Published in final edited form as:

FEBS Lett. 2007 February 20; 581(4): 764–770.

Active $G\alpha_q$ subunits and M3 acetylcholine receptors promote distinct modes of association of RGS2 with the plasma membrane

Michael A. Clark, Pooja R. Sethi, and Nevin A. Lambert

Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, 30912, USA.

Abstract

RGS proteins accelerate the GTPase activity of heterotrimeric G proteins at the plasma membrane. Association of RGS proteins with the plasma membrane can be mediated by interactions with other membrane proteins and by direct interactions with the lipid bilayer. Here we use fluorescence recovery after photobleaching (FRAP) to characterize interactions between RGS2 and M3 acetylcholine receptors (M3Rs), $G\alpha_q$ subunits and the lipid bilayer. Active $G\alpha_q$ and M3Rs both recruited RGS2-EGFP to the plasma membrane. RGS2-EGFP remained bound to the plasma membrane between interactions with active $G\alpha_q$, but rapidly exchanged between membrane-associated and cytosolic pools when recruited by M3Rs.

Keywords

GPCRs; FRAP; RGS proteins; precoupling; amphipathic

1. Introduction

Regulators of G protein signaling (RGS proteins) are integral components of heterotrimeric G protein signaling pathways. RGS proteins accelerate the GTPase activity of G proteins, and are necessary for the rapid time course of physiological signals mediated by G protein-coupled receptors (GPCRs) [1–3]. RGS proteins bind transiently to active (GTP-bound) $G\alpha$ subunits, which are attached to the plasma membrane by posttranslational lipid modifications. Therefore, the physiological actions of RGS proteins usually take place at the plasma membrane. However, RGS proteins are not strongly attached to the plasma membrane by a common targeting motif. Some RGS proteins are attached to the plasma membrane, but a variety of mechanisms have been implicated. These include interactions with G proteins, GPCRs or accessory proteins [4–9], posttranslational lipid modifications [10,11], and direct hydrophobic and electrostatic interactions of amino-terminal amphipathic α -helices with the lipid bilayer [12–15]. Stable attachment of RGS proteins to the plasma membrane would be expected to greatly facilitate catalytic acceleration of GTPase activity, as diffusion between active $G\alpha$ subunits would be limited to two dimensions. Therefore, it is not surprising that membrane attachment has often been found to be important for RGS function in cells [12,14,16].

Corresponding author: Nevin A. Lambert, Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30809 USA, 706-721-6336, 706-721-2347 (fax), nlambert@mcg.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Members of the R4 family of RGS proteins (e.g. RGS2, RGS4, RGS8 and RGS16) have a relatively simple architecture, consisting of a core GTPase-activating (GAP) domain and short amino- and carboxyl-terminal extensions [1,17]. In heterologous expression systems R4 RGS proteins can be recruited to the plasma membrane from intracellular sites by expression of active $G\alpha$ subunits [18]. Plasma membrane localization of these proteins could thus result from direct interactions with $G\alpha$ subunits, or as a result of downstream signaling [15]. In addition, the amino-terminal extensions of several R4 proteins contain an amphipathic α -helix that is thought to bind directly to (and partially insert into) the lipid bilayer [13–15,19,20]. To complicate matters further, several RGS proteins (belonging to R4 and other subfamilies) interact with GPCRs and accessory proteins [4–7], thus providing several potential mechanisms of plasma membrane association, and raising the possibility that RGS proteins participate in stable, preassembled signaling complexes [21–23]. The stability of membrane association promoted by these various mechanisms has not been assessed in intact cells.

The purpose of the current study was to compare membrane association of an R4 RGS protein (RGS2) promoted by active $G\alpha$ subunits and a GPCR, the M3 muscarinic acetylcholine receptor (M3R). We were interested in determining the stability of membrane association promoted by these molecules, and to test the possibility that RGS2 forms stable complexes with GPCR signaling components at the plasma membrane. We made use of a newly-developed technique to assess the stability of membrane protein interactions [24]. This technique involves immobilizing one of a pair of binding partners using avidin-mediated crosslinking, and measuring changes in the mobility of the other. We found that active $G\alpha_q$ variants recruited RGS2 to the plasma membrane, and that RGS2 remained associated with the plasma membrane between transient encounters with these subunits. In contrast, although M3Rs also recruited RGS2 to the plasma membrane, RGS2 exchanged rapidly between membrane-bound and cytosolic pools without diffusing laterally. These results demonstrate two distinct modes of membrane association promoted by $G\alpha$ subunits and GPCRs.

2. Materials and Methods

2.1 cDNA constructs

RGS2-EGFP and EGFP-RGS2 constructs were provided by Peter Chidiac (University of Western Ontario) and Scott Heximer (University of Toronto). $G\alpha_{q19}$ and TRPC5-EGFP were provided by Bruce Conklin (University of California, San Francisco) and David Clapham (Harvard University). All other plasmids were obtained from the University of Missouri-Rolla cDNA resource. CFP-TM- $G\alpha_{q19}$ subunits consisted of (starting at the amino terminus) a signal peptide, enhanced cyan fluorescent protein (ECFP), the amino terminal 103 amino acids of the rat μ -opioid receptor and $G\alpha_{q19}$. CFP-M2Rs and CFP-M3Rs consisted of a signal peptide and ECFP fused to the receptor amino-terminus. $G\alpha_q$ -EGFP consisted of $G\alpha_q$ with EGFP inserted between Y128 and V129 in the helical domain. All constructs were made using an adaptation of the QuikChange (Stratagene) mutagenesis protocol, and were verified by automated sequencing.

2.2 Cell culture and transfection

Cerebellar granule neurons were cultured from neonatal (PN5-8) Sprague-Dawley rats as described previously [25]. All procedures involving animals were carried out in strict compliance with institutional guidelines. Human embryonic kidney (HEK 293) cells and Chinese hamster ovary (CHO) cells (ATCC) were propagated in plastic flasks and on polylysine coated glass coverslips according to the supplier's protocol. Cells were transfected using polyethyleneimine and were used for experiments 12–48 hours later.

2.3 Avidin-mediated crosslinking

Cells were rinsed 3 times in buffer containing 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 12 mM glucose, 0.5 mM CaCl₂, and 0.5 mM MgCl₂ (BE buffer; pH 8.0), and incubated at room temperature for 15 minutes in 0.5 mg ml⁻¹ NHS-sulfo-LC-LC-biotin (Pierce). Cells were washed and incubated for 15 minutes in 0.1 mg ml⁻¹ avidin (Pierce). Control cells were biotinylated but were not exposed to avidin. Avidin crosslinked cells remained viable and endocytosed avidin after several hours, thus all experiments were performed within 1 hour of avidin exposure.

2.4 Imaging

Coverslips bearing control or avidin crosslinked cells were imaged using a Leica SP2 scanning confocal microscope and a 63X, 1.4 NA objective. For translocation experiments images were acquired and analyzed by an experimenter that was blinded to the transfection condition. A 5 µm profile drawn normal to and centered on the plasma membrane was obtained for each cell. Membrane:cytosol ratios were calculated for each cell using the intensity at the center of the profile and the average intensity of the profile segment from 1 to 2 µm from the plasma membrane. For FRAP experiments low intensity illumination was used during a control (prebleach) period, after which a segment of the plasma membrane was photobleached by increasing the laser intensity. Recovery of fluorescence into the bleached segment of plasma membrane was monitored using low intensity illumination. Average pixel intensity in the bleached region was corrected for photobleaching during low intensity illumination, normalized and plotted versus time. Diffusion coefficients (D) were derived by fitting fluorescence recovery curves to the empirical equation: $F(t) = F_i + F_m (1 - [w^2(w^2 + 4\pi Dt)^{-1}]^{0.5})$ where F_i is the fluorescence intensity immediately after photobleaching, F_m is the intensity recovered, w is the width of the bleached plasma membrane, and D is the effective one-dimensional diffusion coefficient.

2.4 Electrophysiology

Perforated patch-clamp recordings were made from isolated neurons (8-12 DIV) expressing EGFP or CHO cells expressing TRPC5-EGFP 16–48 hours after transfection. For recording of standing outward potassium current neurons were held in voltage-clamp mode at -20 mV. Cells were continuously perfused with external solution containing (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 0.5 CaCl₂, 0.5 MgCl₂ (pH 7.8, ~310 mOsm kg⁻¹ H₂O). Patch electrodes (3–6 MΩ) were filled with a solution containing (in mM): 70 K-gluconate, 70 KCl, 0.2 EGTA, 10 HEPES, 3 MgATP and 0.3 Na₂GTP, and 0.03% amphotericin B (pH 7.2, ~295 mOsm kg⁻¹ H₂O). For recording TRP currents CHO cells were held at -75 mV and perfused with a solution containing (in mM): 140 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 2 CaCl₂, 1 MgCl₂ (pH 7.4, ~310 mOsm kg⁻¹ H₂O). Patch electrodes were filled with a solution containing (in mM): 120 Cs-gluconate, 2 MgCl₂, 3 CaCl₂, 5 EGTA, 10 HEPES, 3 MgATP and 0.3 Na₂GTP, and 0.03% amphotericin B (pH 7.2, ~295 mOsm kg⁻¹ H₂O). All recordings were made at room temperature. Solution changes were made using a multiport attachment and perfusion capillary positioned directly in front of the cell under study.

3. Results

3.1 Plasma membrane translocation of RGS2-EGFP and EGFP-RGS2

In order to study the membrane association of RGS proteins we expressed RGS2 with the enhanced green fluorescent protein fused to either its carboxyl- or amino-terminus (RGS2-EGFP and EGFP-RGS2, respectively) together with GPCRs and G proteins known to interact with or promote membrane association of this protein. Previous studies have shown that expression of active or inactive $G\alpha$ subunits redistributes RGS2 from the nucleus and cytosol

to the plasma membrane of mammalian cells [15,26], and that RGS2 can provide GAP activity at GTPase-defective $G\alpha$ subunits [27]. For our experiments we expressed inactive or active mutants of $G\alpha_q$ chimeras containing the final 9 amino acids of $G\alpha_i$ [28]. Using these chimeras allowed us to verify function by activating these subunits with $G\alpha_i$ -linked receptors, thus bypassing endogenous $G\alpha_q$ subunits [25]. In agreement with previous studies, RGS2-EGFP (Fig. 1A) and EGFP-RGS2 (Fig. 1B) translocated to the plasma membrane of HEK 293 cells when they were expressed together with the active mutant $G\alpha_{qi9}$ R183C ($G\alpha_{qi9}^*$; Fig. 1C). However, in contrast to a previous report [26], no plasma membrane recruitment was observed when either RGS2 fusion protein was expressed with inactive $G\alpha_{qi9}$ ($G\alpha_{qi9}$; Fig. 1A, B). We also observed robust plasma membrane translocation of RGS2-EGFP (Fig. 1D) but not EGFP-RGS2 (Fig. 1E) when these proteins were expressed with M3 muscarinic acetylcholine receptors (M3Rs; Fig. 1F). M1, M3 and M5 muscarinic receptors have been shown to bind directly to the amino-terminus of RGS2 [6], thus is it possible that amino-terminal (but not carboxyl-terminal) fusion of EGFP hinders this interaction and thereby prevents membrane translocation. The absence of EGFP-RGS2 recruitment after M3R expression also implies that recruitment of RGS2-EGFP by M3Rs was not the result of constitutive receptor activity and activation of endogenous $G\alpha_q$. M2 muscarinic receptors (M2Rs) do not bind directly to RGS2, and expression of these GPCRs failed to induce membrane translocation of either RGS2-EGFP (Fig. 1D) or EGFP-RGS2 (Fig. 1E). These results are consistent with recruitment of RGS2 to the plasma membrane mediated by specific protein-protein interactions.

3.2 Immobilization of functional CFP-TM- $G\alpha_{qi9}^*$ and CFP-M3R

We next constructed fusion proteins containing $G\alpha_{qi9}$ subunits and M3Rs that could be verifiably immobilized at the plasma membrane by avidin-mediated crosslinking. We extended the amino-terminus of inactive $G\alpha_{qi9}$ and active $G\alpha_{qi9}$ R183C with a transmembrane domain (TM) and cyan fluorescent protein (CFP) to produce CFP-TM- $G\alpha_{qi9}$ and CFP-TM- $G\alpha_{qi9}^*$. Both constructs were fully functional, as both could couple activation of α_2A adrenoreceptors to inhibition of standing outward potassium currents in cultured cerebellar granule neurons (Fig. 2A) [25]. The fact that CFP-TM- $G\alpha_{qi9}^*$ could couple receptor activation to downstream signaling implies that some of these subunits were in the inactive state prior to receptor activation, possibly due to the actions of endogenous RGS2 (or other RGS proteins) on this mutant [27]. The slow reversal of CFP-TM- $G\alpha_{qi9}^*$ -mediated signals (Fig. 2A) is consistent with the impaired (but not null) GTPase activity of these subunits. Fluorescence recovery after photobleaching (FRAP) experiments showed that CFP-TM- $G\alpha_{qi9}^*$ subunits were mobile in the plasma membrane (Fig. 2B). The effective diffusion coefficient (D_{eff}) calculated for these subunits was $0.17 \pm 0.02 \mu m^2 sec^{-1}$ ($n=17$) in biotinylated control cells. CFP-TM- $G\alpha_{qi9}^*$ subunits became immobile after avidin-mediated crosslinking, as indicated by the absence of fluorescence recovery after photobleaching (Fig. 2B). Similarly, we extended the amino-terminus of M3Rs with CFP and verified that these receptors (CFP-M3Rs) could functionally couple to endogenous $G\alpha_q$ subunits and downstream effectors (Fig. 2C). CFP-M3Rs were mobile in the plasma membrane of biotinylated control cells ($D_{eff}=0.18 \pm 0.03 \mu m^2 sec^{-1}$; $n=13$), but became immobile after cell surface biotinylation and avidin-mediated crosslinking (Fig. 2D).

3.3 CFP-TM- $G\alpha_{qi9}^*$ and CFP-M3R promote different modes of association of RGS2-EGFP with the plasma membrane

We next used CFP-TM- $G\alpha_{qi9}^*$ and CFP-M3Rs to recruit RGS2-EGFP to the plasma membrane. Since the CFP moiety on both constructs was extracellular it was not surprising that both recruited RGS2-EGFP in a manner similar to their untagged counterparts. FRAP experiments revealed an effective diffusion coefficient for membrane associated RGS2-EGFP of approximately $0.5 \mu m^2 sec^{-1}$ when it was recruited by either CFP-TM- $G\alpha_{qi9}^*$ or $G\alpha_{qi9}^*$ (Fig. 3A, B). This value is similar to diffusion coefficients calculated for other peripheral

membrane proteins [29]. In order to test the possibility that membrane recruitment by active $G\alpha_q$ involves a direct interaction between RGS2-EGFP and the G protein, we immobilized CFP-TM- $G\alpha_{qi9}^*$ by avidin-mediated crosslinking. This manipulation caused a significant decrease in the apparent mobility of RGS2-EGFP; the effective diffusion coefficient slowed from $0.50 \pm 0.07 \mu m^2 sec^{-1}$ in biotinylated cells ($n=13$) to $0.31 \pm 0.05 \mu m^2 sec^{-1}$ in avidin-crosslinked cells ($n=30$; $P<0.05$; Fig. 3B). This result suggests that membrane-associated RGS2-EGFP spends time bound to immobile CFP-TM- $G\alpha_{qi9}^*$, but is not bound so tightly as to be rendered completely immobile. As expected RGS2-EGFP mobility was not decreased by avidin crosslinking when it was recruited to the plasma membrane by $G\alpha_{qi9}^*$, since this subunit is not exposed to the extracellular space (Fig. 3B).

In contrast to these results, RGS2-EGFP mobility was substantially faster ($D_{eff}=0.87 \pm 0.12 \mu m^2 sec^{-1}$; $n=20$) when it was recruited to the plasma membrane by CFP-M3Rs (Fig. 3B). Moreover, the apparent mobility of RGS2-EGFP was not slowed when CFP-M3Rs were immobilized by avidin-mediated crosslinking ($D_{eff}=0.88 \pm 0.11 \mu m^2 sec^{-1}$; $n=35$; $P>0.05$; Fig. 3B). This result suggests that membrane-associated RGS2-EGFP spends very little time bound to immobile CFP-M3Rs.

Fluorescence recovery after photobleaching can occur due to lateral diffusion of membrane-associated proteins or by exchange of membrane-associated proteins with a cytosolic pool. Therefore, we asked if membrane-associated RGS2-EGFP diffused laterally between encounters with CFP-TM- $G\alpha_{qi9}^*$ and CFP-M3Rs or dissociated from the plasma membrane and was replaced by RGS2-EGFP from the cytosol. To this end we photobleached plasma membrane segments of two different lengths (5 μm and 3 μm) in the same cells and compared the rates of fluorescence recovery in the two segments. If fluorescence recovery results from lateral diffusion, then the rate of recovery will depend on the size bleached segment, with larger segments recovering more slowly. If fluorescence recovery results from exchange with the cytosol, then the rate of recovery will be independent of the segment size. As shown in Figure 3C, RGS2-EGFP fluorescence recovery depended on the size of the bleached plasma membrane segment when this protein was recruited by either CFP-TM- $G\alpha_{qi9}^*$ or $G\alpha_{qi9}^*$ in both biotinylated and avidin crosslinked cells. Under these conditions the ratio of the fluorescence recovery time constants for 5 μm and 3 μm segments was >1.5 . These ratios were similar to the ratios for the transmembrane proteins CFP-TM- $G\alpha_{qi9}^*$ (1.71 ± 0.28 ; $n=9$) and CFP-M3Rs (1.82 ± 0.24 ; $n=13$), which must recover by diffusing laterally. This suggests that RGS2-EGFP diffuses laterally in the plasma membrane between transient encounters with active CFP-TM- $G\alpha_{qi9}^*$ and $G\alpha_{qi9}^*$ subunits.

In contrast, the rate of recovery of RGS2-EGFP fluorescence was independent of the size of the photobleached segment when it was recruited to the plasma membrane by M3Rs. In biotinylated cells and avidin crosslinked cells the ratio of recovery time constants was essentially 1 (Fig. 3C). This result indicates that fluorescence recovery of RGS2-EGFP recruited by CFP-M3Rs does not reflect lateral diffusion in the plasma membrane, and suggests that RGS2-EGFP dissociates from the plasma membrane and is replaced by molecules from a cytosolic pool.

The above results suggested that M3Rs and RGS2-EGFP did not form a tight complex at the plasma membrane. We then asked if this interaction might be different if RGS2-EGFP were recruited to the plasma membrane by the combined presence of active $G\alpha_q$ and CFP-M3Rs. Therefore, we compared RGS2-EGFP mobility in the presence of mobile and immobile CFP-M3Rs in the presence of active $G\alpha_{qi9}^*$. In the presence of mobile CFP-M3Rs and $G\alpha_{qi9}^*$, RGS2-EGFP mobility was slowed to a level comparable to its mobility when recruited by CFP-TM- $G\alpha_{qi9}^*$ ($D_{eff}=0.47 \pm 0.13 \mu m^2 sec^{-1}$; $n=9$), consistent with the idea that membrane association promoted by active $G\alpha_q$ differs substantially from that promoted by M3Rs.

However, immobilization of CFP-M3Rs by avidin crosslinking still had no effect on RGS2-EGFP mobility ($D_{\text{eff}} = 0.53 \pm 0.07 \mu\text{m}^2 \text{sec}^{-1}$; $n=17$; $P>0.05$), suggesting active $G\alpha_q$ did not promote a high affinity interaction between M3Rs and RGS2-EGFP.

Finally, we considered the possibility that agonist-activated CFP-M3Rs might form a higher affinity complex with RGS2-EGFP than would unliganded receptors. Therefore, we compared the fluorescence recovery of membrane-localized RGS2-EGFP after photobleaching in the presence of unliganded or liganded (100 μM carbachol) CFP-M3Rs. The apparent effective diffusion coefficients calculated for RGS2-EGFP were not significantly different under these two conditions ($2.7 \pm 0.5 \mu\text{m}^2 \text{sec}^{-1}$; $n=6$ and $2.9 \pm 0.9 \mu\text{m}^2 \text{sec}^{-1}$; $n=7$ respectively; $P>0.05$). Thus the transient nature of the association of RGS2-EGFP with CFP-M3Rs appears to be independent of activation state of the receptor.

3.4 Mobility of $G\alpha_q$ with immobile CFP-M3Rs and RGS2

Previous studies have suggested that R4-subfamily RGS proteins can form a complex with active or inactive $G\alpha$ subunits and GPCRs, and by doing so may facilitate the interaction between GPCRs and G proteins [21,22]. RGS-mediated facilitation of interactions between GPCRs and inactive G proteins (precoupling) has been suggested as a mechanism for acceleration of signal onset by RGS proteins. We previously observed an acceleration of $G\alpha_q$ signaling mediated by endogenous RGS proteins [25], and were thus interested in the possibility that RGS proteins might facilitate precoupling between $G\alpha_q$ and GPCRs. Therefore, we constructed an EGFP-tagged $G\alpha_q$ subunit ($G\alpha_q$ -EGFP), placing EGFP in the helical domain in order to prevent steric attenuation of receptor and $G\beta\gamma$ coupling, and compared the mobility of this subunit in the presence of mobile and immobile CFP-M3Rs. Immobile CFP-M3Rs slowed the diffusion of $G\alpha_q$ -EGFP, consistent with the possibility that these proteins interacted (precoupled) in the plasma membrane. However, coexpression of untagged RGS2 did not appear to enhance this interaction; $G\alpha_q$ -EGFP mobility was decreased by immobile CFP-M3Rs by the same amount in the absence and presence of coexpressed RGS2 (Fig. 4A, B). Therefore, these results do not support the hypothesis that RGS proteins serve as physical scaffolds for GPCRs and G proteins, at least not for these particular receptors and G proteins.

4. Discussion

RGS proteins encounter active $G\alpha$ subunits at the plasma membrane, thus acceleration of GTPase activity by RGS proteins can be facilitated by stable association of these proteins with the plasma membrane. RGS proteins can bind to the plasma membrane by interacting directly with the lipid bilayer [11,15], or by interacting with signaling proteins (e.g. GPCRs) [30] or intermediate proteins (e.g. spinophilin) [4]. We have compared association of RGS2 with the plasma membrane induced by active $G\alpha_q$ subunits and M3Rs, both of which are known to directly bind to RGS2 [6,31]. Not surprisingly, we found that both of these proteins could recruit RGS2-EGFP to the plasma membrane. However, the stability of membrane association promoted by these two proteins differed markedly.

We found that immobile active $G\alpha_q$ limited the mobility of RGS2-EGFP at the plasma membrane, consistent with the known direct binding interaction between these proteins. In addition, RGS2-EGFP diffused laterally between encounters with active immobile $G\alpha_q$ subunits. This implies that a separate mechanism maintains membrane association of RGS2 after dissociation from active $G\alpha_q$. A likely candidate is direct binding of the amino-terminal amphipathic α helix with the lipid bilayer [15]. If this interpretation is correct, our results imply that membrane association mediated by the amino-terminal α helix orients RGS2 in such a manner that it can still interact with active $G\alpha_q$. Moreover, membrane association mediated by an amphipathic helix is sufficiently stable to keep RGS2 attached to the plasma membrane for long enough periods to allow the protein to act catalytically in two dimensions.

This finding supports previous observations of stable, tonic association of RGS proteins with model membranes and cell membranes mediated by amphipathic helices [15,19,20]. If a direct interaction with the lipid bilayer keeps RGS2 at the plasma membrane as suggested, it will be interesting to determine if physiological changes in the bilayer (e.g. depletion of phosphatidylinositol 4,5-bisphosphate and accumulation of diacylglycerol) will be able to regulate this interaction.

In contrast to these results, we found that immobile M3Rs had no effect on the recovery of RGS2-EGFP fluorescence after photobleaching. This result implies that the lifetime of RGS2-M3R complexes is brief compared to the lifetime of RGS2-G α_q complexes. In addition, RGS2-EGFP did not diffuse laterally in the plane of the plasma membrane if it was recruited there by M3Rs, but instead exchanged with cytosolic RGS2-EGFP. This result implies M3Rs do not promote the direct interaction between the RGS2 amino-terminal alpha helix and the lipid bilayer. Since it is the amino-terminus of RGS2 that interacts with M3Rs [6], it would not be surprising if binding to M3Rs interfered with the more stable direct association of this region with the lipid bilayer. In this manner the two mechanisms of membrane association may be mutually exclusive.

The ability of RGS proteins to bind to several GPCR signaling components raises the possibility that these proteins participate in stable signaling complexes [32–34]. Indeed, it has been suggested that R4 family RGS proteins serve as physical scaffolds linking GPCRs and G proteins, and that this mechanism accounts for acceleration of signal onset by RGS proteins [21,22,35,36]. We previously observed that endogenous RGS proteins accelerate the onset of M3R- and G α_q -mediated signaling [25]. We were, therefore, interested in the possibility that RGS2 might enhance an interaction between M3Rs and inactive G α_q subunits. However, we found that overexpression of RGS2 had no effect on the apparent interaction between M3Rs and inactive G α_q -EGFP. This result suggests that either endogenous RGS proteins were sufficient to stabilize the M3R-G α_q interaction, or that RGS2 does not serve as a physical scaffold for this particular receptor-G protein combination. Previous studies have shown that heterologous expression of RGS proteins accelerates signal onset in HEK 293 cells [21,37], suggesting these cells have a modest supply of endogenous RGS proteins. Therefore, we favor the interpretation that RGS2 does not serve as a physical scaffold between M3Rs and G α_q subunits. This conclusion is strictly limited to the particular receptor (M3R) and RGS protein (RGS2) used in our studies, and additional experiments will be required to confirm the possible role of R4 RGS proteins as physical scaffolds.

In summary, we have provided evidence that active G α subunits and GPCRs promote distinct modes of association of RGS2 with the plasma membrane. Several members of the R4 family of RGS proteins interact directly with GPCRs and also possess an amphipathic amino-terminal alpha helix, thus it is likely that these results will be applicable to other RGS proteins. The functional consequences of the two association modes have yet to be determined. However, it has been suggested that direct and indirect interactions between GPCRs and RGS proteins are necessary for receptor-specific actions of RGS proteins [38]. Our results suggest that specificity conferred by direct protein-protein interactions might, in some cases, come at the expense of stable association with the plasma membrane. In contrast, direct association of RGS proteins with the lipid bilayer might provide stable membrane association and increase catalytic efficiency, but might not allow for receptor-specific effects.

Acknowledgements

We thank Peter Chidiac, Scott Heximer, Bruce Conklin and David Clapham for providing plasmid DNA. Supported by grants from the National Institutes of Health (NS36455 to N.A.L.) and the National Science Foundation (MCB-0620024 to N.A.L.).

References

1. Neubig RR, Siderovski DR. Regulators of G-protein signalling as new central nervous system drug targets. *Nature Reviews Drug Discovery* 2002;1:187–197.
2. Dohlman HG, Thorner J. RGS proteins and signaling by heterotrimeric G proteins. *J Biol Chem* 1997;272:3871–3874. [PubMed: 9064301]
3. Hepler JR. Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* 1999;20:376–382. [PubMed: 10462761]
4. Wang X, et al. Spinophilin regulates Ca²⁺ signalling by binding the N-terminal domain of RGS2 and the third intracellular loop of G-protein-coupled receptors. *Nat Cell Biol* 2005;7:405–11. [PubMed: 15793568]
5. Martemyanov KA, Yoo PJ, Skiba NP, Arshavsky VY. R7BP, a novel neuronal protein interacting with RGS proteins of the R7 family. *J Biol Chem* 2005;280:5133–6. [PubMed: 15632198]
6. Bernstein LS, Ramineni S, Hague C, Cladman W, Chidiac P, Levey AI, Hepler JR. RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq/11alpha signaling. *J Biol Chem* 2004;279:21248–21256. [PubMed: 14976183]
7. Hu G, Wensel TG. R9AP, a membrane anchor for the photoreceptor GTPase accelerating protein, RGS9–1. *Proc Natl Acad Sci USA* 2002;99:9755–60. [PubMed: 12119397]
8. Ballon DR, Flanary PL, Gladue DP, Konopka JB, Dohlman HG, Thorner J. DEP-domain-mediated regulation of GPCR signaling responses. *Cell* 2006;126:1079–93. [PubMed: 16990133]
9. De Vries L, Lou X, Zhao G, Zheng B, Farquhar MG. GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. *Proc Natl Acad Sci USA* 1998;95:12340–5. [PubMed: 9770488]
10. Tu Y, Wang J, Ross EM. Inhibition of brain Gz GAP and other RGS proteins by palmitoylation of G protein alpha subunits. *Science* 1997;278:1132–5. [PubMed: 9353196]
11. De Vries L, Elenko E, Hubler L, Jones TL, Farquhar MG. GAIP is membrane-anchored by palmitoylation and interacts with the activated (GTP-bound) form of G alpha i subunits. *Proc Natl Acad Sci USA* 1996;93:15203–8. [PubMed: 8986788]
12. Srinivasa SP, Bernstein LS, Blumer KJ, Linder ME. Plasma membrane localization is required for RGS4 function in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 1998;95:5584–9. [PubMed: 9576926]
13. Chen C, Seow KT, Guo K, Yaw LP, Lin SC. The membrane association domain of RGS16 contains unique amphipathic features that are conserved in RGS4 and RGS5. *J Biol Chem* 1999;274:19799–806. [PubMed: 10391923]
14. Bernstein LS, Grillo AA, Loranger SS, Linder ME. RGS4 binds to membranes through an amphipathic alpha-helix. *J Biol Chem* 2000;275:18520–6. [PubMed: 10764749]
15. Heximer SP, Lim H, Bernard JL, Blumer KJ. Mechanisms governing subcellular localization and function of human RGS2. *J Biol Chem* 2001;276:14195–14203. [PubMed: 11278586]
16. Saitoh O, Masuho I, Terakawa I, Nomoto S, Asano T, Kubo Y. Regulator of G protein signaling 8 (RGS8) requires its NH2 terminus for subcellular localization and acute desensitization of G protein-gated K⁺ channels. *J Biol Chem* 2001;276:5052–8. [PubMed: 11087736]
17. Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* 2002;54:527–59. [PubMed: 12223533]
18. Chidiac P, Roy AA. Activity, regulation, and intracellular localization of RGS proteins. *Receptors & Channels* 2003;9:135–147. [PubMed: 12775336]
19. Tu Y, Woodson J, Ross EM. Binding of regulator of G protein signaling (RGS) proteins to phospholipid bilayers. Contribution of location and/or orientation to Gtpase-activating protein activity. *J Biol Chem* 2001;276:20160–6. [PubMed: 11274219]
20. Ouyang YS, Tu Y, Barker SA, Yang F. Regulators of G-protein signaling (RGS) 4, insertion into model membranes and inhibition of activity by phosphatidic acid. *J Biol Chem* 2003;278:11115–22. [PubMed: 12538649]
21. Benians A, Nobles M, Hosny S, Tinker A. Regulators of G-protein signalling form a quaternary complex with the agonist, receptor and G-protein: a novel explanation for the acceleration of signalling activation kinetics. *J Biol Chem* 2005;280:13383–94. [PubMed: 15677457]

22. Zhang Q, Pacheco MA, Doupnik CA. Gating properties of GIRK channels activated by Galpha(o)- and Galpha(i)-coupled muscarinic m2 receptors in *Xenopus* oocytes: the role of receptor precoupling in RGS modulation. *J Physiol* 2002;545:355–373. [PubMed: 12456817]
23. Roy AA, Baragli A, Bernstein LS, Hepler JR, Hebert TE, Chidiac P. RGS2 interacts with Gs and adenylyl cyclase in living cells. *Cell Signal* 2006;18:336–48. [PubMed: 16095880]
24. Digby GJ, Lober RM, Sethi PR, Lambert NA. Some G protein heterotrimers physically dissociate in living cells. *Proc Natl Acad Sci USA* 2006;103:17789–94. [PubMed: 17095603]
25. Clark MA, Lambert NA. Endogenous regulator of G-protein signaling proteins regulate the kinetics of Galphaq/11-mediated modulation of ion channels in central nervous system neurons. *Mol Pharmacol* 2006;69:1280–7. [PubMed: 16368893]
26. Roy AA, Lemberg KE, Chidiac P. Recruitment of RGS2 and RGS4 to the plasma membrane by G proteins and receptors reflects functional interactions. *Mol Pharmacol* 2003;64:587–593. [PubMed: 12920194]
27. Ingi T, et al. Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J Neurosci* 1998;18:7178–88. [PubMed: 9736641]
28. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR. Substitution of 3 Amino-Acids Switches Receptor Specificity of G(Q)Alpha to That of G(I)Alpha. *Nature* 1993;363:274–276. [PubMed: 8387644]
29. Kenworthy AK, Nichols BJ, Remmert CL, Hendrix GM, Kumar M, Zimmerberg J, Lippincott-Schwartz J. Dynamics of putative raft-associated proteins at the cell surface. *J Cell Biol* 2004;165:735–46. [PubMed: 15173190]
30. Snow BE, et al. GTPase activating specificity of RGS12 and binding specificity of an alternatively spliced PDZ (PSD-95/Dlg/ZO-1) domain. *J Biol Chem* 1998;273:17749–55. [PubMed: 9651375]
31. Heximer SP, Watson N, Linder ME, Blumer KJ, Hepler JR. RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc Natl Acad Sci USA* 1997;94:14389–14393. [PubMed: 9405622]
32. Siderovski DP, Strockbine B, Behe CI. Whither goest the RGS proteins? *Crit Rev Biochem Mol Biol* 1999;34:215–51. [PubMed: 10517644]
33. Sierra DA, Popov S, Wilkie TM. Regulators of G-protein signaling in receptor complexes. *Trends Cardiovasc Med* 2000;10:263–8. [PubMed: 11282305]
34. Abramow-Newerly M, Roy AA, Nunn C, Chidiac P. RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. *Cell Signal* 2006;18:579–91. [PubMed: 16226429]
35. Benians A, Nobles M, Tinker A. Participation of RGS8 in the ternary complex of agonist, receptor and G-protein. *Biochem Soc Trans* 2004;32:1045–1047. [PubMed: 15506959]
36. Jaen C, Doupnik CA. RGS3 and RGS4 Differentially Associate with G Protein-coupled Receptor-Kir3 Channel Signaling Complexes Revealing Two Modes of RGS Modulation: PRECOUPLING AND COLLISION COUPLING. *J Biol Chem* 2006;281:34549–60. [PubMed: 16973624]
37. Mutneja M, Berton F, Suen KF, Luscher C, Slesinger PA. Endogenous RGS proteins enhance acute desensitization of GABA(B) receptor-activated GIRK currents in HEK-293T cells. *Pflugers Arch* 2005;450:61–73. [PubMed: 15806402]
38. Zeng WZ, et al. The N-terminal domain of RGS4 confers receptor-selective inhibition of G protein signaling. *J Biol Chem* 1998;273:34687–34690. [PubMed: 9856989]

Figure 1.

Active $G\alpha_q$ and M3Rs recruit RGS2-EGFP to the plasma membrane. Average 5 μm intensity profiles of RGS2-EGFP (A) and EGFP-RGS2 (B) fluorescence with coexpressed inactive $G\alpha_{q9}$ or active $G\alpha_{q9}^*$. The plasma membrane is positioned at 0 μm (grey vertical line). Both RGS2 fusion proteins were recruited to the plasma membrane only by active $G\alpha_{q9}^*$, as indicated by a sharp peak in fluorescence (a.u.; arbitrary units). (C) Summary data for experiments shown in panels A and B ($n=41-69$). Average intensity profiles of RGS2-EGFP (D) and EGFP-RGS2 (E) fluorescence with coexpressed M2Rs or M3Rs. Only RGS2-EGFP was recruited to the plasma membrane by M3Rs. (F) Summary data for experiments shown in

panels D and E ($n=37-57$). Bars in panels C and F represent mean $\pm$ S.E.M.; * $P<0.05$ (Student's t-test) compared to inactive G α q β 9 or M2Rs.

Figure 2.

CFP-tagged $G\alpha$ subunits and M3Rs are fully functional, and are immobilized by avidin-mediated crosslinking. (A) Average membrane currents recorded from a primary cerebellar granule neurons expressing inactive CFP-TM- $G\alpha q9$ ($n=4$) or active CFP-TM- $G\alpha q9^*$ ($n=6$) and α_2A adrenoreceptors. Application of norepinephrine ($50 \mu M$) reversibly inhibited standing outward current. Tonic and agonist-evoked inhibition of this current mediated by CFP-TM- $G\alpha q9^*$ implies that only a fraction of these subunits were in the active state at rest. (B) Average membrane currents recorded from CHO cells expressing CFP-M3Rs ($n=6$) or vector (pcDNA3.1; $n=5$) together with TRPC5-EGFP channels. Application of the M3R agonist carbachol ($30 \mu M$) reversibly activated TRPC5-EGFP only in cells expressing CFP-M3Rs. (C) Fluorescence recovery after photobleaching (FRAP) experiments showing CFP-TM- $G\alpha q9^*$ subunits were mobile after biotinylation ($n=17$) as indicated by recovery of fluorescence, but immobile after avidin-mediated crosslinking ($n=11$), as indicated by the lack of fluorescence recovery. (D) CFP-M3Rs were mobile after biotinylation ($n=13$), but immobile after avidin-mediated crosslinking ($n=12$). Photobleaching in panels C and D occurred at time = 5 seconds.

Figure 3.

Active $G\alpha$ subunits and M3Rs promote distinct modes of membrane association of RGS2-EGFP. (A) FRAP experiments comparing RGS2-EGFP mobility at the plasma membrane when this protein was recruited by immobile CFP-TM- $G\alpha_{qi9}^*$ subunits (+avidin; $n=30$), mobile CFP-TM- $G\alpha_{qi9}^*$ subunits (biotin; $n=13$) and mobile $G\alpha_{qi9}^*$ subunits (with avidin but without a TM domain; $n=11$). (B) Summary of experiments similar to panel (A). Only immobile CFP-TM- $G\alpha_{qi9}^*$ subunits significantly slowed recovery of RGS2-EGFP fluorescence, as indicated by a significantly slower effective diffusion coefficient (D_{eff} ; $*P<0.05$; $n=8-30$). The calculated D_{eff} for RGS2-EGFP was not different when this protein was recruited by mobile or immobile CFP-M3Rs ($n=20$ and 35). (C) Ratios of RGS2-EGFP fluorescence recovery time

constants for 5 μm and 3 μm bleached membrane segments. When RGS2-EGFP was recruited to the plasma membrane any $G\alpha q_9^*$ subunit ($n=7-18$) this ratio was significantly different from 1, indicating that fluorescence recovery was due to lateral diffusion. When RGS2-EGFP was recruited to the plasma membrane by CFP-M3Rs ($n=7$ and 16) this ratio was not significantly different from 1, indicating that fluorescence recovery was due to exchange with the cytosol. In all panels bars represent the mean $\pm$ S.E.M..

Figure 4.

RGS2 does not stabilize the interaction between inactive M3Rs and $G\alpha_q$. (A) FRAP experiments comparing $G\alpha_q$ -EGFP mobility at the plasma membrane in the presence of mobile CFP-M3Rs ($n=9$), immobile CFP-M3Rs ($n=19$), and immobile CFP-M3Rs together with RGS2 ($n=22$). (B) Summary of experiments identical to those shown in panel A. Immobile CFP-M3Rs slowed the lateral mobility of $G\alpha_q$ -EGFP, consistent with a transient interaction between the two, but RGS2 did not significantly enhance this effect (n.s., not significant; $P>0.05$).