RGS4 Inhibits G-Protein Signaling in Cardiomyocytes

Praveen Tamirisa, MD; Kendall J. Blumer, PhD; Anthony J. Muslin, MD

**Background**—RGS family members are GTPase-activating proteins for heterotrimeric G_q and G_i proteins. RGS genes are expressed in heart tissue and in cultured cardiomyocytes. There is evidence that altered RGS gene expression may contribute to the pathogenesis of cardiac hypertrophy and failure.

**Methods and Results**—We investigated the ability of RGS proteins to block G-protein signaling in vivo by using a cultured cardiomyocyte transfection system. Endothelin-1, angiotensin II, and phenylephrine signal through G_q or G_i family members and promote the hypertrophy of cardiomyocytes. We found that phenylephrine-mediated and endothelin-1–mediated induction of the atrial natriuretic factor and myosin light chain-2 genes was inhibited in cells that were transfected with RGS4. Phenylephrine-mediated gene induction was not inhibited in cells that were transfected with N128A-RGS4, a point mutant form that lacks GTPase-activating protein activity. Phenylephrine-mediated myofilament organization and cell growth were also blocked in cells by RGS4.

**Conclusions**—These results demonstrate that RGS protein can inhibit G-protein–mediated signaling in vivo and suggest that increased expression of RGS protein may be a counterregulatory mechanism to inhibit G protein signaling.

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**Key Words:** hypertrophy ■ genes ■ growth substances ■ atrial natriuretic factor ■ proteins

Postnatal mammalian cardiomyocytes respond to a variety of stimuli, including mechanical stress, growth factor and hormonal action, and metabolic abnormalities, by increasing their cell size.1–3 Postnatal cardiomyocytes have the capacity to hypertrophy but are unable to proliferate for reasons that are not understood. Cardiomyocyte hypertrophy leads to growth of the entire heart, resulting in thickening of the ventricular chambers. Cardiac hypertrophy is an independent risk factor for the development of serious cardiac arrhythmias4,5 and is associated with diastolic dysfunction that can result in congestive heart failure.6,7

In cultured cardiomyocytes, mechanical stress and ligands such as phenylephrine,8,9 endothelin-1,10 angiotensin II,11 and basic fibroblast growth factor (bFGF)12 promote a hypertrophic response. This response is characterized by an increase in cell size, protein synthesis, and organization of contractile proteins into sarcomeres and by an induction of specific genes.13 Genes that are induced include atrial natriuretic factor (ANF),14 the immediate early proto-oncogene c-fos,15 and myosin light chain-2 (MLC-2).16 There is evidence that the action of these ligands on cultured cells mimics cardiac hypertrophy in whole animals. For example, in aortic-banded rats, treatment with an angiotensin-converting enzyme inhibitor at a dosage that does not reduce blood pressure causes regression of cardiac hypertrophy.17 Treatment of banded rats with the vasodilator hydralazine does not result in regression of hypertrophy.

Three agonists that cause cultured cardiomyocytes to hypertrophy signal through heterotrimeric G proteins. Endothelin-118 and angiotensin II19 bind to 7 transmembrane receptors that are coupled to G_q proteins, whereas phenylephrine binds to α1-adrenergic receptors that are coupled to G_i and G_q proteins.20 It is interesting to note that mechanical stress may lead to the local release of angiotensin II or endothelin-1 in the heart.21 FGF does not signal through heterotrimeric G proteins but instead activates a signaling cascade that includes the proteins FRS2, Grb2, SOS, and the small G protein ras.22

A family of mammalian signaling molecules was recently identified and termed RGS (for regulators of G-protein signaling), based on homology to the budding yeast protein Sst2.23 Genetic studies in yeast revealed that Sst2 negatively regulates signaling by heterotrimeric G proteins. Biochemical studies performed in vitro with purified proteins have demonstrated that RGS proteins have GTPase-activating protein (GAP) activity toward α-subunits of heterotrimeric G proteins of the G_i and G_q families.24–27 RGS proteins promote the rapid deactivation of G proteins by binding to and stabilizing the transition state of α-subunits as they hydrolyze GTP. The biological role of RGS proteins in mammalian tissues is largely unknown.

We have recently demonstrated that the RGS3 and RGS4 genes are expressed in heart.28 We also found that RGS gene expression is enhanced in hypertrophied cultured cardiomyocytes and in cardiac tissues from pulmonary artery–banded mice.28 These experiments suggest that RGS function may be
regulated at the transcriptional level; however, they do not establish whether increased RGS expression promotes, inhibits, or has no effect on the hypertrophic growth program.

We hypothesized that RGS family members are intrinsic cardiac proteins that block hypertrophy by inhibiting signal transduction stimulated by ligands such as phenylephrine and endothelin-1. To test this hypothesis, we overexpressed RGS4 in neonatal rat cardiomyocytes that were subsequently stimulated with hypertrophic ligand. Our results demonstrate that RGS4 can inhibit the action of phenylephrine and endothelin-1 but not bFGF in cultured cardiomyocytes.

**Methods**

**Reagents**

Collagenase was obtained from Wako Pure Chemicals, Inc. Dulbecco’s modified Eagle medium and other tissue culture material was from Life Technologies, Inc. The murine monoclonal anti-myc-1-epitope antibody was obtained from Babco. The rabbit polyclonal anti-RGS4 antibody was generated as previously described. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Phenylephrine, endothelin-1, FGF, human apotransferrin, insulin, bovine serum albumin, and phorbol 12-myristate 13-acetate (TPA) were obtained from Sigma. Tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin was obtained from Molecular Probes, Inc. β-Galactosidase assays were performed with the Galactolight Kit (Promega). Cell lysates were subjected to 1 round of freeze-thawing. Supernatants were used in β-galactosidase assays (Tropix) and luciferase assays (Analytical Luminescence Laboratory) according to the manufacturer’s instructions. All activities were measured in duplicate with a monolight 401 luminometer (Analytical Luminescence Laboratory). Luciferase activity was normalized by β-galactosidase activity for each sample in all experiments to correct for any variation in cardiomyocyte transfection efficiency. Results are expressed as the percent of maximal induction in control versus agonist-treated cells. Results are presented as mean±SE for experiments on 3 separate preparations of cardiomyocytes.

**Expression and Reporter Plasmids**

The open reading frame of rat RGS4 or a point mutant form of RGS4 (N128A) linked in-frame to the myc-1 epitope was subcloned into the pCR3 vector that contains a strong cytomegalovirus (CMV) promoter and is designed for high-level expression in mammalian cells. These vectors have been named pCMV-RGS4-myc and pCMV-N128A-RGS4-myc. The luciferase reporter construct pANF(-638)LΔ9 was kindly provided by Dr K.R. Chien (Department of Medicine, University of California, San Diego). The luciferase reporter construct pMRC-L250(LΔ9) was a gift from Dr A. Thorburn (University of Utah, Salt Lake City). To control for cardiomyocyte transfection efficiency, pON249, a β-galactosidase expression vector under control of the human cytomegalovirus promoter, was used as previously described.

**Myocardial Cell Cultures**

Cultured rat neonatal cardiomyocytes were prepared as described. Briefly, ventricles were obtained from 1- to 2-day-old Sprague-Dawley pups, and cardiomyocytes were isolated by digestion with collagenase (Wako). Cardiomyocytes were separated from nonmyocytes by differential plating. The estimated purity of cardiomyocytes after differential plating was 90% to 95%. Purified cardiomyocytes were plated on collagen-coated dishes at low density (200 cells/mm²) in primary myocyte medium containing Dulbecco’s modified Eagle medium supplemented with 10% donor horse serum, 5% fetal calf serum, glutamine, 0.1 mM L-2-deoxyuridine (BrDU) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide. Twelve hours later, the cells were washed twice with Hanks’ balanced salt solution (without calcium, magnesium, or phenol red) and resuspended in serum-containing medium. Twenty-four hours after plating, the cells were washed and resuspended in serum-free medium (Dulbecco’s modified Eagle medium supplemented with insulin, apotransferrin, bovine serum albumin, BrDU, and antibiotics as above). To induce hypertrophy, phenylephrine (100 μM), endothelin-1 (10 mM), or bFGF (25 ng/mL) was added to the maintenance medium and cells were incubated for ~40 hours.

**Transient Transfection of Cultured Neonatal Rat Ventricular Myocytes**

Ventricular myocytes were plated as described above. Immediately after plating, transient transfection was carried out with the synthetic liposomal agent DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methyl sulfate) (Boehringer Mannheim) according to the manufacturer’s instructions. Briefly, plasmid DNA in 20 mM Hepes was incubated for 15 minutes at room temperature with DOTAP. The reaction mixture was then slowly added to the primary myocyte medium and incubated overnight. For each transfection, 1.5 μg of reporter plasmid, 1.5 μg of β-galactosidase plasmid, and/or 0 to 15 μg of pCMV-RGS4-myc plasmid was used.

**Luciferase and β-Galactosidase Assays**

After agonist stimulation, cells were washed twice with PBS (without added calcium or magnesium) and were lysed with 400 μL of reporter lysis buffer per 3.8 cm² well (Promega). Cell lysates were subjected to 1 round of freeze-thawing. Supernatants were used in β-galactosidase assays (Tropix) and luciferase assays (Analytical Luminescence Laboratory) according to the manufacturer’s instructions. All activities were measured in duplicate with a monolight 401 luminometer (Analytical Luminescence Laboratory). Luciferase activity was normalized by β-galactosidase activity for each sample in all experiments to correct for any variation in cardiomyocyte transfection efficiency. Results are expressed as the percent of maximal induction in control versus agonist-treated cells. Results are presented as mean±SE for experiments on 3 separate preparations of cardiomyocytes.

**Immunofluorescence Microscopy**

Cardiomyocytes were cultured on collagen-precoated, plastic, 2-well chamber slides (Labtek). Cells were washed twice with PBS and fixed in 3% paraformaldehyde. After fixation, cells were permeabilized with 1% Igepal CA-630 (Sigma) in PBS for 5 minutes at room temperature and nonspecific binding sites were blocked with 10% horse serum in 1% Igepal CA-630 in PBS for 10 minutes at room temperature. Primary antibodies were diluted 1:200 in PBS-glycine (10 mM) and incubated at room temperature for 1 hour. Secondary FITC-conjugated or TRITC-conjugated antibodies (1:200 dilution) were used for immunofluorescence and incubated for 30 minutes at room temperature. TRITC-labeled phalloidin was diluted 1:50 in PBS-glycine before use. Cells were washed 3 times with PBS-glycine (10 mM) after each antibody incubation step and were visualized by immunofluorescence microscopy.

**Figure 1.** Subcellular localization of native and transfected RGS4 in rat neonatal cardiomyocytes by use of immunofluorescence microscopy. A, Background fluorescence of cardiomyocytes probed with secondary FITC-conjugated antibody only. Cultured untransfected cardiomyocytes were fixed, permeabilized, and probed with FITC-conjugated anti-rabbit IgG secondary antibody. B, Localization of native RGS4 to the plasma membrane and cytoplasm. Cultured untransfected cardiomyocytes were fixed, permeabilized, and probed with rabbit polyclonal anti-RGS4 antibody, followed by FITC-conjugated anti-rabbit IgG secondary antibody. C, Localization of transfected RGS4-myc to the cytoplasm and plasma membrane of cardiomyocytes. Cultured transfected cardiomyocytes were fixed, permeabilized, and probed with mouse monoclonal anti-myc-1-epitope antibody (Babco), followed by FITC-conjugated antimouse IgG secondary antibody.
Analysis of RGS4\textit{-myc} Expression Levels

To determine the level of RGS4\textit{-myc} expression achieved by transfection with plasmid DNA, cardiomyocytes were transfected with 1.5 \( \mu \text{g} \) of \( \beta \)-galactosidase plasmid and 1.5 \( \mu \text{g} \) of pCMV-RGS4\textit{-myc} and were incubated for \( \sim 40 \) hours. Double-immunofluorescence microscopy was performed by use of anti-\( \beta \)-galactosidase primary antibody to identify transfected cells and by use of anti-RGS4 polyclonal primary antibody to determine RGS4 protein levels in transfected versus nontransfected cells. Digitized immunofluorescent photomicrographs of cells were downloaded onto a Power Macintosh computer and analyzed with NIH Image software. The mean signal intensity of 10 to 15 transfected to nontransfected cells was compared in 2 separate experiments.

Statistical Analysis

Analyses between groups were performed with the use of ANOVA, with the post hoc Scheffé's test, with a value of \( P<0.05 \) considered significantly different.

Results

Subcellular Localization of Native and Transfected RGS4 Proteins in Neonatal Cardiomyocytes

Previous work has demonstrated that heterotrimeric G-protein subunits are primarily localized to the plasma membrane and the cytoplasmic compartments of cultured cells.\textsuperscript{30} To determine the subcellular localization of native RGS4 protein, untransfected cardiomyocytes were probed with purified polyclonal anti-RGS4 antibody followed by secondary antibody (FITC-coupled anti-rabbit immunoglobulin) or with secondary antibody alone (control). Immunofluorescence microscopy revealed that native RGS4 protein was localized to the cytoplasm and plasma membrane (Figure 1, A and B).

To determine the subcellular localization of transfected RGS4, the cDNA encoding RGS4 was linked in-frame to the...
myc-1 epitope and inserted into a mammalian expression vector (pCMV-RGS4-myc). Cardiomyocytes were transfected with this construct, and immunofluorescence microscopy was performed that revealed that RGS4-myc protein was present chiefly in the cytoplasm, with some found in the plasma membrane (Figure 1C).

The level of RGS4 overexpression achieved by transfection with pCMV-RGS4-myc was determined by densitometric analysis of immunofluorescence microscopy images. The signal intensity obtained with anti-RGS4 antibody was compared in cells that were immunoreactive to anti-β-galactosidase antibody (transfected cells) and those that were not immunoreactive to anti-β-galactosidase antibody (non-transfected cells) and revealed that transfection with 1.5 μg pCMV-RGS4-myc resulted in a 2.5- to 3.2-fold increase in RGS4 protein levels (see “Methods”).

**Gene Induction Experiments**

To determine whether RGS4 overexpression could inhibit signaling by growth-promoting ligands, pCMV-RGS4-myc (or empty vector) was triple-transfected with a CMV promoter-β-galactosidase vector and a promoter-reporter construct such as pANF-luc that has ANF promoter sequence linked to cDNA encoding firefly luciferase. After cells were stimulated with ligand, luciferase activity was measured and normalized by β-galactosidase activity (to account for variability in transfection efficiency).

ANF was examined first because ligand stimulation of cardiomyocytes results in robust induction of ANF gene expression. In control cardiomyocytes that were transfected with a CMV promoter-β-galactosidase vector and pANF-luc but not with pCMV-RGS4-myc, stimulation with the α-adrenergic ligand phenylephrine resulted in an 8- to 15-fold induction of ANF gene expression. Phenylephrine-induced ANF gene expression was inhibited in cells that were transfected with 1.5 μg or more pCMV-RGS4-myc plasmid (Figure 2A).

To determine whether the effect of RGS4-myc was specific to its ability to deactivate G proteins, we transfected cardiomyocytes with a construct encoding a point mutant form of RGS4, N128A, which lacks GAP activity when tested in vitro. Phenylephrine-induced ANF gene expression was not reduced in N128A-RGS4-myc cells compared with that in control cells (Figure 2B).

In control cardiomyocytes that were transfected with a CMV promoter-β-galactosidase vector and pANF-luc but not with pCMV-RGS4-myc, stimulation with a second ligand that signals through heterotrimeric G proteins, endothelin-1, resulted in a 5- to 10-fold induction of ANF gene expression. Endothelin-1–induced ANF expression was inhibited in cells that were transfected with 1.5 μg pCMV-RGS4-myc. ANF gene induction as measured by luciferase activity and normalized by β-galactosidase activity was reduced by 60% in RGS4-myc cells (Figure 2C).

To control for nonspecific inhibitory effects of RGS4 overexpression, cardiomyocytes were also stimulated with bFGF. In contrast to phenylephrine and endothelin-1, bFGF does not signal through heterotrimeric G proteins but instead activates intracellular signaling pathways, such as the MAP kinase cascade, using the intrinsic tyrosine kinase activity of the bFGF receptor. In control cardiomyocytes that were transfected with a CMV promoter-β-galactosidase vector and pANF-luc but not with pCMV-RGS4-myc, stimulation with bFGF resulted in a 3- to 4-fold induction of ANF gene expression. As predicted, bFGF-induced ANF gene expression was not blocked in cardio-

![Stimulation of ANF-luciferase](image-url)

**Figure 3.** Lack of inhibition of bFGF-induced ANF-luciferase gene induction by RGS4. Cardiomyocytes were triple-transfected as indicated in Figure 2. 1.5 μg pCMV-RGS4-myc or N128A plasmid was used in each transfection. After 24 hours of incubation in serum, cells were transferred to serum-free media and treated with 25 ng/mL bFGF for 40 hours. Luciferase and β-galactosidase activities were determined and analyzed as indicated in Figure 2. A, bFGF–induced ANF-luciferase gene induction is not inhibited by overexpression of RGS4. B, bFGF–induced ANF-luciferase gene induction is not inhibited by overexpression of N128A-RGS4-myc (N128A).
myocytes that were transfected with 1.5 μg pCMV-RGS4-myc (Figure 3A) or pCMV-N128A-RGS4-myc (Figure 3B), demonstrating that the effect of RGS4-myc was specific to a heterotrimeric G protein–coupled pathway. Furthermore, treatment of cardiomyocytes with the phorbol ester TPA resulted in ANF gene induction that was not inhibited by overexpression of RGS4 (data not shown).

The ability of RGS4 overexpression to block the expression of MLC-2 was also examined. The MLC-2 gene encodes a sarcomeric protein that is associated with cardiac hypertrophy. MLC-2 gene induction occurs as a result of activation of the MAP kinase and other signaling pathways. In control cardiomyocytes that were transfected with a CMV promoter–β-galactosidase vector and pANF-luc but not with pCMV-RGS4-myc, stimulation with phenylephrine resulted in a 3- to 4-fold induction in MLC-2 gene expression as measured by luciferase activity normalized by β-galactosidase activity. Stimulation of control cardiomyocytes with endothelin-1 or bFGF also resulted in a 3- to 4-fold induction of MLC-2 gene expression. Phenylephrine- and endothelin-1–induced MLC-2 gene expression was inhibited in cardiomyocytes that were transfected with 1.5 μg pCMV-RGS4-myc (Figure 4A, 4B). Transfection with 1.5 μg pCMV-RGS4-myc did not inhibit bFGF-induced MLC-2 gene expression (Figure 4C).

Analysis of Myofilament Organization and Cell Size
In cultured cardiomyocytes, mechanical stress and ligands promote a hypertrophic response that includes the organiza-
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Figure 5. Inhibition of phenylephrine-stimulated myofilament organization by RGS4. Cardiomyocytes were double-transfected with 1.5 \( \mu \)g pCMV-RGS4-myc and 1.5 \( \mu \)g pON249. After 24 hours of incubation in serum, cells were transferred to serum-free media and stimulated with 100 \( \mu \)mol/L phenylephrine for 40 hours. Cells were fixed, permeabilized, and processed for immunofluorescent analysis with (1) TRITC-conjugated phalloidin and (2) anti-\( \beta \)-galactosidase primary antibody followed by FITC-conjugated secondary antibody. A, Absence of \( \beta \)-galactosidase immunoreactivity in a nontransfected cardiomyocyte. B, Phenylephrine-induced myofilament organization in the same nontransfected cell depicted in A. C, \( \beta \)-Galactosidase immunoreactivity used to identify transfected cells. Cardiomyocyte surface area was determined with NIH Image software. Results are expressed as mean cell surface area (arbitrary units) of 20 cardiomyocytes for each condition \( \pm \)SEM.

Figure 6. Inhibition of phenylephrine-stimulated cardiomyocyte growth by RGS4. Cardiomyocytes were double-transfected, stimulated with phenylephrine, and processed for immunofluorescent analysis as indicated in Figure 5. \( \beta \)-Galactosidase immunoreactivity was used to identify transfected cells. Cardiomyocyte surface area was determined with NIH Image software. Results are expressed as mean cell surface area (arbitrary units) of 20 cardiomyocytes for each condition \( \pm \)SEM.

Discussion

G proteins are thought to play an important role in the regulation of hypertrophic cardiac growth. RGS family members are GTPase-activating proteins for heterotrimeric G proteins of the \( G_i \) and \( G_q \) but not \( G_s \) families. We have demonstrated that overexpression of RGS4 can inhibit the ability of 2 ligands, phenylephrine and endothelin-1, to induce the cardiomyocyte hypertrophic growth program as assayed by the expression of marker genes, the organization of myofilaments, and by the analysis of cell size. Phenylephrine and endothelin-1 signal through heterotrimeric G proteins of the \( G_i \) and \( G_q \) families, respectively. Overexpression of RGS4 in cardiomyocytes does not inhibit the ability of bFGF, a ligand that does not signal through heterotrimeric G proteins, to increase the expression of the marker genes.

One assumption in this work is that RGS overexpression results in deactivation of heterotrimeric G proteins. It has not been possible to directly measure the activation state of heterotrimeric G proteins in cells, for example, by determining the fraction of \( G_{\text{qG}} \) that is bound to GTP and not GDP. To exclude the possibility that RGS4 inhibits endothelin-1 and phenylephrine-mediated cardiomyocyte hypertrophy by a mechanism separate from its ability to promote GTPase activity, we performed 3 control experiments. In the first we observed that overexpression of N128A-RGS4, a point mutant form of RGS4 that lacks GAP activity, did not inhibit phenylephrine-induced or endothelin-1–induced gene induction. In the second we found that RGS4 overexpression did not block bFGF–induced gene induction in cardiomyocytes. Indeed, others have reported that bFGF does not signal through heterotrimeric G proteins but instead activates the MAP kinase cascade through a pathway that involves FRS2, Grb2, SOS, and ras.\(^{22}\) In the third experiment we observed that RGS4 overexpression did not block phorbol ester–induced gene induction. Short-term administration of phorbol ester results in the direct activation of protein kinase C,
leading to activation of Raf-1 and MAP kinase. These results imply that RGS4 overexpression does not nonspecifically inhibit gene induction in cardiomyocytes and also suggests that RGS4 activity is upstream of ras, Raf-1, and protein kinase C.

To determine the role of RGS proteins in cardiomyocyte hypertrophy, we used the rat neonatal cardiomyocyte model. There are 3 caveats about the rat cardiomyocyte system that should be considered. First, cultured cardiomyocytes can be contaminated with cardiac fibroblasts. To address this issue, we (1) plated purified cardiomyocytes at low density, (2) used selective media, and (3) added 0.1 mmol/L BrdU to the cultures to inhibit nonmyocyte cell proliferation. Second, the transfection efficiency of cultured cardiomyocytes is often low or variable transfection efficiency, we used a CMV promoter and measured β-galactosidase activity in all transfected cells. In our experiments, luciferase data were always normalized by β-galactosidase activity to control for variation in transfection efficiencies. Third, cultured cardiomyocytes may not be an accurate model of cardiac hypertrophy in live animals. It is clear that cultured homogenous isolated cells are not perfectly representative of an intact heart, which is a multi layered complex tissue with many cell types. However, there is considerable evidence that agents that promote hypertrophy in cultured cells, such as endothelin-1, angiotensin II, and mechanical stretch, also play a role in cardiac hypertrophy in intact animals. Confirmation of results obtained in transfected cardiomyocytes will have to be sought in transgenic animals.

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