGTA 1, HEPES 5, ATP 5, GTP 0.2, pH 7.2.

Electrophysiological Recordings from Xenopus Oocytes

All procedures for the use and handling of Xenopus laevis (Xenopus One, Ann Arbor, MI) were approved by the University of South Florida Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Oocytes were injected with a mixture of 5'-capped cRNAs synthesized in vitro from linearized cDNA vectors (mMessage mMachne, Ambion). Experimental groups (~20 oocytes each) were injected with different cRNA mixtures (50 nl final volume) and incubated at 19 °C in parallel for 48–60 h. All groups received cRNAs for the human muscarinic m2 receptor (0.5 ng/oocyte), rat Kir3.1 subunit (0.5 ng/oocyte), and mouse Kir3.2a subunit (0.5 ng/oocyte). Expressions of RGS4(C2V)-FLAG, RGS3s-FLAG, and RGS4-(58–205)-FLAG were varied by including different amounts of cRNA (0, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 ng/oocyte).

ACh-activated Kir3 channel currents were recorded by two-electrode voltage clamp methods from a holding potential of ~80 mV (GeneClamp 500; Axon Instruments). Oocytes were initially superfused with a minimal salt solution (98 mM NaCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.5) and then switched to an isotonic high K + solution (20 mM KCl, 78 mM NaCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.5) to resolve the kinetics of ACh-activated inward Kir3 channel currents. Rapid application and washout of ACh in the high K + solution was performed using a computer-triggered superfusion system (SF-77B; Warner Instruments) (46). To monitor inward rectification of I_{K,ACH}, voltage ramps from ~80 to +20 mV and 1 s in duration were evoked before and during agonist application. All recordings were performed at room temperature (21–23 °C).
Kinetic Analysis of Receptor-activated Kir3 Channel Currents

Time-dependent Kir3 current kinetics were analyzed using nonlinear curve fitting software that fit single exponential functions to derive activation time constants ($\tau_{\text{act}}$) and deactivation time constants ($\tau_{\text{deact}}$) (Clampfit 8.0 software; Axon Instruments). Agonist dose–response relations were analyzed by fitting peak current amplitudes with a Hill function, where the effective concentration producing a 50% response (EC$_{50}$) and Hill coefficient value ($n_H$) were derived from the best fit (Origin 6.0 software, OriginLab Corp.). Pairwise statistical analysis between experimental groups was performed by one-way analysis of variance, where $p < 0.05$ was considered significant.

RESULTS

Comparative RGS Protein Expression in CHO-K1 Cells—To determine whether RGS4 or RGS3s can associate with GPCR-Kir3 channel complexes, we co-expressed N-terminal HA-tagged m2 receptors, C-terminal MYC-tagged Kir3.1/ Kir3.2a channels, with and without C-terminal FLAG-tagged RGS3s or RGS4 in CHO-K1 cells. The HA-m2 receptor or the Kir3.1–MYC subunit was then immunoprecipitated and probed for co-precipitating proteins by Western blot analysis similar to that described by Lavine et al. (35). Initial Western blot analysis of cell lysates reaffirmed previous findings indicating RGS4 protein levels are low and often undetectable (24, 47), and significantly less than RGS3s (Fig. 1A). This has been attributed to the rapid degradation of RGS4 via the ubiquitin/proteasome-dependent N-end rule pathway initiated by arginylation of RGS4 at Cys-2 (24, 25). RGS3s notably lacks this N-terminal cysteine residue. We therefore also compared protein levels of the degradation-resistant RGS4(C2V) mutant (24). As shown in Fig. 1B, the level of RGS4(C2V) protein in the cell lysate was significantly greater than wild type RGS4 and more comparable with the protein levels observed with RGS3s expression. Both RGS3s–FLAG (23.5 kDa) and RGS4(C2V)–FLAG (24.25 kDa) migrated near their calculated molecular weights and were often accompanied by a slightly smaller band of lower intensity that may represent some degree of proteolysis or an alternative translation initiation start site (24, 47). Given the similar and stable expression levels of RGS3s and RGS4(C2V), we routinely used RGS4(C2V) for immunodetection and for comparisons with RGS3s.

Functional tests of co-expressed HA-tagged m2 receptors with Kir3.1–MYC/Kir3.2a channels revealed ACh-elicited inwardly rectifying K$^+$ currents that were indistinguishable from those produced by their untagged counterparts reported previously (42). Comparative analysis of the modulatory effects of FLAG-tagged RGS3s, RGS4, and RGS4(C2V) on the kinetics of I$_{K{ACh}}$ activation and deactivation indicated all three RGS proteins accelerated Kir3 channel gating properties to similar extents (Fig. 1, D and E). This was somewhat unexpected given the large difference in protein expression between RGS4 and RGS4(C2V), and indicates RGS4 protein levels (significantly lower than RGS3s and RGS4(C2V)) are saturating with regard to functional Kir3 channel modulation. Also consistent with our previous study (42), RGS3s–FLAG caused a significant rightward shift in the ACh dose–response relation (Fig. 1F) and reduced the peak I$_{K{ACh}}$ amplitudes by $\sim50\%$ (data not shown).

FIGURE 1. RGS3s, RGS4, and the degradation-resistant RGS4(C2V) mutant are differentially expressed in CHO-K1 cells, yet similarly accelerate the kinetics of muscarinic m2 receptor-activated Kir3 channel currents. A and B, Western blot (WB) analysis of C-terminal FLAG-tagged RGS3s, RGS4, and RGS4(C2V) protein levels in transfected CHO-K1 cell lysates. Cells for each RGS group were co-transfected with the HA-tagged m2 receptor, C-terminal MYC-tagged Kir3.1 subunits, and the Kir3.2a subunit (see "Materials and Methods" for details). Sample lanes were each loaded with $20\,\mu$g of total protein. C, whole-cell recordings of ACh-activated Kir3 channel currents from CHO-K1 cells expressing either no RGS (black traces), RGS3s–FLAG (red trace), or RGS4–FLAG (blue trace), or RGS4(C2V)–FLAG (green trace). The peak current amplitudes with a Hill function, where the effective concentration producing a 50% response (EC$_{50}$) and Hill coefficient value ($n_H$) were derived from the best fit (Origin nonlinear curve fitting software that fit single exponential functions. D and E, RGS3s–FLAG (red), RGS4–FLAG (blue), and RGS4(C2V)–FLAG (green) similarly accelerate the activation and deactivation time course for m2 receptor-activated Kir3 channel currents. Single exponential fits to the activation and deactivation time course were performed to derive the associated time constants, $\tau_{\text{act}}$ and $\tau_{\text{deact}}$, respectively. The ACh concentration dependence of $\tau_{\text{act}}$ is shown for each RGS examined (F). The $\tau_{\text{deact}}$ values are the following the rapid washout of 1 $\mu$M ACh. Values are the mean $\pm$ S.E. ($n=7–9$). F, ACh dose–response relations for ACh-evoked Kir3 channel currents expressing either no RGS (black symbols), RGS3s–FLAG (red symbols), RGS4–FLAG (blue symbols), or RGS4(C2V)–FLAG (green symbols). Mean values were fit with a Hill function (solid curves) to compare EC$_{50}$ values for each condition.

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Differential RGS Interaction with m2 Receptor-Kir3 Channel Complexes—We next immunoprecipitated the HA-tagged m2 receptor and probed for co-precipitating Kir3.1-MYC and RGS-FLAG by Western blot analysis. Shown in Fig. 2A, Kir3.1-MYC readily co-precipitated with the muscarinic m2 receptor demonstrating the presence of stable m2 receptor-Kir3 channel complexes similar to that reported for other GPCRs (35). Interestingly however, although RGS4(C2V) co-precipitated with the m2 receptor-Kir3 channel complex, RGS3s did not. The apparent molecular weights of the immunoprecipitated proteins were consistent with predicted and previously reported values. The immunoprecipitated HA-m2 receptor migrated as three major bands as follows: a low molecular weight band that closely corresponds to the molecular weight of the core protein (52.81 kDa), a higher band (70–75 kDa) that corresponds to glycosylated receptors (48), and an ~150-kDa band that corresponds to m2 receptor dimers (49, 50). The co-precipitated Kir3.1-MYC subunit also migrated close to its calculated molecular weight (57.77 kDa) with a slightly larger band that corresponds to glycosylated Kir3.1 subunits.

We next questioned whether the availability of G proteins might influence the coupling of RGS3s and RGS4 given potential limiting levels of endogenous G proteins present within the CHO-K1 cells. To test this, we examined the effects of co-expressing the Goα2 subunit on RGS co-precipitation with the m2 receptor-Kir3 channel complex. As shown in Fig. 2B, Goα2 expression appeared to slightly enhance wild type RGS4 protein levels, and RGS4 was now detected as a co-precipitating protein with the m2 receptor-Kir3 channel complex. Yet similar to the previous experiments without Goα2 expression, RGS3s also did not co-precipitate with the complex, and RGS4(C2V) was readily detected (Fig. 3A). Levels of RGS3s and RGS4(C2V) protein in the cell lysates were roughly equivalent, indicating the lack of RGS3s association with the complex was not attributable to differences in protein availability. The m2 receptor-Kir3 channel complex could also be immunoprecipitated via the Kir3.1-MYC channel subunit, where the co-precipitating HA-m2 receptor was then detected by Western blot (data not shown). Yet in this configuration, co-expression of Goα2 was found to block immunoprecipitation of Kir3.1-MYC. We speculate that immunoprecipitation via the cytosolic C-terminal Kir3.1-MYC epitope may be disrupted by G protein interactions that map to the Kir3 C-terminal region (39).

To test whether the Kir3.1-MYC or RGS4(C2V)-FLAG co-immunoprecipitations could be due to non-specific interactions with the antibody-conjugated agarose beads, we also performed a series of control experiments where CHO-K1 cells were co-transfected with the untagged m2 receptor, Kir3.1-MYC/Kir3.2a channels, and RGS4(C2V)-FLAG and then processed with anti-HA-agarose beads as shown in Fig. 2. These experiments failed to pull down any detectable Kir3.1-MYC, indicating the co-immunoprecipitation of Kir3.1-MYC is mediated via its interaction with the HA-m2 receptor complex. For RGS4(C2V)-FLAG, we were able to detect some intermittent interaction with the anti-HA beads in two of five experiments, but it was not sufficient to account for the level of co-precipitated RGS4(C2V)-FLAG protein (described below). This nonspecific interaction was not observed with the anti-MYC-agarose beads (cf. Fig. 6B). Thus altogether, the results indicate that RGS4(C2V) and RGS4 can both form a stable interaction with the m2 receptor-Kir3 channel complex, whereas the closely related RGS3s isoform does not.

Structural Determinants of RGS4 Association with m2 Receptor-Goα2-Kir3 Channel Complexes—RGS3s and RGS4 share a high degree of sequence homology within their conserved RGS domain (indeed they are nearest neighbors at 76% similarity), yet have important differences in their N-terminal sequences (Fig. 3A). The N-terminal domain of RGS4 (amino acids 1–57) contains two palmitoylation sites (Cys-2 and Cys-12) (51) and an amphipathic α-helix (amino acids 1–33) (52) that are both highly conserved among two other R4 RGS proteins, RGS5 and RGS16 (53, 54). The amphipathic helix of RGS4 is both necessary and sufficient for membrane association (51, 52) and is conserved in the RGS3s N terminus (Fig. 3A). Yet the RGS3s N terminus lacks the two palmitoylation sites (Cys-2 and Cys-12) that help target RGS16 (and presumably RGS4 and RGS5) to

**FIGURE 2.** Selective association of RGS4 with muscarinic m2 receptor-Kir3 channel complexes. A, immunoprecipitation (IP) of muscarinic m2 receptor-Kir3 channel-RGS complexes. HA-tagged muscarinic m2 receptors were immunoprecipitated from CHO-K1 cells co-expressing Kir3.1-MYC/Kir3.2a channels and either no RGS, RGS3s-FLAG, or RGS4(C2V)-FLAG. Co-precipitating Kir3.1-MYC and RGS-FLAG proteins were then probed by Western blot (WB) analysis in addition to the immunoprecipitated HA-m2 receptor. Western blots for each of the cell lysates are shown in the lower panel to assess the level of RGS-FLAG protein present within each of the cell lysates. B, effects of Goα2 co-expression on RGS coupling to muscarinic m2 receptor-Kir3 channel complexes. A Goα2 expression vector (the PTX-insensitive Goα2(C32G) mutant) was included in the CHO-K1 cell transfections as described in A. Note both RGS4 and RGS4(C2V) co-precipitate with the muscarinic m2 receptor-Kir3 channel complex, whereas RGS3s does not.
cholesterol-rich membrane lipid rafts (55) and enhance RGS GAP activity (51, 52, 56, 57).

Our initial hypothesis was that the RGS4 N-terminal domain was both necessary and sufficient for association of RGS4 with m2 receptor-Gi2-Kir3 channel complexes. To test this hypothesis, RGS4 deletion mutants and RGS3s/RGS4 chimeras (Fig. 3B) were individually co-expressed along with the HA-m2 receptor, the Gi2 subunit, and Kir3.1-MYC/Kir3.2a channels. The HA-m2 receptor was then immunoprecipitated and co-precipitating RGS proteins probed by Western blot. In support of our hypothesis, deleting the N-terminal domain of RGS4 resulted in the complete loss of association with the m2 receptor-Kir3 channel complex (Fig. 3C), as expected with the loss in membrane association (51). Interestingly, however, substituting the RGS3s N-terminal domain (amino acids 1–62) in place of the RGS4 N-terminal domain (R3s-R4-FLAG chimera) also resulted in the complete loss of association with the m2 receptor-Kir3 channel complex (Fig. 3C), indicating the RGS3s N-terminus (e.g. amphipathic helix) is not sufficient for conferring association. Together these results clearly demonstrate that the RGS4 N-terminal domain is necessary for RGS4 coupling to the signaling complex.

To further examine the role of the RGS4 N terminus, we replaced the RGS3 N terminus with the N-terminal domain of RGS4 (with or without the C2V mutation: R4-R3s-FLAG chimera or R4(C2V)-R3s-FLAG chimera), expecting the RGS4 N terminus to be sufficient to confer coupling to the m2 receptor complex. Surprisingly, however, the RGS4 N terminus in the context of the R4-R3s chimeras conferred only a very weak interaction, significantly less than RGS4(C2V) (Fig. 3C), and could be attributable to the nonspecific interactions described earlier. Thus the RGS4 N-terminal domain is clearly necessary for RGS4 coupling to the signaling complex.

FIGURE 3. Structural determinants of RGS4 association with muscarinic m2 receptor-Kir3 channel complexes. A, amino acid sequence alignment of the mouse RGS3 “short” isoform and rat RGS4. Asterisks denote sites of sequence identity, and green residues denote the highly conserved RGS domain. The N-terminal amphipathic α-helical domains are boxed, and the conserved basic residues are highlighted in red, and the palmitoylated RGS4 Cys-2, Cys-12 residues are highlighted in orange. The arrowhead denotes the site for RGS deletions and junction site for RGS chimeras. B, schematic diagram illustrating C-terminal FLAG-tagged RGS proteins constructed and tested for co-precipitation with muscarinic m2 receptor-Kir3 channel complexes. RGS4 regions are in blue, and RGS3s regions are in red. C, the RGS4 N-terminal domain (amino acids 1–57) is necessary for RGS association with muscarinic m2 receptor-Kir3 channel complexes. Six different RGS-FLAG constructs were individually co-expressed with HA-muscarinic m2 receptor, the Gi2(C352G) subunit, and Kir3.1-MYC/Kir3.2a channels in CHO-K1 cells. The HA-m2 receptor was then immunoprecipitated, and co-precipitating Kir3.1-MYC and RGS-FLAG proteins were detected by Western blot (WB). RGSFLAG present in the cell lysates are shown in the lower blot. Faint bands for RGS4-(58–205)-FLAG (lane 2), the R4-R3s-FLAG (lane 5), and R4(C2V)-R3s-FLAG chimera (lane 6) could be detected, yet none of the RGS constructs matched the level of coupling displayed by RGS4(C2V)-FLAG.
level of m2 receptor-Kir3 channel coupling (Fig. 3C), indicating assembly of m2 receptor-Kir3 channel complexes is not affected by RGS association.

**RGS4(C2V) Associates with Multiple GPCR-Kir3 Channel Complexes**—Kir3 channels are functionally coupled to a variety of G\_i/o-coupled receptors in the nervous system and heart (27, 28). To determine whether RGS3s and RGS4 selectively associate with different G\_i/o-coupled receptors known to activate native Kir3 channels, we examined RGS and Kir3 channel co-precipitation with several different HA-tagged GPCRs (serotonin 1A, adenosine A\_1, dopamine D2L, and LPA1 receptors) co-expressed with either G\_i2 or G\_oA. With G\_oA expression, each GPCR tested (serotonin 1A, adenosine A\_1, and LPA1 receptors) co-precipitated Kir3.1-MYC/Kir3.2a channels (Fig. 4A) and behaved just as the muscarinic m2 receptor (cf. Fig. 3). Moreover, each GPCR-Kir3 channel complex demonstrated the same selectivity in associating with RGS4(C2V) but not RGS3s. Wild type RGS4 co-precipitation was not readily detectable as RGS4 expression levels were significantly less than both RGS3s and RGS4(C2V). Similarly with G\_oA expression, each GPCR tested (serotonin 1A, adenosine A\_1, dopamine D2L, and LPA1 receptor) co-precipitated Kir3.1-MYC/Kir3.2a channels and RGS4(C2V), but not RGS3s (Fig. 4B). Thus RGS3s does not interact with a variety of G\_i/o-coupled receptors, whereas RGS4(C2V) coupling is rather promiscuous.

It is worth noting that the immunoprecipitation levels of the different HA-tagged GPCRs varied considerably, with m2 receptors and dopamine D2L receptors being markedly less than serotonin 1A, adenosine A\_1, or LPA1 receptors (Fig. 4). The multiple bands for each HA-GPCR are likely to correspond to post-translational modification(s) (i.e. glycosylation) and receptor oligomerization, as reported for muscarinic receptors (48–50). Bands corresponding to the core monomer receptor protein were readily apparent. The underlying cause for the differences in GPCR expression level is not clear but was not attributable to either the N-terminal HA tag (1× HA versus 3× HA) or the presence of the signal sequence. The differences apparently reflect distinct coding region differences that affect GPCR protein expression levels. The amount of co-precipitating RGS4(C2V) did not correlate with the level of immunoprecipitated HA-GPCR, being relatively constant for each expression condition. This finding suggests RGS4(C2V) coupling was limited by its own expression level or by an endogenous interacting protein(s) (i.e. G protein subunits).
Selective RGS Coupling to GPCR-Kir3 Channel Signaling Complexes

A.

![Image](https://example.com/image1)

B.

![Image](https://example.com/image2)

**FIGURE 5.** Kir3 channels and RGS4(C2V) co-assemble with $G_q$-coupled receptors. Four different HA-tagged GPCRs, the muscarinic m2 receptor (HA-m2R), the serotonin 1A receptor (HA-5-HT1AR), the lysophosphatidic acid 1 receptor (HA-LPA1R), and the muscarinic m1 receptor (HA-m1R), were expressed in CHO-K1 cells with Kir3.1-MYC/Kir3.2a channels, the $G_q$ subunit, and either RGS4(C2V)-FLAG (A) or RGS3s-FLAG (B). Each HA-tagged GPCR was then immunoprecipitated (IP), and co-precipitating (Co-IP) Kir3.1-MYC and RGS-FLAG proteins were detected by Western blot (WB). Kir3.1-MYC and RGS4(C2V)-FLAG co-precipitated with each HA-GPCR (A), whereas RGS3s-FLAG did not couple to any of the GPCR-Kir3 channel complexes (B). The comparative RGS-FLAG present in each of the cell lysates is shown in the lower blots.

Because both RGS3 and RGS4 are also effective GAPs for $G_q$ (8, 58), we also tested whether RGS3s might associate with a GPCR known to couple selectively to $G_q$ subunits, namely the muscarinic m1 receptor. For these experiments we co-expressed $G_q$ and Kir3.1-MYC/Kir3.2a channels, and we tested in parallel three additional GPCRs that display varying degrees of $G_q$ coupling for comparison (LPA1, serotonin 1A, and m2 receptor). Interestingly, the Kir3.1-MYC/Kir3.2a channels co-precipitated with the muscarinic m1 receptor indicating $G_q$-coupled receptors can also form stable complexes with Kir3 channels (Fig. 5). As observed with the $G_{11}$-coupled receptors, RGS3s again failed to couple to the m1 receptor-Kir3 channel complex (or any of the other GPCR-$G_q$-Kir3 channel complexes), whereas RGS4(C2V) associates with the m1 receptor-Kir3 channel complex (Fig. 5). Thus despite the functional effects of RGS3 on Kir3 channel gating kinetics (cf. Fig. 1), RGS3s does not associate with any of the GPCR-Kir3 channel complexes tested in our experiments.

RGS4(C2V) Couples to GPCRs Independent of Co-assembled Kir3 Channels—We next questioned whether RGS4(C2V) association with GPCR-Kir channel complexes was mediated via specific GPCR interactions, by specific Kir3 channel interactions, or by interactions with both. To determine this we 1) co-expressed several GPCRs with RGS4(C2V) in the absence of Kir3 channel expression, and 2) co-expressed RGS4(C2V) with Kir3.1-MYC/Kir3.2a channels in the absence of HA-GPCR expression. As shown in Fig. 6A, immunoprecipitation of each HA-GPCR readily co-precipitated RGS4(C2V) in the absence that might correlate with the differences in RGS precoupling to the signaling complex (data not shown). We therefore questioned whether RGS association with GPCR-Kir3 channel complex was of no functional benefit in CHO-K1 cells because of moderate levels of RGS protein expression and a resulting high degree of RGS collision coupling.

To control and vary the expression levels of RGS3s and RGS4, we turned to the *Xenopus* oocyte system where protein expression levels can be incrementally increased by titrating the amount of injected RGS cRNA (43). Given the similar steady-state protein levels of RGS3s-FLAG and the degradation-resistant RGS4(C2V)-FLAG mutant in CHO-K1 cells, we initially examined the concentration-dependent modulatory effects of these two RGS proteins on m2 receptor-activated Kir3.1/Kir3.2a channels expressed in *Xenopus* oocytes. As shown in Fig. 7, the amount of RGS3s-FLAG cRNA necessary to produce a half-maximal acceleration in the Kir3 channel activation rate was ~100-fold greater than the amount of RGS4(C2V)-FLAG cRNA needed to produce an equivalent effect. Both RGS3s-FLAG and RGS4(C2V)-FLAG produced similar maximal effects on the Kir3 channel activation rate at 10 ng of cRNA/oocyte. To determine whether this functional difference in RGS3s versus RGS4 potency could be attributed to the ability of RGS4(C2V) to precouple with the m2 receptor-Kir3 channel complex, we also compared the potency of the RGS4 N-terminal deletion construct (RGS4-(58–205)-FLAG) that does not associate with the complex (cf. Fig. 3) but still accelerates...
ACh-activated Kir3 currents via RGS domain interactions (59). As shown in Fig. 7, deleting the RGS4 N terminus significantly reduced the potency for accelerating the deactivation rate of Kir3 channels and approached that observed for RGS3s. Similar to RGS3s, RGS4-(58–205) also produced a maximal effect at 10 ng of cRNA/oocyte that was indistinguishable from RGS4(C2V). Together these results reveal a significant functional advantage of RGS4 that is reflected in a greater potency in accelerating the gating kinetics of receptor-activated Kir3 channels through targeted association via its N-terminal domain. Yet uncoupled RGS proteins (i.e. RGS3s) are clearly still capable of accelerating Kir3 channel gating kinetics via a collision coupling mechanism. Thus both of these scenarios, illustrated in Fig. 8, represent distinct and viable mechanisms for RGS modulation of G protein-dependent Kir3 channel gating kinetics yet have significantly different concentration requirements.

FIGURE 6. RGS4(C2V) couples to GPCRs and not the Kir3 channel. A, six different HA-tagged GPCRs (the muscarinic m2 receptor (HA-m2R), the serotonin 1A receptor (HA-5-HT1AR), the lysophosphatidic acid 1 receptor (HA-LPA1R), the adenosine A1 receptor (3HA-A1R), the dopamine D2L (3HA-D2LR), and the muscarinic m1 receptor (HA-m1R), were expressed in CHO-K1 cells with RGS4(C2V)-FLAG in the absence of Kir3.1-MYC/Kir3.2a channel expression. Immunoprecipitation (IP) of each of the HA-GPCRs co-precipitated (Co-IP) RGS4(C2V)-FLAG was determined by Western blot (WB) analysis. B, co-expression of Kir3.1-MYC/Kir3.2a channels and RGS4(C2V)-FLAG in the absence of HA-GPCR. Immunoprecipitation of Kir3.1-MYC failed to co-precipitate RGS4(C2V)-FLAG as determined by Western blot analysis.

FIGURE 7. RGS precoupling reduces the concentration necessary for modulation of muscarinic m2 receptor-activated Kir3 channel currents in Xenopus oocytes. A, ACh-activated Kir3 channel currents recorded from oocytes expressing the muscarinic m2 receptor, Kir3.1/Kir3.2a channel subunits, and equivalent levels of either RGS4(C2V)-FLAG (which precouples to the m2 receptor-Kir3 channel complex) or RGS4-(58–205)-FLAG or RGS3s-FLAG (which do not precouple to the complex). Each RGS was expressed at 1 ng of cRNA/oocyte. A “control” trace from an oocyte not expressing an ectopic RGS protein is shown for comparison (gray traces). Inward Kir3 channel currents were elicited by a 25-s application of 1 μM ACh, from a holding potential of −80 mV. Current amplitudes have been normalized to illustrate kinetic differences in the activation and deactivation time course. B, concentration-dependent effects of RGS4(C2V), RGS4-(58–205), and RGS3s on Kir3 channel deactivation kinetics. The deactivation time course following the rapid removal of 1 μM ACh was fit with a single exponential function to derive deactivation time constants. Separate groups of oocytes injected with increasing amounts of cRNA (0.03–10 ng/oocyte) encoding RGS4(C2V)-FLAG (circles), RGS4-(58–205)-FLAG (squares), or RGS3s-FLAG (triangles) were tested in parallel. A control group that did not receive RGS cRNA (gray bar) was included for comparison. Values represent the mean ± S.E. (n = 8) from two separate batches of oocytes.

**DISCUSSION**

RGS4(C2V) Precouples to Multiple GPCRs—Our findings reported here demonstrate an unexpected promiscuity in the association of RGS4(C2V) with several Gi/o and Gq/11-coupled receptors that assemble with Kir3 channels to form macromolecular signaling complexes. Critical to this observation was the utilization of the degradation-resistant RGS4(C2V) mutant that increased protein expression and reliable detection of RGS4(C2V) in our co-immunoprecipitation assays. RGS4(C2V) demonstrated a strong association with each of the GPCRs tested, yet did not associate with the Kir3 channel, indicating selectivity in its association with different transmembrane proteins. The elevated expression levels of RGS4(C2V) are likely to have masked our ability to detect any differences in the efficacy of RGS4 coupling to different GPCRs as reported in other systems (60, 61).
Selective RGS Coupling to GPCR-Kir3 Channel Signaling Complexes

RGS4 precoupling

![Diagram of RGS4 precoupling]

Nevertheless, the demonstrated ability of RGS4(C2V) to associate with multiple G_{13/11} and G_{13/11} coupled receptors, independent of the Kir3 channel effector, suggests precoupled RGS4-GPCR complexes may participate in the G protein-dependent modulation of native Kir3 channels and several other ion channels regulated by different G_{13/11} and G_{13/11} coupled receptors (e.g., Kir2 and Kir6 channels, KCNQ channels, TRP channels, and voltage-gated calcium channels).

Interestingly, a previous study had found recombinant GST-RGS4 fusion protein to interact in vitro with Kir3 channels expressed in HEK293 cells, suggesting a direct RGS4-Kir3 channel protein-protein interaction (62). In light of our findings, the reported GST-RGS4 interactions may have resulted from endogenous GPCRs co-assembled with the Kir3 channels expressed in HEK293 cells. Alternatively, RGS4 may have direct interactions with Kir3 channels that are not detected in our co-immunoprecipitation experiments but are more apparent using the recombinant RGS4 protein.

Structural Determinants of RGS4(C2V) Coupling to GPCR-Kir3 Channel Complexes—Recombinant RGS4 was recently found to directly interact with the third intracellular loop (i3L) of muscarinic m1 and m5 receptors but not the i3L of m2 receptors (63). Our experiments showing RGS4(C2V) co-precipitation with muscarinic m1 receptors (Fig. 5A) is therefore interpreted as a result, at least in part, of direct protein-protein interactions between RGS4(C2V) and the m1 receptor. The lack of RGS4 interactions with the i3L of m2 receptors (63) suggests other m2 receptor domains may also participate in direct receptor-RGS4 coupling, or alternatively the coupling may be mediated indirectly or cooperatively via interactions with precoupled Ga_{13/11} subunits or other proteins. Recent reports of RGS4 co-precipitating with μ- or δ-opioid receptors from peri-aqueductal gray membranes (64) and involving direct protein-protein interactions between RGS4 and the C-terminal domains of μ- or δ-opioid receptors (65) suggest RGS4 may also directly interact with the C-terminal domain of other GPCRs, including the m2 receptor.

The structural determinants of RGS4 that mediate association with GPCR-Kir3 channel complexes support a critical role for both the N-terminal domain and RGS domain. Deleting the RGS4 N terminus or substituting the RGS3s N terminus (R3s/R4 chimera) both resulted in decoupling from the GPCR-Kir3 channel complex. Unexpectedly, however, substituting the RGS4 N terminus for the RGS3s N terminus was not sufficient to confer coupling to the extent observed with RGS4(C2V). Because the RGS4 N terminus is sufficient to confer membrane association (51) and contains two palmitoylation sites that are expected to facilitate targeting to membrane lipid rafts (55) where GPCRs (66), heterotrimeric G proteins (67), and Kir3 channels localize (68), there are apparent cooperative and selective interactions between the N-terminal and RGS domain of RGS4 that together mediate the high affinity coupling.

Molecular Models of RGS4 Precoupling to GPCR-Kir3 Channel Complexes—Our findings are consistent with the model proposed by Wilkie and colleagues (61) where the RGS4 N terminus provides membrane association and orientation and may also have direct contacts with the GPCR, and the RGS domain interacts with precoupled Ga subunits. As a consequence, receptor-RGS4 association is expected to increase the fraction of precoupled receptor-G protein complexes. Recent fluorescence resonance energy transfer experiments in heterologous expression systems support a stable interaction between RGS proteins (RGS7 and RGS8) and Ga subunits within an agonist-receptor-G-protein quaternary complex (69). Importantly, however, these experiments did not detect fluorescence resonance energy transfer between RGS8 and the GPCR, indicating the RGS-Ga fluorescence resonance energy transfer signals could be potentially derived via a collision-coupled process.
due to high expression (as observed functionally for RGS3s in our CHO-K1 experiments). It will be important to extend our co-immunoprecipitation experiments to RGS8 and other members of the RGS protein family to identify RGS proteins that stably associate with different GPCRs and identify those that do not.

Although we found no evidence for receptor-specific association of RGS4(C2V), wild type RGS4 coupling was low or not detectable for each of the GPCRs tested. This may be in part due to the low RGS4 protein levels caused by the rapid degradation of RGS4 via the N-end rule pathway (24), or alternatively could reflect effects of Cys-2 modifications on coupling to GPCRs. The RGS4 Cys-2 residue is the target of palmitoylation (51), arginylation (24), ubiquitination (24), and oxidation (26), where the RGS4(C2V) mutant would be insensitive to any negative effects of these Cys-2 modifications on GPCR coupling. Future studies exploring the role of the RGS4 Cys-2 site and its modifications on the efficacy of specific GPCR coupling will be needed to resolve this fascinating possibility.

Functional Implications of RGS4 Precoupling Versus RGS3s Collision Coupling—Our initial electrophysiological measurements of the accelerated time course for RGS-modulated Kir3 currents in CHO-K1 cells did not reveal any kinetic advantage for precoupled RGS4 proteins versus uncoupled RGS3s, although the observed steady-state activation properties were consistent with precoupled RGS4 proteins versus uncoupled RGS3s (41, 43, 70). Our RGS dosage experiments in Xenopus oocytes, however, revealed that RGS4(C2V) precoupling provides a nearly 100-fold greater potency in Kir3 channel modulation versus uncoupled RGS3s. These findings illustrate a high level of RGS collision coupling that occurs in our CHO-K1 expression experiments, a possible result of the high protein expression levels produced in this commonly used mammalian expression system. Yet because the comparative levels of endogenous RGS proteins in native cells are not known (46, 71), both mechanisms (precoupling and collision coupling) may be physiologically relevant, with precoupling providing a means for targeting RGS4 to specific membrane microdomains (cf. Fig. 8).

Summary—Our findings demonstrate a strong association between RGS4(C2V) and several GPCRs that are central participants in normal and pathologically altered neuromodulation of membrane excitability. Given the multiple mechanisms affecting RGS4 protein levels, including the recently described impact of the oxidative environment (26), it will be important to determine to what extent these changes in RGS4 concentration and modification affect coupling to different GPCR signaling pathways. Inherited gene mutations that disrupt RGS9-1 association with the rhodopsin signaling complex cause bradyopsia in humans, a nonlethal condition characterized by a significantly reduced temporal resolution in motion detection (72). Acquired or inherited disruptions in RGS4-GPCR coupling may also play a role in a variety of neurological disorders that include schizophrenia (12), Parkinson disease (73), depression, epilepsy, and drug addiction (64). Their potential impact on cardiovascular disease is also becoming increasingly apparent (74).

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