

Published in final edited form as:

Circ Res. 2010 November 26; 107(11): 1350–1354. doi:10.1161/CIRCRESAHA.110.224212.

RGS6/Gβ5 complex accelerates I_{KACH} gating kinetics in atrial myocytes and modulates parasympathetic regulation of heart rate

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Abstract

Rationale—The parasympathetic reduction in heart rate (HR) involves the sequential activation of m₂ muscarinic cholinergic receptors (m₂R), pertussis toxin-sensitive (Gi/o) heterotrimeric G proteins, and the atrial potassium channel I_{KACH}. Molecular mechanisms regulating this critical signal transduction pathway are not fully understood.

Objective—To determine whether the G protein signaling regulator Rgs6/Gβ5 modulates m₂R-I_{KACH} signaling and cardiac physiology.

Methods and Results—Cardiac expression of Rgs6, and its interaction with Gβ5, was demonstrated by immunoblotting and immunoprecipitation. *Rgs6*^{-/-} mice were generated by gene targeting, and the cardiac effects of *Rgs6* ablation were analyzed by whole-cell recordings in isolated cardiomyocytes and electrocardiogram (ECG) telemetry. Loss of Rgs6 yielded profound delays in m₂R-I_{KACH} deactivation kinetics in both neonatal atrial myocytes and adult sino-atrial nodal cells. *Rgs6*^{-/-} mice exhibited mild resting bradycardia and altered heart rate responses to pharmacologic manipulations that were consistent with enhanced m₂R-I_{KACH} signaling.

Conclusions—The cardiac Rgs6/Gβ5 complex modulates the timing of parasympathetic influence on atrial myocytes and heart rate in mice.

Keywords

G protein; muscarinic; knockout; cardiac; GIRK; Regulators of G protein signaling

Introduction

Cardiac output is shaped to a great extent by sympathetic and parasympathetic influences. Parasympathetic input tempers heart rate (HR) and counteracts the pro-arrhythmic effects of sympathetic activation, and is mediated by acetylcholine (ACh)¹. ACh is released from post-ganglionic parasympathetic neurons and binds to m₂ muscarinic receptors (m₂R) on pacemaker cells and atrial myocytes, triggering activation of pertussis toxin-sensitive (Gi/o) heterotrimeric G proteins². Once activated, G proteins dissociate into Gα-GTP and Gβγ subunits, leading to modulation of adenylyl cyclase and multiple ion channels. Central among these reactions is the binding of Gβγ to the atrial potassium channel I_{KACH}, a

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Disclosures. None

heterotetramer composed of Girk1 and Girk4 subunits³. Binding of G $\beta\gamma$ to I_{KACH} enhances its gating which leads to cell hyperpolarization and ultimately, decreased HR⁴.

The duration of G protein signaling is controlled by members of the Regulator of G protein Signaling (RGS) family⁵. RGS proteins stimulate inactivation of G α -GTP, facilitating its re-assembly with G $\beta\gamma$. RGS proteins play a critical role in shaping bradycardic effects of M₂R receptor activation^{6–8}. Indeed, eliminating RGS influence by expressing G α subunits insensitive to RGS action results in a substantial enhancement of I_{KACH} regulation by m₂R signaling, via both G α_o and G α_{i2} pathways^{7,8}. Although more than 30 RGS proteins have been identified, the involvement of specific RGS proteins in the regulation of parasympathetic input is not fully-understood. Here, we report an unexpected role of the Rgs6/G β 5 complex, previously thought to be neuron-specific regulator, in the temporal regulation of m₂R-I_{KACH} signaling.

Methods

Littermate mice were used for all experiments in this study. All procedures were carried out in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. A detailed Methods section is available as Supplementary Material.

Results

Profiling Rgs6 protein expression across mouse tissues revealed its readily detectable levels in the heart in addition to abundant presence in the brain (Fig. 1A; Online Fig. I). Rgs6 protein was enriched in atria, where it was found predominantly in myocytes (Online Fig. II), consistent with a recent report⁹, and similar to the distribution of Girk1, an integral subunit of I_{KACH} (Fig. 1B). To begin exploring the role of Rgs6 in cardiac physiology, we obtained Rgs6^{-/-} mice where exons 5–7 encoding the critical N-terminal portion of the protein were eliminated (Fig. 1C, D). Immunoblotting verified the complete absence of Rgs6 protein in the hearts of Rgs6^{-/-} mice (Fig. 1E).

Rgs6 interacts with the type 5 G protein β subunit (G β 5) and the R7 Binding Protein (R7BP) in the CNS¹⁰ (Fig. 1F). In the mouse heart, however, only G β 5 is available for the interaction with Rgs6 (Fig. 1E). Rgs6 was undetectable in hearts from G β 5^{-/-} mice, indicating that the physical association with G β 5 is critical for the expression and/or stability of Rgs6 (Fig. 1G). Similarly, G β 5 levels were dramatically reduced in the Rgs6^{-/-} heart but not brain, indicating that in the heart Rgs6 is the predominant RGS bound to G β 5. No effect on Rgs6 or G β 5 levels was observed upon elimination of R7BP or Girk4. Notably, we detected no compensatory changes in either Gai/o proteins or RGS4, a protein previously implicated in regulation of the m₂R-I_{KACH} signaling⁶.

Given the co-enrichment of Rgs6 and I_{KACH} in atria and the role of R7 RGS/G β 5 complexes in GPCR-GIRK signaling in the CNS¹¹, we next measured the impact of Rgs6 ablation on m₂R-I_{KACH} signaling in neonatal atrial myocytes, which exhibit robust inward current triggered by the non-selective muscarinic agonist carbachol (CCh). While CCh evoked currents with comparable potency in atrial myocytes from wild-type mice, current deactivation kinetics were notably slower across all CCh concentrations tested in myocytes from Rgs6^{-/-} mice (Online Fig. III). Current activation kinetics were also delayed in Rgs6^{-/-} myocytes, though only for the lower CCh concentrations tested.

We next compared CCh-induced currents in sino-atrial node (SAN) cells, the key anatomic substrate for parasympathetic control of heart rate (Fig. 2). While some differences in the density and kinetics of CCh-induced responses between adult SAN cells and neonatal atrial

myocytes were evident, *Rgs6* ablation correlated with significantly delayed deactivation rates in both cell types. Under the same conditions, no differences in CCh-induced steady-state current density or activation kinetics were observed between genotypes in either atrial myocytes or SAN cells (Fig. 2). Furthermore, deletion of the $G\beta 5$ replicated prolonged deactivation kinetics seen in *Rgs6*^{-/-} myocytes (Fig. 2E), indicating that regulation of the m_2R - I_{KACH} signaling in heart atria is mediated by the *Rgs6*/ $G\beta 5$ complex rather than *Rgs6* by itself.

The striking impact of *Rgs6* ablation on m_2R - I_{KACH} signaling kinetics in atrial myocytes and SAN cells, prompted us to test whether *Rgs6*/ $G\beta 5$ can physically associate with the I_{KACH} channel. In transfected HEK293 cells, we detected robust co-immunoprecipitation of the *Rgs6*/ $G\beta 5$ complex with *Girk4* but not *Girk1* by both forward and reverse precipitation strategies (Fig 3). Thus, the involvement of *Rgs6*/ $G\beta 5$ in m_2R - I_{KACH} signaling is likely aided by a direct protein-protein interaction mediated by the cardiac-specific *Girk* subunit, *Girk4*.

The delay in I_{KACH} deactivation kinetics triggered by *Rgs6*/ $G\beta 5$ elimination is expected to enhance m_2R - I_{KACH} signaling as the channel would stay open longer which would potentiate the parasympathetic regulation of HR. We addressed this possibility by analyzing cardiac function in mice using ECG telemetry, at baseline and following pharmacologic manipulation. Analysis of ECG traces did not reveal gross abnormalities in cardiac physiology in *Rgs6*^{-/-} mice (Fig. 4A and Online Fig. IV). *Rgs6*^{-/-} mice did, however, display a mild resting bradycardia (511 ± 13 vs. 476 ± 4 bpm, $P < 0.05$), consistent with the effect of *Rgs6*/ $G\beta 5$ ablation on m_2R -dependent signaling in atrial myocytes (Fig. 4B). While CCh administration (0.1 mg/kg, i.p.) triggered a rapid decrease in HR in wild-type and *Rgs6*^{-/-} animals, the effect was significantly larger and persisted longer in *Rgs6*^{-/-} mice (Fig. 4C). Similarly, parasympathetic blockade with atropine (1 mg/kg, i.p.) had a positive chronotropic effect in both groups, with a significantly larger effect seen in *Rgs6*^{-/-} mice (Fig. 4D). Importantly, there was no difference in HR immediately following atropine administration, indicating that the bradycardia seen in *Rgs6*^{-/-} mice results from enhanced intrinsic m_2R signaling.

Discussion

Here, we report that *Rgs6*/ $G\beta 5$ negatively regulates m_2R - I_{KACH} signaling in atrial myocytes by accelerating I_{KACH} deactivation kinetics. These observations, together with the effect of *Rgs6* ablation on HR and responses to pharmacologic manipulation, indicate that *Rgs6*/ $G\beta 5$ represents a key node of regulation in the parasympathetic control of cardiac output. Since dysregulation of the parasympathetic tone by deficiencies in I_{KACH} function is increasingly accepted as a major factor in the pathogenesis of the atrial fibrillation¹, our study introduces *Rgs6*/ $G\beta 5$ complex as an attractive candidate for better understanding of cardiac pathophysiology and development of corrective therapies.

Rgs6 belongs to the R7 family of RGS proteins, members of which were thought to be expressed exclusively in the nervous system, where they play roles in nociception, vision, reward behavior and locomotion¹⁰. Although *Rgs6* expression was reported previously in the heart^{9,12,13}, our study documents for the first time the functional relevance of *Rgs6* to cardiac physiology. In the CNS, *Rgs6* forms complexes with two proteins, $G\beta 5$ and *R7BP* that specify its stability, subcellular distribution, and activity¹⁰. Here we show that cardiac *Rgs6* forms a complex with $G\beta 5$, but not with *R7BP* which is undetectable in the heart. The obligate and functionally-relevant nature of the *Rgs6*/ $G\beta 5$ interaction was underscored by the mutual dependence of *Rgs6* and $G\beta 5$ levels on their co-expression, and the phenotypic similarities in m_2R - I_{KACH} signaling in myocytes from *Rgs6*^{-/-} and $G\beta 5$ ^{-/-} mice. In neurons,

Gβ5 recruits R7 RGS proteins to GIRK channels, resulting in accelerated channel kinetics associated with GABA_B receptor activation¹¹. Thus, the present work reveals the conservation of this compartmentalization mechanism by showing that Rgs6/Gβ5 can likewise regulate m₂R-I_{KACH} signaling.

Previous work has identified Rgs4 as a critical regulator of m₂R-I_{KACH} signaling in sinoatrial nodal cells⁶. Indeed, the deficiencies in m₂R-I_{KACH} signaling linked to *Rgs6* ablation reported herein are reminiscent of those reported in *Rgs4*^{-/-} mice⁶. Therefore, murine sinoatrial nodal cells may employ parallel approaches involving Rgs4 and Rgs6/Gβ5 to regulate m₂R-I_{KACH} signaling. It is possible, for example, that Rgs4 and Rgs6/Gβ5 selectively regulate different G protein subtypes involved in I_{KACH} gating. Indeed, studies with knock-in mice expressing RGS-insensitive G proteins reveal a differential contribution of Gα_{i2} and Gα_o to m₂R-dependent actions^{7,8}. Furthermore, Rgs6/Gβ5 shows selectivity towards Gα_o over Gα_{i2} *in vitro*¹⁴. However, while the role of Gα_{i2} in mediating m₂R-I_{KACH} coupling is well established⁸, the involvement of Gα_o in this process is less certain. Moreover, it remains possible that other proteins of more than 30-member RGS family also play roles in this regulation. Delineating the mechanisms of the functional involvement of RGS proteins in controlling m₂R-I_{KACH} signaling in the mouse models and their relevance to human physiology will serve as an exciting future research direction.

Novelty and Significance

What is known?

- Activation of the parasympathetic branch of the autonomic nervous system decreases heart rate via the neurotransmitter acetylcholine.
- Acetylcholine stimulates m₂ muscarinic receptors (m₂R) on sinoatrial nodal cells and atrial myocytes, leading to the G protein-dependent activation of the potassium channel I_{KACH}.
- Modulating m₂R-I_{KACH} signaling can impact heart rate.

What new information does this article contribute?

- The Rgs6/Gβ5 protein complex is an essential modulator of m₂R-I_{KACH} signaling in cardiac myocytes and sinoatrial cells
- Inactivation of the *Rgs6* gene in mice results in a mild bradycardia and an enhanced effect of drug-induced parasympathetic stimulation.

Summary

m₂R-I_{KACH} signaling plays critical role in controlling heart rate and has been implicated in the pathogenesis of atrial fibrillation. The molecular mechanisms regulating this process, however, are not fully-understood. Here, we identify the Rgs6/Gβ5 protein complex as a key regulator of m₂R-I_{KACH} signaling in cardiomyocytes and sinoatrial cells. We show that Rgs6/Gβ5 can physically associate with I_{KACH} and that deletion of the *Rgs6* gene in mice significantly enhances the parasympathetic regulation of heart rate. Thus, Rgs6/Gβ5 may contribute to, or represent a novel therapeutic target for, pathological conditions characterized by abnormal regulation of cardiac output.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of funding. This work was supported by NIH grants DA026405 (KAM), MH061933 (KW), DA011806 (KW), and T32 DA07234 (KA), and a McKnight Land-Grant Award (KAM).

Abbreviations

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|-----------------------|--|
| GIRK channel | G protein-gated inwardly-rectifying K ⁺ channel |
| CCh | carbachol |
| RGS | Regulator of G protein Signaling |
| HR | heart rate |
| ECG | electrocardiogram |
| m₂R | type 2 muscarinic receptor |

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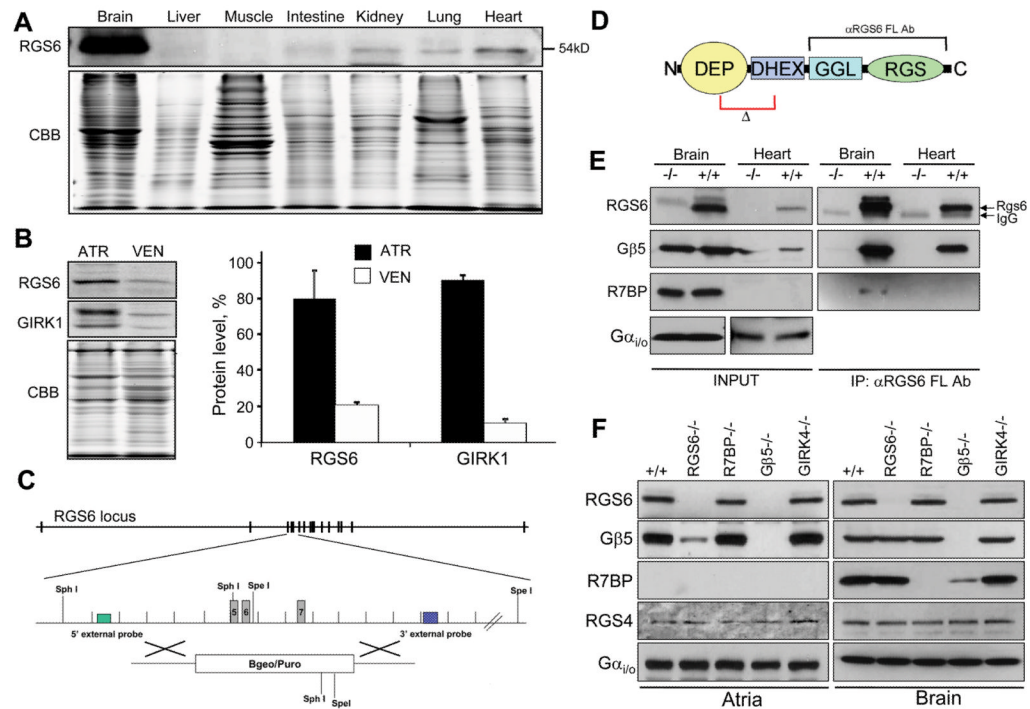


Figure 1. Rgs6 protein level and complex formation in the mouse heart

A, Rgs6 protein levels across mouse tissues as analyzed by Western blotting. Equal amounts of total protein (20 μ g) were loaded in each lane. Coomassie staining (CBB) was used as a loading control. **B**, Rgs6 is co-enriched with GirK1 in the atria. Atrial (ATR) and ventricular (VEN) lysates were analyzed by Western blotting (upper panels). CBB staining confirms equal protein loading. *Graph*: Quantification of Rgs6 and GirK1 band densities. **C**, Strategy for *Rgs6* ablation by homologous recombination. **D**, Structural organization of Rgs6. Frames designate the deleted region (Δ) and recognition site of the RGS6 FL antibody (α RGS6 FL Ab) used throughout the study. Colored boxes designate known structural domains. **E**, Co-immunoprecipitation of Rgs6 with G β 5 and R7BP from heart and brain tissues. **F**, Co-dependence of Rgs6 and G β 5 expression in the heart and brain as analyzed by Western blotting.

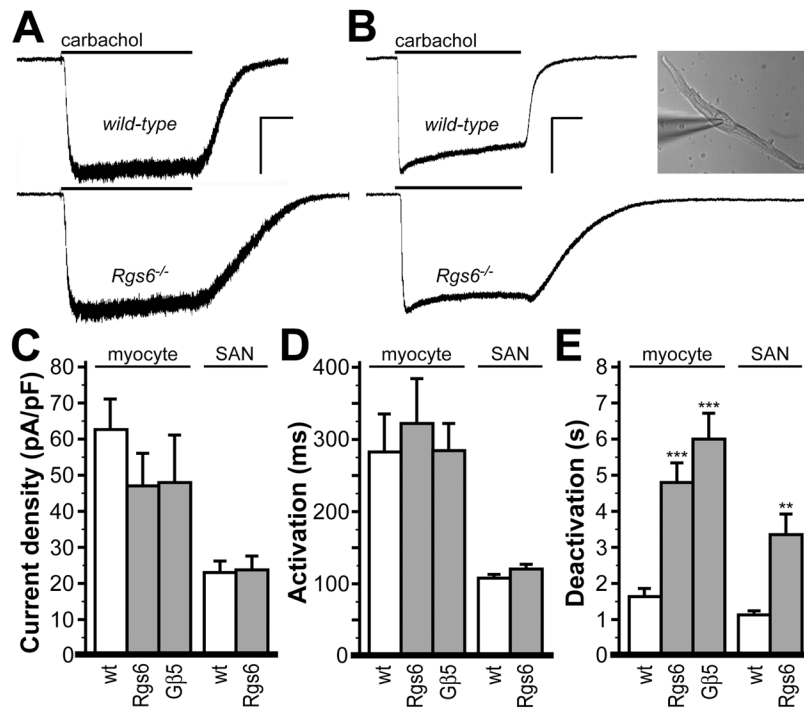


Figure 2. Impact of *Rgs6* ablation on m_2R - I_{KACH} signaling in atrial myocytes and SAN cells
A, Inward currents evoked by CCh (10 μ mol/L) in atrial myocytes from wild-type and *Rgs6*^{-/-} mice. Scale bars: 5 s/200 pA. **B**, Inward currents evoked by CCh (10 μ mol/L) in SAN cells from wild-type and *Rgs6*^{-/-} mice. Scale bars: 5 s/400 pA. Inset: image of the wild-type SAN cell evaluated in the adjacent trace. Summary of steady-state CCh-induced current density (**C**), activation kinetics (**D**), and deactivation kinetics (**E**) in wild-type, *Rgs6*^{-/-}, and *Gβ5*^{-/-} atrial myocytes (n=5–11 per group), and in wild-type and *Rgs6*^{-/-} SAN cells (n=10–12 per genotype). Genotype did not impact current density (atrial myocytes: $F_{2,21}=1.9$, $P=0.18$; SAN cells: $t(20)=0.13$, $P=0.90$) or activation kinetics (atrial myocytes: $F_{2,21}=1.0$, $P=0.38$; SAN cells: $t(20)=1.69$, $P=0.11$), but did influence deactivation kinetics (atrial myocytes: $F_{2,21}=24.8$, $P<0.001$; SAN cells: $t(20)=3.71$, $P<0.01$). Symbols: **, *** $P<0.01$ and 0.001 , respectively, vs. wild-type (within cell type).

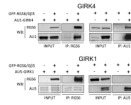


Figure 3. RGS6/G β 5 forms a complex with Girk4

Rgs6 and G β 5 were co-expressed with either AU1-tagged Girk4 or AU5-tagged Girk1 in HEK293 cells. Forward and reciprocal co-immunoprecipitation assays were performed as described in the *Methods* using indicated antibodies. Eluates were analyzed using Western blotting.

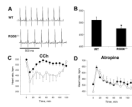


Figure 4. Effect of *Rgs6* ablation on resting HR and muscarinic regulation

A, Baseline ECG recorded during light phase from conscious, unrestrained wild-type (WT, n=5) and *Rgs6*^{-/-} mice (n=5). **B**, Average HR as determined from the analysis of the ECG recordings (6hr). Symbols: * $P < 0.05$ vs. wild-type. **C**, Effect of CCh (0.1 mg/kg, i.p.) on HR, analyzed and plotted as simple moving average with a period of 10 min. HR at 0 min corresponds to the 30-min average baseline HR on the day of the experiment. Two-way ANOVA analysis (genotype and time) of the 120-min post-injection interval revealed main effects of genotype ($F_{1,104}=21.6$; $P < 0.001$). **D**, Effect of atropine (1 mg/kg, i.p.) on HR, analyzed and plotted as simple moving averages with a period of 10 min. No significant difference in HR was observed during the 60-min post-injection interval ($F_{1,56}=0.02$; $P=0.89$).