RGS proteins act as negative regulators of G protein signaling by serving as GTPase-activating proteins (GAP) for α subunits of heterotrimeric G proteins (Gα), thereby accelerating G protein inactivation. RGS proteins can also block Gα-mediated signal production by competing with downstream effectors for Gα binding. Little is known about the relative contribution of GAP and effector antagonism to the inhibitory effect of RGS proteins on G protein-mediated signaling. By comparing the inhibitory effect of RGS2, RGS3, RGS5, and RGS16 on Gα-mediated phospholipase Cβ (PLCβ) activation under conditions where GTPase activation is possible versus nonexistent, we demonstrate that members of the R4 RGS subfamily differ significantly in their dependence on GTPase acceleration. COS-7 cells were transiently transfected with either muscarinic M2 receptors, which couple to endogenous Gαq protein and mediate a stimulatory effect of carbachol on PLCβ, or constitutively active Gαqα, which is inert to GTP hydrolysis and activates PLCβ independent of receptor activation. In M2-expressing cells, all of the RGS proteins significantly blunted the efficacy and potency of carbachol. In contrast, Gαqα-induced PLCβ activation was inhibited by RGS2 and RGS3 but not RGS5 and RGS16. The observed differential effects were not due to changes in M2, Gαq, Gαqα, PLCβ, or RGS expression, as shown by receptor binding assays and Western blots. We conclude that closely related R4 RGS family members differ in their mechanism of action. RGS5 and RGS16 appear to depend on G protein inactivation, whereas GAP-independent mechanisms (such as effector antagonism) are sufficient to mediate the inhibitory effect of RGS2 and RGS3.

Many extracellular stimuli elicit intracellular responses by activating seven-transmembrane receptors that are coupled to heterotrimeric G proteins comprising α and βγ subunits (1, 2). The duration of the response of a cell to external signals is largely determined by the activation/inactivation cycle of G proteins. Activated receptors trigger GTP-for-GDP exchange on Gα subunits, thus dissociating Gα from Gβγ and subsequent activation of downstream effectors (such as enzymes and ion channels). The duration of G protein activation is limited by GTPase activity intrinsic to Gα subunits that catalyzes the conversion of active GTP-bound Gα into inactive GDP-bound Gα, which in turn can reassociate with Gβγ and receptors.

RGS proteins belong to a family of more than 20 proteins with a conserved RGS core domain of ∼120 amino acids that is necessary and sufficient for binding to Gα subunits (3). They are divided into several subfamilies based on their structural similarities, gene organization, and function. RGS proteins act as regulators of G protein signaling by limiting the signals generated by G protein-coupled receptors. They markedly increase the rate at which Gα subunits hydrolyze GTP to GDP, a property that defines them as GTPase-activating proteins (or GAPs) (4). Hastening of Gα inactivation facilitates the reassociation of Gα with Gβγ subunits. As a result, RGS proteins inhibit both Gα- and Gβγ-mediated downstream effects. GAP effects have been described for virtually all proteins featuring an RGS core domain (5). Some RGS proteins can also diminish Gα-mediated signal generation by functionally inhibiting Gα-effector coupling (so-called effector antagonism) (6, 7). The absolute requirement and relative significance of the GAP effect and GAP-independent mechanisms for negative regulation of G protein-mediated signaling pathways by RGS proteins in mammalian cells are still poorly understood.

In the present study, we compared the effect of RGS proteins in a setting where they can exert GAP function with one where GAP function cannot be elicited to test the following hypotheses: (i) that RGS proteins do not necessarily depend on their GAP function to modulate mammalian cell signaling and (ii) that the relative contribution of their GAP and effector antagonistic effects differs among different RGS proteins. We examined several members of the R4 RGS protein subfamily (RGS2, RGS3, RGS5, and RGS16), because they are primarily composed of the RGS core domain and have the shortest N- and C-terminal flanking regions among the entire RGS family. Other RGS subfamilies contain longer N- and C-terminal sequences with various additional structural and functional domains that serve as binding sites for other proteins and impart subfamily specific activities, subcellular localization, or regulation (4). Because RGS3 has a much longer N terminus than all of the other R4 subfamily members (8), we included RGS3s, an RGS3 isoform with a N-terminal flanking region comparable in size to other R4 RGS proteins, in this study (9). All R4 RGS family members interact with Gαq and Gαqα proteins in vitro and accelerate their inactivation (4); for many, inhibition of Gαqα.

The abbreviations used are: GAP, GTPase-activating protein; PLC, phospholipase C; HA, hemagglutinin; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-O-(3-thiotriphosphate).
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and Gq-mediated signaling has been demonstrated in mammalian cells (10). We selected Gq-mediated PLCβ activation as a read-out for the present study, because effector antagonism has been described as a potential mechanism of action for modulation of Gq signaling by RGS proteins (6, 7, 11).

**EXPERIMENTAL PROCEDURES**

**Generation of cDNA Constructs**—The cDNA for mouse HA epitope-tagged, constitutively active G protein αq, Q209L (Goqαq12) was subcloned into pcDNA3. RGS proteins were tagged at their N or C terminus with a FLAG epitope by PCR. RGS2 (11), RGS3s (9), RGS3 and RGS4 (14), RGS5 and RGS16 (15) were used as templates. The PCR products were subcloned into pcDNA3 (Invitrogen) with convenient restriction sites. All of the sequences were confirmed by DNA sequencing. The cDNAs encoding muscarinic M3 receptor and Goq were subcloned into pcDNA3. RGS proteins were tagged at their N or C terminus with a FLAG epitope by PCR. RGS2 (11), RGS3s (9), RGS3 and RGS4 (14), RGS5 and RGS16 (15) were used as templates. The PCR products were subcloned into pcDNA3 (Invitrogen) with convenient restriction sites. All of the sequences were confirmed by DNA sequencing. The cDNAs encoding muscarinic M3 receptor and Goq were subcloned into pcDNA3. RGS proteins were tagged at their N or C terminus with a FLAG epitope by PCR.

**Muscarinic Receptor Binding Assay**—COS-7 cells were rinsed with Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and incubated for 90 min at room temperature with 7.5–4000 pM N-methyl-[3H]scopolamine (84 Ci/mmol; Amersham Biosciences) in the presence or absence of atropine (1 μM) to determine nonspecific binding. The binding reaction was stopped by removing the labeling medium and washing the wells twice with ice-cold PBS, followed by cell lysis in 0.2 M NaOH and 0.1% SDS. For each lysate, the amount of radioactivity was determined by scintillation counting and normalized to the amount of protein present (DC protein assay; Bio-Rad). Saturations unpaired ‘t’ test. GraphPad Prism 4 was used to fit dose-response curves by nonlinear regression and to probe for statistical differences (maximal effect and logEC50) in the presence or absence of exogenous RGS. A p value < 0.05 was considered statistically significant.

**RESULTS**

**Experimental Design**

This study was designed to compare the inhibitory effect of R4 RGS proteins on Goαq-mediated PLCβ activation under conditions where GTPase activation is possible versus nonexistent. This was achieved by transiently transfecting COS-7 cells with either muscarinic M3 receptors or constitutively active Goαq in the presence or absence of RGS2, RGS3, RGS5, or RGS16. Fig. 1A illustrates the two different transfection systems and our rationale for using them in the present study. (i) In M3-transfected COS-7 cells (Fig. 1A, right side), PLCβ activation in response to receptor activation with carbachol was expected to be mediated by endogenous Gq/11 proteins. COS-7 cells were chosen to limit the contribution of Gβγ released upon receptor/G protein activation. COS-7 cells express PLCβ1 and PLCβ2, which are sensitive to Goαq stimulation but relatively insensitive to Gβγ stimulation (17, 18). PLCβ3, an isoform that is very sensitive to Gβγ, is not expressed (19). Receptor-coupled endogenous Goαq proteins exchange GDP for GTP upon activation, whereas inactivation occurs when GTP is hydrolyzed to GDP. GDP proteins could therefore exert an inhibitory effect on receptor-induced Goαq signaling by acting as GAPs, effector an-
agonists, or both. (ii) In contrast, in Goq*-transfected COS-7 cells (Fig. 1A, left side), PLCβ was expected to be directly stimulated by constitutively active Goq*. We used HA-tagged Goq* to distinguish it from endogenous Goq. The internal HA epitope (substitution of amino acids 125–130) does not interfere with Goq* function, including PLCβ activation (20). Importantly, Goq* is inert to GTP hydrolysis (12) and therefore not susceptible to GTPase acceleration by RGS proteins (21). Titration experiments were performed in each setting to alter the ratios between Goq and RGS proteins over a broad range. Different amounts of cDNAs were transfected for Goq* and RGS proteins, and the amount of endogenous Goq available for PLCβ activation was varied by establishing concentration response curves for the muscarinic receptor agonist carbachol.

To compare their relative expression level, all of the RGS proteins were FLAG-tagged at their N terminus. The in vitro function of several R4 RGS proteins appears to be uncompromised by the addition of an N-terminal tag (e.g. (21, 22)). However, because the N terminus of some R4 RGS proteins has structural features important for subcellular targeting (5), we included select C-terminally tagged RGS proteins (RGS2 and RGS3) as controls.

Immunoprecipitation of lysates from 35S-labeled COS-7 cells (Fig. 1B) and Western blotting (see Fig. 5B) yielded bands of the expected molecular weights and demonstrated a dose-dependent increase in RGS protein expression with increasing amounts of RGS cDNA transfected. The overall expression level varied among the different RGS proteins. Importantly, expression of each RGS protein was comparable between cells that were transfected with the M3 receptor and those transfected with Gq* (see below), so that any potential functional differences between M3- and Gq*-expressing cells cannot be attributed to a difference in their amount of cellular RGS protein. Comparing the inhibitory effects of RGS protein in these two experimental settings should therefore provide insight into the functional importance of GTPase activation and effector antagonism for the inhibitory effect of R4 RGS proteins on Goq signaling in mammalian cells.

**Inhibitory Effect of RGS Proteins on M3-induced, Gq-mediated PLCβ Activation**

**Experimental System**—To examine the effect of RGS proteins in a mammalian cell, where they can act as both GAPs and effector antagonists, COS-7 cells were transiently transfected with muscarinic M3 receptors, and total inositol phosphate production was measured in response to carbachol stimulation. Fig. 2A illustrates that exogenous M3 receptor was required for carbachol-induced PLC activation. The lack of endogenous Gq*-coupled muscarinic receptors (such as M1, M4, and M5 receptors) in COS-7 cells was confirmed by negligible N-methyl-[3H]isoprenaline-binding sites in vector-transfected cells (data not shown; see below). The amount of endogenous Goq and PLCβ was sufficient to couple transfected M3 receptors to downstream signaling components, because the stimulatory effect of carbachol on PLCβ in M3-expressing cells was not further enhanced by co-transfection of Goq* and/or PLCβ (Fig. 2A).

Selective coupling of transfected M3 receptors with Goq was confirmed by insensitivity of carbachol-induced PLCβ activation to 12 h of preincubation of COS-7 cells with 100 ng/ml pertussis toxin (Fig. 2B). Pertussis toxin irreversibly inactivates some members of the G protein family (such as Goi/o), but not Goq, by catalyzing ADP-ribosylation at the very C-terminal cysteine in Ga. To demonstrate that under the conditions chosen pertussis toxin completely inactivated pertussis toxin-sensitive G proteins in vivo, in vitro back ADP-ribosylation was performed (Fig. 2B, inset). No ADP-ribosylated Ga subunits were detectable in COS-7 cells that had been pretreated with pertussis toxin. Potential contributions of Goq* proteins to carbachol-induced PLCβ activation, which would be mediated via their βγ subunits (18), was further excluded by a lack of effect...
of co-transfection of a C-terminal region of β-adrenergic receptor kinase (BARK<sub>7</sub>, data not shown), a protein fragment widely used as a Gβγ scavenger (23). Thus, transient transfection of the M<sub>3</sub> receptor was sufficient to achieve carbachol-induced PLCβ activation in COS-7 that was mediated by endogenous G<sub>q</sub> protein (via its α subunit) and was, as such, subject to the regular G protein activation/inactivation cycle.

**Effect of RGS Proteins**—Carbachol dose-dependently increased the activity of PLC in the absence of exogenously expressed RGS proteins (Fig. 3, left panels, open circles). The maximal effect was observed at 100 μM carbachol and amounted to a 12–15-fold rise over basal PLC activity. The EC<sub>50</sub> of carbachol was 0.9 ± 0.1 μM (n = 10 assays). We then compared carbachol-induced PLCβ activation in the absence or presence of different RGS proteins (Fig. 3, filled circles). Expression of RGS2, RGS3 (both isoforms), RGS5, and RGS16 caused a significant blunting of the maximal effect of carbachol (left panels) as well as a modest right shift of the dose-response curve for carbachol (right panels). The maximal activation of PLCβ by carbachol was reduced to 64.2 ± 2.0% of vector-transfected cells (n = 10, RGS2), 58.8 ± 2.3% (n = 3, RGS3<sub>α</sub>), 36.6 ± 2.1% (n = 11, RGS3<sub>β</sub>), 76.7 ± 4.6% (n = 7, RGS5), and 68.8 ± 4.5% (n = 8, RGS16). Compared with vector-transfected control, the EC<sub>50</sub> for carbachol was increased by a factor of 3.4 ± 0.4 (n = 4, RGS2), 7.8 ± 0.9 (n = 3, RGS3<sub>α</sub>), 4.3 ± 0.9 (n = 3, RGS3<sub>β</sub>), 2.2 ± 0.4 (n = 4, RGS5), and 2.9 ± 0.6 (n = 3, RGS16; all p < 0.05 versus vector-transfected COS-7 cells). The inhibitory effect of each RGS protein was concentration-dependent over a wide range of stimulatory input via the M<sub>3</sub> receptor, as shown by titration experiments with increasing amounts of RGS cDNA that were performed at three different carbachol concentrations for each RGS protein (Fig. 4). Fig. 1B illustrates that RGS protein expression rose linearly upon transfection of increasing RGS cDNA amounts. RGS proteins containing the FLAG tag at the N or C terminus yielded comparable results (data not shown), indicating that the epitope tag did not alter RGS protein function.

To determine whether or not changes in the expression level of any of the components in the M<sub>3</sub>/G<sub>q</sub>/PLCβ signaling pathway contribute to the observed effects, receptor binding assays and Western blots were performed. N-Methyl-[<sup>3</sup>H]scopolamine binding was saturable over the concentration range examined, and the experimental data were well fitted by a one-site binding model, as shown for M<sub>3</sub>-transfected COS-7 cells (Fig. 5A). The maximal number of binding sites (B<sub>max</sub>) amounted to 2871 ± 358 fmol/mg protein with a ligand affinity (K<sub>d</sub>) of 308 ± 63 pmol/l (n = 3). B<sub>max</sub> was not significantly changed upon expression of RGS2 (91.5 ± 25.2% of control, n = 3), RGS3 (92.9 ± 19.6%, n = 3), RGS5 (91.7 ± 15.7%, n = 2), and RGS16 (91.6 ± 21.3%, n = 2) and only modestly reduced in RGS3s-expressing cells (82.7 ± 4.0% of control, n = 3, p < 0.05). There was no significant change in K<sub>d</sub> upon co-expression of any of the RGS proteins tested (data not shown). Western blots in Fig. 5B illustrate that the cellular amount of the endogenous G<sub>q</sub> protein, PLCβ<sub>1</sub> and PLCβ<sub>2</sub> was also largely unchanged by the presence or absence of M<sub>3</sub> receptors or RGS proteins. Of note, RGS4 is known to inhibit G<sub>q</sub>-mediated PLC signaling in a variety of cell types and experimental settings (10), and we originally intended to include this well-characterized RGS protein in this study. However, in our hands the expression of RGS4 was rather weak and markedly reduced upon co-expression of the M<sub>3</sub> receptor (Figs. 1B and 5B). At this expression level, no significant inhibitory effect of RGS4 on carbachol-induced PLC activation was observed (data not shown). In summary, RGS2, RGS3 (both isoforms), RGS5, and RGS16 dose-dependently inhibited carbachol-induced, M<sub>3</sub>/G<sub>q</sub>-mediated PLCβ activation, which could not be attributed to changes in the expression level of any of these signaling components.

**Inhibitory Effect of RGS Proteins on G<sub>q</sub>-Mediated PLCβ Activation**

**Experimental System**—To examine the effect of the same RGS proteins under conditions where they cannot hasten G<sub>q</sub> inactivation, COS-7 cells were transiently transfected with constitutively active G<sub>q</sub>Q<sub>209L</sub> (G<sub>q<sub>α</sub></sub>*) in the presence or absence of the different RGS proteins. First, we determined whether transfection of G<sub>q</sub>α and RGS (alone or in combination) affected the expression of endogenous PLCβ and/or their respective expression levels. Fig. 6 shows representative immunoblots from COS-7 lysates that were transfected with decreasing amounts of G<sub>q</sub>α cDNA in the absence or presence of a fixed amount of RGS cDNA. Endogenous PLCβ<sub>1</sub> and PLCβ<sub>2</sub> levels were not changed upon exogenous expression of G<sub>q</sub>α and/or any of the RGS proteins tested (data not shown). However, the expression of G<sub>q</sub>α and select RGS proteins was altered upon co-transfection. The cellular amount of G<sub>q</sub>α was increased upon co-transfection with RGS2, RGS3s, and RGS3, for which the effect was most pronounced. Conversely, RGS2 and RGS3s.
(but not RGS3) were markedly increased in the presence of Gq*. The extent of their up-regulation appeared to correlate with the amount of Gq* expressed (best illustrated in the RGS2 blot). In contrast to RGS2 and RGS3, RGS5 and RGS16 levels were largely comparable in the presence or absence of Gq*, and conversely Gq* was not significantly altered by the presence of exogenous RGS5 or RGS16. Thus, co-expression of Gq* with RGS2 or RGS3 (but not RGS5 and RGS16) caused a reciprocal increase in protein expression, which needs to be taken into account for the interpretation of functional data (see below).

**Effect of RGS Proteins**—As expected for a constitutively active protein, Gq* dose-dependently activated PLCβ in the absence of receptor stimulation (Fig. 7, left panels, open symbols). To assess the potential effector antagonistic effects of RGS2, RGS3, RGS5, and RGS16, two types of titration experiments were performed: (i) increased expression of Gq* in the presence of a fixed amount of RGS protein (Fig. 7, left panels) and (ii) increased expression of RGS protein in the presence of a fixed amount of Gq* (at two different concentrations; Fig. 7, right panels). RGS2 and RGS3 (both isoforms) shifted the Gq* dose-response curve to the right with an estimated 8–10-fold increase in EC50 compared with vector control (left panels, p < 0.05). Upon titration, RGS2 and both isoforms of RGS3 dose-dependently blunted the stimulatory effect of Gq* on PLCβ (right panels). Their inhibitory effect was much more pronounced in cells expressing less Gq* (solid line versus dotted line), in which their maximal effect amounted to 22 ± 1%
RGS2 and RGS3, RGS5 and RGS16 did not significantly alter doses of carbachol (0.1, 10, and 1000 increasing amounts of RGS cDNA (125, 250, and 750 ng/well). Please population (H11005 open bars indicated Gq-mediated PLC in vivo to induce an inhibitory effect on M3-induced, Gq* activation (Fig. 7), despite expression levels that were sufficient to exert an inhibitory effect in a setting where they could not act as GAPs.

**Discussion**

The main finding of this study is that members of the R4 RGS family differ in their dependence on GTPase acceleration as a mechanism of action for their negative regulation of Gq*-mediated signaling in vivo. RGS2 and RGS3 markedly inhibited Gq*-mediated PLCβ activation in the absence of GTPase acceleration, suggesting that GAP-independent mechanism(s) (such as effector antagonism) are sufficient to mediate their inhibitory effect. In contrast, RGS5 and RGS16 were unable to exert an inhibitory effect on Gq*-induced PLCβ activation, i.e. in a setting where they could not act as GAPs.

**Experimental System**—Using several members of the R4 RGS family, we tested the hypotheses that RGS proteins regulating Gq*-mediated signaling do not necessarily depend on their GAP function to modulate cell signaling in vivo and that the relative contribution of their GAP effects and effector antagonism differs among RGS proteins. RGS point mutations devoid of GAP activity could not be utilized to address this question. To our knowledge, all GAP-deficient RGS mutants described so far have significantly reduced Gq* binding affinity (24–26), which in turn reduces not only their GAP activity but also potential effector antagonistic effects. Therefore, we chose to take advantage of a well characterized point mutation in Gaq* (Ga-Q209L) that destroys its GTPase activity and thereby renders it constitutively active (12). Both PLCβ and RGS proteins bind to receptor-activated as well as GTPase-deficient Ga subunits. Importantly, constitutively active Ga subunits (QL mutants) do not regain GTPase function in the presence of a GAP (21). The contact sites of PLCβ on Gaq have been mapped to helices a3 and a4 in the Ras-like domain of Gaq (27), so that the Q209L point mutation located in the switch II region of Gaq is unlikely to markedly alter its binding affinity to PLCβ. However, the contact sites of RGS on Gaq are almost exclusively located in the three switch regions (28), and the in vitro binding affinity of RGS proteins for Ga-GDP-AIF* (mimicking the transitional state of wild-type Ga) is higher than that for Ga-GTP* (mimicking constitutively active GaQL) (4). Potential differences in the binding affinity of RGS proteins for Gaq and Gaq* in vivo can therefore not be excluded. To minimize their potential contribution to the overall effect, Gaq/Gaq* and RGS proteins were titrated individually over a broad range of expression levels.

**Implications of Expression Changes of Key Components of the PLCβ Signaling Pathway**—Cellular amounts of all PLCβ signaling pathway components (both endogenous and exogenous) were carefully monitored for each transfection condition from both experimental systems to determine their potential contributions to the observed changes in PLCβ activity in the presence of a particular RGS protein. In the first experimental model (M3-expressing COS-7 cells), endogenous Gaq levels and M3 receptor density were largely unchanged (Fig. 5). The observed reduction in M3 receptor density in RGS3-expressing cells was very modest and is therefore unlikely to be a major contributor to the pronounced inhibition of PLCβ activity in the presence of RGS3s. The cellular amount of PLCβ1 and PLCβ3 was not altered in any of the conditions tested, which is of critical importance for the interpretation of the data obtained because PLCβ itself is known to act as a GAP on Gaq (29–31).

Although the expression level of transfected RGS proteins was not significantly altered upon co-expression of the M3 receptor (Fig. 5B), there was a marked increase in RGS2 and RGS3s in cells with constitutive PLCβ activation (Fig. 6). This effect was also observed in Gaq*-transfected CHO cells (data not shown) and is therefore not limited to COS-7 cells. Little is known about the regulation of RGS3s expression, but RGS2 expression has been reported to be altered in many different cell types in response to a variety of stimuli (32), including enhanced phosphoinositide signaling (33). Transfection studies do not provide information on gene/protein regulation of endogenous proteins, and the expression of endogenous Gaq could unfortunately not be assessed in this system, because it cannot be distinguished from Gaq* by size. Nevertheless, the fact that only RGS2 and RGS3s but none of the other RGS proteins controlled by the same promoter (including RGS3) were changed in their expression in the presence of Gaq* seems noteworthy, points toward possible post-transcriptional regulation, and may involve protein stabilization (possibly phosphorylation-induced), which was reported for two other RGS proteins (34, 35). Because RGS2 and RGS3s are potent inhibitors of Gaq*-mediated PLCβ activation, their up-regulation likely serves as a negative feedback mechanism enabling the cell to desensitize upon prolonged PLCβ activation. Conversely and consistent with regulatory feedback, Gaq* expression was up-

![Fig. 4. Concentration-dependent effect of RGS proteins on carbachol-induced, Gaq*-mediated PLCβ activity.](image-url)
regulated only in the presence of RGS proteins capable of negatively modulating its effect (i.e. RGS2, RGS3s, and RGS3, for which the effect was most pronounced). Taken together these findings suggest a finely tuned balance between Gαq and RGS proteins that modulate its activity. Further work is needed to understand the mechanism underlying the reciprocal increase in RGS2/RGS3 and Gαq* as well as the differential regulation of the two RGS3 isoforms.

**RGS2 and RGS3 Inhibit Gαq-mediated Signaling in the Absence of GTPase Activation**—RGS2 and RGS3 were capable of markedly inhibiting PLCβ activity in response to stimulation with carbachol via M3/Gαq (Fig. 3) or constitutively active Gαq* (Fig. 7), i.e. independent of their abilities to act as GAPs. We interpret this finding as an indication that mechanisms other than accelerated G protein inactivation (e.g. effector antagonism) are sufficient to mediate their inhibitory effects. RGS2
RGS Inhibition of G_q Signaling: GAP vs. Effector Antagonism

Fig. 6. Characterization of COS-7 cells transfected with G_{q}* and RGS proteins. COS-7 cells were transiently transfected with decreasing amounts of G_{q} cDNA (250 to 4 ng/well in a 12-well plate) in the absence or presence of different RGS cDNAs (each at 500 ng/well). Equal amounts of total cell lysates were analyzed by Western blotting, using antibodies directed against the HA and FLAG epitopes in G_{q} and RGS, respectively. Shown are representative blots from three independent experiments.

was previously reported to be 10–20-fold more potent as a GAP than effector antagonist in vitro (36). The stoichiometry of signaling components cannot be easily controlled in vivo, partly because protein expression can be altered upon co-transfection, as seen in the present study (see above). We varied the stoichiometry of G_{q}*, RGS, and PLC beta by cDNA titrations and demonstrated that RGS2 and both isoforms of RGS3 markedly inhibited G_{q}* induced PLC beta activation over a broad range of expression levels, indicating that they are potent inhibitors of G_{q} signaling in mammalian cells in vivo, even in the absence of GTPase acceleration (Fig. 7). The higher the ratio between RGS2 or RGS3 and G_{q}*, the stronger was their inhibitory effect, consistent with effector antagonism. A direct inhibitory effect of RGS2 and RGS3 on PLC beta, analogous to the interaction between RGS2 and adenyl cyclase (37), is unlikely, because it would be expected to cause a reduction in basal PLC beta activity that was not observed.

To what extent GAP-independent mechanisms (such as effector antagonism) contribute to the overall inhibitory effect of RGS2 and RGS3 when GTPase acceleration is possible (such as in the M_{3} transfection model) cannot be easily discerned from this study. As long as they are bound to G_{q}, they would be expected to have the capacity to functionally inhibit G_{q}-effector coupling. However, the time frames for effector antagonistic (or other GAP-independent) effects are likely much shorter in M_{3}-expressing cells because of the acceleration of GTP hydrolysis by RGS proteins.

Although it was not the goal of this study to directly compare the efficiency of inhibition among different RGS proteins on G_{q} signaling, the relative expression levels of each RGS protein could be estimated using a common FLAG antibody. For example, despite comparatively low expression, RGS2 had a very pronounced inhibitory effect, consistent with RGS2 proteins being very potent inhibitors for G_{q} signaling (38). Similarly, despite its low expression level, RGS3 also potently inhibited receptor-induced PLC beta activation in M_{3}-expressing cells (Figs. 3 and 5B). In fact, its expression level was much lower than that of RGS3, whereas the overall inhibitory effect was almost comparable, consistent with the notion that the C-terminal part of RGS3 contains structural domains important for signal modulation (39, 40). Via its C-terminal region RGS3 can directly interact with G_{q} and inhibit G_{q}-mediated PLC beta activation (41). This potential interaction is unlikely to play a major role in this study, because PLC beta activation is mediated predominantly (if not exclusively) by the alpha subunit of G_{q} in the experimental system used (see above).

RGS5 and RGS16 Require GTPase Activation to Inhibit G_{q}-mediated Signaling—Consistent with previous reports showing that RGS5 and RGS16 can bind to G_{q} and accelerate its GTPase activity in vitro and modulate G_{q}-mediated signaling in vivo (42–44), both RGS5 and RGS16 exerted an inhibitory effect on carbachol-induced PLC beta activation in M_{3}-expressing COS-7 cells (Fig. 3). Endogenous RGS5 was recently shown not to be involved in the negative regulation of M_{3} receptor signaling in rat aortic smooth muscle cells (45). Differences among species, cell types, and experimental approaches (overexpression of exogenous RGS5 versus reduction of endogenous RGS5) likely determine the degree of involvement of RGS5 in regulating receptor-mediated cellular signaling.

The major finding of the present study is that the inhibitory effects of both RGS5 and RGS16 were absent in cells expressing G_{q} despite expression levels sufficient to inhibit G_{q}-induced PLC beta activation (Fig. 7). This finding suggests that (in contrast to RGS2 and RGS3) RGS5 and RGS16 depend on their GTP function to exert an inhibitory effect on G_{q}-mediated signaling. Comparison of the RGS domains of RGS2 and RGS3 with those of RGS5 and RGS16 does not reveal any striking differences that could explain their differential behavior (28, 40). Although the N-terminal extension to the G_q core domain in RGS3 is very different in length and composition from all other R4 RGS proteins, RGS2 is similar in its N-terminal structure to RGS5 and RGS16, including an amphipathic alpha-helical membrane targeting domain (46, 47). However, the N termini of RGS2 and RGS5/RGS16 differ in their overall length (22) and the number of potential palmitoylation sites (48). Because the N terminus of R4 RGS proteins is believed to serve as a scaffold for receptors and signaling proteins (46), it is conceivable that structural differences between RGS2/3 and RGS5/16 contribute to their differential effects. Phosphorylation of RGS proteins may also play a role, but so far little is known about its regulation and functional implications (36, 49, 50). Changes in post-translational modifications on G_{q} subunits, such as an increase in palmitate turnover in constitutively active G_{q}, (51), also have the potential to differentially affect RGS function (52, 53).

Conclusions —The ability of RGS proteins to interact with G protein alpha subunits is central for their ability to negatively regulate G protein-mediated signaling. As RGS proteins bind to G_{q}, they have the potential to act as GTPase-activating proteins and/or as effector antagonists. This study demonstrates that RGS2 and RGS3, but not RGS5 and RGS16, are capable of potently inhibiting G_{q}-mediated PLC beta activation in the ab-
sence of GTPase acceleration. Although information on the relative contribution of GAP-dependent and -independent mechanisms of action to the overall inhibitory effect of RGS proteins cannot be derived from this study, it reveals potentially significant functional differences among closely related R4 RGS family members with considerable structural similarities. Further work is needed to define the underlying mechanism(s)/factor(s), which likely include differences in the interplay between RGS proteins, G proteins, and effectors and in their receptor activation-dependent availability or function. As more mechanistic insights are gained, selective targeting of RGS subsets with similar regulatory mechanisms may become feasible. Additional questions raised by this study are (i) whether accelerated G protein inactivation is also a prerequisite for the inhibitory effect of RGS5 and RGS16 on G<sub>i</sub><sup>q</sup> signaling (22, 42, 44) and (ii) whether a similar differential behavior in RGS function can be found within members of other RGS families.

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