SUPPLEMENT MATERIAL

METHODS

Antibodies, Recombinant Proteins, DNA Constructs. Sheep anti-RGS6 antibodies (α-RGS6-FL) were generated against recombinant fragment containing amino acids 263-472 of mouse RGS6 that was expressed and purified from *E.coli* as described[1]. Antibodies were affinity-purified on the epitope-conjugated column and stored in PBS buffer containing 50% glycerol. Rabbit anti-Gβ5 (SGS) and rabbit anti-R7BP (TRS) antibodies were a generous gift from Dr. William Simonds, NIDDK. Rabbit polyclonal anti-AU1 tag (GenScript, Piscataway, NJ), mouse monoclonal anti-AU5 (MMS-135R; Covance, Princeton, NJ), goat polyclonal anti-RGS4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-PSD95 (Millipore, Billerica, MA) rabbit polyclonal anti-$G\alpha i_{1/2}$ (Thermo Fisher Scientific, Rochford, IL) and rabbit polyclonal anti-$G\alpha_o$ (K20; Santa Cruz Biotechnology, Santa Cruz, CA) were purchased. All general chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Cloning of full-length mouse Gβ5S and RGS6 was described previously[2,3]. The open reading frame of Gβ5S was subcloned into pcDNA3.1/TOPO (Invitrogen) mammalian expression vector, and Rgs6 was cloned into pcDNA3.1NT-GFP-TOPO (Invitrogen) creating an N-terminal fusion with GFP. Cloning of Girk1-AU5 and Girk4-AU1 into mammalian expression vectors has been described[4]. All constructs were propagated using an *E.coli* Top-10 strain (Invitrogen), isolated using Nucleobond kits, (Macherey-Nagel; Bethlehem, PA) and sequenced.

Mouse Strains. The generation of *R7bp*$^{-/-}$[5], *Gβ5*$^{-/-}$[6], and *Girk4*$^{-/-}$[7] mice has been described previously. *Gβ5*$^{-/-}$ mice were generously provided by Dr. Jason Chen (Virginia Commonwealth University). These three strains of mice were out-bred onto the C57BL/6 background for at least 5 generations. Rgs6$^{-/-}$ mice were generated by Lexicon Pharmaceuticals using 129SvEvBrd embryonic stem cells. Chimeric offspring were mated with C57BL/6 strain and the resulting heterozygous progeny were inbred to generate null mutant and wild-type littermates. Mice were housed in groups on a 12h light/dark cycle with food and water available *ad libitum.* All procedures were carried out in accordance with the National Institute of Health guidelines and were granted formal approval by the Institutional Animal Care and Use Committee of the University of Minnesota. All efforts were made to minimize the use of animals in this study, as well as their suffering. All animals used in this study were bred on-site.

Cell culture and transfections. HEK293FT cells were obtained from Invitrogen (Carlsbad, CA) and cultured at 37°C and 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium; GIBCO) supplemented with 100 units of penicillin and 100 mg of streptomycin, 10% FBS, 1x MEM non-essential amino acids (GIBCO; Carlsbad, CA), 1 mM sodium pyruvate and 4 mM L-glutamine. Cells were transfected at ~70% confluency, using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. The ratio of Lipofectamine to DNA used was 6.25 μl : 2.5 μg per 10 cm^2 cell surface. Cells were grown for 24-48 hours post-transfection. Equal amounts of each construct were transfected, balanced when necessary by empty pcDNA3.1 vector.

Immunoprecipitation assays and Western blotting. Cellular and tissue lysates were prepared in immunoprecipitation (IP) buffer (1XPBS (Fisher Scientific), 150 mM NaCl, 1% Triton X-100, protease inhibitors (Roche; Indianapolis, IN) and centrifuged for 15 min at 14,000 x g. Protein concentration was determined in the resulting extracts using BSA assay (Pierce; Rockford, IL) and equal amounts of protein were incubated with 3 μg of antibodies and 10 μl of protein G beads (GE Healthcare; Waukesha,WI) for 1 h at 4^0C. After 3 washes with ice-cold IP buffer proteins bound to the beads were eluted with SDS-sample buffer. Eluates were resolved on 12.5% SDS-PAGE gel, transferred onto PVDF membrane (Millipore (Billerica, MA)) and subjected to Western blot analysis using HRP conjugated secondary antibodies and an ECL West Pico (Pierce) detection system. For quantification, samples were analyzed

by infrared Western blotting using IRDye680 and IRDye800 labeled secondary antibodies (Li-Cor Biosciences; Lincoln, NE) according to the manufacturer's protocol. Detection and quantification of specific bands was performed on an Odyssey Infrared Imaging System (Li-Cor Biosciences). The integrated intensity of each band of interest was measured in a corresponding channel with a top-bottom background setting.

Adult cardiomyocyte isolation. Atrial cardiomyocytes were isolated from adult mice (1 m.o.). Hearts were excised into Tyrode's solution (in mM): 140 NaCl, 5.4 KCl, 1.2 KH2PO4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 5 HEPES, pH 7.4 with NaOH. Atria were dissected, cut into strips and placed into a modified Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES, 0.1% BSA, pH 6.9 with NaOH, with elastase (0.3 mg/ml; Worthington Biochemical Corp.), collagenase II (0.21 mg/ml; Worthington Biochemical Corp.) and proteinase XIV (0.2 mg/ml; Sigma). Tissue was digested at 37°C for 1 hr, with occasional inversion, and then washed twice in a solution containing (in mM): 100 L-glutamic acid/potassium salt, 10 L-aspartic acid/potassium salt, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, 0.1% BSA, pH 7.2 with KOH. Atrial tissue was then triturated in the wash solution, and cell pellet (50g, 5 min) was washed twice with PBS (Fisher Scientific) and lysed in IP buffer (PBS, 150 mM NaCl, 1% Triton, protease inhibitor coctail (Roche).

Whole-cell electrophysiology. Primary cultures of atrial myocytes were generated from neonatal mice (P2-4) as described [8,9], using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp., Lakewood, NJ). Atrial myocytes were used for electrophysiological analysis after 1-3 d in culture. Sinoatrial nodal cells were isolated from adult mice (3 months) as described[10], and used within 8 h of isolation. In brief, hearts were excised into Tyrode's solution (in mM): 140 NaCl, 5.4 KCl, 1.2 KH2PO4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 5 HEPES, pH 7.4 with NaOH. The sinoatrial node (SAN) was identified as the narrow band of tissue located on the inner wall of the right atrium, medial to the crista terminalis and between the superior and inferior vena cava. Two incisions were made to the superficial side of the superior and inferior vena cava, followed by a longer cut along the outer atrial wall, to expose the SAN region. SAN-containing tissue was excised into a modified Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES, 0.1% BSA, pH 6.9 with NaOH, with elastase (0.3 mg/ml; Worthington Biochemical Corp.) and collagenase II (0.21 mg/ml; Worthington Biochemical Corp.). SAN tissue was digested at 37°C for 30 min, with occasional inversion, and then washed three times in a solution containing (in mM): 100 L-glutamic acid/potassium salt, 10 L-aspartic acid/potassium salt, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, 0.1% BSA, pH 7.2 with KOH. SAN tissue was then triturated in the wash solution and plated onto poly-L-lysine coated coverslips for electrophysiological studies.

Coverslips containing atrial myocytes or SAN cells were transferred to a chamber containing a low-K^+ bath solution (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 HEPES/NaOH (pH 7.4). Cardiac cells were visualized using an Olympus IX-70 microscope. The dominant population of atrial myocytes with spherical shape (typical capacitance, 10-20 pF) was targeted for this study. SAN cells were identified as the thin striated cells exhibiting spontaneous contractions (typical capacitance, 25-40 pF) (see **Fig. 2A,** inset). Membrane potentials and whole-cell currents were measured with hardware (Axopatch-200B amplifier, Digidata 1320) and software (pCLAMP v. 9.2) from Molecular Devices (Sunnyvale, CA). Borosilicate patch pipettes (3-5 MΩ) were filled with (in mM): 130 gluconate, 2 $MgCl_2$, 1.1 EGTA/KOH (pH 7.2), 5 HEPES/KOH (pH 7.2), 2 Na_2ATP, 5 phosphocreatine, 0.3 Na-GTP.

Upon achieving whole-cell access, input resistance, capacitance, and resting membrane potentials were measured. Neonatal atrial myocytes and SAN cells from wild-type and knockout mice did not differ with respect to these parameters. CCh-induced currents were measured at a holding potential of -70 mV using a high-K^+ bath solution (in mM): 120 NaCl, 25 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 HEPES/NaOH (pH 7.4). The high-K^+ bath solution (+/-CCh) was applied with an SF-77B rapid perfusion system (Warner Instruments, Inc.; Hamden, CT). In pilot studies, we found no difference in

current amplitudes evoked by 10 and 100 μM CCh, irrespective of genotype or cell type. As such, 10 μM CCh was taken as the saturating CCh concentration for these studies. All currents were low-pass filtered at 1 kHz, sampled at 2 kHz, and stored on computer hard disk for analysis. Steady-state current amplitudes were measured for each experiment by subtracting the baseline current from the current measured just prior to the return to drug-free solution. Activation and deactivation time constants were extracted from appropriate regions of current traces, which were fit with a 1-term Boltzmann equation using the Levenberg-Marquardt search method, sum of squared errors minimization method, and no weighting (**Online Fig. V**). Only those experiments for which the access resistances were stable and low (<15 MΩ) were included in the final analysis.

Telemetry. Wild-type (n=5) and *Rgs6*$^{-/-}$ (n=5) littermates aged 4-5 months were used for *in vivo* ECG monitoring. Biopotential telemetry transmitters ETA-F10 (Data Sciences International; Saint Paul, MN) were implanted intraperitoneally under kethamine/xylazine anesthesia (60 and 12 mg/kg correspondently). ECG leads were externalized and abdominal wall was closed with Prolene 5-0 (Ethicon; Somerville, NJ) incorporating suture rib of the transmitter into the closure. ECG leads were tunneled under the skin into lead II position and sutured to the abdominal wall by Prolene 5-0. Skin incisions were closed using Vicryl 5-0 (Ethicon). Upon termination of anesthesia, animals received a single intraperitoneal injection of ketoprofen (5 mg/kg), followed by administration of ibuprofen and amoxicillin in drinking water during recovery period (days 1-10). Upon recovery, recordings were performed in a scheduled manner, for 20 s each min, following 1-h acclimation using Dataquest ART 4.2 acquisition software (Data Sciences International). On day 11, 6 h of baseline ECG data were recorded. On day 12, after 30 min of baseline recording, animals were injected i.p. with 0.9% saline solution (10 ml/kg) as a vehicle control. Atropine sulfate was injected 2 h later (1 mg/kg, i.p.; Hospira, Lake Forest, IL), followed by 3 h of recording. On day 13, following 30 min of baseline recording, animals were injected first with 0.9% saline solution and 2 h later with CCh (0.1 mg/kg, i.p.; Acros Organics, Geel, Belgium). Recording proceeded for 3 h, after which animals were sacrificed by CO_2 inhalation. Transmitters were explanted, cleaned with 1% Tergazyme enzymatic detergent (Alconox; White Plains, NY), sterilized with Cidex activated dialdehyde solution (Ethicon), and reused.

Statistical Analysis. Statistical analyses were performed using Prism (GraphPad Software, Inc.; La Jolla, CA) and SigmaPlot 11 (Systat Software Inc; San Jose, CA). EC_{50} values were calculated with the Hill coefficient set to 1. The impact of genotype on CCh-induced current responses (steady-state current density and kinetics) was evaluated using one-way (single-saturating concentration study) and two-way (concentration-response study) ANOVA. The impact of genotype on CCh- and atropine-induced heart rate response was evaluated using two-way (time-response study) ANOVA. Tukey's Multiple Comparison (one-way ANOVA) and Bonferroni (two-way ANOVA) post hoc tests were used as appropriate. For all analyses, the level of significance was set at $P < 0.05$.

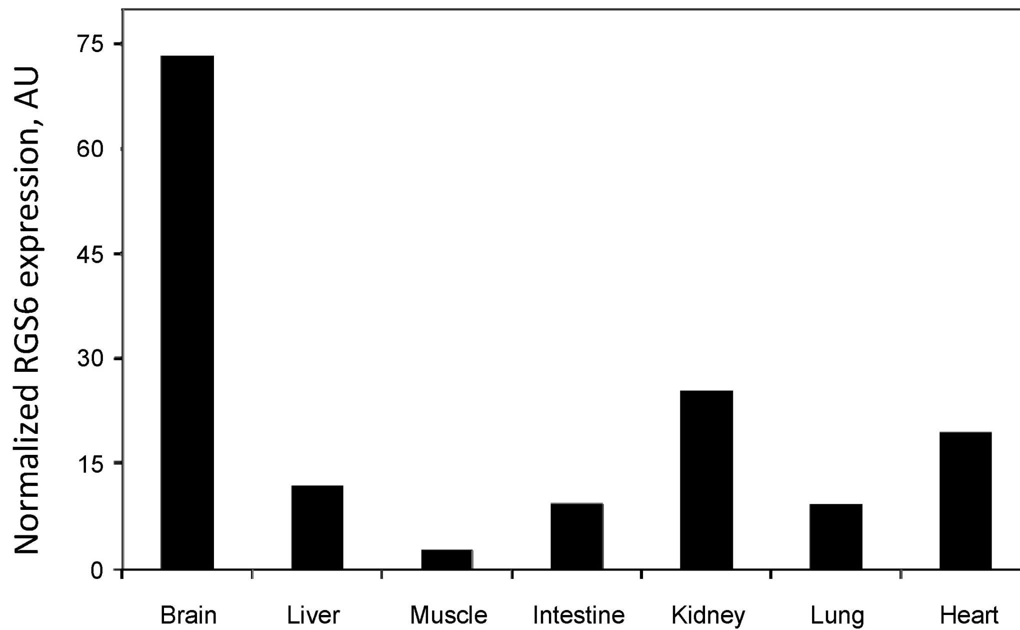
REFERENCES

1. Martemyanov KA, Arshavsky VY. Noncatalytic domains of RGS9-1.Gβ5L play a decisive role in establishing its substrate specificity. *J Biol Chem.* 2002;277:32843-32848.
2. Martemyanov KA, Yoo PJ, Skiba NP, Arshavsky VY. R7BP, a novel neuronal protein interacting with RGS proteins of the R7 family. *J Biol Chem.* 2005;280:5133-6.
3. Martemyanov KA, Hopp JA, Arshavsky VY. Specificity of G protein-RGS protein recognition is regulated by affinity adapters. 2003;38:857-862.
4. Kennedy ME, Nemec J, Clapham DE. Localization and interaction of epitope-tagged GIRK1 and CIR inward rectifier K+ channel subunits. *Neuropharmacology.* 1996;35:831-9.
5. Anderson GR, Lujan R, Semenov A, Pravetoni M, Posokhova EN, Song JH, Uversky V, Chen CK, Wickman K, Martemyanov KA. Expression and localization of RGS9-2/Gβ5/R7BP complex

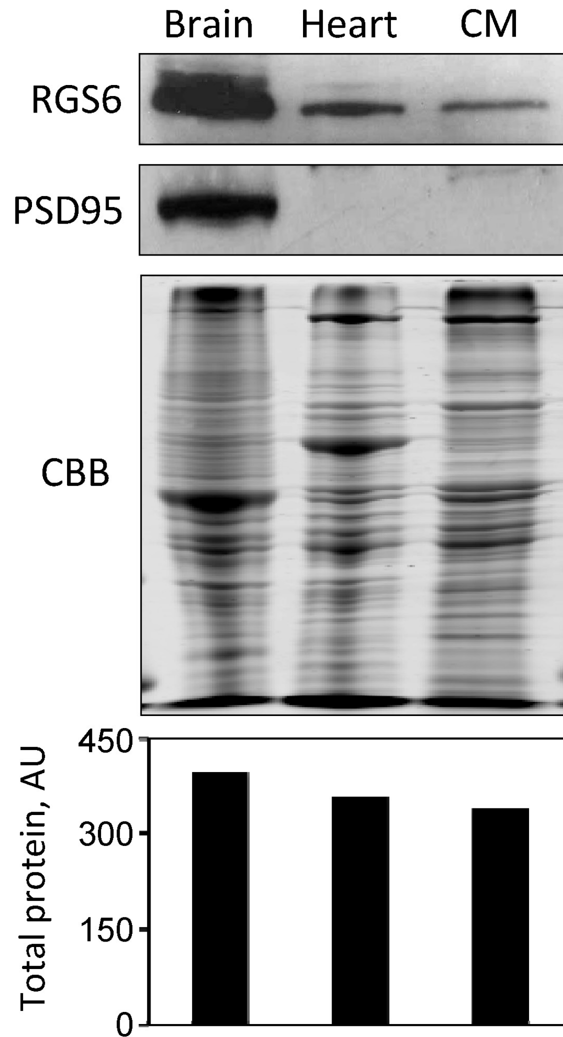
in vivo is set by dynamic control of its constitutive degradation by cellular cysteine proteases. *J Neurosci*. 2007;27:14117-27.

6. Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, He W, Wensel TG, Simon MI. Instability of GGL domain-containing RGS proteins in mice lacking the G protein beta-subunit Gbeta5. *Proc Natl Acad Sci U S A*. 2003;100:6604-9.
7. Wickman K, Nemec J, Gendler SJ, Clapham DE. Abnormal heart rate regulation in GIRK4 knockout mice. *Neuron*. 1998;20:103-14.
8. Bettahi I, Marker CL, Roman MI, Wickman K. Contribution of the Kir3.1 subunit to the muscarinic-gated atrial potassium channel IKACh. *J Biol Chem*. 2002;277:48282-8.
9. Koyrakh L, Roman MI, Brinkmann V, Wickman K. The heart rate decrease caused by acute FTY720 administration is mediated by the G protein-gated potassium channel I. *Am J Transplant*. 2005;5:529-36.
10. Cifelli C, Rose RA, Zhang H, Voigtlaender-Bolz J, Bolz SS, Backx PH, Heximer SP. RGS4 regulates parasympathetic signaling and heart rate control in the sinoatrial node. *Circ Res*. 2008;103:527-35.

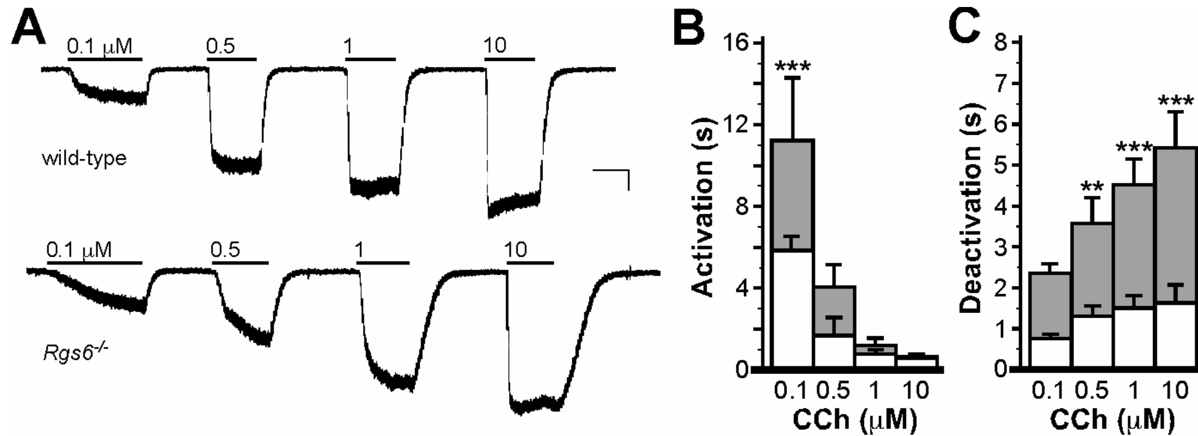
FIGURES



Online Figure I. Quantitative analysis of Rgs6 distribution across tissues. Rgs6 band densities from Western blot experiment presented in Figure 1A have been determined by densitometry using Image J software and normalized to the total protein content determined from CBB stained gel (Figure 1A). Resulting values representing relative abundance of Rgs6 proteins across tissues are plotted as a bar graph.

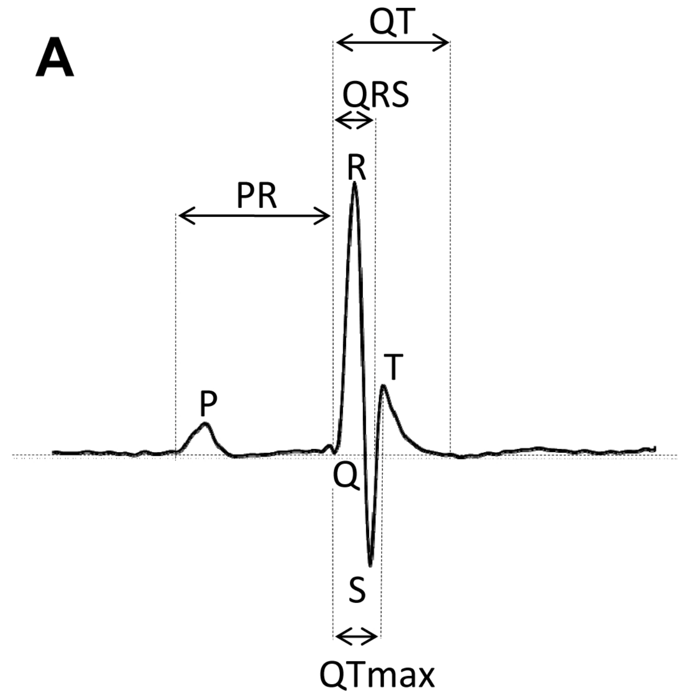


Online Figure II. Rgs6 is present in isolated atrial cardiomyocytes. Cardiomyocytes were isolated from adult mice as described in Methods section above. Following lysis in SDS sample buffer, 25 µg of total protein was loaded on the gel. Rgs6 expression was detected by Western blotting with specific anti-Rgs6-FL antibodies. Brain tissue was used as a control. The absence of the immunoreactivity for the neuronal specific marker PSD95 in isolated cardiomyocyte fraction (CM) demonstrate that Rgs6 is predominantly expressed in the myocytes. Protein loading was verified by Coomasie Brilliant Blue (CBB) following separation on SDS-PAGE gel. Densitometric analysis of the total protein content quantified from the CBB-stained gel is presented in the lower panel.



Online Figure III. Impact of *Rgs6* ablation on m_2R-I_{KACh} signaling in atrial myocytes. A, Inward currents evoked by CCh (0.1-10 μmol/L) in atrial myocytes from wild-type and *Rgs6*$^{-/-}$ mice. Scale bars: 15 s/100 pA. Evoked currents developed gradually at 0.1 μmol/L CCh and saturated at 10 μmol/L CCh (responses to 10 μmol/L CCh are not shown). Half-saturating parameters were for wild-type: EC_{50} = 0.25 to 0.49 μmol/L (95% CI) and for *Rgs6*$^{-/-}$: EC_{50} = 0.21 to 0.62 μmol/L (95% CI). Steady-state current densities did not differ between *Rgs6*$^{-/-}$ (-36±4 pA/pF at 10 μmol/L CCh, n=7) and wild-type (-42±6 pA/pF, n=7) myocytes. **B,** Summary of m_2R-I_{KACh} activation kinetics in atrial myocytes from wild-type and *Rgs6*$^{-/-}$ mice. Main effects of concentration ($F_{3,43}$=25.5; *P*<0.001) and genotype ($F_{1,43}$=13.3; *P*<0.001) were observed, as well as a concentration x genotype interaction ($F_{3,43}$=4.1; *P*<0.05). Symbols: *** *P*<0.001 vs. wild-type (within dose). **C,** Summary of m_2R-I_{KACh} deactivation kinetics in atrial myocytes from wild-type and *Rgs6*$^{-/-}$ mice. Main effects of concentration ($F_{3,44}$=5.5; *P*<0.01) and genotype ($F_{1,44}$=58.9; *P*<0.001) were observed, and there was no concentration x genotype interaction ($F_{3,44}$=1.7; *P*=0.17). Symbols: *,** *P*<0.05 and 0.01, respectively, vs. wild-type (within dose).

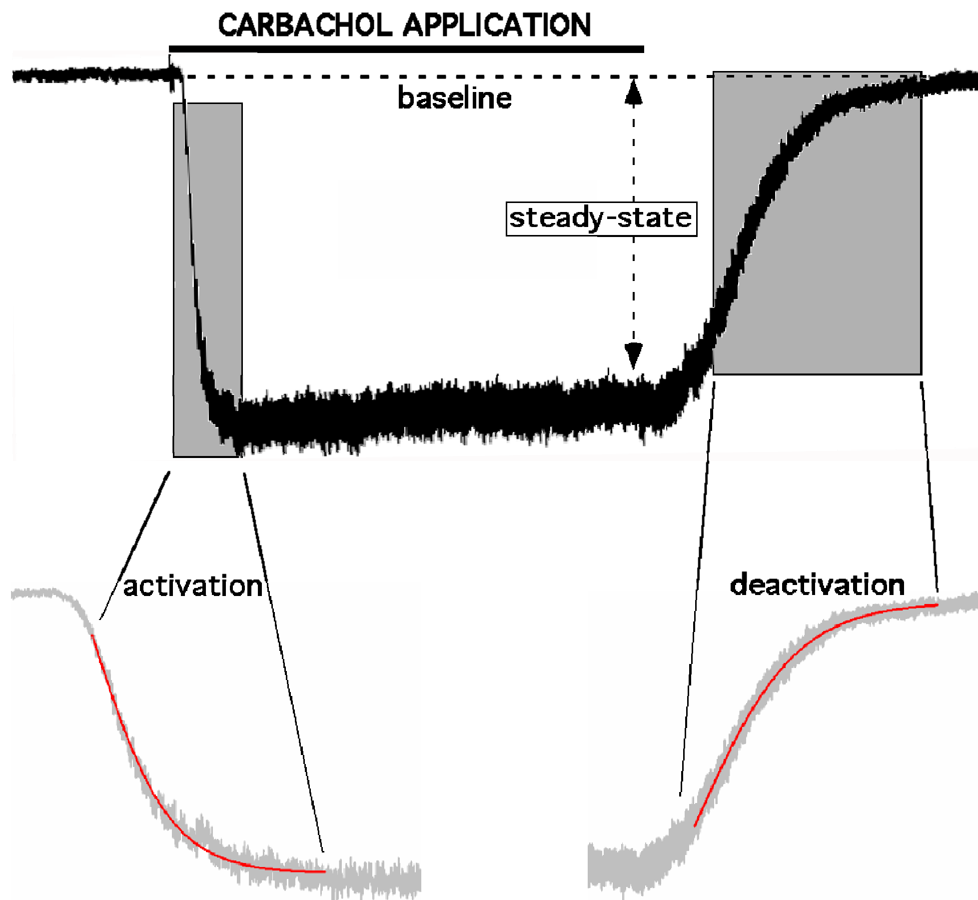
A



B

Interval, ms±SEM	RGS6$^{+/+}$	RGS6$^{-/-}$
PR	39.41±1.08	40.94±2.12
QRS	9.68±0.49	10.14±0.55
QT	44.12±1.91	38.75±2.08
QTmax	12.06±0.60	11.88±0.29

Online Figure IV. Quantitative analysis of ECG intervals in RGS6$^{-/-}$ mice. A, Representative ECG trace obtained from RGS6$^{-/-}$ mice. Peaks and intervals used for the quantitative analysis are annotated. **B,** Quantitative analysis of the ECG traces. Data derived from the analysis of total 500 representative traces from 3 to 5 mice of each genotype. Values were averaged separately for each animal. Group sizes were defined as a number of unique animals used for the analysis (n=3-5). Errors are s.e.m values.



Online Figure V. Depiction of measured parameters for the whole-cell CCh-induced current studies. A typical response of a wild-type neonatal atrial myocyte to 10 μM CCh is shown, with the horizontal line showing the duration of CCh application. Current amplitude and density determinations involved steady-state currents, measured relative to baseline just prior to the removal of CCh. Shaded rectangles identify the regions of the trace used for determination of current activation and deactivation kinetics. The fit curves, derived from a 1-term standard Boltzmann equation, are shown in red.