Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 6159–6163, June 1997
Biochemistry

Attenuation of G_i- and G_q-mediated signaling by expression of RGS4 or GAIP in mammalian cells

CHUNFA HUANG, JOHN R. HEPLER*, ALFRED G. GILMAN, AND SUSANNE M. MUMBY†

Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235

Contributed by Alfred G. Gilman, April 12, 1997

ABSTRACT **Protein regulators of G protein signaling (RGS proteins) were discovered as negative regulators of heterotrimeric G protein-mediated signal transduction in yeast and worms. Experiments with purified recombinant proteins *in vitro* have established that RGS proteins accelerate the GTPase activity of certain G protein α subunits (the reaction responsible for their deactivation); they can also act as effector antagonists. We demonstrate herein that either of two such RGS proteins, RGS4 or GAIP, attenuated signal transduction mediated by endogenous receptors, G proteins, and effectors when stably expressed as tagged proteins in transfected mammalian cells. The pattern of selectivity observed *in vivo* was similar to that seen *in vitro*. RGS4 and GAIP both attenuated G_i-mediated inhibition of cAMP synthesis. RGS4 was more effective than GAIP in blocking G_q-mediated activation of phospholipase Cβ.**

A family of guanine nucleotide-binding regulatory proteins (G proteins) transduces signals across the plasma membrane by sequential interactions with cell surface receptors and appropriate effectors (e.g., enzymes and ion channels; reviewed in ref. 1). These interactions are modulated by nucleotide-driven conformational changes in the α subunits of heterotrimeric G proteins. A ligand-bound receptor catalyzes the exchange of GDP for GTP on the α subunit of its cognate G protein, and the ensuing conformational change in the α subunit promotes its dissociation from the complex of β and γ subunits. These dissociated subunits are then competent to modulate the activity of effectors. The intrinsic GTPase activity of α serves as a molecular clock, returning the protein to the GDP-bound state and allowing reformation of the heterotrimer.

The intrinsic GTPase activities of α subunits measured *in vitro* cannot, in many cases, account for the rapid termination of signaling that occurs *in vivo*. The discovery of a novel family of regulators of G protein signaling (RGS proteins) has provided a model that can help explain this discrepancy because members of this family have recently been shown to accelerate the GTPase activity of certain α subunits (reviewed in ref. 2). Patterns of specificity between individual RGS proteins and subfamilies of G protein α subunits are only beginning to emerge. Among these, GAIP and RGS4 can serve as GTPase-activating proteins (GAPs) for the G_i and G_q but not the G_s or G_{12} subfamilies of α subunits (3–7). To date, such studies have largely been limited to proteins produced and purified from *Escherichia coli* and assayed *in vitro*. These proteins may lack relevant covalent modifications or they may (at high concentrations) display properties that are irrelevant for intact cells. We sought to determine if stable expression of GAIP or RGS4 in HEK293 cells would negatively regulate their endogenous signaling pathways in ways that would be anticipated from the GAP activities of these two RGS proteins *in vitro*.

MATERIALS AND METHODS

Construction of Plasmids. Methods for construction of plasmids have been described (8). PCR was used to generate a new NH_2 terminus for RGS4 with a Myc tag appended for detection of the expressed protein with a commercially available antibody. The sense PCR primer began with an *Nco*I site, followed by codons encoding a Myc epitope tag (underlined), a glycine residue, and the NH_2 terminus of RGS4: 5′-ACATGCCATGGAACAGAAGCTGATCTCCGAAGAGGACCTCAACGGCATGTGCAAAGGACTCGCTGGTCTGCCG-3′. The antisense PCR primer was 5′-GGAAAACCTGATTAACCATGAATGTGGACTGGCA-3′ and contained the unique *Bst*XI site present in RGS4. The PCR product was ligated into the *Nco*I and *Bst*XI sites of pQE60RGS4 (3). The entire coding region of MycRGS4 was then subcloned into the *Eco*RI and *Bam*HI sites in the mammalian expression vector $pCB6^+$ (9). The same restriction enzymes were employed to subclone NH_6GAIP, encoding full-length GAIP with a hexa-histidine tag at its NH_2 terminus, from the pQE60 plasmid (3) into $pCB6^+$. In this vector, the transcription of RGS4 or GAIP cDNA was directed by the cytomegalovirus promoter, whereas a neomycin-resistance gene is under the control of the simian virus 40 early-region promoter.

Stable Transfection of MycRGS4 and NH_6GAIP. cDNA (1 μg/ml; MycRGS4p$CB6^+$ or NH_6GAIPp$CB6^+$) was used for stable transfection. Cultured 293 cells (about 90% confluent) in 6-well plates were transfected with 1 ml of DNA–Lipofectamine mixture per well according to manufacturer's instructions (GIBCO/BRL). After 24 hr, the cells from an individual well were placed in a 150-mm dish and cultured in medium containing 200 μg/ml of G418, which was changed every 3–4 days. G418-resistant clones were isolated after 3–4 weeks and screened by immunoblotting. Clones, including A1 (transfected with $pCB6^+$), A16 (transfected with NH_6GAIPp$CB6^+$), and B12 (transfected with MycRGS4p$CB6^+$), were used for the experiments shown. Results obtained with additional clones are mentioned in the figure legends.

Detection of MycRGS4 and NH_6GAIP Expression. SDS/PAGE sample buffer was added directly to cells grown in 6-well plates. The samples were boiled for 5 min and subjected to SDS/PAGE and Western immunoblotting; relevant proteins were detected by enhanced chemiluminescence (Amersham). The anti-c-Myc monoclonal antibody (9E10), reactive with the Myc tag of RGS4, was purchased from Santa Cruz Biotechnology. The polyclonal antiserum against GAIP (R381) was made in rabbits against a synthetic peptide, YRALLLQGPSQSSSEA, that corresponds to C-terminal amino acid sequence—residues 202–217 of GAIP. The amount of NH_6GAIP in homogenates of transfected cells was estimated by Western immunoblotting by comparison with ho-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RGS, regulators of G protein signaling; GAP, GTPase-activating protein.

*Present address: Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.
†To whom reprint requests should be addressed.

mogenates of control cells to which known amounts of purified NH_6GAIP (3) had been added as standards.

Assay of GAP Activity. Cloned cells were homogenized in buffer containing 50 mM Na-Hepes (pH 8.0), 5 mM EDTA, 2 mM DTT, and protease inhibitors (10 μg/ml leupeptin and lima bean trypsin inhibitor, and 16 μg/ml phenylmethylsulfonyl fluoride, tosyl-lysine-chloromethyl ketone, and tosyl-phenylalanine-chloromethyl ketone). Protein content was determined with a Bio-Rad reagent, with use of BSA as a standard (10). Recombinant myristoylated α_{i1} (220 nM, purified from *E. coli*; ref. 11) was loaded with radioactive GTP by incubation with 1 μM [γ-^{32}P]GTP, 50 mM Na-Hepes (pH 8.0), and 5 mM EDTA at 20°C for 20 min. GAP activity was determined by incubation of 1 μg of homogenate protein with 205 nM [γ-^{32}P]GTP-α_{i1} as substrate at 4°C for 3 min (3). The amount of MycRGS4 in homogenates of transfected cells was estimated by measurement of GAP activity and comparison with homogenates of control cells to which known amounts of purified NH_6RGS4 (3) had been added as standards.

Measurement of cAMP Accumulation in Intact Cells. Nearly confluent cells were labeled with 2 μCi/ml [^{3}H]adenine (26.3 Ci/mmol; DuPont/NEN; 1 Ci = 37 GBq) in DMEM containing 5% fetal bovine serum for 12–18 hr. The cultures were rinsed with isotope- and serum-free DMEM containing 20 mM Na-Hepes (pH 7.4), equilibrated for 30 min in the same medium, and then further incubated in the presence or absence of agonists for 15 min in the same medium containing 1 mM isobutylmethylxanthine. For pertussis toxin treatment, control cells, labeled with [^{3}H]adenine as above, were incubated with or without 100 ng/ml pertussis toxin (List Biological Laboratories, Campbell, CA) for 4 hr (without radioactivity in the medium), and then treated with no β-adrenergic agonist as a control or isoproterenol in the presence or absence of somatostatin for 15 min. Tritiated nucleotides were extracted and separated on Dowex and alumina columns as described (12). Results are reported as radioactivity in cAMP divided by total radioactivity in noncyclic adenine nucleotides.

Measurement of Inositol Trisphosphate in Intact Cloned Cells. Cells (about 75% confluent) in 12-well or 6-well plates were labeled with 5 μCi/ml *myo*-[2-^{3}H]inositol (22.3 Ci/mmol; DuPont/NEN) in 0.5 ml (for 12-well) or 1.0 ml (for 6-well) of inositol-free DMEM containing 10% fetal calf serum for 48 hr. After equilibration for 30 min in inositol-free DMEM containing 20 mM Na-Hepes (pH 7.4) and 20 mM LiCl, cells were incubated in 0.4 ml of equilibration medium with or without agonist as indicated in the figure legends. In some experiments, control cells labeled with [^{3}H]inositol were incubated with or without 100 ng/ml of pertussis toxin for 4 hr and then treated with or without 100 nM bradykinin for 30 sec. After treatment with agonist, 0.4 ml of 10% perchloric acid was added to wells and stored at 4°C for 3–5 hr. The acidified samples were transferred to centrifuge tubes, neutralized by addition of $\approx$350 μl of 2 M KOH with 1 mM EDTA, and centrifuged (12,000 $\times$ *g* for 3 min). The supernatant fractions (0.8 ml) were applied to AG1-X8 anion exchange columns (200–400 mesh, formate form; Bio-Rad). The separation of inositol-containing compounds was achieved essentially as described (13). Elution was with 10 ml of H_2O, 8 ml of 50 mM ammonium formate, and 6 ml each of 0.4 M ammonium formate/0.1 M formic acid and 1.2 M ammonium formate/0.1 M formic acid.

RESULTS

Expression of RGS Proteins in Mammalian Cells. Expression of NH_6GAIP or MycRGS4 in stably transfected 293 cells was detected by Western immunoblotting with antibodies to the C terminus of GAIP (R381) or to Myc, respectively (Fig. 1*A*). In an effort to demonstrate functional expression of the RGS proteins, homogenates of three cell lines were assayed for their ability to stimulate the GTPase activity of exogenously added α_{i1} with [γ-^{32}P]GTP bound to it. Homogenates of the vector control cells had no discernible effect on the GTPase activity of α_i, whereas homogenates from cells expressing NH_6GAIP or MycRGS4 stimulated this activity (Fig. 1*B*). These two lines of evidence demonstrate stable expression of the RGS proteins in the cloned 293 cell lines. The level of expression of NH_6GAIP or MycRGS4 was estimated to be approximately 1 ng of RGS protein per microgram of cellular protein.

Effect of RGS Protein Expression on Hormone-Regulated cAMP Accumulation in Cells. The G protein termed G_s couples stimulatory receptors, such as the β-adrenergic receptor, to adenylyl cyclase. The somatostatin receptor, an example of an inhibitory receptor, is coupled to the enzyme by members of the G_i subfamily. It has been demonstrated that GAIP or RGS4, tested *in vitro* with purified proteins, stimulates the GTPase activity of α_i but not that of α_s (3). As anticipated from these results *in vitro*, treatment of whole cells with the β-adrenergic agonist isoproterenol caused a dose-dependent increase in cAMP accumulation in 293 cells whether or not they express NH_6GAIP or MycRGS4 (Fig. 2*A*). The results of five experiments indicate that neither of these two RGS proteins have a significant capacity to regulate isoproterenol-stimulated

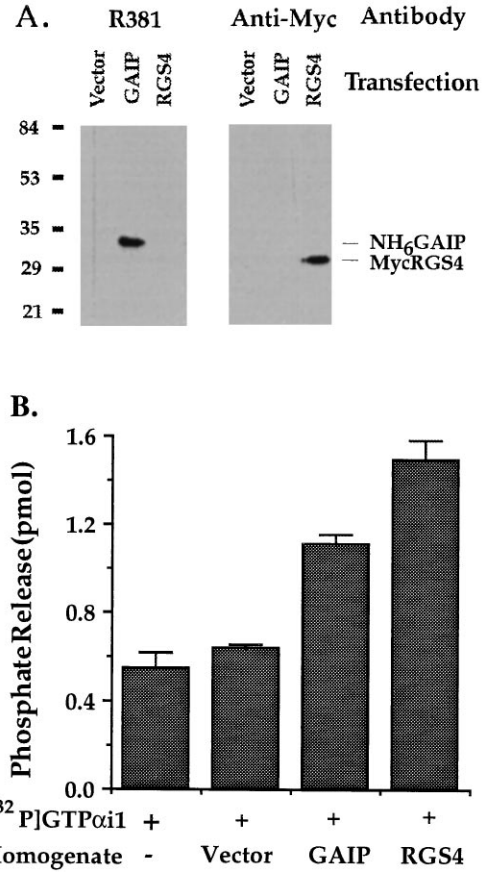

FIG. 1. Expression of RGS proteins in stably transfected 293 cell clones. (*A*) Whole-cell lysates of transfected cells were analyzed by Western immunoblotting. Cells were transfected with empty expression vector or vector including NH_6GAIP or MycRGS4. Expression of NH_6GAIP was detected with a GAIP-reactive antiserum (R381, *Left*) and MycRGS4 with the Myc-reactive antibody (Anti-Myc, *Right*). Numbers at the left represent the migration position of prestained molecular mass standards in kilodaltons. (*B*) Homogenates of cells transfected with the empty vector or with vector encoding NH_6GAIP or MycRGS4 were assayed for GAP activity with [γ-^{32}P]GTP bound to purified α_{i1} as substrate. The bars represent the mean $\pm$ the SD of samples assayed in triplicate. Three independent clones of cells expressing NH_6GAIP or MycRGS4 gave qualitatively similar results.

cAMP accumulation *in vivo* (although in the experiment shown in Fig. 2 there is slightly less cAMP produced in the NH_6GAIP-expressing cells). In contrast, somatostatin-mediated inhibition of cAMP production (induced by isoproterenol) was prevented by the expression of either NH_6GAIP or MycRGS4 (Fig. 2*B*). These data suggest that GAIP and RGS4 have the capacity to stimulate specifically the GTPase activity of α_i but not α_s *in vivo*, in a fashion that is similar to that established *in vitro* (3, 6). The balance between GTP- and GDP-bound α_i would thus be shifted toward the inactive state, resulting in loss of inhibition of adenylyl cyclase activity.

Effect of RGS Protein Expression on Hormone-Stimulated Production of Inositol Trisphosphate by Cells. Bradykinin stimulates production of inositol trisphosphate in a reaction that is catalyzed by phospholipase Cβ. The bradykinin receptor can be coupled to this enzyme by G_q (by the α subunit, α_q) and/or by G_i (by $\beta\gamma$), depending on the cell type. The bradykinin-stimulated increase in inositol trisphosphate synthesis was consistently attenuated in cells expressing MycRGS4 (Fig. 3). Marginal (yet significant) attenuation by NH_6GAIP was detected consistently only at lower concentrations of bradykinin. These data suggest that RGS4 is more effective than GAIP in stimulating the GTPase activity of the G protein that couples the bradykinin receptor to phospholipase C. Such selectivity of RGS4 over GAIP for regulating inositol trisphosphate production in 293 cells is consistent with *in vitro* data, indicating that RGS4 is about 10 times more effective than GAIP in experiments with α_q (6). To verify that the bradykinin receptor is coupled by G_q to inositol trisphosphate production in 293 cells, vector control cells were treated with pertussis toxin. This toxin selectively catalyzes the ADP ribosylation of members of the α_i (but not α_q) subfamily, thereby uncoupling them from their cognate receptors. Treatment of cells with pertussis toxin abolished the G_i-mediated inhibition of cAMP production by somatostatin, thus demonstrating that the toxin was effective (Fig. 4*A*). Toxin treatment did not, however, significantly alter bradykinin-stimulated synthesis of inositol trisphosphate (Fig. 4*B*). These results indicate that the bradykinin receptor in 293 cells is largely

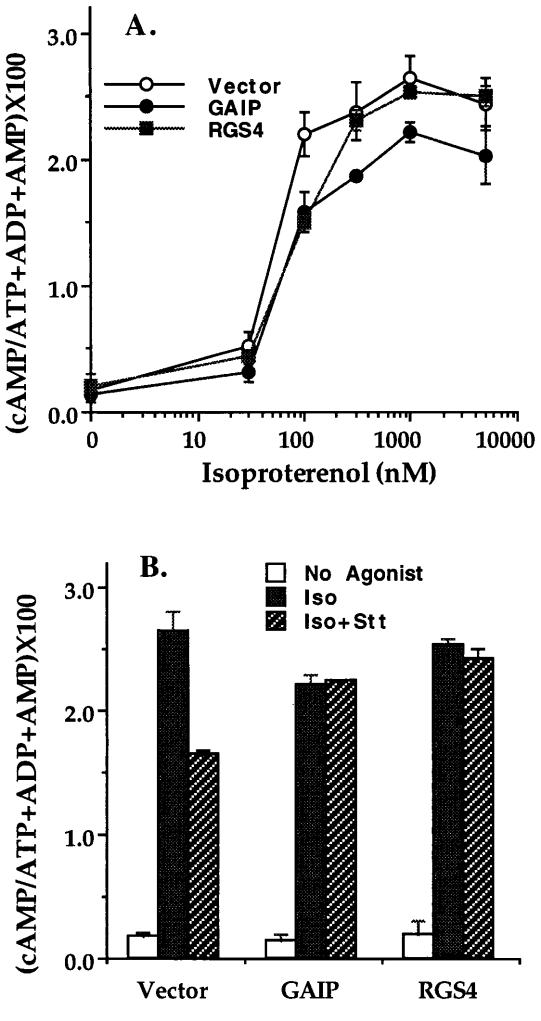

FIG. 2. Effects of isoproterenol and somatostatin on cAMP accumulation in transfected 293 cells. (*A*) Cells were treated for 15 min with varying concentrations of isoproterenol, and the intracellular cAMP concentration was determined. Accumulation of cAMP in response to isoproterenol did not differ significantly between control cells (Vector) and those expressing NH_6GAIP or MycRGS4. (*B*) Cells were treated with 5 μM isoproterenol (Iso) with or without 1 μM somatostatin (Stt) for 15 min. Somatostatin inhibits isoproterenol-stimulated cAMP accumulation in the control cells but is ineffective in cells expressing RGS4 or GAIP. The data points represent the mean, and the error bars represent the range of duplicate samples. Three independent experiments yielded similar results. Two independent clones of cells expressing NH_6GAIP or MycRGS4 gave qualitatively similar results.

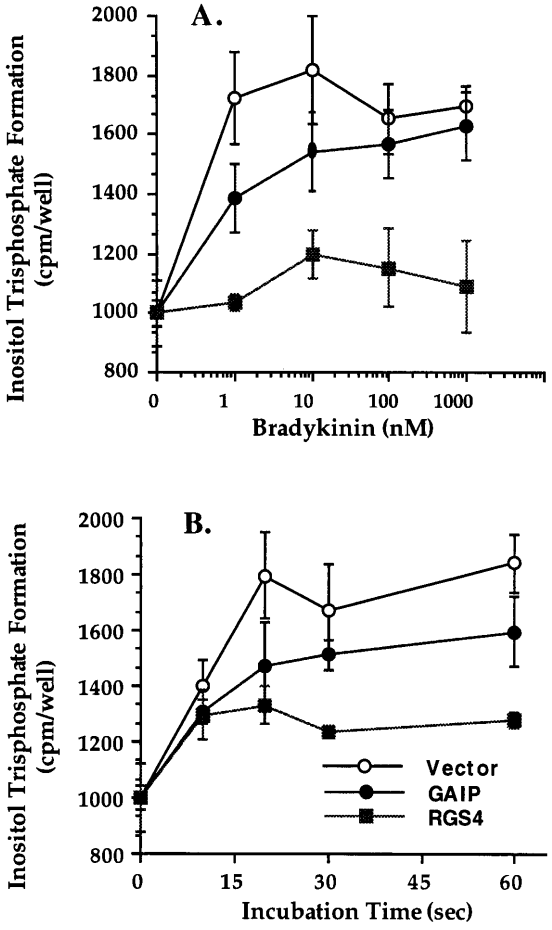

FIG. 3. Hormone-stimulated production of inositol trisphosphate in 293 cells expressing RGS proteins. (*A*) Inositol trisphosphate accumulation was determined after cells were treated for 15 sec with varying concentrations of bradykinin. Expression of MycRGS4 (■) markedly attenuated the bradykinin response at all concentrations of hormone tested relative to control cells (○); the effect of NH_6GAIP (●) was apparent only at lower concentrations. (*B*) Time course of inositol trisphosphate production by cells treated with 100 nM bradykinin (○, control cells) reveals a more prominent inhibition of the response in cells expressing MycRGS4 (■) than in those expressing NH_6GAIP (●). The data represent the mean of triplicates ± SD. Three independent experiments yielded similar results.

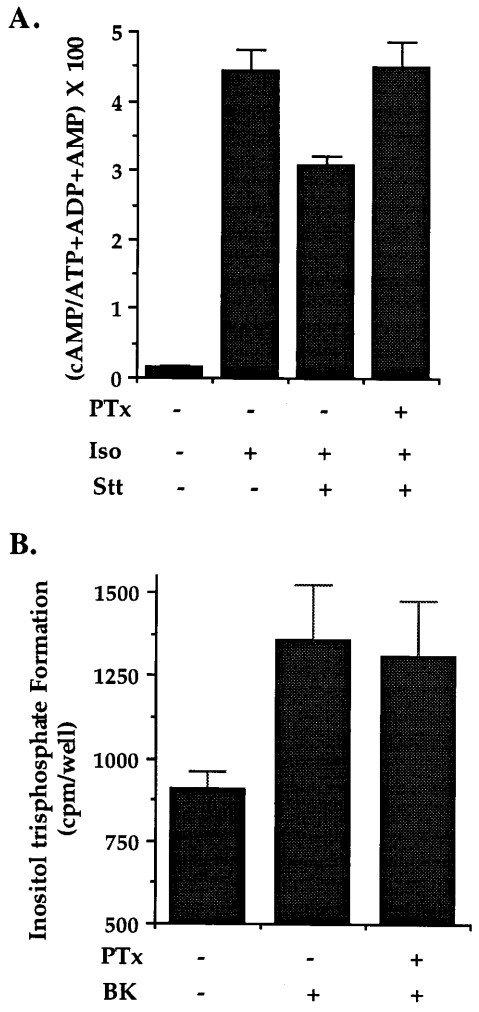

FIG. 4. Effects of pertussis toxin on hormone-regulated cAMP or inositol trisphosphate accumulation in 293 cells. Cells transfected with empty vector were incubated with the appropriate radioactive precursor for 24 or 48 hr and treated with 100 ng/ml pertussis toxin (PTx) for 4 hr and then with the indicated hormone(s) before the cells were lysed. (*A*) Cells were treated with 5 μM isoproterenol (Iso) ± 1 μM somatostatin (Stt) for 15 min. Pertussis toxin prevented the somatostatin-induced inhibition of cAMP accumulation. The bars represent the mean ± SD of five determinations made in two independent experiments. (*B*) Pertussis toxin had no effect on the ability of bradykinin (BK, 100 nM) to stimulate inositol trisphosphate synthesis in cells treated with the hormone for 30 sec. The bars represent the mean ± SD of seven determinations made in two independent experiments.

coupled to phospholipase Cβ by a pertussis toxin-insensitive G protein, almost certainly a G_q family member.

Lack of Effect of RGS Protein Expression on Endogenous Levels of Receptors and G Proteins. We addressed the possibility that expression of NH_6GAIP or MycRGS4 might indirectly affect signal transduction by changing the levels of expression of endogenous receptors or G proteins. Crude membranes prepared from the vector control and RGS protein-expressing cell lines were assayed for G protein content by Western immunoblotting and receptor content by radioligand binding (data not shown). Membranes from NH_6GAIP- and MycRGS4-expressing and control cells displayed similar concentrations of α_q (detected by antiserum W082; ref. 14), α_{i1} and α_{i2} (detected by antiserum B087; ref. 15), and β (detected by the common β antiserum B600; ref. 15). These results suggest that stable and constitutive expression of the RGS proteins did not grossly alter the expression of the endogenous G protein α subunits thought to participate in production of the second messengers that we examined.

Similarly, no statistically significant differences were detected in the specific binding of ^{125}I-labeled [Tyr^{11}]somatostatin to membranes from NH_6GAIP, MycRGS4, or control cells. Binding of biotinylated bradykinin to membranes was assayed indirectly with use of ^{125}I-labeled streptavidin. The specific binding of ^{125}I-streptavidin did not vary significantly between the three cell lines (data not shown). Thus, the concentrations and affinities of the endogenous somatostatin and bradykinin receptors appeared unaltered by the stable and constitutive expression of either tagged RGS4 or GAIP in 293 cells.

DISCUSSION

RGS proteins were discovered as negative regulators of signal transduction in yeast and worms (2), with actions likely exerted at the level of the receptor, the G protein α subunit, or their interaction. *In vitro* experiments with purified recombinant proteins established that RGS proteins have the capacity to accelerate the GTPase activity of certain G protein α subunits and, furthermore, to block the interaction with at least one G_α protein and its effector (2, 7). We find here that either of two representative RGS proteins, GAIP or RGS4, when expressed in a mammalian cell line, attenuates signal transduction mediated by specific endogenous receptors, G proteins, and effectors.

In vitro experiments with GAIP and RGS4 indicated that these two proteins have the capacity to stimulate the GTPase activity of members of the G_i and G_q (but not the G_s or G_{12}) classes of α subunits (3–6). This rather broad selectivity of RGS for G proteins was reflected in the transfected mammalian cells. We presume that expression of GAIP or RGS4 prevented somatostatin-induced inhibition of cAMP accumulation because somatostatin receptors are coupled to adenylyl cyclase by members of the G_i subfamily of α subunits. In contrast (and as anticipated), neither of the two RGS proteins altered the accumulation of cAMP stimulated by isoproterenol, which is mediated by G_s. Expression of RGS4 markedly suppressed the G_q-mediated production of inositol trisphosphate in cells, but the effect of GAIP was marginal. This difference between the two RGS proteins, expressed *in vivo*, may reflect the 10-fold reduced effectiveness of GAIP relative to RGS4 that we observed *in vitro* for interaction with α_q (6). DeVries *et al.* (16) found an interaction between GAIP and α_i in a yeast two-hybrid assay but failed to detect interaction with α_q. Taken together, the data suggest that regulation of the G_q pathway by GAIP, while possible, is unlikely to be the primary function of this particular RGS protein *in vivo*.

Our test for function *in vivo* involved the introduction of a single RGS protein, by stable expression, to the endogenous signaling systems of a mammalian cell line. Other tests in mammalian cells have required the overexpression of not only an RGS protein but also an upstream (receptor) or downstream (kinase) signaling component. The activity of extracellular signal-regulated kinase, which can be stimulated by platelet activating factor by a G protein, was attenuated by RGS1 (introduced by cotransfection of cells with cDNAs for the RGS protein and the kinase) (17). In another study, Neill *et al.* (18) cotransfected COS cells with cDNAs for the gonadotropin-releasing hormone (GnRH) receptor and RGS1, RGS2, RGS3, or RGS4 (18). In contrast to the capacity of RGS4 to inhibit α_q-mediated signaling demonstrated herein, Neill *et al.* (18) found that GnRH-induced increases in inositol trisphosphate were suppressed only in cells transfected with the receptor and RGS3. One explanation for the lack of effect for RGS4 in the GnRH study may be an inadequate level of expression of RGS4. Western and Northern blot data were shown to verify the expression of RGS3 but not the other three

RGS proteins (18). Based on the results reported by Neill *et al.* (18) and our own, it appears likely that both RGS3 and RGS4 have the capacity to regulate negatively G_q-mediated pathways *in vivo*. The broad selectivity of RGS proteins for G proteins that has been reported thus far will be further refined as more is learned about the endogenous expression of these proteins within cells. We envision that cell-type-specific and temporal expression and subcellular localization of endogenous RGS proteins may dictate which G proteins they encounter. Negative regulation of particular signal transduction pathways will be thus modulated in response to altered physiological conditions.

We thank David Berman, Carmen Dessauer, Richard Whisnant, and Tohru Kozasa for reagents, helpful advice, and suggestions and Linda Hannigan for technical assistance. This work was supported by grants from the National Institute of General Medical Sciences, the National Institutes of Health to S.M.M. (GM50515) and A.G.G. (GM34497), and the American Heart Association Texas Affiliate to J.R.H. (94G-112). A.G.G. also acknowledges support from the Raymond and Ellen Willie Distinguished Chair in Molecular Neuropharmacology.

1. Hepler, J. R. & Gilman, A. G. (1992) *Trends Biochem. Sci.* **17,** 383–387.
2. Dohlman, H. G. & Thorner, J. (1997) *J. Biol. Chem.* **272,** 3871–3874.
3. Berman, D. M., Wilkie, T. M. & Gilman, A. G. (1996) *Cell* **86,** 445–452.
4. Berman, D. M., Kozasa, T. & Gilman, A. G. (1996) *J. Biol. Chem.* **271,** 27209–27212.
5. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H. & Blumer, K. J. (1996) *Nature (London)* **383,** 172–175.
6. Hepler, J. R., Berman, D. M., Gilman, A. G. & Kozasa, T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 428–432.
7. Hunt, T. W., Fields, T. A., Casey, P. J. & Peralta, E. G. (1996) *Nature (London)* **383,** 175–177.
8. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
9. Brewer, C. B. (1994) *Methods Cell Biol.* **43,** 233–245.
10. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
11. Linder, M. E. & Mumby, S. M. (1994) *Methods Enzymol.* **237,** 254–268.
12. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548.
13. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212,** 473–482.
14. Gutowski, S., Smrcka, A. V., Nowak, L., Wu, D., Simon, M. I. & Sternweis, P. C. (1991) *J. Biol. Chem.* **266,** 20519–20524.
15. Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G. & Mumby, S. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3675–3679.
16. De Vries, L., Mousli, M., Wurmser, A. & Farquhar, M. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11916–11920.
17. Druey, K. M., Blumer, K. J., Kang, V. H. & Kehrl, J. H. (1996) *Nature (London)* **379,** 742–746.
18. Neill, J. D., Duck, L. W., Sellers, J. C., Musgrove, L. C., Scheschonka, A., Druey, K. M. & Kehrl, J. H. (1997) *Endocrinology* **137,** 843–846.