RGS6, a Modulator of Parasympathetic Activation in Heart


Rationale: Parasympathetic regulation of heart rate is mediated by acetylcholine binding to G protein–coupled muscarinic M2 receptors, which activate heterotrimeric $G_{i/o}$ proteins to promote G protein–coupled inwardly rectifying K$^+$ (GIRK) channel activation. Regulator of G protein signaling (RGS) proteins, which function to inactivate G proteins, are indispensable for normal parasympathetic control of the heart. However, it is unclear which of the more than 20 known RGS proteins function to negatively regulate and thereby ensure normal parasympathetic control of the heart.

Objective: To examine the specific contribution of RGS6 as an essential regulator of parasympathetic signaling in heart.

Methods and Results: We developed RGS6 knockout mice to determine the functional impact of loss of RGS6 on parasympathetic regulation of cardiac automaticity. RGS6 exhibited a uniquely robust expression in the heart, particularly in sinoatrial and atrioventricular nodal regions. Loss of RGS6 provoked dramatically exaggerated bradycardia in response to carbachol in mice and isolated perfused hearts and significantly enhanced the effect of carbachol on inhibition of spontaneous action potential firing in sinoatrial node cells. Consistent with a role of RGS6 in $G$ protein inactivation, RGS6-deficient atrial myocytes exhibited a significant reduction in the time course of acetylcholine-activated potassium current ($I_{\text{KACH}}$) activation and deactivation, as well as the extent of $I_{\text{KACH}}$ desensitization.

Conclusions: RGS6 is a previously unrecognized, but essential, regulator of parasympathetic activation in heart, functioning to prevent parasympathetic override and severe bradycardia. These effects likely result from actions of RGS6 as a negative regulator of G protein activation of GIRK channels. (Circ Res. 2010;107:1345-1349.)

Key Words: RGS6 ■ SA node ■ Heart rate ■ K$^+$ channel ■ G proteins

Since the discovery that acetylcholine (ACh) release from the vagus produces bradycardia, key proteins and mechanisms underlying this action of ACh in heart have been identified. It is now known that ACh binds to muscarinic M2 receptors (M2Rs) that activate heterotrimeric G proteins ($G_{i/o}$) in key pacemaking regions of the heart. Activation of these G proteins causes release of $G$ subunits that bind to and activate G protein–coupled inwardly rectifying K$^+$ (GIRK) channels, which results in a large K$^+$ current (acetylcholine-activated potassium current [$I_{\text{KACH}}$]) and membrane hyperpolarization.

RGS proteins function as GTPase-activating proteins (GAPs) for $G$ subunits, accelerating their conversion to the inactive GDP-bound form. This results in their reassembly with $G$ subunits to form inactive G protein heterotrimers, thereby terminating signaling by both $G$ and $G\beta\gamma$ proteins. Heterologous expression of various members of the RGS protein family with GIRK channels and M2Rs are required to reconstitute the normal activation and deactivation kinetics of native atrial GIRK channels. In vivo evidence for this key role of RGS proteins in controlling ACh-mediated bradycardia was provided using knock-in mice expressing mutant forms of $G_i$ or $G_o$ that cannot be acted on by RGS proteins. Thus, endogenous RGS proteins are required to regulate parasympathetic signaling in heart.

Therefore, it is of considerable importance and interest to identify which specific RGS proteins are responsible for this activity in heart. Here, we show that RGS6 is expressed highly in the heart, including sinoatrial (SAN) and atrioventricular (AVN) nodal regions, leading us to assess its involvement in parasympathetic control of the heart and $I_{\text{KACH}}$ signaling.
Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mice

RGS6+/−/+− mice in a 129/Sv × C57BL6 background, obtained from TIGM, were bred to generate RGS6 knockout (RGS6+/−/−) mice.

Results

Robust Expression of RGS6 in Heart

We cloned RGS6 in 1998 (GenBank accession no. AF073920) and later discovered multiple splice forms of RGS6 in brain.6 Given evidence that RGS6 transcripts are expressed in heart,7 we examined RGS6 protein expression in heart. We found robust expression of a single immunoreactive form of RGS6 in heart, corresponding to that of RGS6L,6 with higher levels of expression in atria compared with ventricles (Figure 1A; Online Figure I, A). RGS4, which has been found to regulate parasympathetic signaling in the SAN,8 was barely detectable in atria and was absent in ventricles. Examination of mRNA levels of RGS6 revealed strong enrichment in the SAN and AVN and higher expression in atrium compared with ventricle (Figure 1B; Online Figure I, B). RGS4 mRNA levels also were enriched in the SAN and AVN and were about two-fold higher than those of RGS6 in these regions. The low or undetectable levels of RGS4 protein in atria and ventricles is consistent with our finding that RGS4 mRNA levels in these tissues are 3% to 6% of those in brain.

To examine the role of RGS6 in heart, we developed RGS6+/−/− mice (Figure 1C, top), which would generate a short truncated form of RGS6L lacking its RGS domain (responsible for GAP activity). A typical genotyping result and immunoblot of RGS6 in atria and ventricles of mice of each genotype is shown (Figure 1C, bottom). RGS6 expression is reduced in atria and ventricles of RGS6+/−/− mice compared with wild-type (WT) mice, illustrating a gene dosage effect in both of these tissues (Online Figure I, A) and is absent in RGS6+/−/− mice.

Gross morphological examination of WT and RGS6+/−/− mice failed to identify any pathology as a result of RGS6 loss (Figure 2A; Online Figure II). Immunohistochemistry confirmed our immunoblotting results demonstrating that RGS6 is expressed more highly in the atrium than the ventricle and is absent in RGS6+/−/− mice (Figure 2B). In agreement with the observed expression of RGS6 mRNA in the SAN and AVN, we found that RGS6 protein is expressed in the SAN and AVN, which was confirmed by using HCN4 as a marker (Figure 2C and 2D). These results demonstrate robust expression of RGS6 in mouse heart, particularly in the SAN and AVN regions, and validate the use of RGS6+/−/− mice to study the functional role of RGS6 in heart.

Loss of RGS6 Provokes Exaggerated Bradycardia and Altered SAN Action Potential Firing

We examined carbachol (CCh)-induced bradycardia in conscious unrestrained WT and RGS6+/−/− mice (Figure 3A and 3B). A dramatic enhancement in CCh-induced bradycardia was seen in RGS6+/−/− mice compared with WT mice, 66% versus 34%, respectively. Similar results were found in anesthetized mice (Online Figure III, A). Thus, RGS6+/−/− mice exhibit enhanced M2R-mediated bradycardia as found in mice expressing RGS-insensitive Gα2.9 Perfused hearts from RGS6+/−/− mice showed a normal chronotropic response to isoproterenol, but an en-

Non-standard Abbreviations and Acronyms

- ACh: acetylcholine
- AVN: atrioventricular node
- CCh: carbachol
- GAP: GTPase-activating protein
- GIRK: G protein–coupled inwardly rectifying K+ channel
- IACh: acetylcholine-activated potassium current
- M2R: muscarinic M2 receptor
- RGS: regulator of G protein signaling
- SAN: sinoatrial node
- WT: wild type

Figure 1. Cardiac expression of RGS6 and characterization of RGS6+/−/− mouse. Protein (A) and mRNA (B) levels of RGS4 and RGS6 in mouse heart tissues (n=3 mice). C, Generation of RGS6+/−/− mice. Top, Illustration (in scale) of targeted region of RGS6 gene (black bars, exons; P1, P2, P3, PCR primers used for genotyping). Lower Left, Typical genotyping result. Lower Right, RGS6 protein expression in hearts of RGS6+/+, RGS6+/−, and RGS6−/− mice.
hanced bradycardia (60% versus 34%, Figure 3C) and atrioventricular block (Online Figure III, B; Online Figure IV, D) in response to CCh compared with WT mice. Consistent with these findings, inhibition of spontaneous action potential firing rates in SAN cells by CCh was significantly enhanced by loss of RGS6 (Figure 3D; Online Figure V). These results show that genetic deletion of RGS6 in heart leads to increases in CCh-mediated bradycardia likely by actions on cardiac pacemaker cells in the SAN, demonstrating that RGS6 is essential for modulation of M2R signaling in heart.

Altered M2R Regulation of $I_{K_{ACh}}$ in RGS6$^{-/-}$ Atrial Myocytes

The role of $I_{K_{ACh}}$ in M2R-mediated bradycardia is well established. Knockout of GIRK1 or GIRK4 channel subunits in mice causes loss of $I_{K_{ACh}}$ in atrial myocytes and severe impairment in vagal-mediated bradycardia. RGS proteins reconstitute the rapid gating kinetics of GIRK channels in atrial myocytes. In view of our findings above, we examined whether atrial myocytes from RGS6$^{-/-}$ mice exhibited al-
tered M2R regulation of GIRK channels. In atrial myocytes from WT mice, application of CCh elicited rapid $I_{\text{KACH}}$ that showed significant desensitization over time, followed by rapid deactivation on removal of CCh (Figure 4A). Atrial myocytes from RGS6/−/− mice exhibited a significant reduction in the time course of activation and deactivation, as well as the extent of $I_{\text{KACH}}$ desensitization (Figure 4A, 4C, and 4D). In some RGS6/−/− cells, we found a smaller amplitude of $I_{\text{KACH}}$, but this effect was not significant (Figure 4B). Thus, RGS6 is required for the normal activation and deactivation kinetics, as well as the rate of desensitization of GIRK channels. These effects are likely mediated by the GAP activity of RGS6 on $G_i$ proteins that activate GIRK channels by releasing $G_{\beta\gamma}$. Indeed, the GAP function of RGS proteins accelerates both the onset of Gi protein-coupled receptor signaling and rate of deactivation.10 Although RGS6 forms a complex with $G_{\beta\gamma}$ in atria, we found no evidence for direct interaction of RGS6 with GIRK1 or R7BP (Online Figure VI).

**Discussion**

This study establishes RGS6 as an essential modulator of parasympathetic activation in heart, where it functions to prevent parasympathetic override and severe bradycardia. Loss of RGS6 was associated with severely exaggerated bradycardia in response to CCh in both mice and isolated perfused hearts, showing that this response was not dependent on effects of RGS6 in tissues beyond the heart. Indeed, RGS6 is expressed highly in heart, especially within the SAN and AVN regions. This is the first demonstration of endogenous expression of RGS6 protein in heart and the only member of the RGS protein family to be detected at the protein level in heart. Loss of RGS6 within SAN cardiac pacemaker cells, which dramatically enhanced CCh inhibition of spontaneous action potential firing, likely accounts for the observed effects on CCh-mediated bradycardia. Our findings support a role for RGS6 as a major negative modulator of M2R signaling in the SAN. It may play other important roles in the heart in view of its expression in both atrium and ventricle. Indeed, we found that RGS6 was expressed in the AVN, and we observed CCh-induced atrioventricular block in RGS6/−/− mice.

We provide the first evidence that RGS6 is required for desensitization and rapid deactivation of GIRK-mediated $I_{\text{KACH}}$ in atrial myocytes, consistent with its role as a GAP for $G_i$.10,11 Delayed deactivation of $I_{\text{KACH}}$ in RGS6-deficient atrial pacemaker cells slows channel closing, prolonging membrane hyperpolarization. This effect would be expected to produce the dramatic increase in CCh-induced bradycardia in RGS6/−/− mice and isolated hearts and enhanced inhibition of spontaneous action potential firing in SAN cells. In fact, the finding that loss of RGS6 has such dramatic effects on GIRK channel deactivation implies that other RGS proteins do not compensate for loss of RGS6 or have a role similar to that of RGS6. We speculate that this may result from lack of expression of other RGS proteins specifically in SAN regions because most RGS proteins are capable GAPs for $G_i$. However, Cifelli et al8 recently reported that RGS4 modulates parasympathetic activation and heart rate control in the SAN based on studies with RGS4/−/− mice. In their study, RGS4 expression measured by $\beta$-galactosidase staining (LacZ in the knockout allele) was limited to the SAN region, in contrast to
the expression of RGS6 protein shown here. Here, we showed enriched expression of RGS6 and RGS4 mRNA in SAN and AVN regions of the heart. We envision 2 possible ways to reconcile our findings with those of Cifelli et al. It is possible that RGS6 and RGS4 are both expressed in the same SAN cells and act on the same G proteins mediating GIRK channel opening as both proteins function as GAPs for $G_{11-3}$ and $G_{o}$. In this case, we might expect to see even greater exaggeration of parasympathetic signaling in mice with combined loss of RGS4 and RGS6. Alternatively, it is possible that RGS6 and RGS4 act in different cells or on different G proteins in vivo; in which case, they might not produce additive effects on their loss.

The present results provide new evidence for an important role of RGS6 in the heart. RGS6 is expressed highly in heart, and its role promotes severe bradycardia during parasympathetic activation. These findings suggest that alterations in RGS6 expression or activity in heart could potentially contribute to diseases such as sick sinus syndrome or other maladies involving abnormal parasympathetic activation in heart. Thus, this work identifies RGS6 as a possible therapeutic target for treatment of such diseases.

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What Is Known?
- Parasympathetic stimulation of the heart is achieved through acetylcholine (ACh) release from the vagus, which binds to G protein–coupled muscarinic M2 receptors (M2Rs) located at pacemaking nodes and electrically conducting portions of the heart.
- Stimulation of M2Rs by ACh results in release of the G$\beta$$\gamma$ component of the heterotrimeric G protein complex associated with the receptor which promotes activation of G protein–coupled inwardly rectifying K$^+$ (GIRK) channels, hyperpolarization of the membrane, inhibition of cell firing, and a net decrease in heart rate.
- Regulator of G protein signaling (RGS) proteins determine the magnitude and duration of the cellular response to G protein–coupled receptor stimulation through inactivation of G proteins and are essential for proper GIRK channel gating kinetics and normal parasympathetic control of the heart.

What New Information Does This Article Contribute?
- RGS6 is expressed robustly in heart, particularly in the sinoatrial node (SAN) and atrioventricular node (AVN), which are known to control heart rate and cardiac contractility.
- RGS6 is required for desensitization and rapid deactivation of M2R GIRK-mediated $I_{K_{GIRK}}$. Channel current and suppression of atrial myocyte membrane excitability.
- Loss of RGS6 is associated with severely exaggerated bradycardia and atrioventricular block in response to parasympathetic stimulation, demonstrating that RGS6 is essential for modulating M2R signaling in heart to prevent parasympathetic override.

Novelty and Significance

Disclosures
None.

References
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Methods

Mice
RGS6<sup>+/−</sup> mice in a 129/Sv x C57BL6 background obtained from TIGM were bred to generate RGS6 knockout (RGS6<sup>−/−</sup>) mice. Experiments were performed using litter matched 2-4 month-old mice. Experiments were performed in agreement with the Guide for the Use and Care of Laboratory Animals.

Atrial Myocyte and SAN Cells
Atrial myocytes were prepared from eight day old pups as described<sup>1</sup>. Preparation of SAN cells from adult mice and recording of spontaneous action potentials in SAN cells were performed as described<sup>2</sup>.

ECG Measurements in Mice
Surface ECG measurements in lightly anesthetized mice (15 μl/g 2.5% Avertin) were recorded as described<sup>3</sup>. Determination of heart rate in conscious unrestrained mice was done using ECG telemetry method as described<sup>4</sup>.

Langendorff-Perfusion
ECG recording from isolated Langendorff-perfused hearts was performed essentially as described<sup>2</sup>. Excised hearts were mounted on a modified Langendorff apparatus (HSE-HA perfusion systems, Harvard Apparatus) and retrograde aortic perfusion was performed at constant pressure of 80 mm Hg with 95% O<sub>2</sub>:5% CO<sub>2</sub> oxygenated Krebs-Henseleit buffer.

Histology, Immunohistochemistry, Immunoblotting and PCR
Gross morphology of hearts derived from four month-old wild-type and RGS6<sup>+/−</sup> mice was examined at the University of Iowa Microscopy core. Immunohistochemistry was performed in frozen sections derived from formaldehyde-perfused mice using antibodies specific for RGS6 and HCN4. The RGS6-specific antibody was developed in our laboratory, RGS4-specific antibody (U1079)<sup>5</sup> was from Dr. Susan Mumby and the HCN4 antibody was from Abcam (ab32675). PCR primers used for genotyping RGS6 mice are P1 (GAG GGA GTC ATC ATC GGT GCC ATC GC), P2 (AAC ATG GTC TGA GAT TGG GAA GAT GTA GCC), and P3 (GAC TCT TTC CAC AAC TAT CCA ACT CAC AAC GT).

Electrophysiological Measurements of I<sub>Kach</sub> in Atrial Myocytes
Whole-cell patch clamp recordings were performed in cultured atrial cardiomyocytes from wild-type and RGS6<sup>+/−</sup> mice. Myocytes were bathed in extracellular buffer composed of (mM) 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES and 10 glucose, pH 7.4. The intracellular/pipette buffer contained (mM) 140 KCl, 5 NaCl, 0.1 CaCl<sub>2</sub>, 10 EGTA, 10 HEPES, 4 Mg-ATP and 0.3 Na-GTP, pH 7.4. I<sub>Kach</sub> were recorded with the application of 20 μM CCh for 10-15 sec at a holding potential of -100 mV using an Axopatch200B amplifier, connected to Digidata 1440A, with the pClamp10 acquisition software (MMolecular Devices) as detailed earlier<sup>6</sup>. Currents recordings were analyzed and figures prepared using Clampfit and Origin 7 software.

Determination of mRNA levels of RGS4 and RGS6 using quantitative real time PCR
Mouse hearts were dissected into four portions, namely SA node, AV node, left atrium and ventricles. Total RNA was isolated from these tissues using a Qiagen RNeasy kit, according to the manufacturer’s recommendation. First strand of cDNA was synthesized from 150ng of total RNA using SuperScriptIII First Strand Synthesis system (Invitrogen, CA). Real time PCR was carried out using iQ™ SYBR® Green Supermix (Bio-Rad, CA), by following the manufacturer’s protocol. To quantitatively determine RNA levels of RGS4 and RGS6, plasmids containing
cDNAs of RGS4, RGS6, and 18S rRNA were diluted to individual concentration series of 50, 5, 5 $\times$ 10$^{-1}$, 5 $\times$ 10$^{-2}$, 5 $\times$ 10$^{-3}$, 5 $\times$ 10$^{-4}$, 5 $\times$ 10$^{-5}$, 5 $\times$ 10$^{-6}$ pg/ul, and were used as quantification standards for determining corresponding mRNA levels. 18S rRNA level was used as internal control to normalize mRNA levels of RGS4 and RGS6. Primers were the following: RGS4 forward, AAC ATT GAC TTC TGG ATC AGC TGT GAG GAG; RGS4 reverse, AGT CCA GGT TCA CCT CTT TTG TTG CTT G; RGS6 forward, ATG GAG GGA GAT ACA CAT TTG AAG ATG CC; RGS6 reverse, CAG CGA CTT TCC CTT CTT CTT GGC C; 18S rRNA forward, CAA AGA TTA AGC CAT GCA TGT CTA AGT ACG C; 18S rRNA reverse, GGC ATG TAT TAG CTC TAG AAT TAC CAC AGT TAT CC; HCN4 forward, GAC AGC GCA TCC ATG ACT ACT ATG AAC AC; HCN4 reverse, TTA AAG TTG ATG ATC TCC TCT CGA AGT GGC.

Co-immunoprecipitation

Mouse atria were harvested and lysed in RIPA buffer. Lysates containing 1 mg protein were pre-cleared at 4ºC for 1.5 h, by incubating with 10 µl of Protein A/G-agarose beads (Santa Cruz Biotechnology Inc., CA) and 0.4 µg of rabbit anti-GFP IgG. Cleared lysates were then incubated at 4ºC for 1.5 h with 4 µg of antibody against RGS6, followed by an additional overnight incubation with 20 µl of Protein A/G-agarose beads (Santa Cruz Biotechnology Inc., CA) at 4ºC. At the end of the incubation, beads were collected by centrifugation at 1,000 x g for 5 min at 4ºC, and washed three times with 1X TTBS (1 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.05% Tween-20). After the final wash, immunoprecipitates were eluted from the beads with 30 µl of 2X SDS-PAGE sample buffer by heating the tube at 95ºC for 10 min, subjected to SDS-PAGE, and transferred to a NC membrane. The resultant membrane was cut into three strips based upon the molecular size and used for detection of GIRK1 and RGS6, G85, and R7BP, respectively. Antibodies were GIRK1 (Alomone Labs, Israel), G85 (generous gift from Dr. William Simonds, NIH), and R7BP (generous gift from Dr. Kirill Martemyanov).

Statistical Analysis

Experimental data were expressed as means ± SE (standard errors of the means). Significance of differences was determined by the unpaired Student’s t test using SigmaPlot (Systat Software, CA); a p < 0.05 was considered as statistically significant.

Online Figure I

A

![Graph A showing relative RGS6 levels in atria and ventricles for RGS6+/-, +/-, and -/- mice.]

B

![Graph B showing relative HCN4 mRNA levels in mouse tissues.]

I. A, Quantification of RGS6 protein levels in hearts of RGS6 +/+, +/-, and -/- mice. Results are expressed as means ± S.E. of three hearts from mice of each RGS6 genotype. B, Expression of HCN4 in mouse tissues. To authenticate the SAN and AVN tissues used in Figure 1B, relative levels of HCN4 mRNA were determined in mouse brain and ventricle, atrium, SAN, and AVN of heart using real time PCR, and normalized to 18S rRNA mRNA level. The value of relative HCN4 mRNA level in brain was set as 1. Results are expressed as means ± S.E. of tissues from three wild-type mice, and the mean values are indicated in the figure.
Online Figure II

II. Loss of RGS6 does not influence heart size. Weights of atria and ventricles, expressed as percentage of body weight, were plotted from five wild-type and five RGS6<sup>−/−</sup> mice. The size of hearts from wild-type and RGS6<sup>−/−</sup> mice are not significantly different.
Online Figure III

**A**

Heart rates in anesthetized wt and RGS6−/− mice (n=5 each) at rest and following CCh (0.1 mg/kg i.p.). *, p<0.05 vs wt.

**B**

Representative ECG traces of isolated perfused hearts from wild-type and RGS6−/− mice at baseline, in the presence of 50 nM isoproterenol (Iso) or 50 nM + 0.5 μM carbachol (CCh). Severe bradycardia and AV block (prolonged PR interval) was noted in RGS6−/− mice when 0.5 μM CCh was infused.

III. Effects of loss of RGS6 on CCh-induced bradycardia. **A**, Heart rates in anesthetized wt and RGS6−/− mice (n=5 each) at rest and following CCh (0.1 mg/kg i.p.). *, p<0.05 vs wt. **B**, Representative ECG traces of isolated perfused hearts from wild-type and RGS6−/− mice at baseline, in the presence of 50 nM isoproterenol (Iso) or 50 nM + 0.5 μM carbachol (CCh). Severe bradycardia and AV block (prolonged PR interval) was noted in RGS6−/− mice when 0.5 μM CCh was infused.
Online Figure IV

A

B

C

D

IV. Effects of loss of RGS6 on QRS duration, PR, and QT intervals in isolated perfused mouse hearts. A, A hypothetical ECG trace is used to show the definition of QRS duration, PR, and QT intervals. ECGs of isolated mouse hearts (n=3) were recorded at basal condition (B), in the presence of 50 nM isoproterenol (C), or 50 nM isoproterenol plus 500 nM carbachol (D). Intervals of PR, QT, and QRS duration were calculated from these ECG traces. Results are means ± S.E. of three measurements. *, p<0.001.
V. Representative recordings of spontaneous action potential firing in SA nodal myocytes isolated from wild-type (wt) and RGS6−/− mice. Recording was done at control, 0.1 μM carbachol (CCh)-treated, and washout conditions.
VI. RGS6 co-immunoprecipitates with Gβ5 but not R7BP or GIRK1 in atrial lysates. Lysates (~1 mg) from wild-type (wt) or RGS6−/− mouse atria were immunoprecipitated using an anti-RGS6 antibody. The precipitates were then resolved using SDS-PAGE and blotted for Gβ5, R7BP, GIRK1, and RGS6. Brain lysate from a wild-type mouse (20 μg) was used as a positive control. NS, non-specific cross-reactive band.