Molecular Mechanisms, and Selective Pharmacological Rescue, of Rem-Inhibited \( \text{Ca}_{v}1.2 \) Channels in Heart

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**Rationale:** In heart, \( \text{Ca}^{2+} \) entering myocytes via \( \text{Ca}_{v}1.2 \) channels controls essential functions, including excitation–contraction coupling, action potential duration, and gene expression. RGK GTPases (Rad/Rem/Rem2/Gem/Kir sub-family of Ras-like GTPases) potently inhibit \( \text{Ca}_{v}1.2 \) channels, an effect that may figure prominently in cardiac \( \text{Ca}^{2+} \) homeostasis under physiological and disease conditions.

**Objective:** To define the mechanisms and molecular determinants underlying Rem GTPase inhibition of \( \text{Ca}_{v}1.2 \) channels in heart and to determine whether such inhibited channels can be pharmacologically rescued.

**Methods and Results:** Overexpressing Rem in adult guinea pig heart cells dramatically depresses L-type calcium current \( (I_{\text{Ca,L}}) \) (90% inhibition) and moderately reduces maximum gating charge \( (Q_{\text{max}}) \) (33%), without appreciably diminishing the physical number of channels in the membrane. Rem-inhibited \( \text{Ca}_{v}1.2 \) channels were supramodulated by BAY K 8644 (10-fold increase) compared to control channels (3-fold increase). However, Rem prevented protein kinase A–mediated upregulation of \( I_{\text{Ca,L}} \), an effect achieved without disrupting the sympathetic signaling cascade because protein kinase A modulation of \( I_{\text{KS}} \) (slow component of the delayed rectifier potassium current) remained intact. In accord with its functional impact on \( I_{\text{Ca,L}} \), Rem selectively prevented protein kinase A– but not BAY K 8644–induced prolongation of the cardiac action potential duration. A GTP-binding-deficient Rem[\( T94N \)] mutant was functionally inert with respect to \( I_{\text{Ca,L}} \) inhibition. A chimeric construct, Rem\(_{65\alpha}\), featuring a swap of the Rem C-terminal tail for the analogous domain from H-Ras, inhibited \( I_{\text{Ca,L}} \) and \( Q_{\text{max}} \) to the same extent as wild-type Rem, despite lacking the capacity to autonomously localize to the sarcolemma.

**Conclusions:** Rem predominantly inhibits \( I_{\text{Ca,L}} \) in heart by arresting surface \( \text{Ca}_{v}1.2 \) channels in a low open probability gating mode, rather than by interfering with channel trafficking. Moreover, Rem-inhibited \( \text{Ca}_{v}1.2 \) channels can be selectively rescued by BAY K 8644 but not protein kinase A–dependent phosphorylation. Contrary to findings in reconstituted systems, Rem-induced ablation of cardiac \( I_{\text{Ca,L}} \) requires GTP-binding, but not membrane-targeting of the nucleotide binding domain. These findings provide a different perspective on the molecular mechanisms and structural determinants underlying RGK GTPase inhibition of \( \text{Ca}_{v}1.2 \) channels in heart, and suggest new (patho)physiological dimensions of this crosstalk. (Circ Res. 2010;107:620-630.)

**Key Words:** L-type calcium channels • Rem • RGK GTPase • cardiac myocytes
by associating with auxiliary CaVβ subunits.12-13 Distinct RGK GTPases (Rad/Rem/Rem2/Gem/Kir subfamily of Ras-like GTPases) are expressed in heart, and changes in their levels occur under different pathological conditions. For example, expression of Rem in ventricular myocytes is downregulated during conditions mimicking inflammation; Gem is upregulated in the failing human heart; and Rad knockout mice develop cardiac hypertrophy.15 Owing to their strong impact on CaV1.2 channels, RGK GTPases may profoundly regulate Ca2+ signaling in heart under both physiological and disease states.

This study focuses on 2 fundamental questions: (1) How do RGK GTPases inhibit whole-cell ICa,L in heart? (2) Can RGK GTPase–inhibited channels be functionally rescued by pharmacological or physiological molecules? Overexpressing Gem or Rad in heart dramatically inhibits ICa,L.16,17 It has been suggested that RGK proteins inhibit ICa,L in heart by interfering with channel trafficking, thereby reducing the surface density of cardiac CaV1.2 channels. However, in other cell types, it has been demonstrated that RGK proteins can inhibit ICa without reducing the surface density of channels.18,19 In HEK 293 cells, Rem inhibits recombinant CaV1.2 channels using 3 separate mechanisms: by reducing surface density of channels, accomplished through enhancing dynamin-dependent endocytosis; by immobilizing CaV channel voltage sensors; and by arresting channels that remain on the surface in a low open probability (Po) gating mode.20 Whether RGK GTPases use a similar multiplicity of mechanisms to inhibit CaV1.2 channels in heart has not been explored. Ambiguity also surrounds the role played by key structural features of RGK GTPases in blocking ICa,L in heart. For example, GTP binding to the NBD is dispensable for Rem inhibition of CaV1.2 and 2 channels reconstituted in HEK 293 cells.20 However, in heart, a GTP-binding-deficient Rad mutant displays a dominant negative effect that results in enhancement of ICa,L.17 It is unknown whether these variations reflect genuine differences among distinct RGK GT-Pases, or whether they are cell type–specific. Similarly, in some systems, membrane targeting of the NBD is necessary and sufficient for Rem inhibition of ICa,L.18,21,22 Whether this rule holds true in heart is unclear. Finally, it is unknown whether RGK GTPase–inhibited CaV1.2 channels are irretrievably lost or whether they can be acutely rescued by agents that normally upregulate ICa,L in heart. Discovering molecules that can functionally rescue RGK GTPase–inhibited CaV1.2 channels may not only refine understanding of the mechanisms underlying this modulation but also have potential therapeutic applications by reversing cardiac dysfunction arising from RGK GTPase-mediated block of cardiac ICa,L.

Here, we find that overexpressing Rem in isolated guinea pig ventricular myocytes markedly inhibits ICa,L (90% reduction) and more modestly decreases maximum gating charge (Qmax) (33% reduction), without appreciably decreasing the surface density of channels. Contrary to results in reconstituted systems, GTP binding to the NBD was necessary for Rem inhibition of ICa,L in heart. However, membrane targeting of the NBD was neither necessary nor sufficient for blocking cardiac CaV1.2 channels. Rem-inhibited CaV1.2 channels could be selectively upregulated by BAY K 8644 but were insensitive to the physiological sympathetic β-adrenergic signaling pathway. The results offer a new perspective on the mechanisms of RGK GTPase inhibition of cardiac ICa,L and reveal an intriguing crosstalk between 2 opposing signaling pathways that converge on CaV1.2 channels in the heart.

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

Construction of Adenoviral Vectors
Adenoviral vectors were generated using the AdEasy XL system (Stratagene, La Jolla, Calif) according to the instructions of the manufacturer. Adenoviral amplification and purification were as described previously.23,24

Cell Culture and Transfection
Primary cultures of adult guinea pig heart cells were prepared as described previously.25 Adult male Hartley guinea pigs were killed with an overdose of isoflurane in accordance with the guidelines of the Columbia University Animal Care and Use Committee. Hearts were excised, and ventricular myocytes were isolated by enzymatic digestion using a Langendorf perfusion apparatus and cultured on laminin-coated coverslips. Myocytes were infected with adenovirus 3 to 5 hours after initial plating.

Immunofluorescence Detection of CaV1.2 Channels
Isolated heart cells plated on glass-bottom 35-mm MatTek dishes were infected with adenovirus. After 24 hours, cells were fixed with 2% paraformaldehyde in PBS and washed with 0.1 mol/L glycine–PBS. Fixed myocytes were permeabilized with 0.2% Triton X-100 in PBS followed by a 1 hour of incubation in blocking solution (3% goat serum/1% BSA/0.1% Triton X-100). Cells were then incubated with anti-CaV1.2 antibody (1:1,000 rabbit polyclonal) in blocking media for 1 hour at room temperature. Cells were then washed and exposed to Alexa Fluor 594–conjugated goat anti-rabbit IgG (H+L) (1:400, Invitrogen Molecular Probes, Carlsbad, Calif) for 1 hour at room temperature in blocking solution. Immunofluorescence detection was performed using a Leica TCS SP2 AOBS MP confocal microscope system (Leica Microsystems, Heidelberg, Germany).
Data acquisition and analysis were performed using Leica LCS imaging software.

**Electrophysiology**

Whole-cell and gating currents were recorded from cultured ventricular myocytes as described previously.23

**Statistics**

Pooled data are presented as means±SEM, and probability values were calculated using Student’s 2-tailed t test; \( P<0.05 \) was considered significant.

**Results**

**Rem Inhibition of Ca\(_{v}\)1.2 Channels in Adult Guinea Pig Heart Cells**

We assessed the impact of Rem on endogenous Ca\(_{v}\)1.2 channels in cultured adult guinea pig ventricular myocytes using adenovirus-mediated somatic gene transfer. Control, uninfected myocytes displayed robust whole-cell Ca\(_{v}\)1.2 currents that activated at a threshold of \(-40\) mV and peaked at 0 mV (Figure 1A through 1C). Myocytes infected with adenovirus coding for cyan fluorescent protein (CFP)-Rem, \( I_{\text{peak}} \) relationship for CFP-Rem myocytes (circles) \((n=7)\). The control data are reproduced (gray trace) to facilitate visual comparison. G through I, Data for myocytes expressing YFP-Rem\(_{265}\). Same format as D through F.

Figure 1. Rem inhibits Ca\(_{v}\)1.2 channels in cultured adult cardiac myocytes. A, Top, Voltage protocol. Bottom, Gray scale image of a cultured guinea pig ventricular myocyte. B, Exemplar whole-cell Ca\(_{v}\)1.2 channel currents from a cultured myocyte. C, Population peak current density versus voltage \( (I_{\text{peak}}-V) \) relationship for uninfected myocytes (squares) \((n=7\) for each point). D, Top, Schematic of CFP-Rem depicting the NBD (hexagon) appended by N- and C termini extensions. Zigzag line represents the membrane-targeting domain in the distal C terminus. Bottom, Confocal image of a myocyte infected with CFP-Rem adenovirus. E, Exemplar whole-cell currents from a myocyte expressing CFP-Rem. F, Population \( I_{\text{peak}} \) relationship for CFP-Rem myocytes (circles) \((n=7)\). The control data are reproduced (gray trace) to facilitate visual comparison. G through I, Data for myocytes expressing YFP-Rem\(_{265}\). Same format as D through F.

Data acquisition and analysis were performed using Leica LCS imaging software.

control uninfected cells \( I_{\text{peak}}=2.67\pm0.35\) pA/pF, \( n=7\), for CFP-Rem-expressing cells; \( I_{\text{peak}}=18.82\pm2.31\) pA/pF, \( n=7\), for control cells; \( P=0.0007\); Online Table I). It has been established that RGK GTPases target the plasma membrane using electrostatic and hydrophobic interactions involving amino acid residues in the distal C terminus.26 In HEK 293 cells, deleting the distal Rem C-terminal extension (generating Rem\(_{265}\); Figure 1G, top) eliminates Rem targeting to the plasma membrane and prevents its capacity to block \( I_{\text{Ca,L}} \).13,22 Similarly, adult guinea pig myocytes infected with yellow fluorescent protein (YFP)-Rem\(_{265}\) adenovirus displayed YFP fluorescence throughout the cytosol and nuclei, without any evident targeting to the sarcolemma or T-tubule network (Figure 1G, bottom; Online Figure I). Most importantly, myocytes expressing YFP-Rem\(_{265}\) exhibited robust whole-cell Ca\(_{v}\)1.2 currents that were essentially the same as those recorded from uninfected cells with respect to amplitude and voltage-dependent properties (Figure 1H and 1I; \( I_{\text{peak}} \) at 0 mV=21.69±1.93 pA/pF, \( n=6\); \( P=0.36 \) compared to uninfected myocytes; Online Table I). Because of its inertness in blocking \( I_{\text{Ca,L}} \), we used cells expressing YFP-Rem\(_{265}\) as effective controls in subsequent experiments.

These results establish that overexpressing Rem in guinea pig ventricular myocytes dramatically decreases \( I_{\text{Ca,L}} \), in agreement with previous observations made using Gem and Rad.16,17 The marked inhibition of \( I_{\text{Ca,L}} \) suggests that in this experimental paradigm, most Ca\(_{v}\)1.2 channels are modified by Rem. This fact greatly simplifies and justifies the use of this experimental system to probe the
mechanistic bases of RGK GTPase inhibition of CaV1.2 channels in heart.

Impact of Rem on $Q_{\text{max}}$ and Surface Density of CaV1.2 Channels in Heart

In HEK 293 cells, Rem inhibits recombinant CaV1.2 channels by 3 distinct mechanisms.20 It has been suggested that Gem and Rad inhibit cardiac $I_{Ca,L}$ by disrupting channel trafficking and, thus, reducing CaV1.2 channel surface density.16,17 However, whether RGK GTPases use multiple mechanisms to inhibit CaV1.2 channels in heart has not been rigorously studied. To address this deficit, we next investigated the impact of Rem on gating charge and subcellular localization of CaV1.2 channels in heart cells (Figure 2).

To isolate CaV1.2 channel gating currents, we used a holding potential of $-50$ mV and blocked ionic currents with 2 mmol/L CdCl$_2$/0.1 mmol/L LaCl$_3$.23 Comparing exemplar ON gating currents between Rem265- and Rem-expressing myocytes indicated that Rem reduced, but did not eliminate this gating parameter (Figure 2A). This impression was confirmed in population ON gating charge versus voltage ($Q_{\text{ON}}$-$V$) relationship for Rem$_{265}$-expressing (squares) ($n=8$ for each point) and Rem-expressing (circles) ($n=7$ for each point) myocytes, *$P<0.05$. Representative confocal images of CaV1.2 immunofluorescence in heart cells expressing YFP-Rem$_{265}$ or CFP-Rem. All fluorescence images were collected at the same gain setting of the microscope. C, Average CaV1.2 immunofluorescence staining intensity measurements ($n=4$ for each condition). Control bar represents analyses of fluorescence images obtained when primary antibody was preincubated with the antigen peptide.

We used immunofluorescence to examine the impact of Rem on CaV1.2 channel subcellular localization in heart cells (Figure 2C). In myocytes expressing YFP-Rem$_{265}$, CaV1.2 channel staining presented as punctate clusters organized along z-lines (Figure 2C, top row). Cells expressing CFP-Rem displayed a CaV1.2 channel staining pattern similar to control cells (Figure 2C, bottom row). The preferential targeting of CFP-Rem to the sarcolemma and T-tubules made it possible to explicitly confirm that CaV1.2 channel staining was present at the cell surface (Figure 2C). Controls in which CaV1.2 antibody was preincubated with immunizing antigen displayed low fluorescence, ruling out nonspecific staining (Online Figure II). Quantifying CaV1.2 immunofluorescence staining across a number of cells indicated no difference between myocytes expressing Rem$_{265}$ or Rem (Figure 2D), in agreement with the impression gathered from visual inspection. These data argue against diminished trafficking of CaV1.2 channels as a dominant mechanism for Rem-induced decrease in cardiac $I_{Ca,L}$.

Thus, under conditions where Rem severely depresses $I_{Ca,L}$ in heart, most CaV1.2 channels remain in the cardiac sarcolemma. In light of this, the moderate reduction in $Q_{\text{max}}$ suggests that Rem partially immobilizes the voltage sensors of sarcolemmal CaV1.2 channels. The data also suggest that Rem-modified CaV1.2 channels in the sarcolemma are either electrically silent or populate a low-$P_o$ gating mode.
Selective Pharmacological Rescue of Rem-Inhibited CaV1.2 Channels in Heart

The notion that Rem-modified CaV1.2 channels remain in the sarcolemma suggested it might be possible to acutely rescue such inhibited channels pharmacologically. We investigated this possibility using the CaV1 channel agonist BAY K 8644. Control myocytes expressing Rem265 showed a rapid and robust increase in current amplitude in response to 1 μmol/L BAY K 8644 at all voltages (Figure 3A through 3C). On average, control cells exhibited a 3-fold increase in currents evoked with a 0-mV test pulse (Figure 3G). Remarkably, cells expressing Rem also responded to 1 μmol/L BAY K 8644, with a large and rapid increase in ICa,L at all voltages (Figure 3D through 3F). In fact, the average fold increase in ICa,L was substantially larger for Rem-expressing myocytes compared to control cells (Figure 3G; fold increase=9.42±1.05, n=6 for control YFP-Rem265-expressing cells; fold increase=3.18±0.46, n=6 for control YFP-Rem265-expressing cells). BAY K 8644 did not increase Qmax in either YFP-Rem265- or CFP-Rem–expressing cells (Online Figure IV), eliminating recovery of Qmax as a potential mechanism to explain the larger effect of BAY K 8644 on cells expressing Rem compared to control.

The pharmacological rescue of Rem-inhibited currents was intriguing and gave reason to wonder whether physiological...
upregulators of Ca\(^{\text{V}}\)\(_{1.2}\) channels could similarly nullify Rem block of I\(_{\text{Ca,L}}\). In heart, activation of \(\beta\)-adrenergic receptors strongly enhances I\(_{\text{Ca,L}}\) via the Gs\(-\alpha\)-cAMP–protein kinase (PK)A signal transduction cascade.\(^{27,28}\) In control myocytes expressing Rem\(_{265}\), exposure to 1 \(\mu\)mol/L isoproterenol rapidly increased I\(_{\text{Ca,L}}\) across all voltages (Figure 4A and 4B). On average, 1 \(\mu\)mol/L isoproterenol resulted in a nearly 3-fold increase in I\(_{\text{Ca,L}}\), similar to the magnitude of response obtained with 1 \(\mu\)mol/L BAY K 8644 (Figure 4E). Surprisingly, in sharp contrast to our observations with BAY K 8644, isoproterenol did not increase I\(_{\text{Ca,L}}\) at any voltage in cells expressing CFP-Rem (Figure 4C through 4E). The same qualitative result was obtained when 1 \(\mu\)mol/L forskolin was used to activate the PKA pathway downstream of the \(\beta\)-adrenergic receptor, at the level of adenyl cyclase (Figure 4E). One possible explanation for the ineffectiveness of the PKA pathway to upregulate Rem-inhibited Ca\(^{\text{V}}\)\(_{1.2}\) channels is that Rem may have an unrecognized ability to eliminate or disrupt this signal transduction cascade in cells. We explored this possibility by evaluating \(\beta\)-adrenergic regulation of I\(_{\text{KS}}\) (slow component of the delayed rectifier potassium current) in control and Rem-expressing myocytes. Under both conditions, 1 \(\mu\)mol/L isoproterenol increased I\(_{\text{KS}}\) to the same extent (Figure 4F and 4G), demonstrating the beta-2-adrenergic receptor signaling pathway remained intact in myocytes expressing Rem. Recently, it has been reported that Rad similarly inhibits \(\beta\)-adrenergic regulation of I\(_{\text{Ca,L}}\) in heart,\(^{29}\) suggesting this may be a general property of all RGK GTPases.

Ca\(^{\text{V}}\)\(_{1.2}\) channels prominently control the cardiac action potential duration (APD) and also provide the trigger Ca\(^{2+}\) for Ca\(^{2+}\)-induced Ca\(^{2+}\) release that underlies cardiac excitation–contraction coupling. Therefore, it might be expected that the divergent effects of BAY K 8644 and forskolin on Rem-inhibited Ca\(^{\text{V}}\)\(_{1.2}\) channels in heart would translate into sharply differing effects of these 2 agents on cardiac excitability and contractility. To directly investigate this, we compared the impact of BAY K 8644 and forskolin on action potentials and Ca\(^{2+}\) transients in myocytes expressing Rem\(_{265}\) and Rem, respectively (Figure 5). Cells expressing Rem played a significantly shortened basal APD compared to control cells expressing Rem\(_{265}\) (APD\(_{90}\)=139.3±17.3 ms, n=6 for Rem-expressing myocytes versus APD\(_{90}=241.9±35.6\) ms, n=5 for Rem\(_{265}\) myocytes, \(P<0.05\), 2-tailed unpaired \(t\) test). In myocytes expressing Rem\(_{265}\), both BAY K 8644 and forskolin markedly increased the APD by 3-fold (Figure 5A and 5C). By contrast, in myocytes expressing Rem, the APD response to forskolin was essentially abolished whereas the response to BAY K 8644 remained intact (Figure 5B and 5C). Similarly, both BAY K 8644 and forskolin significantly increased the amplitude of evoked Ca\(^{2+}\) transients in cells expressing mCherry-tagged Rem\(_{265}\) (Figure 5D; Online Figure V), whereas in cells expressing Rem, the response to forskolin was selectively and significantly muted (Figure 5E; Online Figure V). These results mirror the differential impact of BAY K 8644 and forskolin on I\(_{\text{Ca,L}}\) in Rem\(_{265}\) and Rem-expressing myocytes (Figures 3 and 4).

Overall, these results demonstrate the novel finding that Rem inhibited Ca\(^{\text{V}}\)\(_{1.2}\) channels can be selectively pharmacologically rescued. Furthermore, taken together, the data provide the new insight that Rem-modified Ca\(^{\text{V}}\)\(_{1.2}\) channels in the sarcolemma are arrested in a low-\(P_{\text{e}}\) state, rather than being completely electrically silent.

**Role of GTP Binding and Membrane Localization in Rem Inhibition of Cardiac I\(_{\text{Ca,L}}\)**

We next examined the role played by GTP binding and the Rem C terminus in Rem-mediated inhibition of I\(_{\text{Ca,L}}\) and the loss of sympathetic regulation. To examine the role of GTP binding, we introduced a T94N point mutation into the Rem NBD that is analogous to a mutation which locks Ras in a GDP-bound state.\(^{30}\) When expressed in heart cells, CFP-Rem[T94N] was targeted to the surface sarcolemma, T-tubules, and nucleus (Figure 6A, top; Online Figure I), similar to the subcellular distribution observed with CFP-Rem (Figure 1). However, heart cells expressing CFP-Rem[T94N] displayed robust I\(_{\text{Ca,L}}\) that was indistinguishable from control (Figure 6A and 6B). Furthermore, cells expressing CFP-Rem[T94N] responded robustly to both BAY K 8644 and to PKA activation with...
behaves as if it is functionally inert with respect to CaV1.2 function of CFP-Rem[T94N], which in this context simply translates to being functionally inactive. A, Top, Confocal image showing subcellular localization of CFP-Rem[T94N] in a guinea pig ventricular myocyte. Bottom, Exemplar whole-cell Ca\(_{\text{L}}\),1,2 channel currents from a myocyte expressing CFP-Rem[T94N]. B, Population \(I_{\text{peak}}-V\) relationship for myocytes expressing CFP-Rem[T94N] (black squares) (n=6 for each point). Data from uninfected (gray trace) and Rem-expressing cells (cyan trace) are reproduced from Figure 1 to facilitate visual comparison. C, Top, Exemplar Ca\(_{\text{L}}\),1,2 channel currents from a myocyte expressing CFP-Rem[T94N] before (black trace) and after (red trace) exposure to 1 \(\mu\text{mol/L BAY K 8644}\). Bottom, Diary plot showing time course of the forskolin-mediated increase in \(I_{\text{Ca,L}}\). D, \(I_{\text{peak}}-V\) relationship for myocyte expressing CFP-Rem[T94N] before (black squares) and after (black squares) exposure to 1 \(\mu\text{mol/L BAY K 8644}\). E and F, Impact of forskolin on \(I_{\text{Ca,L}}\) in myocytes expressing CFP-Rem[T94N]. Same format as C and D.

1 \(\mu\text{mol/L forskolin (Figure 6C through 6F). These results differ from data obtained in HEK 293 cells, where Rem[T94N] effectively ablates \(I_{\text{Ca,L}}\). Our data also differ somewhat from a report that the analogous Rad[S105N] exerts a dominant negative effect when expressed in guinea pig and murine cardiac myocytes, resulting in increased \(I_{\text{Ca,L}}\). By contrast, we did not observe a dominant negative function of CFP-Rem[T94N], which in this context simply behaves as if it is functionally inert with respect to Ca\(_{\text{L}}\),1,2 channel modulation.

Previous work has identified the distal C terminus as a locus critical to the Ca\(_{\text{L}}\) channel blocking function of RGK GTPases.\(^{16,21,22}\) Consistent with observations in other cells, deleting the distal Rem C terminus eliminates the capacity to block \(I_{\text{Ca,L}}\) in heart (Figure 1). Previous studies have correlated this functional output of the distal C terminus with its role in autonomously targeting Rem to the plasma membrane. We investigated whether the essential role of the distal Rem C terminus could be substituted by the analogous domain from H-Ras (Figure 7A), which in most cell types functions as a robust membrane targeting module. Unexpectedly, in heart cells, CFP-Rem265-H was not targeted to the sarcolemma (Figure 7B), in sharp contrast to its membrane localization in other cell types (Online Figure VI). Nevertheless, CFP-Rem265-H depressed cardiac \(I_{\text{Ca,L}}\) and Ca\(_{\text{L}}\),1,2 channel \(Q_{\text{max}}\) to the same extent as wild-type Rem (Figure 7B through 7D). Moreover, the remnant current was robustly upregulated by BAY K 8644 (Figure 7E) but almost completely insensitive to PKA activation (Figure 7F). Consistent with these findings, the APD in cells expressing CFP-Rem265-H was largely unaffected by forskolin, but significantly prolonged by BAY K 8644 (Figure 7G). Overall, these data provide the surprising insight that in heart cells membrane targeting of the Rem NBD is neither sufficient (Figure 6) nor necessary (Figure 7) to reconstitute \(I_{\text{Ca,L}}\) inhibition. This finding is opposite to conclusions based on studies from recombinant channels reconstituted in heterologous expression systems.

Discussion
In this work, we have examined the properties of Rem-inhibited Ca\(_{\text{L}}\),1,2 channels in adult heart cells. Our findings differ from prevailing views of the mechanisms underlying RGK GTPase-mediated decrease in cardiac \(I_{\text{Ca,L}}\) and suggest new (patho)physiological ramifications for this potent form of Ca\(_{\text{L}}\),1,2 channel modulation. The results also suggest that RGK GTPases may potentially be exploited as novel tools to probe outstanding biophysical questions related to sympathetically modulated of Ca\(_{\text{L}}\),1,2 channels. We discuss these aspects of our work in relation to previous studies.

Mechanisms and Structural Determinants of the RGK GTPase/Ca\(_{\text{L}}\) Channel Crosstalk in Heart
We found that overexpressing Rem in heart cells markedly decreased \(I_{\text{Ca,L}}\), similar to previous findings with Gem and Rad.\(^{16}\) What is the mechanistic basis of this effect in heart? One prevailing hypothesis is that RGK GTPases inhibit \(I_{\text{Ca,L}}\) in heart cells by interfering with the trafficking of pore-forming \(\alpha_{1C}\) subunits to the sarcolemma, possibly by buffering endogenous Ca\(_{\text{L}}\)\(\beta\) subunits, thereby reducing the surface density of Ca\(_{\text{L}}\),1,2 channels.\(^{16,17}\) In this scenario, the residual currents remaining in the presence of RGK GTPase expression represent the small fraction of wild-type (unbound) channels that make it to the cell surface (Figure 8A; model 1). Our results offer several lines of proof that argue against model 1 as a viable mechanism for Rem-mediated block of \(I_{\text{Ca,L}}\) in heart. First, contrary to the prediction of this model, we find no evidence that the surface density of Ca\(_{\text{L}}\),1,2 channels in heart cells is substantially diminished in the presence of Rem. The second line of evidence relates to the observed responses of Rem-inhibited Ca\(_{\text{L}}\),1,2 channels to BAY K 8644 and PKA activation. Model 1 predicts that the residual currents recorded in the presence of Rem would have the same magnitude of response to BAY K 8644 and PKA activation as channels from control cells (ie, a similar fold increase in \(I_{\text{Ca,L}}\)). Instead, we observed that Rem-inhibited channels were supramodulated by BAY K 8644 and insensi-
tive to PKA activation compared to control channels. The results similarly argue against a model in which Rem-modulated channels remain on the surface but are completely electrically silent (Figure 8A; model 2), because, in this scenario, the remnant current would still be provided by unmodified wild-type channels. Rather, the findings are consistent with the residual currents observed in Rem-expressing myocytes arising from Rem-bound low-$P_o$ channels on the cell surface (Figure 8A; model 3). In this scenario, the enhanced fold increase in current amplitude in response to BAY K 8644 observed in Rem-modified channels is explained by the larger dynamic range available resulting from their lower basal $P_o$. It is not clear from our data precisely how Rem arrests CaV1.2 channels in a low-$P_o$ mode in the sarcolemma. An attractive hypothesis is that this is related to the decrease in $Q_{\text{max}}$ which, in the absence of a decrease in the physical number of surface channels, suggests a Rem-induced partial immobilization of voltage sensors. Deciphering precisely how Rem reduces channel $P_o$ and $Q_{\text{max}}$ remains an important challenge for the future.

Based on the assumption that Rem[T94N] is deficient in binding GTP, our results indicate that GTP binding to the NBD is necessary for Rem inhibition of CaV1.2 channels in heart. By contrast, several studies in other cell types have shown that GTP binding is dispensable for RGK block of CaV1 and CaV2 channels. This discrepancy appears to reflect a genuine difference among cell types because it has also been shown that GTP-binding-deficient Rad does not inhibit CaV1.2 channels in heart. Another sharp distinction from previous studies concerns the role of membrane targeting in $I_{\text{Ca,L}}$ inhibition. Studies on recombinant channels reconstituted in heterologous systems, or endogenous CaV2.2 channels in sympathetic cervical ganglion neurons, have indicated that membrane targeting is necessary and/or sufficient for RGK GTPases to inhibit $I_{\text{Ca,L}}$. By contrast, we find that in heart cells membrane targeting of the Rem NBD is neither necessary nor sufficient for blocking $I_{\text{Ca,L}}$. Overall, these distinctions emphasize the idea that RGK proteins use diverse mechanisms and determinants to inhibit $I_{\text{Ca,L}}$ and that different cell types may use unique subsets of the available toolkit to achieve CaV channel block.

Surprisingly, Rem265-H was not targeted to the sarcolemma in heart cells. In other cell types, the H-Ras C terminus is posttranslationally processed in a sequence of enzyme-catalyzed steps that convert it into an effective membrane-targeting module: farnesylation of the C-terminal CAAX prenylation motif at the cysteine residue followed by proteolysis of the AAX sequence; carboxymethylation of the farnesylated cysteine; and, finally, palmitoylation of the 2 cysteine residues upstream of the farnesylated cysteine (Fig-
Figure 8. Conceptualization of mechanism underlying Rem inhibition of cardiac CaV1.2 channels. A, Schematic showing alternative models that could explain Rem inhibition of CaV1.2 channels in heart. Under control conditions a cadre of CaV1.2 channels are present on the cardiac cell surface and give rise to a robust I_{Ca,L} on membrane depolarization. Overexpressing Rem or other RGK GTPases dramatically, but not completely, decrease I_{Ca,L}. There are 3 candidate mechanisms for this effect: model 1, Rem reduces surface density of channels by disrupting trafficking; model 2, Rem renders a subpopulation of channels on the sarcolemma electrically silent; and model 3, Rem arrests virtually all surface channels in a low-Po gating mode. In models 1 and 2, the remnant current seen with Rem overexpression in myocytes represents a small fraction of unmodified wild-type channels. The pattern of responses of Rem-modified channels to BAY K 8644 and PKA activation indicate that the remnant current does not emanate from wild-type channels, thus discounting models 1 and 2. Our results are consistent model 3 being the dominant mechanism of Rem inhibition of I_{Ca,L} in heart. B, Conceptualization of impact of Rem, BAY K 8644, and PKA activation on modal gating activity of CaV1.2 channels in heart.

Potential Use of Rem As a Tool to Explore Biophysical Mechanisms of CaV1.2 Channel Modulation

A fortuitous outcome from these studies concerns the potential to exploit Rem as a tool to probe outstanding questions concerning the mechanisms underlying CaV1.2 channel modulation by Ca^{2+} channel agonists and sympathetic activation. Previous single-channel studies have characterized distinct gating modes of cardiac CaV1.2 channels including: mode 0a, which is characterized by brief infrequent openings; mode 1, which features frequent millisecond openings; and mode 2, wherein the channels display long openings with brief
closures (Figure 8B). In control channels, both BAY K 8644 and PKA increase the propensity for mode 2 openings from the dominant mode 1 gating mode (Figure 8B).34,35 Whether these 2 modulators use common transduction events to achieve CaV1.2 channel enhancement is unknown. Although we have not directly measured single-channel events here, the whole-cell data indicate Rem arrests channels in a low-Po mode. We conceptualize this low-Po mode as being akin to the described previously mode 0a. The ability of Rem to selectively prevent PKA-mediated upregulation, while preserving BAY K 8644 modulation (Figure 8B), may provide a discriminating tool to probe the structural determinants leading to the 2 forms of CaV1.2 channel modulation.

Regarding PKA modulation of CaV1.2 channels, fundamental mechanistic questions remain unresolved despite intense study by many groups over almost 2 decades.25,36–39 For example, the precise phosphorylation site(s) responsible for channel upregulation remains unclear, and how this posttranslational modification is transduced into a functional effect on channel gating is unknown. The ability of Rem to block PKA-mediated enhancement of ICa,L at the level of CaV1.2 channels could provide a unique tool to gain new mechanistic insights into this physiologically important form of channel regulation. Potentially, Rem could nullify PKA modulation of ICa,L, either by preventing phosphorylation of the functionally relevant residues or by directly opposing the conformational change involved in the transduction event. Future work focusing on these questions will likely shed new insights into the mechanisms underlying PKA modulation of cardiac CaV1.2 channels.

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Disclosures
None.

References

Novelty and Significance

What Is Known?

- RGK GTPases use multiple mechanisms to inhibit L-type calcium current (I_{Ca,L}) in reconstituted systems.
- Overexpressing RGK GTPases in heart cells inhibits I_{Ca,L}.

What New Information Does This Article Contribute?

- Rem GTPase inhibits I_{Ca,L} in heart cells by arresting calcium channels in a low open probability state, without changing the number of channels at the cell surface.
- Rem-inhibited I_{Ca,L} in heart cells can be selectively rescued with BAYK 8644 but not PKA-dependent phosphorylation.
- The structural determinants of Rem necessary and sufficient for I_{Ca,L} inhibition in heart differ from those in reconstituted systems.

Calcium ions entering via the L-type calcium channels in heart cells are essential for cardiac contractility and rhythm. Therefore, molecules that regulate L-type channel activity are critical determinants of the heartbeat under both physiological and disease conditions. RGK proteins are Ras-like GTPases that are the most powerful intracellular blockers of L-type calcium channels known. Although these proteins are found in heart, and their levels altered in cardiac diseases, the precise mechanisms by which they inhibit L-type calcium channels are unclear. Here we show that 1 RGK GTPase, Rem, inhibits L-type channels in heart cells by arresting them in a low open probability state, without changing the number of channels at the cell surface. Moreover, the Rem-inhibited channels can be completely rescued by L-type channel agonists but not by the physiological sympathetic adrenergic pathway. Finally, we found that the structural determinants of Rem that are necessary and sufficient for L-type channel inhibition differ from those found in other cell preparations. The study has implications for the future design of novel calcium channel blockers and suggests the RGK GTPase/L-type calcium channel signaling axis as a potential therapeutic target in cardiac diseases for which enhancing L-type calcium current would be beneficial.
Molecular Mechanisms, and Selective Pharmacological Rescue, of Rem-Inhibited Cav1.2 Channels in Heart

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Materials and Methods

Construction of adenoviral vectors
Adenoviral vectors were generated using the AdEasy XL system (Stratagene, La Jolla, CA, USA). Briefly, CFP-Rem or YFP-Rem265 were cloned into shuttle vectors pShuttle-CMV, then linearized with Pme I and transformed into BJ5183-AD-1 competent cells. Recombinants were identified by restriction digestion and then produced in bulk using the recombination-deficient XL10-Gold strain. Purified adenoviral plasmid DNA was digested with Pac I and then used for transfection. 60 mm diameter dishes containing Ad-293 cells at ∼70% confluency were transfected with 3 μg viral DNA using Fugene (Roche Molecular Biochemicals, Indianapolis, IN, USA). Following observation of cytopathic effects (CPE) usually after 7-10 days, cells were harvested and subjected to three freeze-thaw cycles, followed by centrifugation to remove cellular debris, and the resulting supernatant (2 ml) used to infect a 10 cm dish of 90% confluent Ad-293 cells. Following observation of CPEs after 2-3 days, cell supernatants were used to infect 10 25-cm plates of Ad-293 cells, and viruses harvested and purified by CsCl gradient centrifugation. Viral particle numbers determined by absorbance at 260 nm were in the order of 10^{11}-10^{12} particles ml^{-1}, with a ∼30:1 particle to plaque-forming unit ratio.

Cell culture and transfection
Adult male Hartley guinea pigs were killed with an overdose of isoflurane in accordance with the guidelines of the Columbia University Animal Care and Use Committee. Hearts were excised and ventricular myocytes isolated by enzymatic digestion using a Langendorff perfusion apparatus. Healthy, rod-shaped cardiomyocytes were cultured on laminin-coated coverslips at a density of ∼50 000 cells ml^{-1}, in 35 mm tissue culture dishes. Cells were initially maintained in Medium 199 (Life Technologies, Carlsbad, CA, USA) supplemented with 5 mM carnitine, 5 mM creatine, 5 mM taurine, 100 μg ml^{-1} penicillin-streptomycin and 10 % fetal bovine serum to promote attachment to the coverslips. After 3-5 h, the culture medium was switched to serum-free Medium 199. Cultures were equilibrated with 5 % CO2 and 95 % air at 37 °C in a water-jacketed CO2 incubator. Cells were transfected 3-5 h after initial plating by adding 15-25 μl of the appropriate virus stock (∼10^{11}-10^{12} viral particles ml^{-1}) directly onto the cells, in a final volume of 1-2 ml. In all cases, robust transgene expression was explicitly verified by monitoring CFP or YFP fluorescence ∼24 h after infection.

Immunofluorescence detection of Ca_{v}1.2 channels
Isolated heart cells plated on glass-bottom 35 mm MatTek dishes were infected with adenovirus. After 24 h, cells were fixed with 2% paraformaldehyde in PBS and washed with 0.1 M glycine-PBS. Fixed myocytes were permeabilized with 0.2% Triton X-100 in PBS followed by a 1 h incubation in blocking solution (3% goat serum/1% bovine serum albumin/0.1% Triton X-100). Cells were then incubated with anti-Ca_{v}1.2 antibody (1:1000 rabbit polyclonal) in blocking media for 1 h at room temperature. Cells were then washed and exposed to Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) (1:400, Invitrogen Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature in blocking solution. Immunofluorescence detection was performed using a Leica TCS SP2 AOBs MP confocal microscope system (Leica Microsystems, Heidelberg, Germany). Data acquisition and analysis were done using Leica LCS imaging software.

Electrophysiology
All electrophysiological recordings were acquired at room temperature 24-48 h after transfection by using an EPC8 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) controlled by PULSE software (HEKA Electronics). For whole-cell voltage-clamp recordings in cultured heart cells, electrodes fashioned from borosilicate glass capillaries (World Precision Instruments, MTW 150-F4) were filled with an internal solution containing (mM): 150 cesium methanesulphonate (Cs-MeSO_{3}), 5 CsCl, 10 HEPES, 10 EGTA, 1 MgCl_{2}, 4 MgATP (pH 7.2, adjusted with CsOH). Typically, pipettes had
Supplement Material

resistances of 1.5-2 MΩ. For formation of gigaohm seals and initial break-in to the whole-cell voltage clamp configuration, heart cells were perfused with normal Tyrode solution containing (mM): 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 10 glucose (pH 7.4, adjusted with NaOH). Following successful break-in the perfusing medium was switched to an external recording solution containing (mM): 155 N-methyl-D-glucamine aspartate (NMG-Asp), 5 BaCl₂, 10 4-aminopyridine, 10 HEPES (pH 7.4, adjusted with NMG). To measure gating currents, ionic currents were blocked by adding 2 mM CdCl₂ and 0.1 mM LaCl₃ to the bath solution. Signals were filtered at 5 kHz (step currents) or 10 kHz (gating currents) (four-pole Bessel filter), and sampled at 25 kHz. Data traces were acquired at a repetition interval of 20 s. Leaks and capacitive transients were subtracted by a P/8 protocol (ionic currents), or a P/-8 protocol (gating currents) from a −90 mV holding potential. Action potentials were recorded in current-clamp mode, sampled at 5 kHz and filtered at 2 kHz. Patch pipettes were filled with (in mmol/L): 120 KCl, 1 MgCl₂, 5 Na₂ATP, 10 HEPES, 0.5 EGTA, and 0.01 CaCl₂ (pH 7.4, adjusted with KOH). Heart cells were perfused with normal Tyrode solution. Action potentials were initiated by brief depolarizing current pulses (300 to 700 pA; 5 ms duration). Action potential durations (APD) were measured at 50% repolarization.

Ca²⁺ transient measurements
Cultured cardiac myocytes were infected with adenovirus encoding mCherry-tagged Rem265 or Rem for 24-48 hrs. Cells were loaded with 5 μM of the cell membrane permeable Ca²⁺ indicator fluo-4-AM for 20 min at room temperature, washed with tyrode solution, and incubated for a further 30 min. Line scan imaging was performed on a Leica TCS SP2 AOBS MP confocal microscope system (Leica Microsystems, Heidelberg, Germany). Fluo-4 was excited with the 488 nm line of an argon laser, and the emitted fluorescence was collected through a 515 nm long pass emission filter. Cells were electrically field stimulated (20 V) at a frequency of 1 Hz using a Myopacer Field Stimulator (Ion Optix Corporation, Milton, USA). Fluorescence images were analyzed with Image J 1.4 (NIH).

Statistics
Data were analyzed off-line using PulseFit (HEKA), Microsoft Excel and Origin software. Statistical analyses were performed in Origin using built-in functions. Pooled data are presented as means ± s.e.m., and P values were calculated using Student’s two-tailed t test; P < 0.05 was considered significant.
Online Figure I. Sub-cellular localization of CFP-tagged Rem and derivatives in live guinea pig ventricular myocytes. Confocal images and line scans showing fluorescence intensities. Targeting of CFP-Rem and CFP-Rem[T94N] to the surface sarcolemma and t-tubules is evident from the sharp peaks in fluorescence intensities that occur at ~ 1.8 μm intervals. By contrast, YFP-Rem$_{265}$ and CFP-Rem$_{265}$-H do not localize to the sarcolemma and t-tubules.
Online Figure II. Negative control images for Ca\textsubscript{v}1.2 channel immunofluorescence staining experiments. Immunofluorescence images obtained from a myocyte when the primary Ca\textsubscript{v}1.2 antibody was pre-incubated with the antigen peptide. All fluorescence images were collected at the same gain setting of the microscope as other immunofluorescence experiments.
Online Figure III. Functional rescue of Rem-inhibited channels by FPL 64176. (A) Exemplar CaV1.2 channel currents from control YFP-Rem\textsubscript{265} myocytes before and after exposure to 2 \(\mu\)mol/L FPL 64176. (B) Diary plot of FPL 64176-induced up-regulation of exemplar control CaV1.2 channel currents. (C, D) Data for myocytes expressing CFP-Rem. Same format as A, B.
Online Figure IV. BAY K 8644 does not increase $Q_{\text{max}}$ of Rem-inhibited CaV1.2 channels. (A) Population $Q_{\text{ON}}$-V relationships for Rem265- (□, $n = 6$ for each point) and Rem-expressing (●, $n = 5$ for each point) myocytes in the presence of 1 μM BAY K 8644. (B) Bar chart showing lack of impact of BAY K 8644 on CaV1.2 channel $Q_{\text{max}}$ from cells expressing either Rem265 or Rem.
Online Figure V. Impact of BAY K 8644 and forskolin on on Ca$^{2+}$ transients in myocytes expressing mCherry-tagged Rem$^{265}$ and Rem. Population data showing the differential impact of forskolin and BAY K 8644 on fluo-4-reported Ca$^{2+}$ transients from Rem$^{265}$- and Rem-expressing guinea pig ventricular myocytes. * $P < 0.05$ compared to Rem$^{265}$. # $P < 0.05$ compared to Rem.
Online Figure VI. Exemplar image from HEK293 cells expressing YFP-Rem$_{265}$-H. Confocal images show that YFP-Rem$_{265}$-H is targeted to the cell membrane of HEK293 cells.
Online Table I. Effects of Rem and various derivatives on gating properties of cardiac Ca\textsubscript{v}1.2 channels

<table>
<thead>
<tr>
<th>Condition</th>
<th>(G_{\text{max}}) pA/pF/mV\textsuperscript{-1}</th>
<th>(V_{1/2}) mV</th>
<th>(V_{\text{rev}}) mV</th>
<th>(k) mV</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.36±0.03</td>
<td>-18.1±2.2</td>
<td>53.6±1.3</td>
<td>3.71±0.62</td>
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<tr>
<td>YFP-Rem\textsubscript{265}</td>
<td>0.38±0.02</td>
<td>-16.8±0.8</td>
<td>53.9±0.9</td>
<td>5.49±0.21*</td>
<td>6</td>
</tr>
<tr>
<td>CFP-Rem</td>
<td>0.08±0.01*</td>
<td>-8.42±2.4*</td>
<td>50.7±4.0</td>
<td>18.5±1.4*</td>
<td>7</td>
</tr>
<tr>
<td>CFP-Rem\textsubscript{265-H}</td>
<td>0.09±0.01*</td>
<td>-5.93±3.3*</td>
<td>49.7±2.1</td>
<td>18.5±2.8*</td>
<td>6</td>
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<tr>
<td>CFP-Rem\textsubscript{T94N}</td>
<td>0.34±0.03</td>
<td>-12.5±2.0</td>
<td>53.9±1.4</td>
<td>6.10±0.72*</td>
<td>6</td>
</tr>
</tbody>
</table>

*\(P < 0.05\), compared to the control group.