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Differential coupling of RGS3s and RGS4 to GPCR-GIRK channel signaling complexes

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Differential Coupling of RGS3s and RGS4 to GPCR-GIRK Channel Signaling Complexes

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

Aquest treball està dedicat a la meva família, especialment a la memòria de la meva mare (Joana Peraire) i al meu pare (Joan Jaén). Moltes gràcies per tot el que meu ajudat al llarg de la meva vida.

Veus mama, per fi he acabat els estudis, sembla mentida, no?
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Differential Coupling of RGS3s and RGS4 to GPCR-GIRK
Channel Signaling Complexes

Cristina Jaén

ABSTRACT

‘Regulators of G protein signaling’ (RGS proteins) modulate the G protein cycle by enhancing the GTPase activity of Gα subunits. These changes accelerate the kinetics of ion channel modulation by Gαi/o-coupled receptors (GPCRs) such as the G protein-gated inward rectifier K⁺ (GIRK/Kir3) channel. My experiments indicate that a single cerebellar granule (CG) neuron, a cell type that endogenously expresses GIRK channels is able to express a wide variety of RGS proteins. I selected two of them, which are widely expressed and transcriptionally regulated during pathophysiologic conditions, to compare their functional properties. I originally described the differential modulatory effects of two RGS proteins, the RGS3 short isoform (RGS3s) and RGS4, on muscarinic m2 and serotonin 1A receptor-coupled Kir3.1/Kir3.2a channels expressed in Chinese hamster ovary (CHO-K1) cells. Both RGS3s and RGS4 accelerated GIRK activation and deactivation current kinetics in a similar way. However, only RGS3s significantly decreased the maximal GIRK current (I_{max}) elicited by ACh
(~45% inhibition) and significantly increased the EC$_{50}$ for both GPCRs. The hypothesis that emerged from this initial study was that the distinct RGS4 N-terminal domain mediated a direct coupling of RGS4 to GPCR-GIRK channel signaling complexes that was not shared by RGS3s. To test this hypothesis, I epitope-tagged several GPCRs, the Kir3.1 subunit, RGS3s, RGS4, and several deletion mutants and chimeras for co-immunoprecipitation experiments. Using an epitope-tagged degradation resistant RGS4 mutant RGS4(C2V), I detected co-precipitation of different GPCR-GIRK channel complexes with RGS4 but not RGS3s.

The functional impact of RGS4 coupling to the GPCR-Kir3 channel complex versus uncoupled RGS3s was not apparent in recordings from CHO-K1 cells presumably due to a high degree of RGS collision-coupling. Controlled expression in *Xenopus* oocytes revealed a 30-fold greater potency for RGS4 in the accelerating GIRK channel gating kinetics.

In summary, these findings demonstrate that one of the ways for the cell to achieve signaling pathway specificity may be through selective coupling of the different GPCR-effector-RGS protein complexes.
CHAPTER 1
INTRODUCTION AND BACKGROUND

G protein coupled receptors (GPCRS)

G-protein coupled receptors (GPCRs) are transmembrane receptors that mediate most of their intracellular actions through pathways involving activation of G-proteins. GPCRs have an extracellular N terminus, a cytoplasmic C terminus and 7 transmembrane \( \alpha \)-helices connected by three intracellular loops and three extracellular loops. They are also called heptahelical receptors or serpentine receptors. The extracellular receptor surface is critical for ligand binding and the intracellular surface is involved in G-protein recognition and activation (Wess, 1997). GPCRs are some of the oldest receptors devoted to signal transduction present throughout the evolutionary process, they appear in plants, yeast, slime mold, protozoa, diploblastic metazoa as well as vertebrates (Bockaert and Pin, 1999). The superfamily of GPCR is the largest gene family found so far, more than 1000 human genes have been identified for GPCRs, which include the m2 muscarinic receptor, the serotonin 1A receptor, the \( \alpha 2 \) adrenergic receptor, the D2-dopaminergic receptor, the opioid receptors, the A1 adenosine receptor, the lysophosphatidic acid 1 receptor, and the gamma-
aminobutyric acid type B (GABA<sub>B</sub>) receptor. GPCRs have a wide variety of ligands such as small biogenic amines (for example, 5-hydroxytryptamine (5-HT), dopamine, acetylcholine, epinephrine/norepinephrine, histamine), hormones, chemokines, local mediators, the amino acid L-glutamate, peptides, polypeptides, nucleotides, prostanoids, calcium ions, and lipids. GPCRs also play fundamental roles in sensory systems mediating vision, smell, and taste by responding to light, odorants, and taste stimuli (Wess, 1998). It has been estimated that about 80% of known hormones and neurotransmitters activate cellular signal transduction mechanisms through GPCRs (Birnbaumer 1990), and GPCRs represent 30-45% of current drug targets (Drews et al., 2000; Hopkins and Groom, 2002). GPCRs are susceptible to post-translational modifications, they can be palmitoylated, phosphorylated, and glycosylated. All of these modifications are important for the proper channel function, mediating trafficking, desensitization, and coupling (Daaka et al., 1997; Duvernay et al., 2005; Qanbar and Bouvier, 2003).

GPCRs show selective coupling to G-proteins, for example, muscarinic m1, m3, and m5 couple to the Gq/G11 family of G-proteins whereas m2 and m4 subtypes preferentially interact with the Gi/o family (Gainetdinov and Caron, 1999; Offermanns et al., 1994). Recent studies are challenging the classical idea that GPCRs act as monomers, and the stoichiometry is one receptor and one G protein coupling. Now it seems that a stoichiometry of 2 GPCRS and 1 G protein is more correct (Bulenger et al., 2005). Not only homo-heteromerization of
GPCRs is important and necessary for receptor trafficking and maturation, but it seems to be important for receptor selectivity as well. Chemokine receptors can form homo- and heterodimers. Depending on their composition they activate either Gi signaling pathway (homodimers) or Gq/11 (heterodimers) (Mellado et al., 2001). The diversity and physiological importance of GPCRs are increasing due to splice isoforms from already characterized GPCRs that show differential tissue specificity (Cole and Schindler, 2000; Huang et al., 2004; Mohler et al., 2001; Zhang et al., 2004).

**Guanine nucleotide-binding proteins (G proteins)**

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of α, β and γ subunits. They transduce extracellular signals received by 7-transmembrane receptors into intracellular signals through effector activation (Neer, 1995). Upon GPCR activation, the α subunit of the G protein exchanges its bound GDP for GTP. This causes the βγ subunit to dissociate from the Gα−GTP subunit, and either Gα−GTP or Gβγ or both act as downstream effectors in enhancing the receptor-mediated signal. The duration of G-protein coupled signaling is controlled by the lifetime of GTP-bound Gα subunit. Termination of the G protein cycle occurs when the intrinsic GTPase activity of the Gα subunit hydrolyzes the GTP and Gα-GDP reassociates with its Gβγ subunit (Sadja et al., 2003). Mammalian genes for 16 Gα, 5Gβ, and 12 Gγ subunits have been identified, as well as many splice variants for these genes.
G proteins are divided into four families based on their Gα subunit: Gs, Gi/o, Gq/11, and G12/13. The Gi/o group is composed of three distinct αi (αi1, αi2, and αi3), two splice forms of αo (αoA and αoB), two splice forms of αt, αgust, and αz (Wess, 1998). The different Gα subunits are determinant for receptor coupling specificity. For example, serotonin 1A, 1B, and muscarinic m2 receptors can couple with Gi1 but not Gi, meanwhile adenosine A1 receptor can couple to both of them (Slessareva et al., 2003).

The Gs subunit stimulates adenylyl cyclase (AC) increasing the intracellular concentration of cyclic adenosine-3',5'-monophosphate (cAMP). Gq subunit activates phospholipase C-β (PLC-β) catalyzing hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP2) to form second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The Gi/o subunits have several functions: Gi1 activates cGMP phosphodiesterase, Go modulates Ca2+ channel function, and Gi subtypes inhibits AC (Cabrera-Vera et al., 2003).

Initially, Gβγ subunits were thought to be as passive elements just binding to Gα subunits. Now it is known that Gβγ regulate several effectors such as K+ and Ca2+ channels, PLCβs, and PI3 kinase (Cabrera-Vera et al., 2003).

**Regulator of G protein signaling (RGS) proteins**

Regulators of G protein signaling (RGS) proteins speed up passage through the G-protein cycle by increasing the intrinsic GTPase activity of Gα subunits, thereby accelerating the reassociation of the ‘inactive’ heterotrimeric
complex $G_{\alpha(GDP)}\beta\gamma$ (Ross and Wilkie, 2000). RGS proteins are characterized by a highly conserved ‘RGS domain’ of ~ 125 a.a. that confers direct binding to $G_\alpha$ subunits and is flanked by less conserved N- and C-terminal domains of variable length (Tesmer et al., 1997). The first mammalian RGS proteins were reported about ten years ago (Druey et al., 1996; Vries et al., 1995). More than 20 mammalian RGS genes have been identified to date and are classified into six subfamilies (RZ, R4, R7, R12, RA, RL) based on sequence homology within the RGS domain (Hollinger and Hepler, 2002; Ross and Wilkie, 2000).

Alternative splice isoforms from the already characterized RGS proteins have been described, some of them having specific functions (Hollinger and Hepler, 2002). Thus far, there are four RGS3 splice isoforms that differ in their N-terminal domain: C2PA-RGS3, the largest one (Kehrl et al., 2002). PDZ-RGS3, that binds through the PDZ domain to the Ephrin B receptor and through the RGS domain regulates the migration response of cerebellar granule cells mediated by SDF-1 chemoattractant (Lu et al., 2001). RGS3T is a truncated form of RGS3, it is localized at the nuclear level and is involved in apoptosis (Dulin et al., 2000). And RGS3s “short” is highly expressed in heart, brain and lungs, and inhibits chemotactic responses of B lymphocytes (Reif and Cyster, 2000).

RGS proteins show distinct tissue distribution (Gold et al., 1997; Grafstein-Dunn et al., 2001), they also have specificity towards $G_\alpha$ subunits. For example RGS9 interacts with $G_{i_\alpha}$, meanwhile RGS4 interacts with $G_{i/o}$ and $G_q$. RGS2 interacts preferentially with $G_q$ (Hollinger and Hepler, 2002).
RGS proteins can be phosphorylated and palmitoylated, these posttranslational modifications produce a variety of effects including alterations in subcellular localization, protein stability and alterations in GAP activity (Riddle et al., 2005).

Originally RGS proteins were thought to be only GTPase-activating (GAPs) proteins. However, increasing evidence is pointing to a bigger role in signal transduction. Some RGS are able to interact with other proteins via non-RGS domains, such as RGS12 that with the PDZ domain binds to IL-8 receptor and through its PTB domain is able to bind to N-type Ca\(^{2+}\) channels. PDZ-RGS3s binds via the PDZ domain the Eprin B receptor. RGS3 and RGS7 can bind 14-3-3 proteins when phosphorylated (Hollinger and Hepler, 2002). RGS4 appears to regulate G\(_{\alpha}\) function based in recognition of receptors rather than association with G\(_{\alpha}\). In pancreatic acinar cells carbachol, bombesin, and cholecystokinin (CCK) activate Gq/11 pathways via specific GPCRs. Deletion of the RGS4 N-terminus eliminated the receptor selectivity and reduced the potency of the inhibition (Zeng et al., 1998). In another study, RGS1 and RGS16 also displayed receptor selectivity, whereas RGS2 displayed no preference among the three receptors (Xu et al., 1999). In striatum, RGS9-2 modulates Ca\(^{2+}\) channel inhibition in a GPCR specific manner (Cabrera-Vera et al., 2004).

RGS proteins are emerging as attractive therapeutic targets (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002; Riddle et al., 2005; Zhong and Neubig, 2001). RGS2 appears to be linked to cardiovascular diseases. RGS2
knockout mice exhibit a severe cardiovascular phenotype (Heximer et al., 2003), and overexpression of RGS2 is seen in individuals with Bartter’s/Gitelman’s Syndrome (Calo et al., 2004). RGS4 has been linked to schizophrenia (Chowdari et al., 2002; Mirnics et al., 2001). In addition, an RGS9 mutation has been identified as the cause of a pathological condition, where patients with mutations in either RGS9-1 or R9AP (RGS9 anchor protein) have slow photoreceptor deactivation and difficulty in adjusting to changes in light levels, as well as in seeing low-contrast moving objects (Nishiguchi et al., 2004).

**G protein coupled inward rectifying potassium (GIRK) channel**

G protein-gated inward rectifying potassium (GIRK) are selective K\(^{+}\) ion channels “opened” by a direct interaction with G\(βγ\) subunits (Logothetis et al., 1987). GIRK channels belong to the K\(^{+}\) inward rectifier (Kir) channel family, that is divided into 6 main subfamilies (Kir1.0-kir6.0), so they can also be named Kir3 channels (Doupnik et al., 1995). This family is characterized for having “inward rectification” which means that these channels allow potassium ions to flow through them more readily into the cell than out of the cell at hyperpolarized membrane potentials (Hille, 2001). GIRK channels are activated by GPCRs that couple to G\(αi/o\) (Dascal, 1997) and inhibited by receptors that couple to G\(αq\) (Lei et al., 2001). A large number of agonists can activate GIRK channels through Gi/o receptors such as acetylcholine (Dascal et al., 1993), adenosine (Leaney and Tinker, 2000), dopamine (Inanobe et al., 1999; Leaney and Tinker, 2000),
GABA (Leaney and Tinker, 2000), serotonin (Dascal et al., 1993; Karschin et al., 1991), norepinephrine (Lim et al., 1995; Mullner et al., 2000), somatostatin (Takano et al., 1997), and LPA (Itzhaki Van-Ham et al., 2004) The activation of these channels by PTX-sensitive G protein coupled receptors cause membrane hyperpolarization. The physiological role of GIRK channels is to maintain the resting membrane potential near the potassium equilibrium potential, and to slow pacemaker action potential frequency and heart rate (Breitwieser and Szabo, 1985; Kurachi et al., 1986; Logothetis et al., 1987; Pfaffinger et al., 1985). They are also involved in the slow phase of inhibitory postsynaptic potentials (Doupnik et al., 1995; Luscher et al., 1997; Nichols and Lopatin, 1997; Stanfield et al., 2002). The sequestration of Gβγ by GDP-bound Gα subunit will close the channel. The kinetics of activation and deactivation of GIRK channels therefore reflect the kinetics of the G protein cycle.

The properties of GIRK channel gating can be modulated by a variety of factors such as phosphatidylinositol-4,5-biphosphate (PIP2), Na⁺, Mg²⁺, oxidation-reduction, phosphorylation, and acidification (Sadja et al., 2003).

Four GIRK subunits have been found in mammals (GIRK1,2,3,4 or Kir3.1,2,3,4), another subunit GIRK5 has been characterized in Xenopus oocytes (Yamada et al., 1998). Furthermore, Kir3.2 has at least three different isoforms generated by alternative splicing named Kir3.2a, Kir3.2b, and Kir3.2c (Wei et al., 1998). Each GIRK subunit has intracellular N and C termini, two transmembrane domains and one “P-loop” that is considered the K⁺ channel “signature
sequence” (Hille, 2001). Functional GIRK channels are heterotetramers. Neuronal GIRK channels are composed of Kir3.1, Kir3.2, Kir3.3 subunits, whereas cardiac channels are formed by Kir3.1 and Kir3.4 subunits. GIRK1/2 was found to be the dominant heterotetramer mainly detected in brain (Kofuji et al., 1995; Liao et al., 1996). Only GIRK1 and GIRK4 subunits are distributed in atrial and sinoatrial node cells of the heart (Krapivinsky et al., 1995a), which are involved in the regulation of heart rate (Mark and Herlitze, 2000).

**Coupling of GPCRs to G proteins and GIRK channels**

This cartoon depicts the signaling pathway that couples GPCRs with GIRK channels.

Upon GPCR activation, the $\alpha_i/o$ subunit of the G protein exchanges its bound GDP for GTP. This causes the $\beta\gamma$ subunit to dissociate from the $G\alpha$–GTP subunit, and bind to the GIRK channel, opening the channel and allowing $K^+$ to
flow out of the cell and hyperpolarizing the membrane potential. The duration of G protein coupled signaling is controlled by the lifetime of GTP-bound Gα subunit. Termination of the G protein cycle occurs when the intrinsic GTPase activity of the Gα subunit hydrolyzes the GTP, and Gα-GDP reassociates with its Gβγ subunit. RGS proteins speed up the termination of the G protein signal by enhancing the intrinsic GTPase activity of the Gα/i/o subunit, therefore accelerating the closing of the GIRK channel.

Based on functional findings, a model that postulated the assembly of m2, RGS4, G protein and GIRK channel was proposed (Doupnik et al., 1997; Zhang et al., 2002). Recently, it has been shown that several GPCRs such as dopamine 2, dopamine 4 and beta2 adrenergic receptors form stable complexes with Kir3 channels in COS-7 and HEK 293 cells and brain tissue (Lavine et al., 2002). Some in vitro experiments indicate that RGS2 is able to bind to the third intracellular loop of the Gq/11-coupled m1 muscarinic receptor (Bernstein et al., 2004).

My work described here confirms the existence of these supracomplexes composed of GPCR-G protein-RGS4-GIRK channels, indicating that indeed RGS proteins are more than simply GAPs and serve as anchoring proteins in the assembly of these signaling complexes.
CHAPTER 2
GENERAL METHODS AND MATERIALS

Cerebellar granule neuron cultures

Cerebellar granule (CG) neurons from neonatal rat pups were both amiable to enzymatic isolation and primary culture for experimental manipulations. To isolate and culture postnatal day 4–6 rat CG neurons, I used a protocol modified from (Slesinger and Lansman, 1991). Following ip injection of sodium pentobarbital (4 mg/100 g body weight) to induce deep anesthesia, rat pup cerebella (2–4) were removed rapidly and placed in a 35-mm culture dish containing ice-cold calcium and magnesium-free (CMF) Tyrode’s solution (in mM): 136.9 NaCl, 5.4 KCl, 6.0 NaHCO₃, 0.33 Na₂HPO₄, 5.5 D-glucose, 5.0 HEPES, at pH 7.4 (NaOH), containing 100 U/ml penicillin and 0.1 mg/ml streptomycin. The tissue was minced, washed with CMF Tyrode’s solution, and then digested with 0.5 ml of trypsin/EDTA solution (GIBCO 25300-054) for 10 min at room temperature. The digestion was stopped by placing on ice and adding “isolation medium” that consisted of modified Eagle’s medium with Earle’s salts (GIBCO 11095-080) supplemented with 10% heat-inactivated horse serum, 25 mM KCl, 6 mg/ml d-glucose, 2 mM glutamine, 0.5 U/ml DNase I, 0.5
U/ml penicillin, and 0.5 g/ml streptomycin. The digested tissue was then triturated using a 1-ml sterile pipette, and the dispersed cells plated at low density on poly-l-lysine-coated 35-mm Corning cell culture dishes. The cells were incubated for 5 h at 37° in a 5% CO₂ atmosphere, and then the culture medium was changed to serum-free Neurobasal-A medium (GIBCO 10888-022) with B-27 supplement and 25 mM KCl, 2 mM glutamine, 0.5 U/ml penicillin, and 0.5 g/ml streptomycin. The cerebellar cell cultures were then maintained in a humidified incubator at 37° with a 5% CO₂ atmosphere for 24–48 h before extensive neurite outgrowth took place. All procedures for the use and handling of rats were approved by our institutional animal care and used in accordance with NIH guidelines.

**Single cell harvesting for RT-PCR analysis**

Single rat CG neurons were harvested from culture dishes using a micropipette (15–30 µm tip diameter) fabricated from borosilicate glass tubes (1.5 mm outside diameter, 0.86 mm inside diameter, GC150F-10, Warner Instruments) by a programmable microelectrode puller (P-97, Sutter Instruments). Latex gloves were worn throughout the handling and harvesting procedure to minimize potential sources of contamination. The micropipette was attached to a microelectrode holder used for patch-clamp recordings, allowing application of negative or positive pressure via an attached syringe. The culture dishes were first washed with a solution consisting of (mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 D-glucose, 5 HEPES, at pH 7.4 (NaOH) at room temperature
(~ 23°C). A single CG neuron having bipolar morphology (Fig. 3.1A) was then drawn into the micropipette by negative pressure. The micropipettes were not filled with solution initially, but contained 5 µl of the external solution after the cell had been harvested. The contents of the micropipette were then expelled into a PCR tube by positive pressure and the tube was placed on ice. For each experiment, four to six single cells were harvested and tested in parallel with negative and positive controls. Two negative control samples were a 5 µl sample of RNase-free H₂O and a 5 µl sample of the external solution. Positive controls included poly(A)⁺ mRNA from neonatal rat whole brain (0.5–5.0 ng). Experiments were generally repeated three times from separate dissections/cultures for each RGS examined to account for animal, culture, and cell variability.

**Design of intron-spanning gene-specific primers**

The RT-PCR approach utilizes gene-specific primers that selectively amplify mRNA transcripts from a specific RGS gene from a single cell. Because all mammalian RGS genes are poly-intronic (Sierra et al., 2002), intron-spanning primers were designed to distinguish mRNA-derived PCR products from genomic DNA-derived products (Doupnik et al., 2001). At the time of my original study, sequence information for rat RGS genes was limiting so mouse and human RGS sequences were used as alternatives for primer design. The effectiveness of each RGS primer set was confirmed by positive controls using
samples of rat brain poly(A) mRNA. A full list of the RGS primer sequences is provided (see Table 2.1).

**Single cell reverse transcriptase PCR (RT-PCR) analysis**

One-step RT-PCR was carried out using the intron-spanning gene-specific primers according to the manufacturer’s protocol (OneStep RT-PCR, Qiagen Inc.). For each 5 µl sample, 20 µl of a RT-PCR master mix was added and contained the following: forward and reverse primers at 0.6 µM each (final concentration), dNTPs at 400 µM each (final concentration), Omni-script and Sensiscript reverse transcriptases, HotStar Taq DNA polymerase, RNase inhibitor, and a buffer solution containing Tris–Cl, KCl, (NH₄)₂SO₄, MgCl₂, and dithiothreitol. Concentrations of enzymes and buffer components were as recommended by the manufacturer (1X concentration, Qiagen, Inc.) and included the 1X “Qsolution,” which effectively reduced nonspecific bands produced by mispriming events. Each 25 µl sample was then placed in a PCR thermocycler (GeneAmp 2400, PE Biosystems, Inc.) for the following temperature protocol: 50° for 30 min (reverse transcription), 95° for 15 min (activation of HotStar Taq polymerase), 45 cycles of 94° for 30s (melt), 3–4° below the primer annealing temperature for 30s, and 72° for 60–90s (extension). At the end of the cycling period, samples were held at 72° for 10 min (final extension) and the reaction was stopped by cooling to 4°. According to the manufacturer (Qiagen Inc.), this PCR cycling protocol (45–50 cycles) was expected to allow detection of mRNA
transcripts in the general range of 10 to 100 copies per cell. The PCR samples were then analyzed by 2% agarose gel electrophoresis, and the products were visualized by ethidium bromide staining and UV illumination. Gel images were captured using a digital gel-imaging system (Biolmager, Genomic Solutions Inc.) and were scored for positive or negative expression based on visual detection of the expected gel band.
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<th>Species</th>
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<th>Location (ATG + 1)</th>
<th>Annealing Temp. (°C)</th>
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Table 2.1 Gene Specific Primers for Single-Cell RT-PCR
Chinese hamster ovary (CHO-K1) cells for heterologous expression

Chinese hamster ovary (CHO-k1) cells are a commonly used mammalian expression system. CHO-K1 cells are very robust and easy to grow in culture conditions, they also display a high cotransfection efficiency using cationic lipid-based transfection methods, a critical attribute for reconstituting expression of multiple protein within a single cell (Ehrengruber et al., 1998). CHO-K1 cells have a round geometry and small size making them well suited for whole-cell patch-clamp recordings (Doupnik et al., 1997; Ehrengruber et al., 1998; Jaen and Doupnik, 2005). CHO-K1 cells do not express endogenous GIRK channel subunits, yet they do express various GPCRs (Schonbrunn, 2004) and RGS proteins. The endogenous expression of RGS mRNA in CHO-K1 cells has been partially characterized (RGS1, RGS2, RGS3, RGS4, RGS10, RGS16, and RGS19) with RGS2 being significantly expressed, RGS4 not expressed, and the others being expressed at moderate to low levels based on RT-PCR analysis (Boutet-Robinet et al., 2003; Takesono et al., 1999).

Heterologous expression of wild type cDNAs in CHO-K1 cells

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. One day after low density plating in 35 mm dishes, cells were transfected with DNA-liposome complexes composed of lipofectamine (Invitrogen, Carlsbad, CA)
and a mixture of cDNAs cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). 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 per dish was as follows; 0.2 µg rat Kir3.1 (GenBank accession # NM_031610), 0.2 µg mouse Kir3.2a (GenBank accession # NM_010606), 0.2 µg GPCR either human muscarinic m2 receptor (GenBank accession # NM_000739) or human 5-HT1A receptor (GenBank accession # NM_000524), with or without 1.0 µg RGS either mouse RGS3s (GenBank accession # NM_134257) or rat RGS4 (GenBank accession # NM_017214), with 0.1 µg enhanced green fluorescent protein (GFP) cDNA (pGreenlantern-1, GIBCO) included as a reporter gene (Doupnik et al., 1997; Doupnik et al., 2004). The transfected cells were incubated overnight in serum-free OPTI-MEM media (Invitrogen). Twenty-four to thirty-six hours after transfection, single GFP-positive cells were selected for electrophysiological recordings. The RGS3s cDNA clone was generously provided by Drs Karin Reif and Jason Cyster (University of California, San Francisco) (Reif and Cyster, 2000). All other cDNA clones were as described elsewhere (Doupnik et al., 1997; Doupnik et al., 2004). For pertussis toxin (PTX) pre-treatment experiments, transfected CHO-K1 cells were incubated overnight (12-18 h) with 100 ng/ml PTX (P-7208, Sigma Chemical).
Construction of epitope-tagged expression vectors

**N-terminal-tagged GPCR's** - Complimentary DNA's encoding the human muscarinic m2 receptor (Genbank Accession # NM_000739), human serotonin 1A receptor (Genbank Accession # NM_000524), and mouse lysophosphatidic acid (LPA1/edg2) receptor (Genbank Accession # NM_010336) were "tagged" at their N-termini with the hemagglutinin (HA) sequence (YPYDVPDYA). The HA tag was preceded by a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) for efficient membrane targeting (Guan et al., 1992). The signal sequence and HA tag sequence were introduced by annealing two complimentary oligonucleotide primers (Sigma-Genosys) that contained a 5' **Hind** III restriction site followed by a Kozak translation initiation sequence (GCCGCCCACC), the 16 a.a. signal sequence, the 9 a.a. HA sequence, and finally a 3' **Xba** I restriction site. The annealed duplex was then cut with **Hind** III and **Xba** I, 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 LPA1 receptor were then amplified by PCR and cloned in-frame at the **Xba** I site of the N-terminal HA-tag 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 due to the **Xba** I sequence. The human adenosine A1 receptor (Genbank Accession # AY136746), human dopamine D2L receptor (Genbank Accession # NM_000795), and human muscarinic m1 receptor (Genbank Accession #
AF498915) were obtained from the University of Missouri, Rolla cDNA Resource Center (www.cdna.org) and contained N-terminal triple (3X) HA tags, and were cloned into the pcDNA3.1(+) vector.

**C-terminal-tagged Kir3 channels** - The rat Kir3.1 channel subunit (Genbank Accession # 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 duel expression vector where Kir3.1-MYC expression was driven by the CMV promoter. Mouse Kir3.2a (Genbank Accession # 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 construct yielded expression of both Kir3 channel subunits from a single DNA plasmid.

**C-terminal-tagged RGS proteins** - Rat RGS4 (Genbank Accession # NM_017214) and mouse RGS3s (Genbank Accession # NM_134257) were tagged at their C-termiini 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 CMV promoter. Enhanced green fluorescent protein, GFP(S65T) (pGreenlantern-1, GIBCO), 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 CMV promoter driven site of GFP-pBudCE4.1 vector.

The sequence 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).

**Transfection of epitope-tagged cDNAs in CHO-K1 cells**

The transfection was very similar to the one previously described. In this case, for electrophysiological experiments, cells were plated at low density on 35 mm culture dishes, and 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 8X. Transfected CHO-K1 cells were incubated 24-36 hr in serum-free OPTI-MEM media (Invitrogen). For some experiments, mammalian expression vectors
containing different $G_\alpha$ subunit cDNA’s ($G_{\alpha i2}^{C352G}$, $G_{\alpha oA}^{C351G}$, or $G_{\alpha q}$) were included (1.6 $\mu$g for 100 mm dish).

**Immunoprecipitation and co-immunoprecipitation**

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 $\mu$l 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-$\beta$-D-maltoside (MP Biomedicals), and a protease inhibitor cocktail (Complete Mini EDTA-free, Roche). The crude cell lysate was then left end-over-end rotating at 4°C for 30 minutes to solubilize cell membranes. Afterwards, the sample was spun for 10 minutes 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 $\mu$l or ~600 $\mu$g) were transferred to spin columns and either anti-HA or anti-MYC agarose beads added (10 $\mu$g) followed by end-over-end rotation for 4 hours at 4°C. The columns were then spun to remove the cell lysate, and the beads then washed three times with extraction buffer (500 $\mu$l each). The immunoprecipitated proteins bound to the agarose beads were then
eluted 3X (10 µl each) with pH 2.8 elution buffer (Pierce). The acidic protein sample was then immediately neutralized with 1.5 µl of 1M 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 5X 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 minutes 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 glycine polyacrylamide gels (BIO-RAD) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore).

PVDF membranes were first incubated for 1 hr in blocking buffer (5% nonfat dry milk powder in TBS with 0.05% Tween 20), then incubated overnight at 4°C with the appropriate primary antibody; (1:1000) HRP-conjugated anti-HA 12CA5 antibody (Roche); (1:1000) HRP-conjugated anti-MYC 9E10 antibody (Roche); (1:1000) HRP-conjugated anti-FLAG M2 antibody, or 5-10 µg/ml anti-FLAG M2 antibody (F-3165 Sigma-Aldrich). For anti-FLAG immunodetection using the non-HRP conjugated antibody (F-3165), membranes were washed in blocking buffer (5X) and subsequently incubated for 1 hour with an HRP-conjugated goat anti-mouse secondary antibody diluted 1:10,000 in blocking buffer (sc-2318 Santa Cruz). Following all antibody incubations, PVDF
membranes were washed 4 times (15 minutes each) with TBS containing 0.05% Tween 20, followed by 2 times (20 minutes each) with TBS. HRP-immunoreactive protein bands were then resolved by enhanced chemiluminescence (Luminol, Santa Cruz), 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 cerebellar granule neurons**

Critical to resolving RGS modulated GIRK current kinetics in mammalian cells is establishing an electrophysiology setup capable of rapid solution changes for agonist application and washout during whole-cell voltage-clamp recording. I currently use the SF-77B Fast-Step perfusion system (Warner Instruments) that consists of a 3-barrel array made of 700 µm square capillary tubes, delivering gravity-driven flow of 3 independent solutions in parallel. Each barrel can receive input via a manifold connecting up to six different solution reservoirs to expand the solution testing capability. The movement and position of the barrel array is computer controlled, having a limiting step-speed of ~240 ms, though can be as fast as 120-140 ms at the highest flow rates I can generate (~75 cm column height). These solution exchange rates are comparable to some (Breitwieser and Szabo, 1988; Bunemann et al., 1996), though somewhat slower than the 10-50 ms time constants reported by others using similar configurations (Karschin et al., 1991; Sodickson and Bean, 1996). Nonetheless, they are sufficient to
temporally resolve the GIRK current kinetics observed at room temperature (22-24°C) with and without RGS co-expression. Cerebellar granule neurons are selected for electrophysiological recordings using standard whole-cell tight-seal patch clamp methods (Hamill et al., 1981). Cells are initially washed and placed in an external solution that consists of (in mM): NaCl 145, KCl 5, CaCl₂ 2, MgCl₂ 1; glucose 10, HEPES 5 (pH = 7.4). After gigaseal formation and breaking into the cell for whole-cell recording, 2 min is allowed to permit equilibration of the intracellular solution. The composition of the internal pipette solution is (in mM): KCl 120, NaCl 10, MgCl₂ 5, EGTA 1, HEPES 5, ATP 5, GTP 0.2 (pH = 7.2). First after breaking into the cell, the membrane capacitance (a direct measure of cell surface area) is determined via amplifier compensation, and later used to express the maximal current amplitude as a current density (pA/pF) for cell-to-cell comparisons. Agonist-evoked inward K⁺ currents are recorded from a holding potential of -100 mV, which is sufficiently negative to the experimentally set K⁺ equilibrium potential (E_K = -40 mV).

Thus after establishing the whole-cell recording and clamping the membrane potential to -100 mV, the cell is initially superfused with a “high K⁺” solution composed of (in mM): NaCl 125, KCl 25, CaCl₂ 2, MgCl₂ 1; glucose 10, HEPES 5 (pH = 7.4). The solution is applied via one of the 700 µm square capillary tubes positioned next to the cell and connected to a 20 ml syringe reservoir where the flow rate is gravity controlled by adjusting the syringe height. After a stable baseline holding current is established, the agonist is applied (in
the high K⁺ solution) via the step movement of the barrel array so that agonist flow via the adjacent barrel is positioned in line with the recorded cell. We typically apply the agonist for 15s to minimize receptor desensitization, followed by agonist washout with the step movement back to the high K⁺ solution barrel (see Figure 2.1B). Voltage-clamp recordings are performed using an Axoclamp 1D amplifier (Axon Instruments). Current signals are sampled and digitized via a Digidata 1200B A/D board that also synchronizes digital output signals to the SF-77B Fast-step controller. Axon pCLAMP8.0 software is used to trigger the perfusion barrel movements along with 500 ms voltage ramps (- 100 to +50 mV) evoked before and during agonist application to assess the voltage-dependent properties of the agonist-evoked currents. Characteristic features of GIRK currents include steep inward rectification and K⁺ selectivity (i.e. a reversal potential near the E_k). The analog current signals are low-pass filtered with the amplifier’s integrated 4-pole Bessel filter at a corner frequency of 50 Hz, and then digitally sampled at 100 Hz. The time constants for GIRK current activation (τ_{act}) and deactivation (τ_{deact}) are derived by fitting a single exponential function to the rising or decaying portion of the current (Figure 3.1D/E) using non-linear least-squares curve-fitting software (Clampfit 8.0).

**Electrophysiological recordings from CHO-K1 cells**

Electrophysiological recordings from CHO-K1 cells were performed as explained for CG granule neurons. In this case, GFP-positive cells were identified
by epi-fluorescence microscopy using an inverted microscope (Nikon Diaphot with CF N Plan Fluor Ph 20X objective) equipped with a mercury lamp and GFP filter set (Endow GFP, Chroma Technology Corp.). Rapid application and washout of different agonist (ACh or 5-HT in high K+ solution) concentrations was performed using the multi-barrel perfusion system (SF-77B, Warner Instruments) (Doupnik et al., 2004), see Figure 2.1A,B
**Figure 2.1.** Whole cell patch-clamp recording of receptor activated GIRK currents. (A) CHO-K1 cells twenty-four hours after DNA transfection visualized under phase-contrast (upper panel) and epifluorescence (lower panel) microscopy. Cells were transfected with EGFP, GIRK channel subunits (Kir3.1/Kir3.2a) and the muscarinic m2 receptor. (B) Alignment and movement of perfusion barrels for rapid solution exchange. Upper panel shows the three-barrel array positioned with the patch clamped cell (see patch electrode) being superfused with the high K⁺ solution (washing solution). Flow through both the middle and right barrels is continuous and gravity driven. Lower panel shows the position of the barrels following computer-controlled movement (700 µm), where the agonist barrel is now aligned with the recorded cell.
**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 NIH guidelines. Oocytes were injected with a mixture of 5’ capped cRNA’s synthesized *in vitro* from linearized cDNA vectors (mMessage mMachine, 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 hrs. All groups received cRNA’s 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). Expression of RGS4(C2V)-FLAG and RGS3s-FLAG was varied by including different amounts of cRNA (0, 0.03, 0.1, 0.3, 1.0, 3.0, 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 at pH 7.5), then switched to an isotonic high K⁺ solution (20 mM KCl, 78 mM NaCl, 1 mM MgCl₂, and 5 mM HEPES at 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)(Doupnik et al., 2004). 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 GIRK 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 GIRK current amplitudes with the 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.). For comparison of GIRK current amplitudes across cells, agonist-evoked currents from each cell were normalized to the measured cell membrane capacitance ($C_m$) determined during capacitive current compensation. The normalized current amplitudes are expressed as GIRK current density (pA/pF).

**Statistical Analysis**

Pairwise statistical analysis between experimental groups was performed by one-way ANOVA (analysis of the variance) test where $p<0.05$ was considered significant.
CHAPTER 3
PROFILE OF RGS GENE EXPRESSION IN CEREBELLAR GRANULE NEURONS

INTRODUCTION

G-protein-gated inwardly rectifying potassium (GIRK) channels are K⁺-selective ion channels “opened” by a direct interaction with Gβγ subunits (Logothetis et al., 1987). Physiologically, GIRK channels play an instrumental role in suppressing membrane excitability during the activation of G-protein-coupled receptors (GPCRs) in neurons, cardiomyocytes, and endocrine cells (Stanfield et al., 2002; Yamada et al., 1998). Functional GIRK channels in mammals are now known to be heterotetramers composed of Kir3.1, Kir3.2, Kir3.3, and Kir3.4 subunits (Stanfield et al., 2002; Yamada et al., 1998). Neuronal GIRK channels are more diverse than cardiac GIRK channels, having an overlapping expression of Kir3.1, Kir3.2, and Kir3.3 in different regions of the brain. Kir3 subunits 1, 2, and 3 are highly expressed in the cerebellum (Karschin and Karschin, 1997). Furthermore, they are expressed in the distal part of the CG neuron’s dendrites, at the level of the glomeruli where mossy fibers and
cerebellar granule (CG) cells synapse (Ponce et al., 1996). It is also known that expression of RGS mRNA in brain shows a distinct regional distribution detected by *in situ* hybridization (Gold et al., 1997; Grafstein-Dunn et al., 2001).

Neuronal GIRK channels have only recently been studied in CG neurons, prompted largely from the discovery of the mouse *weaver* gene that contains a point mutation in the Kir3.2 subunit that disrupts K\(^+\) selectivity causing CG cell death and phenotypic ataxia (Kofuji et al., 1996; Patil et al., 1995; Slesinger et al., 1996; Surmeier et al., 1996). The objective of this work is to discern which RGS genes are expressed in a CG neuron that are likely to be involved in the modulation of endogenous CG neuron’s GIRK channels.
RESULTS

Measuring native GIRK channel gating properties in CG neurons.

Neonatal rat CG neurons exhibited baclofen-evoked GIRK currents that were sustained in primary culture and could be characterized electrophysiologically (Figure 3.1). GABA$_B$ receptor-activated GIRK currents in rat CG neurons displayed rapid activation and deactivation kinetics (Figure 3.1F) suggesting modulation by endogenous RGS proteins.
Fig. 3.1. Quantitative analysis of native GIRK currents recorded from rat cerebellar granule (CG) neurons. (A) Phase-contrast image of a typical rat CG neuron maintained in primary culture and selected for electrophysiological recordings. (B) GABA$_B$ receptor agonist baclofen (100 µM) evoke characteristic GIRK currents from CG neurons. Baclofen-activated GIRK currents display steep inward rectification (C) and rapid activation (D, F) and deactivation kinetics (E, F). Data are means ± SEM. Dashed lines in F refer to time constants for solution exchange and represent the limit of resolving kinetic events.
Single Cell RT-PCR analysis of Endogenous RGS Expression

To discern which RGS proteins were expressed endogenously in rat CG neurons, single cell RT-PCR methods were developed for detecting and profiling RGS expression in primary culture neurons. CG neurons were abundant and could be distinguished in culture by their relative small size and simple bipolar morphology compared to other cells present in the culture dish. Positive Kir3.2 expression in CG neurons confirmed GIRK channel expression in these cells. A great diversity of RGS proteins expressed in the granule neurons was found. In some cases, RGS expression was very consistent throughout the different dissections, RGS proteins like RGS5, RGS11 and RGS9 were never detected in the granule neurons, but others like RGS2, RGS10, RGSz2 and RGS4 were almost always detected. However, the detection levels of others like RGS6, RGS7 and RGS8 changed greatly from experiment to experiment, making it difficult to extrapolate any conclusion (Fig. 3.2). GIRK2a was used as both granule neuron marker and control of the efficacy of the sampling method (91% efficiency). Results indicated that CG neurons expressed at least 13 different RGS genes, and each RGS subfamily (R4, R7, R12, and RZ) was represented (Fig 3.3). Although a profile of protein expression was not correlated with mRNA data and relative RGS protein levels were unknown, the single cell RT-PCR results clearly indicate that numerous RGS proteins are likely to be present in these native GIRK-expressing cells.
Figure 3.2. Separation of RT-PCR products by agarose gel electrophoresis. RT-PCR was performed on rat CG neurons as described in the text. Results using selected intron-spanning RGS-selective primer sets (Doupnik et al., 2001), as well as primer sets for GIRK2 (Kir3.2a) are shown. Negative controls included water and external solution (5 µl), and positive controls included postnatal poly(A)+ mRNA of brain (0.5–5.0 ng). The predicted molecular size for each RGS PCR product is indicated on the right of each gel.
Figure 3.3. Profile of RGS expression in rat CG neurons. The percentage of cells sampled and positive for RGS expression by RT-PCR analysis is shown for each RGS within the R4, R7, R12, and RZ subfamilies examined. The number of cells tested for each RGS ranges from 8 to 24 and is from at least two separate cultures.
DISCUSSION

My results were in agreement with published results obtained by in situ hybridization indicating multiple RGS’s are expressed in brain (Gold et al., 1997; Grafstein-Dunn et al., 2001). However, my data were obtained from a singular type of cell compared to the in situ experiments where the data were obtained from a wide variety of tissue.

I also compared the CG results with the RGS gene profile performed in rat atrial myocytes (Doupnik et al., 2001). Both native cell systems expressed RGS genes from each RGS subfamily (R4, R7, R12, and RZ). Intestingly, the profile of RGS expression in cardiac myocytes and CG neurons had some differences: there was more expression of RGS genes in CG neurons, at least 13 compared to 7 in myocytes. Also, the percentage of some RGS expression differed between the two cell types, for example RGS6 had an expression of ~95% in atrial myocytes in contrast to the ~ 30% in CG neurons. This higher expression of RGS genes in CG neurons compared to atrial myocytes also correlated with the $\tau_{\text{deact}}$ of native GIRK channels recorded from both cell types, being faster in the CG neurons (Doupnik et al., 2004).
My data indicate that CG neurons in culture conditions express endogenous and functional GIRK channels and that the fast $\tau_{\text{deact}}$ observed is likely due to the high expression of endogenous RGS.

My original goal of discerning the unique/s RGS proteins involved in the modulation of the GIRK channels clearly was not feasible due to the great variety of RGS proteins present in the CG neurons. For this reason, I decided to focus my studies on two RGS proteins. I chose RGS3s and RGS4 which were expressed in CG neurons and atrial myocytes, and performed the rest of the experiments in an heterologous system where I could have better control of the components of the signaling pathway.
CHAPTER 4

NEURONAL KIR3.1/KIR3.2A CHANNELS COUPLED TO SEROTONIN 1A AND MUSCARINIC M2 RECEPTORS ARE DIFFERENTIALLY MODULATED BY THE ‘SHORT’ RGS3S ISOFORM

INTRODUCTION

Gβγ-gated inwardly rectifying K⁺ channels (GIRKs) are expressed predominantly in brain, heart, and endocrine tissue and suppress cell excitability during neurotransmitter and hormone activation of pertussis toxin (PTX)-sensitive G protein-coupled receptors (GPCRs) (Stanfield et al., 2002). Consistent with this, gene knockout of neuronal GIRK channel subunits promote spontaneous and pharmacologically induced seizures and hyperactivity in mice (Blednov et al., 2001; Signorini et al., 1997). The recent discovery of neuronal GIRK channel involvement in drug-induced analgesia further highlight the physiological role of GPCR-activated GIRK channels and their modulators in the nervous system (Blednov et al., 2003).

The temporal gating properties of receptor-activated GIRK currents are determined by the kinetic properties of the G protein cycle and dramatically
accelerated by ‘regulators of G protein signaling’ (RGSs) (Breitwieser and Szabo, 1988; Doupnik et al., 1997; Saitoh et al., 1997). RGS proteins speed up passage through the G-protein cycle by increasing the intrinsic GTPase activity of Gα subunits, thereby accelerating the reassociation of the ‘inactive ‘heterotrimeric Gα(GDP)βγ complex (Ross and Wilkie, 2000). RGS proteins are characterized by a highly conserved ‘RGS domain ‘of ~ 125 a.a. that confers direct binding to Gα subunits and is flanked by less conserved N- and C-terminal domains of variable length (Tesmer et al., 1997). More than 20 mammalian RGS genes have been identified to date and are classified into six subfamilies based on sequence homology within the RGS domain (Hollinger and Hepler, 2002; Ross and Wilkie, 2000).

The divergent amino terminal region of the R4 subfamily of RGS proteins has been implicated in (1) mediating RGS selective coupling to GPCRs (Zeng et al., 1998), (2) facilitating functional α2 adrenergic receptor-GIRK channel coupling in rat sympathetic neurons (Jeong and Ikeda, 2001), and (3) promoting translocation of GPCR-RGS complexes to the plasma membrane (Roy et al., 2003; Saitoh et al., 2002). Thus the divergent RGS amino terminus may provide a means to confer selective RGS coupling to different GPCR-effector signaling complexes (Hollinger and Hepler, 2002).

I report here the functional properties of an alternatively spliced ‘short isoform’ of mouse RGS3 (RGS3s) (Reif and Cyster, 2000) on neuronal GIRK channels (Kir3.1/Kir3.2a) coupled to either serotonin 1A (5-HT_{1A}) receptors or
muscarinic m2 receptors in Chinese hamster ovary cells (CHO-K1). Mammalian RGS3 isoforms are expressed in both brain and heart (Druey et al., 1996; Koelle and Horvitz, 1996), and alternatively spliced RGS3 transcripts generate at least four different protein isoforms having different amino terminal domains that share a common RGS domain (Kehrl et al., 2002). The unique amino terminal region of mouse RGS3s is 21 amino acids long and comparable in size to the 33 amino acid N-terminus of RGS4. Yet RGS3s lacks the two Cysteines (C2 and C12) that are conserved in some members of the R4 subfamily including RGS4 and are susceptible to plamitoylation (Druey et al., 1999; Hiol et al., 2003; Srinivasa et al., 1998). I therefore questioned whether the variant RGS3s isoform differentially affects GIRK channel gating properties compared to RGS4 (Doupnik et al., 1997). My findings demonstrate RGS3s differentially modulates GPCR-GIRK channel complexes and suggest that different RGS N-termini may influence the agonist sensitivity and magnitude of GIRK channel activation in a GPCR-dependent manner.
RESULTS

Properties of 5-HT\textsubscript{1A} and m2 receptor coupled GIRK currents reconstituted in CHO-K1 cells

Co-expression of neuronal Kir3.1/Kir3.2a channels in CHO-K1 cells with either the 5-HT\textsubscript{1A} receptor or the muscarinic m2 receptor produced agonist-evoked currents that were dose-dependent and displayed strong inward rectification (Fig. 4.1). To resolve the temporal and steady-state kinetic features of the receptor-activated GIRK currents, 5-HT and ACh were rapidly applied and washed out using concentrations ranging from 10^{-9} to 10^{-4} M. The reversal potentials for the 5-HT and ACh-evoked currents were both near the experimentally preset K\textsuperscript{+} equilibrium potential (-40 mV) consistent with K\textsuperscript{+}-selective GIRK channels (Fig. 4.1B). Both the 5-HT-activated GIRK currents (I\textsubscript{K,5-HT}) and Ach-activated GIRK currents (I\textsubscript{K,ACh}) displayed a similar activation and deactivation time course following agonist washout (Fig. 4.1A). Notably, however, the steady-state dose-dependence of 5-HT versus Ach-activated GIRK currents indicated a significantly higher potency for 5-HT versus ACh (Fig. 4.1C). The EC\textsubscript{50} value for 5-HT was 24±8 nM (n=5) compared to the Ach EC\textsubscript{50} value of 820±162 nM (n=10). This difference in EC\textsubscript{50} values indicate either a higher
number of 5-HT$_{1A}$ receptors being expressed compared to m2 receptors and/or a
greater efficacy in 5-HT$_{1A}$ receptor versus m2 receptor signaling. The maximal
GIRK current density at saturating concentrations of receptor agonist (10 µM)
was comparable indicating equivalent Kir3.1/Kir3.2a channel expression with the
two GPCRs; maximal I$_{K,5-HT}$ 90.8±15.4 pA/pF (n=8), maximal I$_{K,ACh}$ 79.2±8.2
pA/pF (n=11). Other than differences in agonist dose-dependence, the temporal
kinetic features of I$_{K,5-HT}$ and I$_{K,ACh}$ were indistinguishable.
Figure 4.1. Functional coupling of 5-HT$_{1A}$ receptors and muscarinic m2 receptors to Kir3.1/Kir3.2a channels expressed in CHO-K1 cells. (A) Representative whole-cell recordings from two separate cells expressing human 5-HT$_{1A}$ receptors (upper trace) or human muscarinic m2 receptors (lower trace). Cells were voltage clamped at -100 mV during a 15 s rapid application and washout of receptor agonist (10 µM 5-HT or ACh) indicated by the horizontal bars. Voltage ramps from -100 to +50 mV were evoked before and during agonist application to assess the voltage dependence of the agonist-evoked currents. (B) Inward rectification of 5-HT (open circles) and ACh-evoked GIRK currents (filled circles). Ramp currents preceding agonist application were digitally subtracted from ramp currents evoked during agonist application as shown in (A). Both $I_{K,5-HT}$ and $I_{K,ACh}$ displayed strong inward rectification and reversal potentials near the predicted Nernst potential for potassium (-40 mV). (C) Dose-response relations for 5-HT (open circles) and ACh-activated GIRK currents (filled circles). Receptor-activated GIRK currents from varying agonist concentrations applied to the same cell were normalized to the maximal amplitude recorded from each individual cell. Data are the mean±SE from GFP-positive CHO-K1 cells co-transfected with cDNA vectors encoding rat Kir3.1, mouse Kir3.2a, and either the human 5-HT$_{1A}$ receptor or the human muscarinic m2 receptor, with GFP included as a reporter. The solid curves represent Hill functions fit to the mean data points.
A. 5-HT 10 µM

B. $V_m$ (mV)

C. [Agonist] (log M)
Effects of PTX pretreatment on 5-HT\textsubscript{1A} and m2 receptor coupled GIRK currents

Both 5-HT\textsubscript{1A} receptors and m2 receptors are capable of coupling to all PTX-sensitive G\textalpha i/o subunits, and coupling in CHO-K1 cells is reportedly limited to endogenous expression of G\textalpha i2 and G\textalpha i3 subunits where G\textalpha i2 protein levels predominate (G\textalpha i2>>G\textalpha i3 by 8:1) (Dell'Acqua et al., 1993; Raymond et al., 1993). Pretreatment of cells with PTX (100 ng/ml) completely abolished the ACh-evoked GIRK currents (n=5) and significantly reduced the 5-HT-evoked GIRK currents ~80% (PTX-treated 20±5 pA/pF, n=3; non-treated 94±14 pA/pF, n=8). Thus endogenous PTX-sensitive G\textalpha i subunits mediate the coupling of m2 receptors and 5-HT\textsubscript{1A} receptors to GIRK channels in CHO-K1 cells, although the residual 5-HT-evoked GIRK current following PTX pretreatment may reflect a small degree of ‘promiscuous’ 5-HT\textsubscript{1A} receptor coupling to PTX insensitive G proteins.

Comparison of RGS3s and RGS4 effects on muscarinic m2 receptor-coupled GIRK currents

I next compared the modulatory effects of RGS3s and RGS4 on m2 receptor activated Kir3.1/3.2a channels in relation to cells that were not transfected with exogenous RGS (control). Shown in Fig. 4.2, the activation and deactivation kinetics of ACh-evoked GIRK currents were accelerated by either RGS3s or RGS4 expression compared to the control cells. Kinetic analysis
indicated RGS3s accelerated the GIRK deactivation time course somewhat greater than RGS4 (RGS3s \( \tau_{\text{deact}} = 0.75 \pm 0.04 \) s, \( n=8 \); RGS4 \( \tau_{\text{deact}} = 1.32 \pm 0.11 \) s, \( n=5 \)), although the effects of RGS3s on the GIRK activation kinetics were equivalent to RGS4 (Fig. 4.2C). The most striking difference between RGS3s and RGS4 was a significant reduction of GIRK current amplitude (~45% decrease at 100 \( \mu \text{M} \)) and a 6-fold shift in the ACh dose-response curve associated with RGS3s expression (Fig. 3.2D,E). With RGS3s, the Ach EC\(_{50}\) was 5.1 \( \pm 0.6 \) \( \mu \text{M} \) (\( n=8 \)) compared to 0.9 \( \pm 0.2 \) \( \mu \text{M} \) (\( n=10 \)) for control cells. By comparison, RGS4 did not significantly affect the maximal GIRK current density as observed previously in Xenopus oocytes (Doupnik et al., 1997), and caused a smaller shift in the ACh EC\(_{50}\) value (2.0 \( \pm 0.5 \) \( \mu \text{M} \), \( n=6 \)) from the control group (Fig. 4.2D,E). Since the ACh dose-response curve with RGS3s expression did not demonstrate saturation (Fig. 4.2D), GIRK current responses to 100 \( \mu \text{M} \), 1 mM, and 10 mM ACh were also compared in a separate set of cells (\( n=9 \)). These experiments confirmed that 100 \( \mu \text{M} \) Ach was indeed a saturating concentration, as maximal GIRK responses to 1 mM (95\%2%) and 10 mM Ach (95\%2%) were not significantly different than 100 \( \mu \text{M} \) Ach (93\%2%). Altogether these findings indicate RGS3s and RGS4 both accelerate GIRK channel gating kinetics, but differentially affect steady-state m2 receptor-GIRK channel coupling properties.
Figure 4.2. Comparative effects of RGS3s versus RGS4 on muscarinic m2 receptor-coupled Kir3.1/Kir3.2a channels expressed in CHO-K1 cells. (A) Representative ACh-activated GIRK currents elicited from three separate expression conditions; either without exogenous RGS expression (control traces), with exogenous RGS4 expression (RGS4 traces), or with exogenous RGS3s expression (RGS3s traces). GIRK currents evoked by a range of ACh concentrations for each cell are superimposed for comparison after baseline adjustment of the holding current immediately preceding each Ach application. ACh applications were 15 s in duration and separated by a ~1 min washout period. (B) Deactivation kinetics of RGS-accelerated GIRK currents. Upper panel: deactivation time constants (τ_{deact}) derived from control (filled bar), RGS3s (grey bar), and RGS4 (open bar) groups following 10 μM ACh-evoked GIRK currents. Lower panel: comparison of t_{deact} values following three different ACh concentrations with either RGS3s (grey bars) or RGS4 (open bars) expression. Data are the mean±SE where * indicates P<0.05. (C) Activation kinetics of RGS-accelerated GIRK currents. Comparison of activation time constants (τ_{act}) derived from control (filled bar), RGS3s (grey bar), and RGS4 (open bar) groups with increasing ACh concentrations. (D) ACh dose-response relations for control (filled squares), RGS3s (grey triangles), and RGS4 (open circles) groups. GIRK currents were normalized to cell membrane capacitance and expressed as a current density (pA/pF) for group comparisons. (E) Normalized ACh dose-response curves from data presented in (D). GIRK current amplitudes were normalized to the maximal amplitude recorded from each cell (100 μM ACh) and fit with a Hill function to derive EC_{50} values and Hill coefficients.
Comparison of RGS3s and RGS4 effects on serotonin 1A receptor-coupled GIRK currents

I next examined the effects of RGS3s and RGS4 on 5-HT$_{1A}$-coupled GIRK currents to determine whether the different effects of RGS3s and RGS4 observed with m2 receptor coupling were similarly conferred with 5-HT$_{1A}$ receptors. Shown in Fig. 4.3, co-expression of either RGS3s or RGS4 significantly accelerated the activation and deactivation time course of 5-HT-activated GIRK currents. Kinetic analysis of both the GIRK activation and deactivation time course indicated the accelerating effects of RGS3s and RGS4 were not significantly different from each other (Fig. 4.3B,C). Interestingly, neither RGS3s nor RGS4 affected the maximal GIRK current density although both appeared to have subtle effects that were not statistically significant (Fig. 4.3D). Similar to m2 receptor coupling, RGS3s significantly shifted the 5-HT dose-response curve yet RGS4 did not. For RGS3s, the 5-HT EC$_{50}$ was 128±36 nM (n=5) compared to 30±9 nM (n=4) for the control cells and 48±6 nM (n=9) with RGS4 expression. Thus RGS3s, in contrast to RGS4, displays GPCR dependence in that it dramatically reduces steady-state m2 receptor-activated GIRK currents but not 5-HT$_{1A}$ receptor-coupled currents.
Figure 4.3. Comparative effects of RGS3s versus RGS4 on serotonin 1A (5-HT$_{1A}$) receptor-coupled Kir3.1/Kir3.2a channels expressed in CHO-K1 cells. (A) Representative 5-HT-activated GIRK currents elicited from three separate cell conditions, either without exogenous RGS expression (control traces), with exogenous RGS4 expression (RGS4 traces), or with exogenous RGS3s expression (RGS3s traces). GIRK currents evoked by a range of 5-HT concentrations for each cell were superimposed for comparison after baseline adjustment of the holding current immediately preceding each agonist application. 5-HT applications were 15 s in duration and separated by a ~1 min washout period. (B) Deactivation kinetics of RGS-accelerated GIRK currents. Upper panel: deactivation time constants ($\tau_{\text{deact}}$) derived from control (filled bar), RGS3s (grey bar), and RGS4 (open bar) groups following 10 µM 5-HT-evoked GIRK currents. Lower panel: comparison of $\tau_{\text{deact}}$ values following three different 5-HT concentrations with either RGS3s (grey bars) or RGS4 (open bars) expression. Data are the mean±SE where * indicates P<0.05. (C) Activation kinetics of RGS-accelerated GIRK currents. Comparison of activation time constants ($\tau_{\text{act}}$) derived from control (filled bar), RGS3s (grey bar), and RGS4 (open bar) groups with increasing 5-HT concentrations. (D) 5-HT dose-response relations for control (filled squares), RGS3s (grey triangles), and RGS4 (open circles) groups. GIRK currents were normalized to cell membrane capacitance and expressed as a current density (pA/pF) for group comparisons. (E) Normalized 5-HT dose-response curves from data presented in (D). GIRK current amplitudes were normalized to the maximal amplitude recorded from each cell (10 µM 5-HT) and fit with a Hill function to derive EC$_{50}$ values and Hill coefficients.
Effects of RGS3s and RGS4 on basal GIRK channel activity

I also analyzed the effects of RGS3s and RGS4 on receptor-independent basal GIRK current amplitudes as reflected in the holding currents in 25 mM K+ solution. Previous reports indicate RGS3 (original 519 a.a. isoform) and RGS4 cause a significant increase in basal GIRK current activity when expressed in either CHO or HEK293 cells by apparently increasing the availability of free Gβγ subunits via RGS sequestration of Gα subunits (Bunemann and Hosey, 1998). This finding is in contrast to observations in the oocyte expression system, where RGS3 and RGS4 reduce I_{K,basal} amplitudes by apparently shifting the equilibrium of Gα subunits towards their GDP-bound state due to RGS-enhanced GTP hydrolysis and effectively sequestering free Gβγ dimers that cause basal GIRK channel activity (Doupnik et al., 1997). In the CHO-K1 experiments reported here, expression of Kir3.1/Kir3.2a channels significantly increased the I_{K,basal} amplitude compared to nontransfected CHO-K1 cells (Table 4.1), thus demonstrating a significant level of receptor-independent ‘basal’ GIRK channel activity in the absence of exogenous RGS expression. Comparison of I_{K,basal} amplitudes from the control groups (RGS-) with co-expression of either RGS3s or RGS4 did not reveal a significant difference with either m2 receptor or 5-HT_{1A} receptor expression (Table 4.1). Thus the effects reported by (Bunemann and Hosey, 1998) may result from significantly higher RGS protein levels produced with their transfection methods, since expression conditions that elevate RGS4
levels in oocytes has also been reported to increase basal GIRK channel activity (Keren-Raifman et al., 2001)

**Table 4.1** Effects of RGS3s and RGS4 on basal GIRK channel activity in CHO-K1 cells

<table>
<thead>
<tr>
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<th>( I_{k,\text{basal}} ) (pA/pF)¹</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Non-transfected CHO-K1 cells</td>
<td>-10 ± 4 (n=6)</td>
</tr>
<tr>
<td>Muscarinic m2 receptor + Kir3.1/Kir3.2a</td>
<td>-130 ± 17 (n=10)</td>
</tr>
<tr>
<td>Serotonin 1A receptor + Kir3.1/Kir3.2a</td>
<td>-84 ± 23 (n=11)</td>
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¹ Data are resting membrane currents in 25 mM K⁺ at a holding potential of -100mV divided by the cell membrane capacitance
Effects of RGS3s and RGS4 on acute desensitization of GIRK currents

In the absence of RGS co-expression, GIRK currents modestly desensitize during the short 15 s agonist application period (<10% of their amplitude). Co-expression of RGS4 causes a significant increase in the rate of ‘acute’ desensitization which is attributable to the accelerated rate of signal termination during sustained receptor activation (Chuang et al., 1998; Doupnik et al., 1997). Shown in Fig. 4.4, comparisons of the extent of acute desensitization with RGS3s versus RGS4 expression during the 15 s agonist application period indicate equivalent effects on both $I_{K,5-HT}$ and $I_{K,ACh}$. These findings are consistent with the rate of acute GIRK current desensitization being closely correlated with $G\alpha$ GTPase activity and best reflected in the GIRK deactivation rates (Chuang et al., 1998; Leaney et al., 2004). As shown earlier for RGS3s and RGS4 (Figs. 4.2 and 4.3), both RGS proteins accelerate GIRK deactivation rates to a similar degree.
Figure 4.4. Acute GIRK current desensitization associated with different GPCR-RGS coupling conditions. (A) Comparative effects of RGS3s (red trace) and RGS4 (blue trace) on acute desensitization of 5-HT$_{1A}$ receptor-activated GIRK currents without exogenous RGS expression (control, black trace). Peak amplitudes of the superimposed recordings were normalized for kinetic comparisons. Right panel: the percent desensitization was quantified by measuring the percent decline in the peak GIRK current amplitude measured at the end of the 15 s application period, as denoted by the application “window” (dotted box in left panel). Data are the mean±SE where * indicates a P<0.05 for comparisons between the control and RGS groups. (B) Comparative effects of RGS3s (red trace) and RGS4 (blue trace) on acute desensitization of muscarinic m2 receptor-activated GIRK currents without exogenous RGS expression (control, black trace). Right panel: quantification of acute GIRK desensitization was determined as described in (A).
A. 5-HT (10µM) GIRK Desensitization (%)

B. ACh (10µM) GIRK Desensitization (%)

Control RGS3s RGS4

5 s

Legend:

* p < 0.05
DISCUSSION

The goal of this study was to evaluate the modulatory effects of a recently identified ‘short’ RGS3 isoform on neuronal GIRK channels activated by different GPCRs in a mammalian cell expression system (CHO-K1 cells). The RGS3s mRNA transcript is abundant in mouse brain and heart (Reif and Cyster, 2000) and therefore may modulate GPCR regulation of neuronal and cardiac cell excitability. The effects of RGS3s were assessed in comparison to the closely related and previously studied RGS4 protein (Doupnik et al., 1997; Zhang et al., 2002) that is co-expressed with RGS3 in individual neurons and atrial cardiomyocytes (Doupnik et al., 2004; Doupnik et al., 2001). The major finding of my experiments is that RGS3s modulates GIRK channels in a GPCR-dependent manner, whereas RGS4 modulated GIRK channels similarly for both of the GPCRs studied. RGS3s significantly reduced GIRK current amplitudes with m2 receptor coupling and shifted the steady-state agonist dose-response relations, whereas RGS4 affected m2 receptor-activated GIRK currents similar to that observed with 5-HT\textsubscript{1A} receptors. These results may indicate RGS3s has distinct interactions with muscarinic m2 versus 5-HT\textsubscript{1A} receptor complexes, whereas RGS4 interacts similarly with both GPCR-GIRK channel complexes. There are several possible RGS-affected cellular processes that may contribute toward the modulatory differences that we have identified and are briefly discussed below.
GTPase accelerating activity of RGS proteins

The GTPase accelerating activity of RGS proteins is mediated by direct interactions between the RGS domain and the Go subunit (Ross and Wilkie, 2000), and differences in RGS modulation of GIRK channels can reflect differences in RGS-Go subunit selectivity (Doupnik et al., 1997; Zhang et al., 2002). Although RGS3 and RGS4 both interact with Go/i/o and Go/q/11 subunits, RGS3 displays a higher affinity for Go/11 versus Go/i3 (Dulin et al., 1999; Neill et al., 1997) and RGS4 shows preferential interactions with Go/i/o subunits versus Goq (Berman et al., 1996a). So for the Go-i-coupled receptors examined in my CHO-K1 experiments, these preferred RGS-Go associations would generally favor greater accelerated GIRK deactivation rates with RGS4 compared to RGS3s. Yet to the contrary, these kinetic differences were not observed and in fact RGS3s accelerated the GIRK deactivation rate somewhat greater than RGS4. Thus differences in RGS3s versus RGS4 affinity for Go/i subunits are not apparent in the accelerated GIRK channel gating properties that reflect RGS-enhanced GTPase accelerating activity and seem unlikely to explain my findings.

RGS membrane association

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Several members of the R4 subfamily, including RGS4, enhance membrane binding through a mechanism requiring their short N-terminal domain. These RGS proteins (RGS2, RGS4, RGS5, RGS8, RGS16, RGS18) possess N-terminal palmitoylated cysteine residues and a conserved basic amphipathic α-helix that confers membrane association and orientation that enhances their GTPase activating activity (Bernstein et al., 2000; Chen et al., 1999; Heximer et al., 2001; Saitoh et al., 2001; Tu et al., 2001). The RGS4 N-terminus also contains a ubiquitin degradation signal (Davydov and Varshavsky, 2000). Yet for both RGS4 and RGS8, deleting the N-terminal domain does not significantly affect RGS-accelerated activation and deactivation kinetics for GPCR-activated GIRK channels expressed in Xenopus oocytes, indicating RGS domain-Go interactions are sufficient for these kinetic effects (Inanobe et al., 2001; Saitoh et al., 2001). However, deleting the RGS8 N-terminus does reduce acute desensitization during dopamine D2 receptor GIRK channel activation (Saitoh et al., 2001) which is attributable to RGS-enhanced GTPase activity (Chuang et al., 1998). Remarkably, a ‘short’ RGS8 splice variant (RGS8s) differing only by the first 7-9 N-terminal residues shows diminished effects on GIRK activation and deactivation kinetics and altered selectivity for Gq-coupled receptor signaling (Saitoh et al., 2002). Furthermore, overexpression of the RGS8 N-terminal domain (1-5 a.a.) in rat sympathetic neurons dramatically accelerates α2-adrenergic receptor activation of heterologously expressed GIRK channels, supporting an important role of the RGS8 N-terminus in facilitating receptor-GIRK
channel coupling (Jeong and Ikeda, 2001). In my comparison of RGS3s and RGS4 on GIRK kinetics reported here, despite their divergent N-terminal sequences, both displayed similar accelerating effects on GIRK activation and deactivation kinetics and equivalent effects on acute desensitization of receptor-activated GIRK currents. Therefore, apparently, differences in RGS3s and RGS4 N-terminal domains do not confer obvious kinetic differences in receptor-dependent GIRK channel gating, despite their differential effects on receptor-dependent steady-state gating properties.

RGS-mediated translocation of GPCRs

RGS4 is predominantly a cytosolic protein recruited to membranes by interactions with G protein subunits (Druey et al., 1998). RGS-specific translocation from the cytosol to the plasma membrane involves direct interactions with the GPCR complex and is determined in part by the relative affinity of the RGS-Gα subunit interaction (Masuho et al., 2004; Roy et al., 2003). It remains unclear whether cytosolic RGS proteins can incorporate into mature GPCR-GIRK channel complexes already located at the plasma membrane, or whether they co-assemble within the GPCR-GIRK channel complexes synthesized and assembled within the endoplasmic reticulum and Golgi apparatus (Lavine et al., 2002). Current evidence, however, clearly indicate RGS4 facilitates trafficking and recruitment of G proteins (Chuang et al., 1998) and m2 receptor Gαi2 complexes (Roy et al., 2003) from intracellular pools to
the plasma membrane, and thereby increases the density of functional receptors at the plasma membrane. For RGS8, deletion of the N-terminal domain (ΔN-RGS8) prevents G protein-induced subcellular translocation of ΔN-RGS8 to the plasma membrane (Saitoh et al., 2001). Similarly, translocation of the original RGS3 isoform from the cytosol to the plasma membrane also occurs but in an agonist- and Ca\(^{2+}\)-dependent manner (Dulin et al., 1999). The Ca\(^{2+}\)-dependent translocation of RGS3 was recently shown to be mediated by Ca\(^{2+}\) binding to an EF-hand motif located in the N-terminus of RGS3, which is not present in the shorter N-terminus of RGS3s (Tosetti et al., 2003). Together, these findings suggest RGS4 may translocate m2 receptor/G\(\alpha_i\) complexes to the plasma membrane more effectively than RGS3s, due either to a lower RGS3s-G\(\alpha_i\) affinity and/or a reduced efficacy of the RGS3s N-terminal domain in the translocation process. The consequence in either case would be a lower cell surface concentration of receptors with RGS3s expression, which is consistent with the reduced GIRK current responses and rightward shift in the ACh dose response curve observed with RGS3s expression. What is puzzling with this working hypothesis is why the RGS3s effect on steady-state receptor-dependent GIRK activation properties are more prominent for m2 receptors and less so for 5-HT\(_{1A}\) receptor complexes.

**Direct RGS-GPCR interactions**
Similar to my observations reported here, different R4 RGS proteins exhibit GPCR-selective modulation of Gq/11-coupled (Wang et al., 2002; Xu et al., 1999) and other Gi/o-coupled signaling pathways (Ghavami et al., 2004). Moreover, the N-terminal domains of RGS3, RGS4, and RGS8 have been shown to affect the selective regulation among Gq/11-coupled receptors (Chatterjee et al., 1997; Saitoh et al., 2002; Zeng et al., 1998). Recently, the N-terminus of RGS2 was shown to have direct and selective interactions with the 3rd intracellular (i3) loop of Gq/11-coupled muscarinic receptors in vitro (Bernstein et al., 2004). Full-length RGS4 also interacts with the i3 loop of Gq/11-coupled muscarinic m1 and m5 receptors, but similar to RGS2, does not interact with the i3 loops of Gi/o-coupled m2 or m4 receptors (Bernstein et al., 2004). Thus direct interaction of RGS4 with the m2 receptor remains to be resolved, yet apparently does not involve interactions with the i3 loop. Given the divergent nature of the RGS3s N-terminal domain compared to RGS4, differential interactions of the RGS3s N-terminus with GPCRs seems plausible and could thereby affect the efficacy of receptor translocation to the plasma membrane and/or G protein activation in a GPCR-selective manner. The intrinsic G protein coupling properties of different GPCRs may also impact RGS interactions within the signaling complex. In the absence of overexpressed RGS proteins in CHO-K1 cells, GIRK channels activated by m2 receptors and 5-HT_{1A} receptors displayed significantly different agonist potencies with 5-HT being ~30-fold more potent than Ach (cf. Fig. 3.1). Differences in receptor expression, cell surface density,
and receptor translocation (i.e. 5-HT$\textsubscript{1A}$$>$m2 receptors) are all possible contributors to this observed difference as discussed above. The 5-HT$\textsubscript{1A}$ receptor-coupled GIRK channels also displayed a PTX-insensitive component that may reflect promiscuous G protein coupling or coupling of residual G$\alpha$ subunits (not ADP-ribosylated) due to a higher coupling affinity. Reconstitution experiments comparing GPCR-G$\alpha$ binding affinities recently found agonist-bound 5-HT$\textsubscript{1A}$ receptors to have a 12-fold higher affinity for G$\alpha$i1(GDP)$\beta\gamma$ compared to agonist-bound m2 receptors (Slessareva et al., 2003), indicating agonist-activated 5-HT$\textsubscript{1A}$ receptors have an intrinsically higher efficacy for G$\alpha$i(GDP)$\beta\gamma$ coupling compared to muscarinic m2 receptors. GPCR differences in intrinsic G protein coupling (i.e. precoupling) and the influence of associated RGS proteins are important considerations for future mechanistic investigations (Shea and Linderman, 1997). From our results described here, RGS3s and RGS4 could produce equivalent modulatory effects on 5-HT$\textsubscript{1A}$ receptor-coupled GIRK channels due to a higher degree of G protein precoupling compared to m2 receptors (Zhang et al., 2002).

In summary, I compared the functional properties of the RGS3s isoform and RGS4 due to their expression in brain and heart (Kehrl et al., 2002; Reif and Cyster, 2000) and in native GIRK-expressing neurons and atrial myocytes (Doupnik et al., 2004; Doupnik et al., 2001). The GPCR dependent effects of RGS3s observed on neuronal GIRK channel function raise new questions regarding RGS-dependent modulation of GPCR-GIRK channel complexes.
To test if there is a selective interaction among RGS-GIRK-GPCR complexes I next generated several RGS chimeras and deletion constructs, epitope tagged them, and by co-immunoprecipitation detected the possible interactions.
CHAPTER 5

RGS4 DIRECTLY ASSOCIATES WITH MULTIPLE GPCR-KIR3 CHANNEL SIGNALING COMPLEXES

INTRODUCTION

RGS4, a member of the “Regulators of G protein Signaling” gene family (Hollinger and Hepler, 2002; Ross and Wilkie, 2000), is abundantly expressed in the mammalian brain and peripheral nervous system (Druey et al., 1996; Gold et al., 1997; Koelle and Horvitz, 1996). Functionally, RGS4 augments the GTPase activity of Gi/o and Gq/11 proteins and accelerates the termination of G protein-coupled receptor (GPCR) signaling (Berman et al., 1996b; Hepler et al., 1997; Mukhopadhyay and Ross, 1999; Watson et al., 1996). Genetic linkage and association analysis has identified the human RGS4 gene as a major susceptibility locus (chromosome 1q21-q22) for schizophrenia (Brzustowicz et al., 2000; Chowdari et al., 2002), where gene profiling studies have shown RGS4 expression to be the most significantly reduced gene in the prefrontal cortex of schizophrenic subjects (Mirnics et al., 2001). These findings, together with the potential role of RGS4 in regulating several neurotransmitter systems known to
affect symptoms of schizophrenia (hallucinations, delusions, and depression), implicate RGS4 in the etiology of schizophrenia (Harrison and Weinberger, 2005). Decreased RGS4 levels are also reported to correlate with the reduced cholinergic signaling found in Alzheimer’s disease (Muma et al., 2003).

Aside from its potential role in neurological disease and disorders, RGS4 is a highly regulated modulator that provides adaptive capabilities during various levels of cell signaling (Chidiac and Roy, 2003). At the transcriptional level, brain RGS4 mRNA levels are dynamically regulated by neurotransmitter activation of different GPCRs (Geurts et al., 2002; Geurts et al., 2003; Taymans et al., 2003), several drugs of abuse (cocaine, morphine, and amphetamines) (Bishop et al., 2002; Garnier et al., 2003; Gold et al., 2003), stress and glucocorticoids (Ni et al., 1999), and electroconvulsive seizures (Gold et al., 2002). At the post-translational level, RGS4 protein is rapidly degraded via the ubiquitin-dependent N-end rule pathway, a process initiated by arginylation of Cys2 by arginyltransferases (Davydov and Varshavsky, 2000; Lee et al., 2005) and tightly coupled to the oxidative environment (Hu et al., 2005). Together these findings illustrate the multiple layers of regulation that determine the RGS4 protein concentration level that modulates Gi/o and Gq/11 signaling in the brain.

One of the key effectors for Gi/o and Gq/11–coupled receptors that modulates neuronal excitability is the G protein-gated inwardly rectifying K+ (Kir3/GIRK) channel (Stanfield et al., 2002; Yamada et al., 1998). Kir3 channels in hippocampal neurons are localized to dendrites, dendritic spines, and the cell
soma (Drake et al., 1997) and thus well positioned for suppressing excitation following activation by pertussis toxin (PTX)-sensitive Gi/o-coupled receptors as evidenced in seizure-prone Kir3.2 knockout mice (Luscher et al., 1997; Signorini et al., 1997). In contrast to activation by Gi/o–coupled receptors, Kir3 channels are inhibited by PTX-insensitive Gq/11-coupled receptor signaling causing enhanced neuronal excitability (Nakajima et al., 1988). Kir3 channels can form stable macromolecular signaling complexes containing Gi/o– or Gs-coupled receptors (Lavine et al., 2002), heterotrimeric G proteins (Clancy et al., 2005; Huang et al., 1995; Ivanina et al., 2004; Krapivinsky et al., 1995b), and multiple kinases and phosphatases (Nikolov and Ivanova-Nikolova, 2004). Since RGS4 significantly accelerates both the activation and deactivation time course for Gi/o-coupled receptor-activated Kir3 channel currents without compromising current amplitude (Doupnik et al., 1997), it has been questioned whether RGS4 directly binds to GPCR-Kir3 channel complexes as a means of efficacious modulation and targeting specificity (Zhang et al., 2002). It is shown here that RGS4 directly interacts with several GPCR-Kir3 channel complexes comprised of either Gi/o or Gq/11-coupled receptors expressed in CHO-K1 cells. RGS4 coupling is mediated through interactions with the GPCR versus the Kir3 channel, and displays specificity since a closely related RGS homolog (RGS3s) (Jaen and Doupnik, 2005; Reif and Cyster, 2000) does not interact with any of the GPCR-Kir3 channel complexes tested.
RESULTS

RGS4 and RGS3s protein expression in CHO-K1 cells.

To determine whether RGS4 or RGS3s directly associate with GPCR-Kir3 channel complexes I 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. Initial western blot analysis of cell lysates reaffirmed previous findings (Krumins et al., 2004) indicating RGS4 protein levels are low and often undetectable, and significantly less than RGS3s (Figure 5.1A). 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 Cys2 (Davydov and Varshavsky, 2000; Lee et al., 2005). RGS3s notably lacks this N-terminal cysteine residue. I therefore also compared protein levels of the degradation-resistant RGS4(C2V) mutant (Davydov and Varshavsky, 2000). As shown in Figure 5.1B, the level of RGS4(C2V) protein in the cell lysate was significantly greater than wildtype RGS4 and more comparable to 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 alternative translation initiation start site (Krumins et al., 2004). Given the similar and stable expression levels of RGS3s and RGS4(C2V), RGS4(C2V) was routinely used 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 were indistinguishable from those produced by their untagged counterparts reported previously (Jaen and Doupnik, 2005). 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 (Figure 5.1D,E). This was somewhat unexpected given the large difference in protein expression between RGS4 and RGS4(C2V), and suggests RGS4 protein levels (significantly lower than RGS3s and RGS4(C2V)) are saturating with regards to functional Kir3 channel modulation. Also consistent with my previous study (Jaen and Doupnik, 2005), RGS3s-FLAG caused a significant rightward shift in the ACh dose response relation (Figure 5.1F) and reduced peak $I_{K,ACh}$ amplitudes by ~50% (data not shown).
Figure 5.1. RGS3s, RGS4, and the degradation-resistant RGS4(C2V) mutant are differentially expressed in CHO-K1 cells, yet similarly affect muscarinic m2 receptor-activated Kir3 channel current kinetics. (A & B) Western blot 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 subunit, and the Kir3.2a subunit (see Methods for details). Sample lanes were each loaded with 20 µg 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), RGS4-FLAG (blue trace), or RGS4(C2V)-FLAG (green trace). The peak currents have been normalized to compare the RGS-dependent effects on Kir3 channel gating kinetics during and after a 15 s application of 1 µM ACh. The current deflections before and during ACh application are responses to voltage ramps (-100 mV to +50 mV) used to monitor inward rectification. The holding membrane potential was -100 mV in all cases. (D & 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 time constants, $\tau_{\text{act}}$ and $\tau_{\text{deact}}$, respectively. The ACh concentration-dependence of $\tau_{\text{act}}$ is shown for each RGS examined (panel F). The $\tau_{\text{deact}}$ values are following the rapid washout of 1 µM ACh. Values are the mean±SEM (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.
Differential RGS interaction with m2 receptor-Kir3 channel complexes.

HA-tagged m2 receptor was immunoprecipitated and probed for co-precipitating Kir3.1-MYC and RGS-FLAG by western blot analysis. Shown in Figure 5.2, 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 (Lavine et al., 2002). Interestingly though, while RGS4(C2V) readily 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 two major bands, one molecular weight band that closely corresponded to the calculated molecular weight (52.81 kDa) and a higher band (70-75 kDa) that corresponds to glycosylated receptors (van Koppen and Nathanson, 1990). The co-precipitated Kir3.1-MYC subunit also migrated close to its calculated molecular weight (57.77 kDa).

I next questioned whether the availability Gi proteins might influence the coupling of RGS3s and RGS4 to m2 receptor-Kir3 channel complexes given potential limiting levels of endogenous Gi proteins present within the CHO-K1 cells. To test this, I examined the effects of co-expressing the Gαi2 subunit on RGS co-precipitation with the m2 receptor-Kir3 channel complex. As shown in Figure 5.3, Gαi2 expression appeared to slightly enhance wildtype 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 G\(\alpha\)i2 expression, RGS3s again did not co-precipitate with the complex and RGS4(C2V) was readily detected (Figure 5.3). 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. These experiments clearly demonstrate that RGS4 and RGS4(C2V) can form a stable interaction with m2 receptor-Kir3 channel complexes and that the closely related RGS3s isoform does not interact with the same complex.

The m2 receptor-Kir3 channel complex could also be immunoprecipitated via the Kir3.1-MYC channel subunit, where the co-precipitating m2 receptor was then detected by western blot (data not shown). Yet in this configuration, co-expression of G\(\alpha\)i2 blocked immunoprecipitation of Kir3.1-MYC. I speculate that immunoprecipitation via the cytosolic C-terminal Kir3.1-MYC epitope may be perturbed by cytosolic G protein interactions that map to the Kir3 C-terminus (Clancy et al., 2005).
Figure 5.2. Selective association of RGS4 with muscarinic m2 receptor-Kir3 channel complexes. HA-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. Coprecipitating Kir3.1-MYC and RGS-FLAG proteins were then probed by western blot analysis. Western blot of the level of RGS-FLAG protein present within each of the cell lysates is shown in the lower panel. Note that lane one (sham), which is a negative control, from CHO-K1 cells transfected only with empty vector shows no unspecific binding of proteins in the cell lysate to the agarose beads as well as any detection of co-immunoprecipitation. However, in the cell lysate, FLAG antibody shows some unspecific binding to proteins that are present in all conditions even those without RGS-FLAG protein expressed; those proteins have higher molecular weight than the RGS-FLAG proteins. Nevertheless, looking at the appropriate molecular weight between 20-25 kDa, specific RGS-FLAG detection from the cell lysates can be observed only in the conditions that were transfected with RGS-FLAG proteins.
Figure 5.3. Effects of Gai2 co-expression on RGS coupling to muscarinic m2 receptor-Kir3 channel complexes. A Gai2 expression vector (the PTX-insensitive Gai2(C352G) mutant) was included in the CHO-K1 cell transfections as described in figure 5.2. Note both RGS4 and RGS4(C2V) coprecipitate with the muscarinic m2 receptor-Kir3 channel complex, whereas RGS3s does not.
Structural determinants of RGS4 binding to m2 receptor-Gαi2-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 (Figure 5.4A). The N-terminal domain of RGS4 (aa 1-57) contains two palmitoylation sites (Cys2, Cys12) (Srinivasa et al., 1998) and an amphipathic alpha-helix (a.a. 1-33) (Bernstein et al., 2000; Tu et al., 1999; Tu et al., 2001) that are both highly conserved among two other R4 RGS proteins, RGS5 and RGS16 (Chen et al., 1999; Druey et al., 1999). The amphipathic alpha-helix of RGS4 is both necessary and sufficient for membrane association (Bernstein et al., 2000; Srinivasa et al., 1998) and is conserved in the RGS3s N-terminus (Figure 5.4A). Yet the RGS3s N-terminus lacks the two palmitoylation sites (Cys2, Cys12) that help target RGS16 (and presumably RGS4 and RGS5) to cholesterol-rich membrane lipid rafts (Hiol et al., 2003) and enhances RGS GAP activity (Bernstein et al., 2000; Srinivasa et al., 1998; Tu et al., 1999; Tu et al., 2001).

My initial hypothesis was that the RGS4 N-terminal domain was both necessary and sufficient for association of RGS4 with m2 receptor-Gαi2-Kir3 channel complexes. To test this hypothesis, RGS4 deletion mutants and RGS3s/RGS4 chimeras (all FLAG-tagged at the C-terminus) were individually co-expressed along with the HA-m2 receptor, the Gαi2 subunit, and Kir3.1-MYC/Kir3.2a channels (Figure 5.4B). The HA-m2 receptor was then
immunoprecipitated and co-precipitating RGS proteins probed by western blot. In support of my hypothesis, deleting the N-terminal domain of RGS4, RGS4(58-205)-FLAG, resulted in the loss of association with the m2 receptor-Kir3 channel complex (Figure 5.4C) as expected with a loss of membrane association (Srinivasa et al., 1998). Yet interestingly, substituting the RGS3s N-terminal domain (a.a. 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 (Figure 5.4C) suggesting palmitoylation of RGS4 Cys2 and Cys12 may also be necessary. Together these results clearly demonstrate that the RGS4 N-terminal domain is necessary for coupling to the signaling complex.

Surprisingly, however, substituting the RGS4 N-terminal domain (with or without the C2V mutation) in place of the RGS3s N-terminal domain (R4-R3s-FLAG chimera or R4(C2V)-R3s-FLAG chimera) conferred only very weak interactions with the m2 receptor-Kir3 channel complex, significantly less than RGS4(C2V) (Figure 5.4C). Thus the RGS4 N-terminal domain is clearly necessary for association with the m2 receptor-Kir3 channel complex, however the remaining RGS domain and/or C-terminus of RGS4 is also necessary for efficient high-affinity coupling. Note that the expression of these various RGS constructs had no effect on the level of m2 receptor-Kir3 channel coupling (Figure 5.4C), indicating assembly of m2 receptor-Kir3 channel complexes is not affected by RGS association.
**Figure 5.4.** 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 alpha helical domains are boxed and the conserved basic residues highlighted in red, and the palmitoylated RGS4 C2, C12 residues 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, RGS3s regions are in red. (C) The RGS4 N-terminal domain (a.a. 1-57) is necessary for RGS association with muscarinic m2 receptor-Kir3 channel complexes. Six different RGS-FLAG constructs were individually coexpressed with HA-muscarinic m2 receptors, the Goi2(C352G) subunit, and Kir3.1-MYC/Kir3.2a channels in CHO-K1 cells. The HA-m2 receptor was then immunoprecipitated and coprecipitating Kir3.1-MYC and RGS-FLAG proteins detected by western blot. RGS-FLAG 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.
RGS4(c2v) associates with multiple GPCR-Kir3 channel complexes

Kir3 channels are functionally coupled to a variety of Gi/o-coupled receptors in the nervous system and heart (Stanfield et al., 2002; Yamada et al., 1998). To determine whether RGS3s and RGS4 selectively associate with different Gi/o-coupled receptors known to activate native Kir3 channels, I examined RGS and Kir3 channel co-precipitation with several different HA-tagged GPCRs (serotonin 1A, adenosine A1, dopamine D2L, and LPA1 receptors) co-expressed with either Gαi2 or GαoA. With Gαi2 expression, each GPCR tested (serotonin 1A, adenosine A1, and LPA1 receptors) co-precipitated Kir3.1-MYC/Kir3.2a channels (Figure 5.5 and Figure 5.6) and behaved just as the muscarinic m2 receptor (cf, Figure 5.3). Moreover, each GPCR-Kir3 channel complex demonstrated the same selectivity in associating with RGS4(c2v) but not RGS3s. Wildtype RGS4 coupling was not readily detectable as RGS4 expression levels were significantly less than both RGS3s and RGS4(c2v).
Figure 5.5. RGS4(C2V) associates with multiple Gi-coupled receptor-Kir3 channel complexes. Three different HA-tagged GPCRs, the adenosine A1 receptor (3HA-A1R), the serotonin 1A receptor (HA-5-HT1AR), and the lysophosphatidic acid 1 receptor (HA-LPA1R), were expressed in CHO-K1 cells with Kir3.1-MYC/Kir3.2a channels, the Gai2(C352G) subunit, and either no RGS, RGS3s-FLAG, RGS4-FLAG, or RGS4(C2V)-FLAG. Each HA-tagged GPCR was then immunoprecipitated (IP) and co-precipitating (Co-IP) Kir3.1-MYC and RGS-FLAG proteins detected by western blot (WB). Kir3.1-MYC and RGS4(C2V)-FLAG co-precipitated with each HA-GPCR.
Similarly with GαoA expression, each GPCR tested (serotonin 1A, adenosine A1, dopamine D2L, and LPA1 receptor) co-precipitated Kir3.1-MYC/Kir3.2a channels and RGS4(C2V), but not RGS3s (Figure 5.6). Thus RGS3s does not directly interact with a variety of Gi/o-coupled receptors, whereas RGS4(C2V) coupling is rather promiscuous.

It is worth noting that the immunoprecipitation levels of the different HA-tagged GPCR proteins varied considerably, with m2 receptors and dopamine D2L receptors being markedly less than serotonin 1A, adenosine A1, or LPA1 receptors (Figure 5.6). The underlying cause for these differences are not clear, and was not attributable to either the N-terminal HA tag (1X-HA versus 3X-HA) or the presence of the signal sequence. The differences apparently reflect distinct coding region differences that affect GPCR protein expression levels. The level of co-precipitating RGS4(C2V) did not correlate with the level of immunoprecipitated HA-GPCR, being somewhat constant for each expression condition and indicates the fraction of associated RGS4(C2V) differed for each GPCR.
Figure 5.6. RGS4(C2V) associates with multiple Go-coupled receptor-Kir3 channel complexes. Effects of GoA subunit expression on RGS coupling to different GPCR-Kir3 channel complexes. Five different HA-tagged GPCRs, the muscarinic m2 receptor (HA-m2R), the serotonin 1A receptor (HA-5-HT1A R), the lysophosphatidic acid 1 receptor (HA-PA1R), the adenosine A1 receptor (3HA-A1R), and the dopamine D2L (3HA-D2L R), were expressed in CHO-K1 cells with Kir3.1-MYC/Kir3.2a channels, the GaoA(C351G) subunit, and either RGS4(C2V)-FLAG (left panel) or RGS3s-FLAG (right panel). Kir3.1-MYC and RGS4(C2V)-FLAG co-precipitated with each HA-GPCR, whereas RGS3s-FLAG did not couple to any of the GPCR-Kir3 channel complexes.
Since both RGS3 and RGS4 are also effective GAPs for G\(\alpha_q\) (Hepler et al., 1997; Scheschonka et al., 2000), I also tested whether RGS3s might associate with a GPCR known to couple selectively to G\(\alpha_q\) subunits, namely the muscarinic m1 receptor. For these experiments I co-expressed G\(\alpha_q\) and Kir3.1-MYC/Kir3.2a channels, and tested in parallel three additional GPCRs that display varying degrees of Gq coupling for comparison (LPA\(_1\), serotonin 1A, and m2 receptor). Interestingly, Kir3.1-MYC/Kir3.2a channels co-precipitated with the muscarinic m1 receptor indicating Gq-coupled receptors can also form stable complexes with Kir3 channels (Figure 5.7). As observed with the Gi/o-coupled receptors, RGS3s again failed to couple to the m1 receptor-Kir3 channel complex (or any of the other GPCR-G\(\alpha_q\)-Kir3 channel complexes) whereas RGS4(C2V) directly interacted with the m1 receptor-Kir3 channel complex (Figure 5.7). Thus despite the functional effects of RGS3s on Kir3 channel gating kinetics (cf. Figure 5.1), RGS3s does not directly couple to any of the GPCR-Kir3 channel complexes tested in my experiments.
Figure 5.7. Kir3 channels and RGS4(C2V) co-assemble with Gq-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-LP1A1R), the muscarinic m1 receptor (3HA-m1R), were expressed in CHO-K1 cells with Kir3.1-MYC/Kir3.2a channels, the Gαq subunit, and either RGS4(C2V)-FLAG (panel A) or RGS3s-FLAG (panel B). Each HA-tagged GPCR was then immunoprecipitated (IP) and co-precipitating (Co-IP) Kir3.1-MYC and RGS-FLAG proteins detected by western blot (WB). Kir3.1-MYC and RGS4(C2V)-FLAG co-precipitated with each HA-GPCR (panel A), whereas RGS3s-FLAG did not couple to any of the GPCR-Kir3 channel complexes (panel B). The RGS-FLAG present in each of the cell lysates is shown in the lower blots.
RGS4(c2v) couples to GPCRs independent of co-assembled Kir3 channels

The next question was whether RGS4(c2v) association with GPCR-Kir channel complexes was mediated via specific GPCR interactions, by direct Kir3 channel interactions, or by interactions with both. To determine this I 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 Figure 5.8A, immunoprecipitation of each HA-GPCR readily co-precipitated RGS4(c2v) in the absence of Kir3 channel expression. Thus the GPCR alone is sufficient, and the Kir3 channel not necessary for RGS4(c2v) coupling to GPCR complexes. Shown in Figure 5.8B, in the absence of HA-GPCR expression, immunoprecipitation of Kir3.1-MYC/Kir3.2a channels failed to co-precipitate RGS4(c2v).
Figure 5.8. 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 coprecipitated (Co-IP) RGS4(C2V)-FLAG as 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.
Functional impact of direct RGS4 coupling to GPCR-Kir3 channel complexes

RGS3s, RGS4, and RGS4(C2V) each accelerated the activation and deactivation gating kinetics of Kir3.1/Kir3.2 channels to equivalent extents in CHO-K1 cells despite differences in their physical coupling to m2 receptor-Kir3 channel complexes (cf. Figure 5.1). Examination of the accelerating effects of each of the N-terminal deletion constructs (RGS4(58-205) and RGS3s(63-192)) and RGS chimeras (R3s-R4 chimera and R4-R3s chimera) on ACh-activated Kir3 channel currents recorded from CHO-K1 cells also failed to identify any functional difference that might correlate with the differences in RGS precoupling to the signaling complex (data not shown). Therefore questioned whether direct RGS interaction with GPCR-Kir3 channel complexes in CHO-K1 cells was of no functional benefit due to saturating levels of RGS protein expression and high degree of RGS3s ‘collision coupling’.

To control and vary the expression levels of RGS3s and RGS4, the Xenopus oocyte system was used because in that system protein expression levels can be incrementally increased by titrating the amount of injected cRNA (Zhang et al., 2002). Given the similar steady-state protein levels of RGS3s-FLAG and the degradation-resistant RGS4(C2V)-FLAG mutant in CHO-K1 cells, those were the RGS proteins used in the oocyte system. Concentration-dependent modulatory effects of these two RGS proteins on m2 receptor-activated Kir3.1/Kir3.2a channels expressed in Xenopus oocytes were examined.
Shown in Figure 5.9, the amount of RGS3s-FLAG cRNA necessary to produce a maximal acceleration in the Kir3 channel deactivation rate (10 ng/oocyte), was 30 times greater than the amount of RGS4(C2V)-FLAG cRNA necessary to produce an equivalent effect (0.3 ng/oocyte). The derived EC$_{50}$ values similarly indicate there is a 30-fold greater potency for RGS4(C2V) (EC$_{50}$, 0.12 ng cRNA/oocyte) versus RGS3s (EC$_{50}$, 3.3 ng cRNA/oocyte). These results reveal the primary functional impact of direct RGS4 coupling is a greater potency in accelerating the gating kinetics of receptor-activated Kir3 channels through targeted association.
Figure 5.9. Differential potency of RGS3s and RGS4(C2V) in accelerating the deactivation kinetics 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 either RGS3s-FLAG (red traces) or RGS4(C2V)-FLAG (green traces) at two different expression levels (1 and 10 ng cRNA/oocyte). 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 RGS3s-FLAG (red symbols) and RGS4(C2V)-FLAG 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 RGS3s-FLAG (red symbols) or RGS4(C2V)-FLAG (green symbols) were tested in parallel. Values represent the mean±SEM (n=8) from two separate batches of oocytes. Mean time constant values for RGS3s-FLAG (red symbols) and RGS4(C2V)-FLAG (green symbols) were fit with a modified Hill function to derive the effective concentration of cRNA (ng/oocyte) producing 50% of the maximal acceleration in current deactivation (EC$_{50}$).
DISCUSSION

RGS4\((c2v)\) precouples to multiple GPCRs

My findings reported here demonstrate a remarkable 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 enabled reliable detection of RGS4\((c2v)\) in my co-immunoprecipitation assays. RGS4\((c2v)\) demonstrated a strong interaction with each of the GPCRs tested, but did not directly interact with the Kir3 channel, indicating selectivity in association with different transmembrane proteins. A previous study had found recombinant GST-RGS4 fusion protein to interact \textit{in vitro} with Kir3 channels expressed in HEK293 cells, suggesting a direct RGS4-Kir3 channel interaction (Fujita et al., 2000). In light of my findings, the GST-RGS4 interactions may have been with endogenous GPCRs co-assembled with the Kir3 channels expressed in HEK293 cells. Alternatively, RGS4 may have interactions with Kir3 channels that are not detected in our co-immunoprecipitation experiments, but more apparent using the recombinant RGS4 protein. The association of RGS4\((c2v)\) to multiple Gi/o and Gq/11-coupled receptors independent of the Kir3 channel effector, suggests
precoupled RGS4-GPCR complexes are likely to participate in the G-protein-dependent modulation of several other known ion channel effectors regulating neuronal excitability (e.g. Kir2 and Kir6 channels, KCNQ channels, TRP channels, and voltage-gated Ca\textsuperscript{2+} channels).

**Structural determinants of RGS4 coupling to GPCRs**

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 (Bernstein et al., 2004). My experiments showing RGS4(C2V) co-precipitates with muscarinic m1 receptors 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 (Bernstein et al., 2004) suggests other m2 receptor domains may also participate in direct receptor-RGS4 coupling, or alternatively the coupling could be mediated indirectly via interactions with precoupled G\textalpha{i/o} subunits or other proteins. Recent reports of RGS4 co-precipitating with \mu- or \delta-opioid receptors from periaqueductal gray membranes (Garzon et al., 2005) and involving direct interactions between RGS4 and the C-terminal domains of \mu- or \delta-opioid receptors (Georgoussi et al., 2005) suggests RGS4 may also directly bind to 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 of the RGS4 N-terminal domain,
since deleting the N-terminus and substituting the RGS3s N-terminus (R3s/R4 chimera) resulted in decoupling from the GPCR-Kir3 channel complex. Since the RGS4 N-terminus confer membrane association (Srinivasa et al., 1998) and contains two palmitoylation sites that are expected to facilitate targeting to membrane lipid rafts (Hiol et al., 2003) where GPCRs (Papoucheva et al., 2004), heterotrimeric G proteins (Moffett et al., 2000), and Kir3 channels localize (Delling et al., 2002), there are apparent cooperative and selective interactions involving the RGS4 N-terminus and the RGS4 RGS domain that together mediate the high affinity coupling. My findings are consistent with the model proposed by Wilkie and colleagues (Zeng et al., 1998), where the RGS4 N-terminus directly interacts with the GPCR and the RGS domain interacts with the precoupled Gα subunit. Thus receptor-RGS4 association is expected to increase the degree of precoupled receptor-G protein complexes. Recent fluorescence resonance energy transfer (FRET) experiments support a stable interaction between RGS proteins (RGS7 and RGS8) and Gα subunits within an agonist-receptor-G-protein quaternary complex (Benians et al., 2005). Importantly, however, these experiments did not detect FRET between RGS8 and the GPCR, indicating the RGS-Gα FRET signals could be potentially derived via a collision-coupled process. 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 I found no evidence for receptor-specific association of RGS4(C2V), wildtype 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 (Davydov and Varshavsky, 2000), or alternatively could reflect effects of Cys2 modifications on coupling to GPCRs. The RGS4 Cys2 residue is the target of palmitoylation (Srinivasa et al., 1998), arginylation (Davydov and Varshavsky, 2000), nitrosylation (Hu et al., 2005), and oxidation (Hu et al., 2005), where the RGS4(C2V) mutant would be insensitive to any negative effects of Cys2 modifications on GPCR coupling. Future studies exploring the role of the RGS4 Cys2 site and its modifications on the efficacy of specific GPCR coupling will be needed to resolve this fascinating possibility.

**Implications of RGS4 precoupling versus RGS3s collision-coupling**

My initial electrophysiological measures of RGS3s- versus RGS4-dependent modulation of Kir3 currents in CHO-K1 cells did not reveal any functional advantage for precoupled RGS4 proteins versus uncoupled RGS3s. Yet RGS dosage experiments in *Xenopus* oocytes clearly demonstrated that RGS4(C2V) precoupling provides a 30-fold greater potency in Kir3 channel modulation versus uncoupled RGS3s. These findings illustrate the high level of RGS collision-coupling that occurs in the CHO-K1 expression experiments, a likely result of the high protein expression levels produced in this commonly used
mammalian expression system. Functional assays probing for RGS-GPCR coupling specificity using similar assay systems are likely biased for ‘false-positives’ due to the high degree of RGS collision-coupling and the generally low selectivity of several RGS proteins towards Gi/o, Gq/11, and Gz subunits. Experimental protocols implementing RGS dosage in live cell assays should help resolve RGS selectivity in the modulation of specific GPCR signaling pathways.
The original purpose of this study was to characterize the specific RGS proteins that modulate endogenous neuronal GIRK channels. Initially I used CG neurons, a native cell type that endogenously expresses GIRK channels. In order to determine the endogenous RGS proteins that were likely to be involved in GIRK channel modulation, a RGS expression profile was performed. This work has shown that CG neurons can express at least 13 RGS genes. Comparison of RGS gene expression profiles from different native cell types (i.e. CG neurons vs cardiac myocytes) can give us an indication of which RGS proteins may be physiologically important for each cell type.

It has been demonstrated that GIRK channels can form stable signaling complexes with GPCRs (Lavine et al., 2002), and multiple RGS proteins are expressed within single GIRK-expressing neurons and atrial myocytes (Doupnik et al., 2004; Doupnik et al., 2001; Gold et al., 1997).

How are cells able to specifically activate a determined signaling pathway?
One way of signaling pathway specificity may come through selective interaction of RGS proteins with various GPCR-effector signaling complexes.

To test this hypothesis I studied RGS3s and RGS4, two RGS’s whose mRNA levels are transcriptionally regulated in the nervous system during pathophysiologic conditions (Costigan et al., 2003).

My findings demonstrate a tight coupling between RGS4 and several GPCRs that are central participants in normal and pathologically altered neuromodulation. Interaction among the different GPCRs-GIRK-RGS proteins seems to be very specific, since only RGS4 was able to co-immunoprecipitate with the GPCR-GIRK channel signaling complexes and RGS3s was not (Figure 6.1).

My results highlight the importance that selective RGS-GPCR interactions may have physiologically. The functional impact of RGS4(C2V) precoupled to the GPCR-Kir3 channel complex was a 30-fold greater potency in the acceleration of Kir3 channel gating kinetics, compared to the uncoupled (or collision coupled) RGS3s. This disparity in potency observed between RGS4(C2V) and RGS3s is probably due to the coupling of RGS4(C2V) to the GPCR-GIRK signaling complex. Further experiments in the oocyte system using the different chimeras and deletion constructs are needed to corroborate this hypothesis.

Given the multiple mechanisms affecting RGS4 protein levels, it will be important to determine to what extent these changes in RGS4 concentration affect coupling to different GPCR signaling pathways. Recently, a mutation in an
RGS protein, RGS9-1 that is involved in the rhodopsin signaling complex has been shown to be the cause of a disease (bradyopsia) in humans, a non-lethal condition characterized by an inability to resolve rapidly changing visual scenes (Nishiguchi et al., 2004).

In summary, my findings demonstrate that RGS4, a highly regulated modulator and susceptibility gene for schizophrenia, is an integral component of multiple GPCR-Kir3 channel complexes affecting a wide range of neurotransmitter-mediated events in the nervous system. Acquired or inherited disruptions in RGS4-GPCR coupling may also be critical for a variety of neurological disorders that may include schizophrenia, depression, epilepsy, and drug addiction. Future experiments in native tissue are needed to detect the location and identification of the distinct RGS proteins involved in the coupling to the different GPCR-effector signaling complexes. Although my experiments have been performed in an heterologous system, the interactions reported here may be physiologically important for several reasons: 1) RGS4 has been reported to co-immunoprecipitate with µ- and δ-opioid receptors from periaqueductal gray membranes (Garzon et al., 2005), 2) the functional impact that precoupled RGS4 has in contrast to uncoupled RGS3s in the *Xenopus* oocyte experiments once the protein concentration of both RGS was reduced and tritated 3) The precoupling is RGS protein specific, being only RGS4 able to associate to the different GPCR-GIRK channel complexes.
Figure 6.1. Differential coupling of RGS proteins to GPCR-GIRK channel signaling complexes. (A) RGS4 couples to GPCR-GIRK channel signaling complexes, interacting with the GPCR and not with the GIRK subunit. (B) RGS3s does not couple to the GPCR-GIRK channel signaling complexes.
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