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Characterization and Comparison of RGS2 and RGS4 as GTPase-Activating Proteins for m2 Muscarinic Receptor-Stimulated G_i

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ABSTRACT

RGS2 and RGS4 were studied for their effects as GTPase activating proteins (GAPs) on receptor-activated G_i in a novel steady-state assay using membranes from Sf9 cells quadruply infected with baculoviruses encoding the m2 muscarinic receptor, $G_{\alpha i2}$, $G_{\beta 1}$, and $G_{\gamma 2}$. In the presence of the muscarinic agonist carbachol, regulator of G protein signaling 2 (RGS2) and RGS4 each produced up to a 10-fold increase in agonist-dependent GTPase activity. The observed K_m for GTP in this system was increased in the presence of RGS4. NaCl and KCl inhibited the GAP activities of both RGS2 and RGS4, although they had no effect on GTPase activity in the absence of RGS proteins. $MgCl_2$ had a complex effect on GTPase activity, with optimal RGS2 and RGS4 GAP activities occurring, respectively, at high micromolar and low millimolar concentrations of free Mg^{2+}. The concentration dependence of RGS GAP activity was assessed, and RGS4 was found to be more potent than RGS2 by up to an order of magnitude. This direct observation confirms a similar difference in potency found when these two RGS proteins were compared for their ability to inhibit signaling downstream of G_i (Heximer et al., 1999). RGS2 yielded Hill coefficients greater than 2.0, suggesting that it may bind in a positively cooperative manner to oligomeric structures containing more than one G protein. Furthermore, RGS4 yielded a bell-shaped dose-dependence under low magnesium (0.5 mM) conditions, which is also consistent with the idea of RGS cooperativity.

Heterotrimeric G proteins are activated by seven transmembrane spanning receptors, which promote the dissociation of GDP and thereby allow intracellular GTP to bind. G proteins are deactivated by the hydrolysis of GTP to GDP and inorganic phosphate, and the rate of that reaction can be increased up to 2 orders of magnitude by GTPase activating proteins (GAPs) (Wilkie and Ross, 2000). Some effectors act as GAPs toward the G proteins that activate them; for example, phospholipase Cβ (Chidiac and Ross, 1999) and p115Rho-GEF (Kozasa et al., 1998), respectively, increase the rates of GTP hydrolysis by G_q and G_{12}. However, most GAPs for heterotrimeric G proteins have no known effector function and belong to the regulators of G protein signaling (RGS) protein family. Most RGS proteins are GAPs for members of the G_i family (Hepler, 1999), and a subset of those also act as GAPs for G_q (Ingi et al., 1998; Scheschonka et al., 2000).

The most direct way to measure RGS GAP activities is via increases in the rates at which their targeted G proteins hydrolyze GTP. With isolated G_α subunits, GTP turnover is limited by the dissociation of GDP, and therefore the rate of GTP hydrolysis needs to be measured under pre–steady-state conditions using G protein prebound to GTP (Wang et al., 1998a). Alternatively, GDP dissociation is not rate-limiting in the presence of activated receptors, and RGS GAP effects therefore can also be observed under steady-state conditions with receptor-coupled G proteins in the presence of agonist (Wang et al., 1998a). Although the pre-steady-state method is advantageous in that it allows changes in G protein GTP hydrolysis rate constants to be quantified unambiguously and also excludes the possible confounding effects of auxiliary proteins, the steady-state method may be more biologically relevant. As an alternative to assaying GTP hydrolysis, evidence of RGS protein GAP activity can also be observed via downstream events such as second messenger generation or mitogen-activated protein kinase activity; however, changes in these endpoints can also arise from other processes, such as steric effects or competition between RGS and effector proteins for activated G proteins (Hepler et al., 1997).

RGS2 is unique among the RGS proteins in its apparent selectivity toward G_q. Indeed, initial findings showed that the pre-steady-state GTPase activities of isolated G_i family

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ABBREVIATIONS: GAP, GTPase activating protein; RGS, regulator of G protein signaling; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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members ($G_{\alpha i1}$ and $G_{\alpha o}$) were not stimulated by RGS2 (Ingi et al., 1998), even at protein concentrations up to 3000-fold higher than necessary to detect the effects of RGS4 (Heximer et al., 1997). This lack of effect can be attributed to three amino acid substitutions in the RGS domain of RGS2, because an RGS2 triple mutant (C106S,N184D,E191K) containing the corresponding residues from RGS4 increased the rate of hydrolysis by G_i to the same extent that RGS4 did (Heximer et al., 1999). Notwithstanding its failure to act as a GAP on isolated $G_{i\alpha}$, however, wild-type RGS2 has been found to inhibit cellular signaling events downstream of receptor-activated G_i (Herlitze et al., 1999; Heximer et al., 1999; Ingi et al., 1998; Potenza et al., 1999) and also to increase steady-state G_i GTPase activity in a reconstituted system containing agonist-activated m2 muscarinic receptor and phospholipids (Ingi et al., 1998). A possible explanation for the discrepancy between the pre–steady-state and other assays is that the additional proteins present in the latter experiments, namely receptor and $G_{\beta\gamma}$, may promote interactions between RGS2 and $G_{i\alpha}$. Because free $G_{\beta\gamma}$ actually inhibits RGS GAP effects on isolated Gα (Chidiac and Ross, 1999; Wang et al., 1998b), it follows that receptors may support or promote RGS–G protein interactions. This view is supported by the identification of putative receptor binding domains in the C- and N-terminal regions of RGS4 (Zeng et al., 1998) and by the finding that the potencies of RGS proteins in attenuating signaling via G_q can vary depending on the identity of the activating receptor (Xu et al., 1999). Thus RGS proteins may be targeted *in vivo* to receptor–G protein complexes in addition to free G proteins.

Some studies have found RGS2 to be without effect on events subsequent to G_i activation by receptors, whereas other RGS proteins assayed in parallel have been inhibitory (Doupnik et al., 1997; Bowman et al., 1998; Reif and Cyster, 2000). The lack of inhibition by RGS2 in these cases suggests that its affinity for G_i is low compared with that of other RGS proteins. Taken from extrapolations from yeast pheromone response assays, RGS4 has been estimated to be approximately 8-fold more potent than RGS2 in blocking or deactivating G_i (Heximer et al., 1999). However, the potency of RGS2 as a GAP for G_i has never been established. For the present study, we developed an assay to measure and compare RGS2 and RGS4 GAP activities in a cellular membrane environment using postnuclear membranes from Sf9 cells infected with baculoviruses encoding the m2 muscarinic receptor plus $G_{\alpha i2}$, $G_{\beta 1}$, and $G_{\gamma 2}$.

Materials and Methods

Plasmids encoding histidine-tagged RGS proteins were a generous gift from Dr. John Hepler (Emory University, Atlanta, GA). Baculoviruses encoding $G_{\alpha i2}$, $G_{\beta 1}$, $G_{\gamma 2}$, and the m2 muscarinic receptor (tagged at the N terminus with a c-myc epitope) were kindly provided by Dr. Terry Hebert (Montreal Heart Institute, Montreal, Quebec). [γ^{32}P]GTP was purchased from ICN Pharmaceuticals (Costa Mesa, CA), and [^{3}H]quinuclidinyl benzilate was purchased from PerkinElmer Life Sciences (Boston, MA). GTP, ATP, carbachol, atropine, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical (St. Louis, MO). Leupeptin and aprotinin were purchased from Roche Diagnostics (Indianapolis, IN). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Receptor and G Protein Expression in Sf9 Cells and Membrane Preparation. Sf9 insect cells at a density of 2×10^6 cells/ml were infected with baculoviruses encoding the following: N-terminal c-myc–tagged m2 muscarinic receptor, $G_{\alpha i}$, $G\beta_1$, and $G\gamma_2$. For some experiments, one or more viruses were omitted. After 48 h of infection, the cells were centrifuged at 228*g* for 5 min, resuspended in phosphate-buffered saline, and recentrifuged. The resulting pellets were resuspended in one third of the original volume of lysis buffer (20 mM Tris, pH 8.0, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin). After a 15-min incubation on ice, the cells were lysed with the use of a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada), followed by a 10-min centrifugation at 500*g*. The supernatant was retained and centrifuged for 30 min at 48,000*g*. The supernatant was discarded and the pellets resuspended in 0.01 volume of lysis buffer, then placed in aliquots and stored at −80°C. This procedure yielded 10.4 ± 3.0 fmol of m2 muscarinic receptor per milligram of membrane protein, as determined by the specific binding of [^{3}H]quinuclidinyl benzilate.

RGS Protein Purification. For RGS4, 4 L of LB broth with ampicillin (0.05 mg/ml) were inoculated with *Escherichia coli* NH6 RGS4 pQE60 and incubated with vigorous aeration at 37°C until mid-log phase (optical density at 600 nm of 0.55). A 3-h induction was commenced by adding 1 mM isopropylthio-β-D-galactoside. The bacteria were harvested and resuspended in 60 ml of buffer A (0.05 M HEPES, pH 8.0, 0.1 M NaCl, 0.02 M β-mercaptoethanol, 1% Triton X-100, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin) and then snap frozen to −80°C. After thawing, 0.2 mg/ml lysozyme was added, and the suspension was mixed and incubated on ice for 30 min followed by the addition of 25 μg/ml DNase in the presence of 0.5 mM $MgCl_2$ for 20 min. The mixture was centrifuged at 140,000*g* for 30 min at 4°C, and the volume of the supernatant increased to 100 ml with buffer A supplemented with glycerol and imidazole (final concentrations of 20% and 0.02 M, respectively). Equilibrated Ni-nitrilotriacetic acid affinity resin (1.5 ml) was added and gently rotated in the cold for 90 min and then loaded onto a 5-ml column, washed with 30 ml of buffer A with 0.5 M NaCl, and then washed with 30 ml of buffer A with no Triton X-100. RGS4 was eluted with 0.2 M imidazole. A Superdex 75 HR 10/30 column (Amersham Biosciences Inc., Piscataway, NJ) was equilibrated with buffer B (0.05 M HEPES, pH 8.0, 0.15 M NaCl, 0.001 M DTT, 0.1 mM PMSF). The RGS4 fraction was loaded and eluted in a clean peak. The fractions were pooled, placed in aliquots, and stored at −80°C. For RGS2, a similar method was used in the purification process. LB broth (4 L) with ampicillin (0.05 mg/ml) were inoculated with *E. coli* BL 21 (DE3) RGS2.H10 pET 19b. The same procedure for the purification of RGS4 was followed except that the concentration of NaCl was 0.5 M throughout the imidazole-elution step and then reduced to 0.25 M for the final gel-filtration step and subsequent pooling of peak fractions. For both RGS2 and RGS4, the pooled Superdex fractions were estimated to be >95% pure as determined by Coomassie staining.

Assay of GTP Hydrolysis. The steady-state hydrolysis of [γ^{32}P]GTP by Sf9 membranes was measured in the absence and presence of purified RGS proteins. Unless indicated otherwise, 50-μl reaction mixtures containing 20 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, plus 10 to 50 mM NaCl and 10 mM $MgCl_2$ [7.5 mM free Mg^{2+}, as calculated using the program "Bound and Determined" (Brooks and Storey, 1992)], were incubated at 30°C for 15 min with 1 μM GTP, 500 μM ATP, [γ^{32}P]GTP (1×10^6 cpm/assay), either 100 μM carbachol or 10 μM atropine, and membranes (2 μg/assay). The assay was stopped by adding 950 μl of ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4; the mix was then centrifuged, and the level of $^{32}P_i$ in the resulting supernatant was determined by liquid-scintillation counting. The nonspecific membrane GTPase signal was estimated by adding 1 mM of unlabeled GTP to the above assay mix, and this value was subtracted from the total counts per minute. In each experiment, separate controls were carried out to determine the GTPase activity attributable to trace contaminants in the purified RGS protein preparations. This was taken as the difference in $^{32}P_i$ between samples with and without maximal levels of RGS2 or RGS4,

but lacking membranes, and then scaled according to RGS protein concentration at each assay point; the scaled value was then subtracted from experimental measurements of membrane-dependent GTPase activity as appropriate. Typically, the GTPase activity of the purified RGS proteins contributed less than 2% to the total signal. Agonist-dependent GTPase activity was taken as that observed in the presence of the muscarinic receptor agonist carbachol minus that in the presence of the antagonist atropine.

Analysis of Data. In the determination of K_m for GTP, the receptor-specific signal at multiple GTP concentrations was estimated by fitting data acquired in the presence of atropine to a straight line and then subtracting that from data acquired in the presence of carbachol. The value of K_m for GTP for receptor-specific GTPase activity was determined by Lineweaver-Burk analysis. Analyses of RGS2 and RGS4 dose-response data were carried out by nonlinear regression using the fitting program SigmaPlot 4.0 (SPSS Science, Chicago, IL). RGS4 data acquired in the presence of 7.5 mM free magnesium and RGS2 data were analyzed according to the four-parameter Hill equation included with the program. RGS4 in the presence of 0.5 mM magnesium yielded a bell-shaped dose-response pattern, and these data were analyzed as the sum of two rectangular hyperbolae plus a nonspecific component (eq. 1):

$$y = ns + max_1[x]/K_{up} + [x]) + max_2[x]/(K_{down} + [x]) \quad (1)$$

where ns is the nonspecific signal, K_{up} and K_{down} correspond to the midpoints, whereas max_1 and max_2 correspond to the y values of the rising and descending rectangular hyperbolae, respectively. In some experiments, max_2 was poorly defined, and its value was constrained during the fitting procedure such that its absolute value would not exceed that of max_1. Throughout the text, mean values are reported ± S.D.

Results and Discussion

RGS proteins limit receptor signaling by accelerating GTP hydrolysis by G proteins, thereby shortening the lifespan of the activated GTP-bound state. This is likely to be their primary function *in vivo*, yet relatively few studies have measured RGS effects on GTP hydrolysis by receptor-activated G proteins. In general, the detection of receptor-stimulated G protein GTPase activity can be hampered by high background activities arising from endogenous nucleotidases in subcellular preparations. To avoid this shortcoming, GTPase assays can be carried out using purified receptors and G proteins co-reconstituted into phospholipid vesicles (Ingi et al., 1998; Mukhopadhyay and Ross, 1999), although such an approach is technically demanding. As a simpler alternative, we functionally reconstituted m2 muscarinic receptors and heterotrimeric G_{i2} in Sf9 cells by coinfecting with baculoviruses encoding the receptor and the three G protein subunits.

Sf9 insect cells are useful for the study of G protein-linked receptors because of the high levels of functional protein that can be expressed and post-translationally modified. Although the endogenous G protein complement in these cells can be sufficient for the measurement of receptor-stimulated second messenger production (Chidiac et al., 1994), measuring changes in the nucleotide binding activity of G proteins is facilitated by the coexpression of the latter with a given receptor (Barr et al., 1997). We could not detect carbachol-stimulated GTPase activity in membranes prepared from cells infected only with baculoviruses encoding the m2 muscarinic receptor, but a clear agonist signal did emerge with cells additionally infected with baculoviruses encoding the three G protein subunits $G_{\alpha i2}$, $G_{\beta 1}$, and $G_{\gamma 2}$ (Fig. 1A). Using this paradigm, we also were able to observe an effect of purified RGS4 on the steady-state GTPase activity of receptor-coupled G_i (Fig. 1B). If any G protein subunit was omitted, GAP activity was decreased or eliminated (data not shown).

Similar to previous findings with GAP effects on receptor-activated G proteins (Biddlecome et al., 1996; Cavalli et al., 2000), the K_m of m2 muscarinic receptor-activated G_{i2} for GTP was increased in the presence of RGS4 (Fig. 2). Such observations are consistent with an increase in the rate of GTP hydrolysis with no change in nucleotide binding properties, as discussed previously (Cavalli et al., 2000). Alternatively, the increase in K_m could conceivably reflect an RGS4-related decrease in the GDP dissociation rate constant or GTP association rate constant, but this seems unlikely because a previous study showed RGS4 to have no effect on nucleotide exchange with isolated $G_{\alpha i1}$ (Berman et al., 1996).

Effects of Salts on RGS GAP Activities. NaCl and KCl both were found to have inhibitory effects on RGS4 GAP activity (Fig. 3, A and B), with 250 mM of either salt decreasing RGS4 GAP activity by approximately 75%. Similarly, both salts also decreased the GAP activity of RGS2 (Fig. 3, C and D). These inhibitory effects seem to be related to RGS GAP activity rather than to either the intrinsic GTPase activity of the G protein or the agonist-stimulated GTPase activity, because both of those rates were essentially constant over the entire range of NaCl and KCl concentrations tested. To minimize the inhibitory effects of NaCl, which was

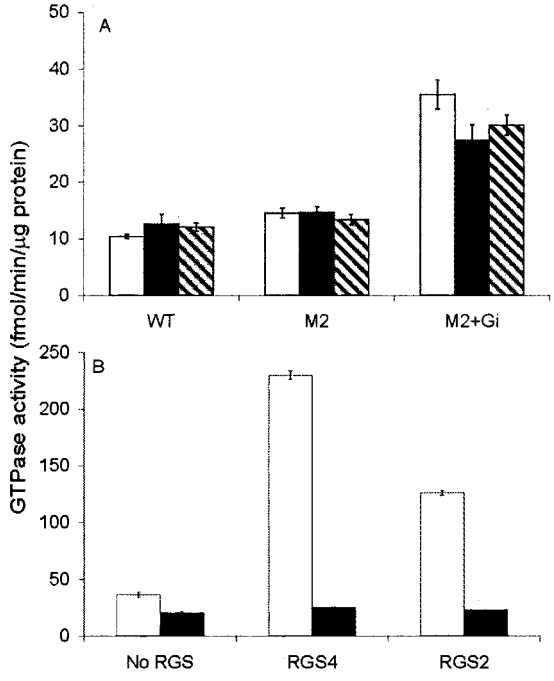

Fig. 1. Stimulation of GTP hydrolysis by m2 muscarinic receptor and RGS proteins. GTPase activity was measured in the absence of drug (striped bars), in the presence of the agonist carbachol (□), or in the presence of the antagonist atropine (■). A, membranes were prepared from Sf9 cells infected with wild-type baculovirus (WT), baculovirus encoding the m2 muscarinic receptor (M2), or baculoviruses encoding the receptor plus $G_{\alpha i2}$, $G_{\beta 1}$, and $G_{\gamma 2}$ (M2 + Gi) were prepared as described and were assessed for GTPase activity as indicated. B. Membranes from Sf9 cells infected with baculoviruses encoding the receptor and three G protein subunits were assayed for GTPase activity alone and in the presence of 5 μM of either RGS2 or RGS4, as indicated.

present in the purified protein stocks, concentrations of the salt were kept at or below 50 mM in all further experiments.

$MgCl_2$ had complex effects on the behavior of the G protein and its responsiveness to RGS proteins. Basal, carbachol-stimulated GTPase activities, and RGS2 GAP activity all plateaued between approximately 50 μM and 1 mM free Mg^{2+} and decreased at higher concentrations (Fig. 4). In contrast, RGS4-stimulated GTPase activity was still maximal at 5 mM free Mg^{2+}. Further experiments did show a minor decrease with RGS4 at concentrations of the ion in excess of 10 mM (data not shown). Biphasic effects of $MgCl_2$ on G protein activities have been described previously (Northup et al., 1982; Higashijima et al., 1987), and the differences in the $MgCl_2$ sensitivities of RGS2 and RGS4 recall comparable differences among RGS proteins in their interactions with Gαz as identified by Wang and coworkers (Wang et al., 1998b). From these results, it seems that $MgCl_2$ interacts directly with some or all RGS proteins.

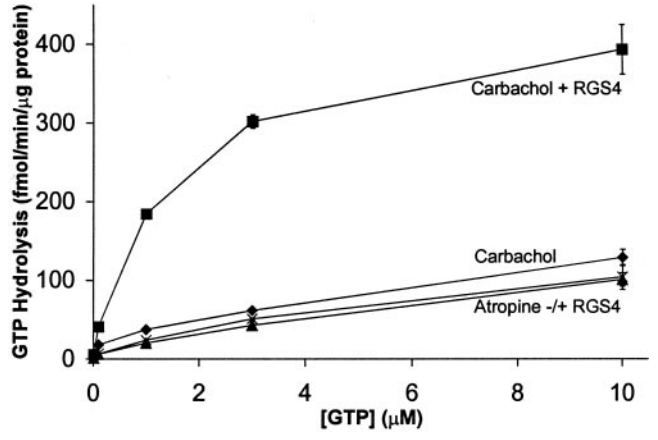

Fig. 2. Effect of RGS4 on GTP concentration dependence. GTPase activity was measured at multiple GTP concentrations in the presence of atropine (▲), carbachol (♦), atropine plus 400 nM RGS4 (×), or carbachol plus 400 nM RGS4 (■). The value of K_m for GTP was determined by Lineweaver-Burk analysis as described under *Materials and Methods,* and the means of this experiment plus two others were calculated to yield values of 130 ± 80 nM in the presence of carbachol alone and 500 ± 80 nM in the presence of carbachol plus RGS4.

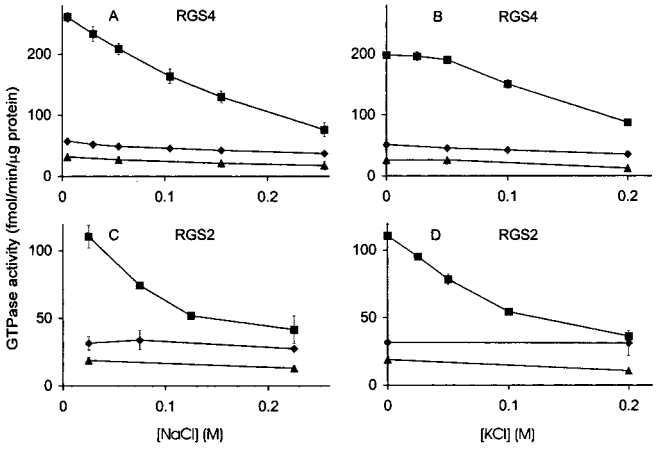

Fig. 3. Effects of NaCl and KCl on RGS2 and RGS4 GAP activity. GTPase activity was measured at the indicated concentrations of NaCl (A and C) or KCl (B and D) in the presence of atropine (▲), carbachol (♦), or carbachol plus RGS protein (■) (A and B: 400 nM RGS4; C and D: 3 μM RGS2).

Concentration dependence of RGS2 and RGS4 GAP Activities. The results shown in Fig. 5 indicate by direct observation that (1) RGS2 acts as a G_i GAP in a membrane environment containing an activated G_i-coupled receptor and (2) the potency of RGS2 is lower than that of RGS4. Assays of RGS concentration dependence were carried out at two different levels of $MgCl_2$. At 7.5 mM free magnesium, RGS4 was approximately 5 times as potent as RGS2 in stimulating agonist-dependent GTPase activity (Table 1). RGS4 increased the agonist-dependent signal approximately 10-fold, whereas the highest concentrations of RGS2 tested increased it by a factor of 5 (Fig. 5A). At 0.5 mM free magnesium, the maximal effects of RGS2 and RGS4 were similar, approximately 10-fold greater than the agonist-dependent GTPase signal (Fig. 5B). It is difficult to compare concentration dependence under these conditions because the shapes of the curves are different; however, the midpoint of the ascending phase (K_{up}, Table 1) suggests a 9-fold greater potency of RGS4. The increased maximal effect of RGS2 relative to RGS4 at the lower magnesium concentration is consistent with the patterns of magnesium dependence observed in Fig. 4.

Previous studies have shown that RGS2 does not interact with isolated $G_{i\alpha}$ (Heximer et al., 1997, 1999; Ingi et al., 1998) but that a GAP effect does occur in the presence of activated m2 muscarinic receptor (Ingi et al., 1998). The present results show that RGS2 is capable of increasing G_i GTPase activity to approximately the same extent as RGS4, albeit with a lower potency. In assays of the inhibition of yeast mating-pheromone signaling via G_i, Heximer and coworkers (1999) concluded that RGS4 was 8 times as potent as RGS2. This agrees well with our findings, and thus it seems likely that the observed inhibition of yeast pheromone signaling (Heximer et al., 1999) was the result of RGS GAP activity on receptor-activated G_i. The relatively low potency of RGS2 as a G_i GAP may explain its observed lack of effect on receptor-promoted G_i signaling in some studies (Doupnik et al., 1997; Bowman et al., 1998; Reif and Cyster, 2000).

In the present study, the concentrations of RGS2 used were limited by the purified protein stocks, and therefore it is

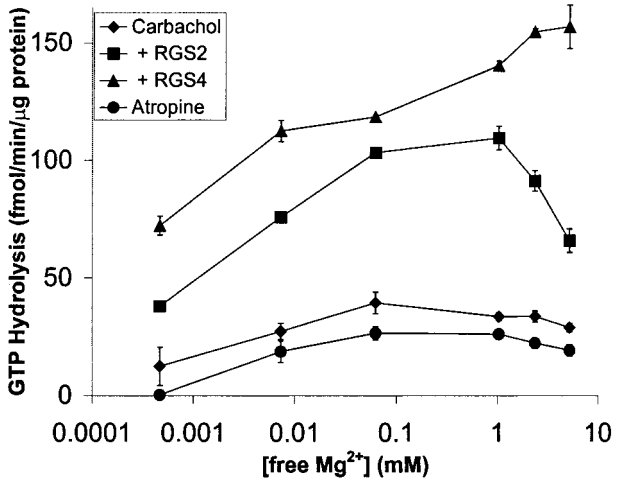

Fig. 4. Effect of $MgCl_2$ on GTPase activity. GTPase activity was measured at increasing concentrations of $MgCl_2$, and the calculated concentrations of free Mg^{2+} are shown on the abscissa. Assays were carried out in the presence of atropine (●), carbachol (♦), or carbachol plus 2 μM of either RGS2 (■) or RGS4 (▲).

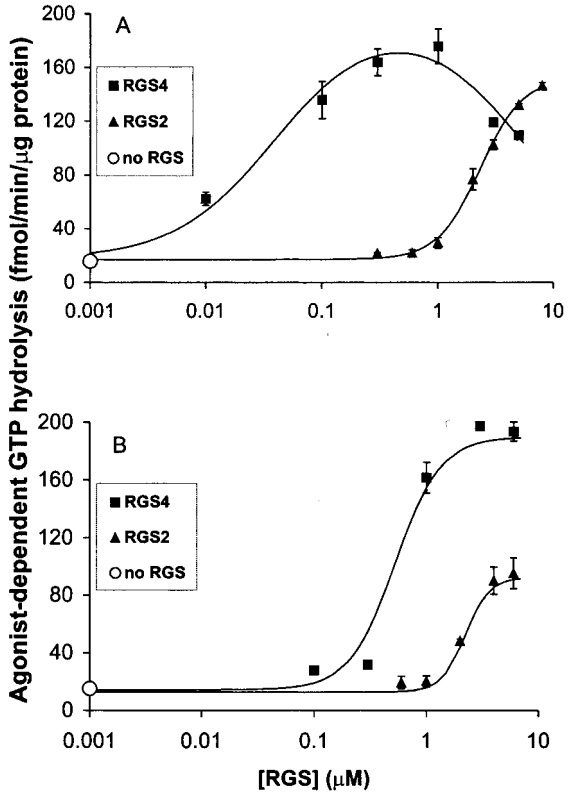

Fig. 5. Concentration dependence of RGS2 and RGS4 GAP activities at high and low magnesium levels. Agonist-dependent GTPase activities were measured in the presence of 7.5 mM (A) and 0.5 mM (B) calculated free Mg^{2+} (total added $MgCl_2$ was 10 mM and 2 mM, respectively) in the absence of RGS protein (○) or at the RGS2 (▲) and RGS4 (■) concentrations indicated on the abscissae. Data were fitted to either eq. 1 (RGS4 in B) or the Hill equation (others), and average parameters from these and replicate data sets are presented in Table 1.

possible that submaximal RGS2 effects may have been attained even at the highest levels tested. In particular, RGS2 activity at high magnesium was notably lower than that of RGS4. Still, the GAP effect of RGS2 seems to be complete. Regardless of $MgCl_2$ concentration, we observed a shoulder at the upper end of the range of RGS2 concentrations tested, implying a plateau in activity. Moreover, when plotted on linear coordinates, these data yielded a sigmoidal pattern, a hallmark of positive cooperativity. Accordingly, fits to the Hill equation yielded Hill coefficients of greater than 2 for RGS2 at the lower magnesium concentration tested and greater than 3 at the higher magnesium concentration.

Patterns inconsistent with simple Michaelis-Menten enzyme kinetics were observed for the concentration dependence of both RGS2 and RGS4. The observed Hill coefficients for RGS2 point to the existence of at least two positively cooperative binding sites. RGS4 at low magnesium levels yielded a bell-shaped pattern, with an initial stimulatory phase at lower concentrations followed by an inhibitory phase at higher concentrations (to our knowledge, this marks the first observation of a negative RGS effect on GTPase activity); although other explanations are possible (Pliska, 1994), bell-shaped dose-response curves can potentially be accounted for by the existence of stimulatory and inhibitory sites of interaction coexisting on the same protein structure (Jarv, 1995). Thus, assuming that a single G protein molecule can bind to only one RGS molecule at a time (Tesmer et al., 1997), the observed behaviors of both RGS2 and RGS4 imply that at least two RGS equivalents may bind to a structure containing multiple copies of G_i. Similarly, the observed inhibition of receptor-stimulated, G_q-mediated Ca^{2+} signaling in pancreatic acinar cells, in which RGS4 yielded dose-response curves that went from essentially no response to a maximal effect over a concentration range of approximately 10-fold (Xu et al., 1999), would seem to be inconsistent with Michaelis-Menten kinetics and indicative of positive cooperativity.

Heterotrimeric G proteins are not known to form higher-order structures by themselves; however, there is increasing evidence for complex protein structures that could incorporate more than one G protein, an idea proposed originally by Rodbell (1980). It is now well established that G protein-coupled receptors, including m2 muscarinic receptors, can form oligomers (Wreggett and Wells, 1995; Hebert and Bouvier, 1998). Accordingly, the binding of agonists to the m2 muscarinic receptor is consistent with cooperativity and points to the existence of receptor oligomers (Wreggett and Wells, 1995; Chidiac et al., 1997), and the G proteins coupled to these receptors similarly bind to guanine nucleotides in a cooperative manner (Chidiac and Wells, 1992).

Taken together, the available data imply that multiple equivalents of an RGS protein can interact with functional signaling complexes containing multiple copies of both receptor and G protein. The present findings thus support the notion that agonist signals are transduced through multimeric signaling complexes containing multiple receptors and G proteins, and that these complexes may contain or interact with other signaling or regulatory proteins. The formation of such complexes may be further promoted or stabilized by scaffolding proteins. At least two RGS proteins also have putative scaffolding functions (Wilkie and Ross, 2000), suggesting an additional role for this protein family in modulating the behavior of receptor signaling complexes. Future work in our laboratory and others should help to reveal the

TABLE 1

Concentration-dependence of RGS effects on steady-state GTPase activity. Carbachol-dependent GTPase activity was measured at multiple concentrations of RGS2 and RGS4 at free magnesium concentrations of 0.5 and 7.5 mM, as shown in Fig. 5. Data from individual dose-response curves for RGS2 and for RGS4 at 7.5 mM free magnesium were fitted to the Hill equation. RGS4 in the presence of 0.5 mM free magnesium yielded bell-shaped curves, which were individually analyzed as the sum of two rectangular hyperbolae. The parameters presented are mean values ± S.D.

	$[Mg^{2+}]_{free}$	EC_{50}	n_H	K_{up}	K_{down}	n
	mM	*μM*		*μM*	*μM*	
RGS2	0.5	1.3 ± 0.6	2.6 ± 0.5			5
RGS2	7.5	2.2 ± 1.2	3.5 ± 0.6			4
RGS4	0.5			0.14 ± 0.08	4.0 ± 3.0	6
RGS4	7.5	0.49 ± 0.07	1.8 ± 0.6			5

functional roles played by RGS proteins within these signaling systems.

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Correction to "Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G_i "

In the above article [Cladman W and Chidiac P (2002) *Mol Pharmacol* **62:**654–659], there were two errors.

In the first error, the equation under *Materials and Methods* was incorrect. It should have read:

$$y = ns + \max_1[x]/(K_{up} + [x]) + \max_2[x]/(K_{down} + [x])$$

In the second error, the legend to Fig. 5 was incorrect. The corrected text of the legend appears below:

Fig. 5. Concentration dependence of RGS2 and RGS4 GAP activities at high and low magnesium levels. Agonist-dependent GTPase activities were measured in the presence of 0.5 mM (A) and 7.5 mM (B) calculated free Mg^{2+} (total added $MgCl_2$ was 2 mM and 10 mM, respectively) in the absence of RGS protein (○) or at the RGS2 (▲) and RGS4 (■) concentrations indicated on the abscissae. Data were fitted to either eq. 1 (RGS4 in A) or the Hill equation (others), and average parameters from these and replicate data sets are presented in Table 1.

We regret these errors and apologize for any confusion or inconvenience they may have caused.