Rem-GTPase regulates cardiac myocyte L-type calcium current

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Rationale: The L-type calcium channels (LTCC) are critical for maintaining Ca²⁺-homeostasis. In heterologous expression studies, the RGK-class of Ras-related G-proteins regulates LTCC function; however, the physiological relevance of RGK-LTCC interactions is untested.

Objective: In this report we test the hypothesis that the RGK protein, Rem, modulates native Ca²⁺ current (I_{Ca,L}) via LTCC in murine cardiomyocytes.

Methods and Results: Rem knockout mice (Rem⁻⁻) were engineered, and I_{Ca,L} and Ca²⁺-handling properties were assessed. Rem⁻⁻ ventricular cardiomyocytes displayed increased I_{Ca,L} density. I_{Ca,L} activation was shifted positive on the voltage axis, and β-adrenergic stimulation normalized this shift compared with wild-type I_{Ca,L}. Current kinetics, steady-state inactivation and facilitation was unaffected by Rem⁻⁻. Cell shortening was not significantly different. Increased I_{Ca,L} density in the absence of frank phenotypic differences motivated us to explore putative compensatory mechanisms. Despite the larger ICa,L density, Rem⁻⁻ cardiomyocyte Ca²⁺ twitch transient amplitude was significantly less than that compared with wild type. Computer simulations and immunoblot analysis suggests that relative dephosphorylation of Rem⁻⁻ LTCC can account for the paradoxical decrease of Ca²⁺ transients.

Conclusions: This is the first demonstration that loss of an RGK protein influences I_{Ca,L} in vivo in cardiac myocytes.

Keywords: L-type calcium channel, Ras-GTPase, calcium current, patch-clamp

Introduction

L-type Ca channels (LTCC) are critical for maintaining Ca-homeostasis, providing trigger Ca for Ca-induced Ca-release, and regulating electrical function in cardiac myocytes. Therefore, precise control of LTCC function has profound implications for cardiac myocyte physiology. LTCC exist as a multi-protein complex in cardiac myocytes.¹ Thus, understanding of LTCC function requires evaluation of LTCC Ca current (I_{Ca,L}) in native complexes. Cardiac myocyte LTCC consists of the main pore-forming subunit in complex with accessory proteins including Ca₂β family members, α₂δ and calmodulin (CaM; reviewed in ref. 2). In addition, we, and others have recently extensively characterized the ability of the RGK-family of monomeric G-proteins to modulate I_{Ca,L}.³⁻⁶

The RGK family of Ras-related monomeric G-proteins includes Rem, Rem2, Rad and Gem/Kir.⁷ Rem and Rad are expressed in myocardium.⁸⁻⁹ Rad co-expressed with recombinant LTCC show block of current,¹⁰ and therefore, reduction of Rad in vivo is expected to increase I_{Ca,L}. Rad is downregulated in experimental induced heart failure (thoracic aorta constriction, TAC) in mice.¹¹ Rad knockout mice show no functional phenotype at baseline, but following TAC CaMKII increases.¹¹ Rad knockdown via RNAi results in an increase of I_{Ca,L}, Ca transients and contractility.¹²

Heterologous expression studies document Rem blockade of I_{Ca,L},⁵,¹⁰,¹³⁻¹⁵ however, there are no reports that assess in vivo Rem function. Overexpression of Rem, and other RGK proteins, results in prominent blockade of cardiac myocyte I_{Ca,L}.⁴,¹⁶ Therefore, we tested the hypothesis that depletion of Rem results in an increase of I_{Ca,L}. In this report, we examined cardiac myocyte I_{Ca,L} in Rem-null mice (Rem⁻⁻). Cardiac myocyte I_{Ca,L} density increases in Rem⁻⁻ compared with that from wild-type mice. Nevertheless, Rem-deletion results in no frank cardiac phenotype at baseline.
The increased $I_{Ca,L}$ density may be offset by a positive shift of the steady-state activation curve. Thus, our data support the notion that compensatory changes in Ca homeostasis preserve cardiac function.

### Results

**$I_{Ca,L}$ in Rem$^{-/-}$ cardiac myocytes.** Rem overexpression blocks $I_{Ca,L}$ in heterologous expression systems, and in cardiac myocytes. These results suggested that Rem governs $I_{Ca,L}$ in vivo. To test this idea, we generated a Rem1 knockout mouse (Fig. 1). Rem null mutant mice (Rem$^{-/-}$) were born at the expected Mendelian ratio, and grew to adulthood without showing any discernible abnormalities.

To examine the contribution of Rem to LTCC function, we measured $I_{Ca,L}$ in Rem$^{-/-}$ cardiac myocytes. The expectation is that $I_{Ca,L}$ should increase in the absence of Rem blockade of current. As the analog traces of Figure 2A shows the $I_{Ca,L}$ density was significantly greater in Rem$^{-/-}$ compared with age-matched wild-type controls (peak $I_{Ca,L}$ density amplitudes at +10 mV: -19.0 ± 0.5 pA/pF (n = 55) for Rem$^{-/-}$ and -16.0 ± 1.0 pA/pF (n = 40) for the age-matched wild-type controls; p < 0.05). The current-voltage relationships [I(V)] show that the differences in calcium current densities were statistically significant in the membrane potential range between 0 mV and +45 mV (Fig. 2B). These data are consistent with Rem governing $I_{Ca,L}$ amplitude in cardiomyocytes.

The maximal $I_{Ca,L}$ was greater in Rem$^{-/-}$ compared with wild-type control, and examination of the I(V) curves reveal a shift in the voltage dependence of $I_{Ca,L}$ (Fig. 2B). Figure 2C shows that the midpoint of activation for Rem$^{-/-}$ $I_{Ca,L}$ is significantly shifted relative to control myocytes (-7.2 ± 0.3 mV and -3.3 ± 0.2 mV at wild-type and Rem$^{-/-}$ myocytes respectively, p < 0.05). The voltage dependence of steady-state inactivation was measured by a double pulse protocol. As Figure 2C demonstrates Rem deletion does not affect the voltage dependence of steady-state inactivation.

Thus the theoretical steady-state window Ca-current was reduced in Rem$^{-/-}$ cardiac myocytes (Fig. 2C and shaded area). Taken together, there is an increase of maximal-activated $I_{Ca,L}$ that is potentially compensated by a shift of steady-state activation.

$I_{Ca,L}$ kinetics are key determinants of total Ca$^{2+}$ entry into the cytosol, and in heterologous expression systems Rem alteration of kinetics depends on exogenous CaM expression. To evaluate whether Rem alters native $I_{Ca,L}$ we measured $I_{Ca,L}$ kinetics in cardiomyocytes. $I_{Ca,L}$ kinetics were unaffected by Rem$^{-/-}$. The effect of Rem on the time dependent inactivation of $I_{Ca,L}$ was determined by bi-exponential fitting to the decay of current. The $\tau_1$ values were 24.7 ± 2.2 ms and 25.5 ± 1.8 ms for wild type and Rem$^{-/-}$, respectively. The $\tau_2$ values were 5.0 ± 0.8 ms and 5.3 ± 0.6 ms (Fig. S1). As expected, the fast and slow component amplitudes of Rem$^{-/-}$ were significantly larger than that of the age-matched wild-type controls (p < 0.05) but the relative amplitudes of the fast/slow components were not different between Rem$^{-/-}$ and control. The effect of Rem was also tested on the facilitation of $I_{Ca,L}$. During these measurements $I_{Ca,L}$ was elicited by 16 consecutive pulses at 0.5 Hz to 0 mV from -80 mV holding potential. The current amplitudes were normalized to the $I_{Ca,L}$ amplitude elicited by the first pulse. Facilitation was not significantly different for Rem$^{-/-}$ (8 ± 4%) compared with that in wild-type cardiac myocytes (6 ± 3%; Fig. S2).

$\beta$-adrenergic stimulation increases Rem blockade of $I_{Ca,L}$ in heterologous expression systems. Also, it is well-established that $\beta$-adrenergic stimulation increases the $I_{Ca,L}$ amplitude, and causes a negative shift of $I_{Ca,L}$ activation. Thus, in the absence of Rem, we predict there is a greater dynamic range of $I_{Ca,L}$ responsiveness to $\beta$-adrenergic stimulation. To test this hypothesis, we superfused isoproterenol onto cardiomyocytes during the whole-cell recording. The $I_{Ca,L}$ response to isoproterenol was greater for Rem$^{-/-}$ compared with wild-type cardiac myocytes. 10$^{-4}$ M isoproterenol caused a 28 ± 4% and 22 ± 3% increase in $I_{Ca,L}$ amplitude of Rem$^{-/-}$ and wild-type myocytes, respectively. Isoproterenol
treatment caused no significant shift in the midpoint ($V_{1/2}$) of steady-state inactivation curve. In the presence of $10^{-8}$ M isoproterenol inactivation $V_{1/2}$ was $-29.8 \pm 1.0$ mV for Rem$^-$ and $-28.9 \pm 0.5$ mV for wild-type cells. Isoproterenol shifted the voltage dependence of steady-state activation by $-6.2 \pm 1.0$ mV for Rem$^-$, but only $-3.9 \pm 1.3$ mV for wild type (Fig. 3). As a consequence, isoproterenol-modulated channel voltage-dependence was no longer significantly different between Rem$^-$ and wild type. These data are consistent with the notion that Rem serves as a governor of $\beta$-adrenergic stimulated $I_{Ca,L}$.

The Na$^+$-Ca$^{2+}$ exchange current ($I_{NCX}$) is the major efflux pathway that homeostatically balances cytosolic Ca-entry via $I_{Ca,L}$. The increased peak $I_{Ca,L}$ can be offset by a positive shift of LTCC activation. Therefore, as a surrogate for assessing net trans-sarcolemmal $I_{Ca,L}$ entry we measured $I_{NCX}$. $I_{NCX}$ was recorded as a Ni$^{2+}$-sensitive current using the descending limb of a ramp pulse changing from +60 to -100 mV. Outward and inward $I_{NCX}$ were determined at +40 and -80 mV, respectively (Fig. 4). Rem$^-$ have a significantly smaller inward $I_{NCX}$ ($0.90 \pm 0.16$ pA/pF) than for the wild type ($1.92 \pm 0.4$ pA/pF; $p < 0.05$, n = 7; Fig. 4B). The mean outward current was larger in wild-type

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**Figure 2.** $I_{Ca,L}$ density for wild-type and Rem$^-$ cardiac ventricular myocytes. (A) Representative original traces from wild-type and Rem$^-$ mice. (B) Average $I_{Ca,L}$-V relationships for wild-type and Rem$^-$ cardiac myocytes. (C) The voltage dependence of steady-state activation and steady-state inactivation of $I_{Ca,L}$ for wild-type and Rem$^-$ cardiac myocytes. $V_n$ of steady-state activation is significantly shifted +4 mV for Rem$^-$ compared with wild type. $V_n$ was obtained from fitting the current-voltage data as in (B) to a modified Boltzmann distribution of the form: $I(V) = G_{max} \times (1 + \exp(V_n - V)/k)$, where $G_{max}$ is maximal conductance, $E_{rev}$ is reversal potential, $V_n$ is activation midpoint potential and $k$ is the slope factor. The voltage dependence of steady-state inactivation of $I_{Ca,L}$ was measured by a double-pulse protocol. The overlap of steady-state activation and steady-state inactivation curves (cross-hatched) illustrates decrease of window $I_{Ca,L}$ in Rem$^-$ compared with wild-type cardiac myocytes.

**Figure 3.** Isoproterenol normalizes the shift of activation $V_n$ for Rem$^-$ compared with wild type. (A) Activation curves for wild-type $I_{Ca,L}$ in control bath and 10 nM isoproterenol ($V_n$ for control and isoproterenol were $-7.2 \pm 0.3$ mV and $-11.7 \pm 0.3$ mV, respectively; $p < 10^{-3}$; n = 12). (B) Activation curves for Rem$^-$ $I_{Ca,L}$ in control bath, and following 10 nM isoproterenol ($V_n$ for control and isoproterenol were $-3.3 \pm 0.2$ mV and $-9.4 \pm 0.3$ mV, respectively; $p < 10^{-4}$; n = 24).
shortening was faster in Rem -/- compared with wild-type myocytes (Fig. S3). The time to peak values of Rem -/- and wild type were 110 ± 4 ms and 132 ± 7 ms (n = 21; p < 0.05), while the half relaxation time was 77 ± 5 ms and 124 ± 16 ms (n = 21; p < 0.05), respectively. 10 nM isoproterenol treatment decreased the time-to-peak values compared with normal pre-drug treatment values in both animal groups (Fig. S3B). Isoproterenol treatment reduced the half relaxation time by 20 ± 8 ms in wild-type animals (n = 21; p < 0.05), but we did not observe significant change of this parameter in of Rem-/- (Fig. S3B). Cell shortening kinetics is a measure of a complex process. Shortening time reflects Ca-induced Ca release, Ca diffusion and contractile apparatus response. Taken together these results suggest that the effect of the chronic absence of Rem extends beyond modulation of ICa,L.

Computer simulations. To explain our results we employed a newly developed computational model of mouse ventricular myocytes. Our approach was to re-adjust LTCC-related parameters based on data from I Ca,L recordings in Rem -/- vs. wild-type myocytes, and then have the model generate Ca2+ transients. Figure 5E shows the activation and steady-state inactivation curves for I Ca,L superimposed with the model output. The computer simulations quantitatively capture the resulting decrease of 1-Hz stimulated Ca transients (Fig. 5F). In addition, the tendency for the mean sarcoplasmic reticulum Ca reduction was also present in the model output. Cell-size adjustments had only modest effects on Ca transients (data not shown). These results are consistent with the contention that a +4 mV shift of LTCC activation results in a decreased Ca-transient, despite a larger maximal conductance.

Compensatory responses. The positive shift of basal I Ca,L activation suggests that Ca,1.2 protein might be less phosphorylated in Rem -/- compared with wild type. The Ca,1.2 distal carboxyl terminus (DCT) Ser1928 is a known substrate for the
β-adrenergic-PKA signaling axis in cardiomyocytes. Cardiomyocyte lysates show that DCT migrates at about 37 kDa. The specificity for the antibody used to probe for Ser1928 is demonstrated in Figure S4. Fractional DCT/S1928/total-DCT was significantly less in Rem−/− (51 ± 3%; n = 4) compared with that in wild-type ventricular lysates (78 ± 4%; n = 4; p < 0.002; Fig. 6A). We also tested phospholamban (PLB) phosphorylation to assess whether this was a general elevation of PKA signaling. PLB-Ser16 is a PKA substrate site, and there was no difference in PLB-Ser16 between wt and Rem−/− (Fig. 6B and C). PLB-Thr17 is an established CaMKII substrate. Although the mean PLB-Thr17 was greater in wt than Rem−/−, the differences were not statistically significant (Fig. 6B and C).

We postulated that an increase of I_{Ca,L} density in the absence of Rem expression, could in theory, be offset by a compensatory decrease of CaV1.2 protein expression. To test this straightforward notion, we evaluated immunoblots from isolated ventricular membrane fractions. We observed a significant decrease of full-length CaV1.2 protein for Rem−/− compared with wild type (74 ± 4%; p < 0.01; Fig. 6D). The other major RGK protein in the myocardium, Rad, was not different in Rem−/− hearts (Fig. S5).

**Discussion**

Overexpressed Rem blocks I_{Ca,L} in myocytes suggesting that endogenous Rem might provide tonic blockade of I_{Ca,L}. Thus, eliminating Rem expression was expected to increase I_{Ca,L}. The major finding of this study is that Rem knockout results in an increased I_{Ca,L} density. This is the first time that RGK GTPase modification of I_{Ca,L} has been shown following in vivo knockout. Rem deletion also resulted in a depolarizing shift of channel activation, and this shift was normalized to control by β-adrenergic stimulation.

Rem is one of three RGK proteins (Rem, Rad, and Gem) expressed by cardiac myocytes. To date, all RGK proteins share the functional property of blockade of I_{Ca,L} when overexpressed in either cardiac myocytes or other cells, including those expressing recombinant proteins. In contrast, loss-of-function studies reveal that Rad knockdown via shRNA or Rad dominant negative mutant overexpression leads to increased I_{Ca,L} density. A Rad-null mouse has been described in reference 11, but unfortunately no information regarding I_{Ca,L} is available in these studies. At baseline, Rad-null mice have no phenotypic differences from control cardiac myocytes and hearts; however, in view of our present findings it would be reasonable to re-evaluate Rad-null mice. Such a study might shed light on possible non-redundant functions of Rad vs. Rem in cardiac myocytes.

A partial decrease of Rad via shRNA increases I_{Ca,L} and leads to a similar increase of SR Ca-release. The parallels between depressing Rad function and the present report of Rem knockout are partial. Elimination of Rem also increases I_{Ca,L} density, but does not alter SR Ca-load. However, twitch Ca release was
difference in relative phosphorylation status of CaV1.2 and PLB can reside in a number of factors, including but not limited to differential accessibilities to PKA and differential modulation by a variety of phosphatases.

We used CaV1.2-Ser1928 phosphorylation as a surrogate for the capacity of LTCC to serve as a substrate for PKA activity. The function of CaV1.2-Ser1928 P is controversial, but the conservation of Ser1928 across species indirectly argues for physiological relevance. Two recent studies argue convincingly that Ser1700P and Ser1704P confer β-adrenergic receptor initiated modulation, whereas Ser1928P is not necessary for ICa,L modulation. Nonetheless, data are also convincing that Ser1928 is a substrate for PKA in cardiac myocytes.

The mechanism for Rem alteration of ICa,L has been the focus of several recent studies. Early suggestions were that Rem blocks current via ‘sponging’ Caβ; however, several lines of evidence argue in favor of a model whereby a Rem-LTCC interaction occurs at the cell surface. Despite RGK overexpression, CaV1.2 can be detected at the cell surface in heterologous expression systems, neurons, and other excitable cells. In addition, Ca2+-channel agonists causes rescue of ICa,L despite RGK overexpression in cardiac myocytes. Thus, it follows that in the absence of Rem, CaV1.2 channels at the surface are unmasked. This does not preclude alternative mechanisms of RGK modulation of LTCC function, particularly changes in

diminished. There are at least two non-competing mechanistic interpretations of how Ca-transients can be diminished despite elevated ICa,L density in Rem−/− cardiac myocytes. Rem may interfere with excitation-contraction coupling directly; alternatively, ICa,L driven by the action potential may be reduced in Rem−/− cardiac myocytes. Computer simulations support this notion. The +4 mV activation shift is sufficient to decrease ICa,L, despite an elevated maximally-activated conductance, and this activation shift may responsible for the reduced twitch Ca amplitude. Interestingly the reduced twitch Ca amplitude did not cause a change of shortening amplitude. The activation shift in Rem-null cardiac myocytes is consistent with relatively fewer de-phosphorylated LTCC. Elevated ICa,L leads to cardiac hypertrophy. Thus, it is plausible to view the shift of ICa,L activation as a compensatory protective effect against increased LTCC Gmax in the absence of Rem expression.

Other compensatory responses that might occur in Rem−/− mice are more complex. For example, there is an elevated in vivo heart rate (data not shown) consistent with elevated basal sympathetic tone. In this light, the relative de-phosphorylation of CaV1.2 could protect against Ca-overload via hyper-active ICa,L. However, we could not detect a change in PLB phosphorylation status, despite assessing conditions that preserved pentameric PLB. Note that such conditions show more pronounced changes in the phosphorylation status of Ser16 and Thr17. The difference in relative phosphorylation status of CaV1.2 and PLB can reside in a number of factors, including but not limited to differential accessibilities to PKA and differential modulation by a variety of phosphatases.

We used CaV1.2-Ser1928 phosphorylation as a surrogate for the capacity of LTCC to serve as a substrate for PKA activity. The function of CaV1.2-Ser1928P is controversial, but the conservation of Ser1928 across species indirectly argues for physiological relevance. Two recent studies argue convincingly that Ser1700P and Ser1704P confer β-adrenergic receptor initiated modulation, whereas Ser1928P is not necessary for ICa,L modulation. Nonetheless, data are also convincing that Ser1928 is a substrate for PKA in cardiac myocytes.

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Thus, it follows that in the absence of Rem, CaV1.2 channels at the surface are unmasked. This does not preclude alternative mechanisms of RGK modulation of LTCC function, particularly changes in
stability of LTCC complexes on the plasma membrane whereby Rem promotes Ca$_{\text{a,1.2}}$ internalization.\textsuperscript{15} We measured less total Ca$_{\text{a,1.2}}$ protein concomitantly with more maximally activated \(I_{\text{Ca,L}}\). This would be consistent with less Ca$_{\text{a,1.2}}$ internalization in the absence of Rem expression. Unfortunately we were not able to directly assess endogenous Ca$_{\text{a,1.2}}$—this will be an important prediction to evaluate in future studies. To counterbalance higher maximal \(I_{\text{Ca,L}}\), the activation curve shift prevents excessive Ca$^{2+}$ load as discussed above. It will be interesting to explore additional compensatory mechanisms possibly operating to maintain Ca-homeostasis. In summary our data are consistent with Rem null resulting in increased \(I_{\text{Ca,L}}\); this is the opposite result to that in response to Rem overexpression in cardiac myocytes.\textsuperscript{16}

In conclusion, the major finding of this study is that Rem-null mice display increased \(I_{\text{Ca,L}}\). The increased \(I_{\text{Ca,L}}\) density is accompanied by a positive shift of the activation curve resulting in a predicted, yet paradoxical decrease of \(I_{\text{Ca,L}}\) entry at voltages corresponding to the plateau of the action potential. Thus, Rem modulates \(I_{\text{Ca,L}}\) in vivo, and cardiac myocytes compensate for modulated \(I_{\text{Ca,L}}\) to maintain Ca-homeostasis.

**Methods**

**Generation of Rem$^{-/-}$ mice.** Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee. A targeting vector (Fig. 1) containing approximately 11.4 kb of the murine Rem1 gene was constructed from 129/Sv strain mouse genomic DNA. Additional details are provided in the Supplemental Methods.

**Adult ventricular myocyte cell isolation, electrophysiology and calcium imaging.** Single ventricular myocytes from female mice were enzymatically isolated following a modified AFCS protocol P00000125 as previously described in reference 30. External Ca was added incrementally back to the solution to 2.0 mM. Only rod-shaped, quiescent myocytes with clear edges were selected for current recording.

Ionic currents were recorded from Ca-tolerant female mouse ventricular cells at 37°C, 1–6 h post-isolation. Currents were recorded with the whole-cell configuration of the patch-clamp technique.

\(I_{\text{Ca,L}}\) was elicited by 500 ms long depolarizations to various test potentials arising from the holding potential of -40 mV. The stimulation frequency was 0.2 Hz. Peak current amplitude was defined as a difference between the peak value of \(I_{\text{Ca,L}}\) and its pedestal measured at the end of the pulse. \(I_{\text{Ca,L}}\) was normalized to cell capacitance.

\(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) current was recorded in voltage-clamped ventricular myocytes as described previously in reference 31. Briefly, NCX current was recorded using ramp pulses (shown in inset to Fig. 3). Outward and inward NCX currents were determined during the descending limb of the ramp at +40 and -80 mV, respectively. After taking the control record in K$^-$-free solution, the cell was superfused with 10 mM NiCl$_2$ in order to fully block the current. Thus, total NCX current was determined at both membrane potentials as a Ni$^{2+}$-sensitive current.

The measurements of intracellular calcium concentration were done on isolated cardiac myocytes. The cells were loaded with 2 \(\mu\)M fura-2-a.m. for 45 min. All recordings were performed at 20–22°C. Cells were paced for a minimum of 2 min at 1 Hz before recordings to allow steady-state Ca loading. At the end of each measurement, the intracellular calcium store was emptied by a pulse of 5 mM caffeine. During the caffeine pulse the cells were not stimulated. Cell shortening was recorded simultaneously using IonOptix myocam and software.

Additional recording conditions are presented in the Supplemental Methods. In all recordings the mouse of origin genotype was encoded to blind the investigator.

**Computer simulations.** Computer modeling was performed using the framework of the previously developed electrophysiology model for murine ventricular myocytes at 35°C. The difference in cell volume between the wild type and Rem$^{-/-}$ was calculated based on average measurements of the cell surface area and cell length and under the assumption that the myocytes are cylindrical in shape. The volumes of the junctional SR, network SR and dyadic space were adjusted according to their percentages (0.35, 1.05 and 0.1%, respectively) of the myoplasmic volume. The differences in the cell capacitance, myoplasmic volume and the volumes of the intracellular compartments between the wild type and Rem$^{-/-}$ were incorporated into the model during parameterization of the \(I_{\text{Ca,L}}\).

Parameters for \(I_{\text{Ca,L}}\) were fitted to experimental measurements obtained from the wild type and Rem$^{-/-}$, including the voltage-dependence of activation and inactivation kinetics, the current-voltage relationship, and the decay kinetics of the current, as described previously in reference 31. All other parameters for the wild-type and the Rem$^{-/-}$ models were unchanged from the existing model.\textsuperscript{19}

**Western blot analysis.** Hearts were excised from wild-type and Rem$^{-/-}$ mice and the left ventricles were isolated. Additional details are in Supplemental Methods. Anti-pan phospholamban (PLB) was obtained from Thermo Scientific and S16\textsuperscript{22} and T176\textsuperscript{22}-phospholamban antibodies were purchased from Badrilla Ltd. (UK). The Ca$_{\text{a,1.2}}$ distal carboxyl terminus (DCT) antibodies (pan-DCT and S1928\textsuperscript{-DCT}) custom designed (ECM Biosciences). Figure S4 shows a representative blot demonstrating phospho-selectivity of the S1928\textsuperscript{-antibody}; for these experiments a group of treated cells was exposed to 10 \(\mu\)M forskolin and 30 \(\mu\)M 3-isobutyl-1-methylxanthine (IBMX) for 10 min immediately prior to cell harvest. The Rad antibody was the kind gift of Dr. CR Kahn (Joslin Diabetes Center, Harvard Medical School). The anti-Rem antibody was monoclonal anti-Rem (2AE4) from Gamma-One Laboratories. All antibodies were diluted in 5% milk blocking buffer and incubated either over night at 4°C or for at least 1 h at room temperature with rocking. The membranes were washed and incubated with secondary antibody for use on the Li-Cor Odyssey Imaging System and analyzed using their software.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

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