Phospholamban Knockout Breaks Arrhythmogenic Ca\textsuperscript{2+} Waves and Suppresses Catecholaminergic Polymorphic Ventricular Tachycardia in Mice


Rationale: Phospholamban (PLN) is an inhibitor of cardiac sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase. PLN knockout (PLN-KO) enhances sarcoplasmic reticulum Ca\textsuperscript{2+} load and Ca\textsuperscript{2+} leak. Conversely, PLN-KO accelerates Ca\textsuperscript{2+} sequestration and aborts arrhythmogenic spontaneous Ca\textsuperscript{2+} waves (SCWs). An important question is whether these seemingly paradoxical effects of PLN-KO exacerbate or protect against Ca\textsuperscript{2+}-triggered arrhythmias.

Objective: We investigate the impact of PLN-KO on SCWs, triggered activities, and stress-induced ventricular tachyarrhythmias (VTs) in a mouse model of cardiac ryanodine-receptor (RyR2)-linked catecholaminergic polymorphic VT.

Methods and Results: We generated a PLN-deficient, RyR2-mutant mouse model (PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{+/−}) by crossbreeding PLN-KO mice with catecholaminergic polymorphic VT–associated RyR2-R4496C mutant mice. Ca\textsuperscript{2+} imaging and patch-clamp recording revealed cell-wide propagating SCWs and triggered activities in RyR2-R4496C\textsuperscript{+/−} ventricular myocytes during sarcoplasmic reticulum Ca\textsuperscript{2+} overload. PLN-KO fragmented these cell-wide SCWs into mini-waves and Ca\textsuperscript{2+} sparks and suppressed the triggered activities evoked by sarcoplasmic reticulum Ca\textsuperscript{2+} overload. Importantly, these effects of PLN-KO were reverted by partially inhibiting sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase with 2,5-di-tert-butylhydroquinone. However, Bay K, caffeine, or Li\textsuperscript{+} failed to convert mini-waves to cell-wide SCWs in PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{+/−} ventricular myocytes. Furthermore, ECG analysis showed that PLN-KO mice are not susceptible to stress-induced VTs. On the contrary, PLN-KO protected RyR2-R4496C\textsuperscript{mutant} mice from stress-induced VTs.

Conclusions: Our results demonstrate that despite severe sarcoplasmic reticulum Ca\textsuperscript{2+} leak, PLN-KO suppresses triggered activities and stress-induced VTs in a mouse model of catecholaminergic polymorphic VT. These data suggest that breaking up cell-wide propagating SCWs by enhancing Ca\textsuperscript{2+} sequestration represents an effective approach for suppressing Ca\textsuperscript{2+}-triggered arrhythmias. (Circ Res. 2013;113:517-526.)

Key Words: Ca\textsuperscript{2+} leak • Ca\textsuperscript{2+}-triggered arrhythmias • Ca\textsuperscript{2+} waves • phospholamban • ryanodine receptor calcium release channel • sarcoplasmic reticulum

In the heart, excitation–contraction coupling is mediated by a mechanism known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release.\textsuperscript{1,3} In this process, membrane depolarization activates the voltage-dependent L-type Ca\textsuperscript{2+} channel (LTCC), resulting in a small influx of external Ca\textsuperscript{2+} into the cytosol. This Ca\textsuperscript{2+} then binds to the cardiac Ca\textsuperscript{2+} release channel/ryanodine receptor (RyR2) and opens the channel, leading to a large release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR). In addition to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, SR Ca\textsuperscript{2+} release has long been known to occur spontaneously under conditions of SR Ca\textsuperscript{2+} overload in the absence of membrane depolarizations.\textsuperscript{4-9} A number of conditions, including excessive β-adrenergic stimulation, Na\textsuperscript{+} overload, elevated extracellular Ca\textsuperscript{2+} concentrations, and fast pacing, can result in SR Ca\textsuperscript{2+} overload, which,
in turn, can trigger spontaneous SR Ca\textsuperscript{2+} release in the form of propagating Ca\textsuperscript{2+} waves.\textsuperscript{3-9} These spontaneous Ca\textsuperscript{2+} waves (SCWs) can also alter membrane potential via activation of the electrogenic Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), leading to delayed afterdepolarizations (DADs), triggered activities, and triggered arrhythmias.\textsuperscript{8,10-12} In fact, SCW-evoked DADs are a major cause of ventricular tachyarrhythmias (VTs) in heart failure.\textsuperscript{13-14} SCW-evoked DADs also underlie the catecholaminergic polymorphic ventricular tachycardia (CPVT) associated with mutations in RyR2 and calsequestrin (CASQ2).\textsuperscript{15} CPVT-causing RyR2 or CASQ2 mutations have been shown to enhance the propensity for SCWs and DADs.\textsuperscript{15} Considering their critical role in arrhythmogenesis, suppressing SCWs represents a promising therapeutic strategy for the treatment of Ca\textsuperscript{2+}-triggered arrhythmias.

Because RyR2 mediates SCWs, inhibiting the RyR2 channel would be effective in suppressing SCWs. Indeed, reduction of RyR2 activity by tetracaine has been shown to inhibit spontaneous Ca\textsuperscript{2+} release.\textsuperscript{16} Furthermore, flecainide, a Na\textsuperscript{+}-channel blocker, has recently been shown to suppress SCWs in cardiac cells and CPVT in both mice and humans by modifying the gating of the RyR2 channel.\textsuperscript{17-19} Flecainide reduces the duration and increases the frequency of openings of the RyR2 channel. Similarly, we have recently shown that carvedilol, a nonselective β-blocker, also reduces the duration and increases the frequency of RyR2 openings, in addition to suppressing SCWs and CPVT, in mice.\textsuperscript{20} Interestingly, by modifying the gating of RyR2, flecainide increases the frequency and reduces the mass of Ca\textsuperscript{2+} sparks without affecting the SR Ca\textsuperscript{2+} content.\textsuperscript{18} These actions of flecainide effectively break up cell-wide propagating spontaneous Ca\textsuperscript{2+} release events (mini-waves or Ca\textsuperscript{2+} sparks).\textsuperscript{18,19} These observations have led to the suggestion that breaking up SCWs by modifying RyR2 gating represents an effective approach to suppressing SCW-evoked DADs and triggered arrhythmia.\textsuperscript{19}

The sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a) in the heart also plays a critical role in determining the initiation and propagation of SCWs.\textsuperscript{21-25} Increasing the activity of SERCA2a by removing phospholamban (PLN), an inhibitor of SERCA2a, has been shown to raise the SR Ca\textsuperscript{2+} load and to markedly enhance the frequency and amplitude of Ca\textsuperscript{2+} sparks.\textsuperscript{26-28} Interestingly, despite severe SR Ca\textsuperscript{2+} leak, no spontaneous cardiac arrhythmias in PLN-knockout (PLN-KO) mice have been reported. Furthermore, cell-wide propagating SCWs were hardly observed or were frequently aborted in PLN-KO cardiomyocytes.\textsuperscript{29} These observations raise the important question regarding whether accelerating SR Ca\textsuperscript{2+} uptake by removing PLN is proarrhythmic or antiarrhythmic. On the one hand, PLN-KO elevates SR Ca\textsuperscript{2+} content and increases SR Ca\textsuperscript{2+} leak, which would enhance the propensity for Ca\textsuperscript{2+}-leak-induced DADs. On the other hand, PLN-KO aborts SCWs, which would suppress SCW-induced DADs and triggered activities. To address this seemingly paradoxical question, we used PLN-KO mice along with the CPVT RyR2-R4496C mutant mice that are prone to SCWs and DAD-evoked VTs.\textsuperscript{20,26,27} We examined the impact of PLN-KO on the spatial and temporal properties of SCWs and the occurrence of triggered activities in ventricular myocytes expressing the RyR2-R4496C mutant. We also determined the effect of PLN-KO on the susceptibility to stress-induced VTs in the CPVT RyR2-R4496C mutant mice. We found that the removal of PLN breaks SCWs and suppresses triggered activities in the RyR2-R4496C mutant ventricular myocytes, in addition to reducing stress-induced VTs in the RyR2-R4496C mutant mice. These data are consistent with the notion that breaking up propagating SCWs by accelerating SR Ca\textsuperscript{2+} uptake is effective in suppressing Ca\textsuperscript{2+}-triggered arrhythmias.

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
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<tr>
<td>AP</td>
<td>action potential</td>
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<tr>
<td>CaMKII</td>
<td>calmodulin-dependent protein kinase II</td>
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<td>CASQ2</td>
<td>calsequestrin</td>
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<td>CPVT</td>
<td>catecholaminergic polymorphic ventricular tachycardia</td>
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<tr>
<td>DAD</td>
<td>delayed afterdepolarization</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>LTCC</td>
<td>L-type Ca\textsuperscript{2+} channel</td>
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<tr>
<td>NCX</td>
<td>Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger</td>
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<tr>
<td>PLN</td>
<td>phospholamban</td>
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<tr>
<td>RyR2</td>
<td>ryanodine receptor</td>
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<td>SCWs</td>
<td>spontaneous Ca\textsuperscript{2+} waves</td>
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<tr>
<td>SERCA2a</td>
<td>sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>IBHQ</td>
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**Figure 1.** Phospholamban (PLN)-knockout breaks cell-wide propagating spontaneous Ca\textsuperscript{2+} waves in isolated ventricular myocytes. Ventricular myocytes were isolated from ryanodine receptor (RyR2)-R4496C\textsuperscript{−/−} mutant mice, PLN-deficient, RyR2-R4496C\textsuperscript{−/−} mice (PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−}), or PLN\textsuperscript{−/−} mice, and loaded with the fluorescent Ca\textsuperscript{2+} indicator dye fluo-4 acetoxy methyl ester. The fluo-4-acetoxy methyl-loaded cells were perfused with Krebs-Ringer-HEPES buffer containing 6 mmol/L extracellular Ca\textsuperscript{2+} to induce sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} overload. Store overload–induced spontaneous SR Ca\textsuperscript{2+} release events were detected by line-scan confocal Ca\textsuperscript{2+} imaging. Representative line-scan images of spontaneous Ca\textsuperscript{2+} release in isolated RyR2-R4496C\textsuperscript{−/−} (n=39; A), PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−} (n=43; B), and PLN\textsuperscript{−/−} (n=9; C) ventricular myocytes are shown.
Methods
To determine whether removal of PLN alters the spatial and temporal profiles of intracellular Ca$^{2+}$ signaling in RyR2 R4496C$^{-/}$ mutant ventricular myocytes, we crossbred the RyR2-R4496C$^{-/}$ mutant mice with the PLN-KO mice (PLN$^{-/-}$) to produce a PLN-deficient mouse line expressing the RyR2 R4496C$^{-/}$ mutation (PLN$^{-/-}$/RyR2-R4496C$^{-/}$). Detailed methods are provided in the Online Data Supplement.

Results
PLN-KO Breaks Cell-Wide Propagating SCWs in Isolated RyR2-R4496C$^{-/}$ Mutant Ventricular Myocytes
Cardiomyocytes display SCWs propagating throughout the entire cell under conditions of SR Ca$^{2+}$ overload. Interestingly, PLN-KO markedly alters the pattern of spontaneous Ca$^{2+}$ release by breaking up the cell-wide propagating SCWs into multiple, localized mini-waves and sparks. To determine whether PLN-KO is also able to break up cell-wide propagating SCWs in ventricular myocytes harboring a CPVT-causing RyR2 mutation, R4496C, which is prone to inducing spontaneous Ca$^{2+}$ release, we crossbred the PLN-KO mice (PLN$^{-/-}$) with the RyR2-R4496C$^{-/}$ mutant heterozygous mice (RyR2-R4496C$^{+/}$) to generate double-mutant mice, PLN$^{-/-}$/RyR2-R4496C$^{+/}$ (Figure 1A). Ventricular myocytes were isolated from the PLN$^{-/-}$/RyR2-R4496C$^{+/}$ and PLN$^{-/-}$/RyR2-R4496C$^{-/}$ mice, loaded with fluo-4 acetoxymethyl ester (AM), and perfused with elevated extracellular Ca$^{2+}$ (6 mmol/L) to induce SR Ca$^{2+}$ overload and SCWs. Intracellular Ca$^{2+}$ dynamics were monitored using line-scan confocal Ca$^{2+}$ imaging. As shown in Figure 1A, SCWs in RyR2-R4496C$^{-/}$ ventricular myocytes originated from the middle (or either end) of the cell and propagated across the entire cell, similar to those reported previously. On the other hand, SCWs in the PLN$^{-/-}$/RyR2-R4496C$^{-/}$ ventricular myocytes frequently and simultaneously occurred at multiple sites and aborted shortly after their initiation without propagating across the entire cell. They appeared as short-lived mini-waves or clusters of Ca$^{2+}$ sparks (Figure 1B). Similar spontaneous Ca$^{2+}$ release events were also detected in ventricular myocytes from PLN$^{-/-}$ mouse hearts (Figure 1C), consistent with those shown previously. Furthermore, this impact of PLN-KO was not limited to SCWs induced by elevated external Ca$^{2+}$. We found that PLN-KO also breaks SCWs induced by isoproterenol (Online Figure I). Considered together, these observations indicate that PLN-KO is able to break up cell-wide SCWs in the RyR2-R4496C$^{-/}$ mutant ventricular myocytes.

PLN-KO Fragments Cell-Wide Propagating SCWs in Ventricular Myocytes in Intact RyR2-R4496C$^{-/}$ Hearts
The markedly altered spatial and temporal profiles of intracellular Ca$^{2+}$ dynamics in PLN$^{-/-}$/RyR2-R4496C$^{-/}$ or PLN$^{-/-}$ ventricular myocytes may have resulted from cellular damage during cell isolation. To avoid this potential problem, we carried out line-scan confocal Ca$^{2+}$ imaging of epicardial ventricular myocytes in intact hearts. Thus, the distinct features of spontaneous Ca$^{2+}$ release in isolated PLN$^{-/-}$/RyR2-R4496C$^{-/}$ or RyR2-R4496C$^{-/}$ hearts (Figure 2A, top) after interruption of electric pacing, SCWs occurred at 1 or 2 sites and propagated throughout the entire cell in ventricular myocytes in intact RyR2-R4496C$^{-/}$ hearts. Analysis of the spatially averaged fluorescence showed well-separated spontaneous Ca$^{2+}$ release events with amplitudes similar to those of stimulated Ca$^{2+}$ transients (Figure 2A, bottom). On the other hand, spontaneous Ca$^{2+}$ release in ventricular myocytes in intact PLN$^{-/-}$/RyR2-R4496C$^{-/}$ (Figure 2B, top) or PLN$^{-/-}$ (Online Figure II, top) hearts frequently occurred at multiple sites as mini-waves or clusters of Ca$^{2+}$ sparks. Analysis of spatially averaged fluorescence showed numerous spontaneous Ca$^{2+}$ release events with amplitudes much smaller than those of the stimulated Ca$^{2+}$ transients (Figure 2B; Online Figure II, bottom). This pattern of spontaneous Ca$^{2+}$ release observed in ventricular myocytes in the intact PLN$^{-/-}$/RyR2-R4496C$^{-/}$ or PLN$^{-/-}$ heart is very similar to that seen in isolated cells (Figure 1). Thus, the distinct features of spontaneous Ca$^{2+}$ release in isolated PLN$^{-/-}$/RyR2-R4496C$^{-/}$ or
PLN−/− myocytes reflect the intrinsic properties related to intracellular Ca²⁺ handling of these cells, rather than reflecting the consequences of cellular damage during cell isolation.

To further assess the spatial and temporal properties of spontaneous Ca²⁺ release in ventricular myocytes in intact RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/−, and PLN−/− hearts, we analyzed all spontaneous Ca²⁺ release events (Figure 2A and 2B; Online Figures II [middle] and III) and classified them into 3 categories: waves, mini-waves, and sparks, based on their total fluorescence/event. As shown in Figure 3, RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/−, and PLN−/− ventricular myocytes displayed very different distributions of spontaneous Ca²⁺ release events. In RyR2-R4496C+/− ventricular myocytes, 93% of the total spontaneously released Ca²⁺ was released in the form of Ca²⁺ waves, whereas mini-waves and Ca²⁺ sparks together consisted of only 7% of the total spontaneously released Ca²⁺ (Figure 3A and 3D). In contrast, a majority of the spontaneously released Ca²⁺ in PLN−/−/RyR2-R4496C+/− or PLN−/− cells was released as mini-waves (77% to 74%), whereas Ca²⁺ waves and sparks consisted of 20% to 25% and 3% to 2% of the total released Ca²⁺ respectively (Figure 3B–3D). Furthermore, the occurrence of Ca²⁺ waves was significantly greater in RyR2-R4496C+/− cells than in PLN−/−/RyR2-R4496C+/− or PLN−/− cells (Figure 3D). In contrast, the occurrence of mini-waves and Ca²⁺ sparks was significantly greater in PLN−/−/RyR2-R4496C+/− or PLN−/− cells than in RyR2-R4496C+/− cells (Figure 3E–3G). In other words, RyR2-R4496C+/− ventricular myocytes exhibited predominantly mini-waves and Ca²⁺ sparks, with few Ca²⁺ waves (Figure 3A–3C).

We next determined and compared the properties of Ca²⁺ waves, mini-waves, and Ca²⁺ sparks in ventricular myocytes in intact RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/−, and PLN−/− hearts. We found that the amplitude, full duration at half maximum, and rate of rise of Ca²⁺ waves or mini-waves are significantly greater in RyR2-R4496C+/− cells than in PLN−/−/RyR2-R4496C+/− or PLN−/− cells (Figure 4A and 4B). On the other hand, the amplitude and duration of Ca²⁺ sparks are significantly smaller in RyR2-R4496C+/− cells than in PLN−/−/RyR2-R4496C+/− or PLN−/− cells. Consistent with previously reported data, PLN-KO increased the amplitude and decreased the full duration at half maximum of stimulated Ca²⁺ transients (Figure 2; Online Figure IV). Taken together, our single-cell and intact heart Ca²⁺-imaging studies demonstrate that PLN-KO suppresses SCWs in RyR2-R4496C+/− mutant ventricular myocytes by breaking up cell-wide propagating SCWs into mini-waves and Ca²⁺ sparks and by reducing the amplitude, duration, and rate of rise of SCWs.

**PLN-KO Suppresses Triggered Activities in RyR2-R4496C+/− Ventricular Myocytes**

Spontaneous SR Ca²⁺ release can lead to DADs, and DADs can trigger action potentials (APs) when the amplitude of a DAD reaches the threshold for Na⁺ channel activation. Whether spontaneous Ca²⁺ release can generate DADs with amplitudes that are sufficient to trigger APs depends on the amplitude and rate of rise of the spontaneous Ca²⁺ release. The substantially different spatial and temporal properties of spontaneous Ca²⁺ release events in RyR2-R4496C+/− hearts with or without phospholamban (PLN). Line-scan confocal images were digitized and spontaneous Ca²⁺ release events were detected and classified using a custom-made program as described in Methods in the Online Data Supplement. Distribution of spontaneous Ca²⁺ release events in RyR2-R4496C+/− hearts (A), PLN−/−/RyR2-R4496C+/− (B), and PLN−/− (C) hearts according to their total fluorescence. Three types of spontaneous Ca²⁺ release events (Ca²⁺ sparks, mini-waves, and waves) were classified based on the size of the total fluorescence (Methods in the Online Data Supplement). The red line represents a Gaussian fit of the distribution of Ca²⁺ waves in RyR2-R4496C+/− hearts. **D.,** The overall contribution (%) of sparks, mini-waves, and waves in RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/−, and PLN−/− hearts. The occurrence (events/scan) of Ca²⁺ waves (E), mini-waves (F), and Ca²⁺ sparks (G) in RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/−, and PLN−/− hearts. Data shown are mean±SEM from 19 (PLN−/−), 39 (RyR2-R4496C+/−), and 43 (PLN−/−/RyR2-R4496C+/−) line-scan images (P<0.001).
Ca\(^{2+}\) release in RyR2-R4496C\(^{-/-}\) and PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) cells raise the important question of whether PLN-KO can also affect the occurrence of triggered activities. To address this question, we perfused ventricular myocytes isolated from the RyR2-R4496C\(^{-/-}\) and PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) mice with 6 mmol/L extracellular Ca\(^{2+}\) to induce SR Ca\(^{2+}\) overload and spontaneous Ca\(^{2+}\) release. We then recorded the membrane potential in these cells using the perforated patch current clamp technique. As shown in Figure 5, RyR2-R4496C\(^{-/-}\) ventricular myocytes displayed frequent DADs and spontaneously triggered APs (Figure 5A-a, 5C, and 5D), which is consistent with the results reported previously.\(^{31}\) Interestingly, under the same conditions, PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) ventricular myocytes showed a large number of small DADs, but little or no triggered APs (Figure 5B-a, 5C, and 5D). Thus, these observations indicate that PLN-KO suppresses the occurrence of triggered APs in RyR2-R4496C\(^{-/-}\) ventricular myocytes.

Considering the close link between SCWs and triggered activities,\(^{10,34}\) the lack of triggered APs in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) cells is probably attributable to the absence of SCWs in these cells. To test this possibility, we mimicked the action of PLN by partially inhibiting SERCA2a with 2,5-di-tert-butylhydroquinone (tBHQ; 5 \(\mu\)mol/L), a SERCA2a inhibitor. As shown in Figure 5E, partial inhibition of SERCA2a by tBHQ in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) ventricular myocytes converted multiple and frequent mini-waves into cell-wide propagating SCWs similar to those observed in RyR2-R4496C\(^{-/-}\) ventricular myocytes. Importantly, the tBHQ treatment increased the occurrence of triggered APs (Figure 5B-b, 5C, and 5D) in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) ventricular myocytes. However, the tBHQ treatment did not markedly affect the occurrence of DADs or triggered APs in RyR2-R4496C\(^{-/-}\) cells (Figure 5A-b, 5C, and 5D). Therefore, these data suggest that PLN-KO suppresses triggered activities by breaking up cell-wide SCWs.

### Role of RyR2, LTCC, NCX, and SR Ca\(^{2+}\) Load in Breaking Cell-Wide SCWs in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) Ventricular Myocytes

The conversion of mini-waves to cell-wide SCWs by tBHQ in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) cells also suggests that enhanced SERCA2a activity as a consequence of PLN-KO is an important determinant of the occurrence of mini-waves. However, PLN-KO may also lead to compensatory changes in the expression of Ca\(^{2+}\)-handling proteins, which may in turn contribute to the genesis of mini-waves in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) cells. To test this possibility, we assessed the expression levels of RyR2, LTCC, SERCA2a, and NCX proteins in the RyR2-R4496C\(^{-/-}\) and PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) hearts using immunoblotting analysis. As shown in Figure 6A, there were no significant differences in their expression levels, except for RyR2 that exhibited a slightly higher (\(\approx 10\%; P<0.05\)) expression in PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) hearts than in RyR2-R4496C\(^{-/-}\) hearts.

PLN-KO may also possibly break SCWs by altering the activities of LTCC, RyR2, or NCX, in addition to that of SERCA2a. For instance, mini-waves could result from reduced activity of LTCC or RyR2, which would reduce Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release and thus suppress the propagation of Ca\(^{2+}\) waves. Furthermore, mini-waves could also result from increased activity of NCX, which would enhance Ca\(^{2+}\) release in RyR2-R4496C\(^{-/-}\) and PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\) cells.

Figure 4. Effect of phospholamban (PLN)-knockout on spontaneous Ca\(^{2+}\) release in intact ryanodine receptor (RyR2)-R4496C (RC) mutant hearts. Spontaneous Ca\(^{2+}\) release events in intact RyR2-R4496C\(^{-/-}\), PLN\(^{-/-}/\text{RyR2-R4496C}^{-/-}\), and PLN\(^{-/-}\) hearts were divided into Ca\(^{2+}\) waves (A), mini-waves (B), and Ca\(^{2+}\) sparks (C) as described in Figure 3, and their amplitude (top), full duration at half maximum (FDHM; middle), and rate of rise (bottom) were compared. Data shown are mean±SEM from 19 to 43 line-scan images (*P<0.01, **P<0.001, versus RC\(^{-/-}\)).
removal and thus reduce SR Ca\(^{2+}\) content and SR Ca\(^{2+}\) release. To test these possibilities, we assessed the effects of Bay K 8644 (an LTCC agonist), caffeine (an agonist of RyR2), and Li\(^{+}\) (an inhibitor of NCX) on spontaneous SR Ca\(^{2+}\) release in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) ventricular myocytes. In sharp contrast to tBHQ, Bay K, caffeine, or Li\(^{+}\) failed to convert miniwaves into cell-wide SCWs in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) cells (Figure 6B–6D).

The SR Ca\(^{2+}\) content is also a critical determinant of SCWs.\(^35,36\) Accordingly, we determined the SR Ca\(^{2+}\) content in RyR2-R4496C\(^{+/-}\), PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\), and PLN\(^{-/-}\) cells. We found that PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) and PLN\(^{-/-}\) cells displayed significantly higher SR Ca\(^{2+}\) content than RyR2-R4496C\(^{+/-}\) cells (Figure 6E). Thus, enhanced SERCA2a activity, rather than reduced SR Ca\(^{2+}\) content, decreased LTCC or RyR2 activity, or increased NCX activity, is a major contributor to the breakup of cell-wide SCWs.

**PLN-KO Protects the RyR2-R4496C\(^{+/-}\) Mice From Stress-induced VTs**

The RyR2-R4496C mutant mice are highly susceptible to CPVT, which is caused by DAD-induced triggered activities.\(^20,30-32\) The lack of triggered activities in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) ventricular myocytes after SR Ca\(^{2+}\) overload raises the possibility that PLN-KO may also suppress CPVT. To test this possibility directly, we recorded ECGs in wild-type littermates, RyR2-R4496C\(^{+/-}\), RyR2-R4496C\(^{+/-}\), PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\), PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\), and PLN\(^{-/-}\) mice before and after the injection of a mixture of caffeine and epinephrine. Similar to the results reported previously,\(^20\) caffeine and epinephrine induced long-lasting VTs in RyR2-R4496C\(^{+/-}\) mice but not in their wild-type littermates (Figure 7). The RyR2-R4496C\(^{+/-}\) homozygous mice are especially vulnerable to stress-induced VTs, displaying sustained VTs for the entire 30-minute period of recording after the injection of the triggers.\(^20\) Remarkably, caffeine and epinephrine induced little or no VTs in PLN\(^{-/-}\) or PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) mice and induced only short-lasting VTs in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) mice (Figure 8). These data indicate that PLN-KO mice are not susceptible to CPVT and that PLN-KO protects the RyR2-R4496C\(^{+/-}\) mutant mice from stress-induced VTs.

**PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) Mice Display No Severe Defects in Cardiac Structure**

Enhanced SR Ca\(^{2+}\) leak as a result of overexpression of the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in the heart has been shown to cause severe heart failure and dilated cardiomyopathy.\(^37,38\) It would be of interest to determine whether enhanced SR Ca\(^{2+}\) leak as a result of PLN-KO could induce severe structural changes in the heart. To this end, we performed echocardiography on conscious RyR2-R4496C\(^{+/-}\), PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\), and PLN\(^{-/-}\) mice. We found that the RyR2-R4496C\(^{+/-}\) mutation itself did not induce gross changes in cardiac structure and function (Online Table I), which is in agreement with previous reports.\(^30,31\) We also found no severe structural defects in the PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) or PLN\(^{-/-}\) hearts, despite the chronic SR Ca\(^{2+}\) overload and the enhanced spontaneous Ca\(^{2+}\) leak (mini-waves and Ca\(^{2+}\) sparks) in the PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) or PLN\(^{-/-}\) cardiomyocytes. This is consistent with previous observations that PLN\(^{-/-}\) mice show enhanced myocardial contractility but no gross defects in cardiac structure.\(^26,39,40\) There are, however, some small differences between PLN\(^{-/-}\)/RyR2-R4496C\(^{+/-}\) and wild-type mice and between PLN\(^{-/-}\) and wild-type mice (Online Table I). Thus, as...
superseding arrhythmias by breaking Ca\textsuperscript{2+} waves

with PLN\textsuperscript{−/−} hearts, PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{+/-} hearts show no severe defects in cardiac structure.

**Discussion**

A novel and surprising finding of the present study is that, despite severe SR Ca\textsuperscript{2+} leak, PLN-KO mice are not susceptible to stress-induced VTs. In fact, on the contrary, PLN-KO protects a mouse model harboring the CPVT-causing RyR2-R4496C mutation from stress-induced VTs. Single-cell and intact heart Ca\textsuperscript{2+} imaging show that PLN-KO effectively breaks cell-wide propagating SCWs into mini-waves and Ca\textsuperscript{2+} sparks. Furthermore, PLN-KO markedly suppresses SCW-evoked triggered activities in RyR2-R4496C mutant ventricular myocytes. These observations indicate that spontaneous SR Ca\textsuperscript{2+} leak, in the forms of mini-waves and Ca\textsuperscript{2+} sparks (leaky SR) without generating cell-wide propagating SCWs, is not necessarily linked to triggered activities and triggered arrhythmias. Our data suggest that breaking up cell-wide propagating SCWs into mini-waves and Ca\textsuperscript{2+} sparks is protective against Ca\textsuperscript{2+}-triggered arrhythmias.

An important question is how PLN-KO rescues the CPVT phenotype of the RyR2-R4496C mutant mice despite severe diastolic SR Ca\textsuperscript{2+} leak. Increased SR Ca\textsuperscript{2+} leak is often observed in cardiomyocytes derived from heart failure specimens and is thought to be a major cause of Ca\textsuperscript{2+}-triggered arrhythmias.\textsuperscript{12-14} This is because diastolic SR Ca\textsuperscript{2+} leak can alter the membrane potential through the activation of the electroneutral NCX, resulting in DADs. These DADs can potentially trigger ectopic APs, which in turn can lead to triggered arrhythmia.\textsuperscript{8,10-12} However, whether a DAD is able to trigger an AP depends on its amplitude. An AP is triggered when the amplitude of a DAD reaches the activation threshold for Na\textsuperscript{+} channels. Furthermore, the amplitude of a DAD is dependent on the amplitude and rate of rise of spontaneous SR Ca\textsuperscript{2+} release.\textsuperscript{10,14} A total SR Ca\textsuperscript{2+} release of 50% to 70% of the SR Ca\textsuperscript{2+} load is required to generate DADs with amplitudes sufficient to produce an AP.\textsuperscript{10} Therefore, the small diastolic SR Ca\textsuperscript{2+} leak in the form of brief, localized Ca\textsuperscript{2+} sparks or even mini-waves themselves are unlikely to produce DADs...
with amplitudes that are high enough to cause triggered activities. The SR Ca\textsuperscript{2+}-overload-induced cell-wide propagating SCWs are however capable of producing triggered activities.

In accordance with this view, we detected a large number of small DADs but only a few triggered APs in PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{+/−} mice. Furthermore, we found that the SR Ca\textsuperscript{2+} load or altered RyR2, LTCC, or NCX activity due to transforming mini-waves to cell-wide propagating SCWs by partially inhibiting SERCA2a with tBHQ. However, increasing the activity of LTCC with Bay K or the activity of RyR2 with caffeine or decreasing the activity of NCX with Li\textsuperscript{+} failed to convert mini-waves to cell-wide SCWs in PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−} ventricular myocytes. Furthermore, we found that the SR Ca\textsuperscript{2+} content was elevated in PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−} ventricular myocytes compared to that in RyR2-R4496C\textsuperscript{−/−} cells. Thus, enhanced SERCA2a activity as a result of PLN-KO probably contributes to the breakup of cell-wide SCWs in PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−} ventricular myocytes, rather than reduced SR Ca\textsuperscript{2+} load or altered RyR2, LTCC, or NCX activity due to potential PLN-KO-induced compensatory changes. The enhanced SERCA2a activity as a result of PLN ablation would result in a rapid resequstration of the released Ca\textsuperscript{2+} into the SR. This would effectively buffer or reduce the cytosolic Ca\textsuperscript{2+} level, which is important for the propagation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, thus limiting the spatial spread of Ca\textsuperscript{2+} waves.\textsuperscript{29} This effect on SCWs would reduce the amplitude of DADs and thus decrease the propensity for triggered APs and triggered arrhythmias. Interestingly, Davia et al\textsuperscript{30} have shown that adenovirus-mediated overexpression of SERCA2a in adult rabbit ventricular myocytes reduced the occurrence of aftercontractions. Our findings are consistent with those of Davia et al\textsuperscript{31} and further show that enhanced SERCA2a activity suppresses triggered activities by breaking up cell-wide SCWs.

Although PLN-KO is effective in suppressing stress-induced VTs in the CPVT RyR2-R4496C mutant mice, whether PLN-KO would be beneficial in suppressing stress-induced VTs in other animal models or in humans with CPVT remains to be determined. Numerous studies have investigated the impact of PLN-KO on heart failure and cardiomyopathies, albeit not specifically on stress-induced arrhythmias.\textsuperscript{32–44} For example, PLN-KO has been shown to rescue the heart failure and dilated cardiomyopathy phenotypes in a mouse model in which the cytoskeletal, muscle-specific LIM protein is ablated.\textsuperscript{42} PLN-KO has also been shown to reverse the cardiac hypertrophy phenotype in a mouse model with calsequestrin overexpression.\textsuperscript{45} However, PLN-KO does not rescue cardiac dysfunction in all mouse models of heart failure and cardiomyopathies tested.\textsuperscript{46–47} For instance, despite the rescue of SR Ca\textsuperscript{2+} handling, PLN-KO exaggerates heart failure and mortality in CaMKII\textsuperscript{δ}-overexpressing mice.\textsuperscript{46} PLN deficiency in the CaMKII\textsuperscript{δ}-overexpressing mice was suggested to result in markedly increased SR Ca\textsuperscript{2+} load despite enhanced diastolic SR Ca\textsuperscript{2+} leak due to CaMKII\textsuperscript{δ}-dependent hyperphosphorylation of RyR2. The combination of increased SR Ca\textsuperscript{2+} load and enhanced SR Ca\textsuperscript{2+} leak predisposes cardiomyocytes to cell death and other Ca\textsuperscript{2+}-mediated abnormalities. Similarly, the combination of enhanced SR Ca\textsuperscript{2+} load as a result of overexpression of the skeletal muscle SR Ca\textsuperscript{2+}/ATPase (SERCA1a) or PLN-KO and increased SR Ca\textsuperscript{2+} leak as a consequence of CASQ2-KO leads to myocyte apoptosis, dilated cardiomyopathy, and early mortality.\textsuperscript{48} On the other hand, we found that the PLN-KO RyR2-R4496C mutant mice show no severe structural and functional defects. Thus, unlike the results in the CaMKII\textsuperscript{δ}-overexpressing or CASQ2-KO mice, PLN-KO does not lead to cardiac dysfunction in the PLN\textsuperscript{−/−}/RyR2-R4496C\textsuperscript{−/−} mice despite enhanced spontaneous SR Ca\textsuperscript{2+} release. The exact reasons for this discrepancy are not clear. Spontaneous SR Ca\textsuperscript{2+} release in the CaMKII\textsuperscript{δ}-overexpressing or CASQ2-KO mice may be much severer than that in the RyR2-R4496C\textsuperscript{−/−} mice. Consistent with this view, both CaMKII\textsuperscript{δ}-overexpressing and CASQ2-KO mice, but not RyR2-R4496C\textsuperscript{−/−} mice, exhibit dilated cardiomyopathy, heart failure, or hypertrophy.\textsuperscript{38,49} Thus, it is possible that the enhanced SERCA2a activity as a result of PLN-KO may not be able to compensate fully for the much severer SR Ca\textsuperscript{2+} leak caused by CaMKII\textsuperscript{δ} overexpression or CASQ2-KO, leading to chronic diastolic SR Ca\textsuperscript{2+} leak, cardiomyopathies, and heart failure. Therefore, whether PLN-KO produces beneficial effects would be dependent on...
the cause and severity of the defects of the disease model. Furthermore, in contrast to the observations in PLN-KO mice, PLN deficiency in humans as a result of nonsense mutations is associated with severe dilated cardiomyopathy and heart failure.\textsuperscript{49} Hence, the beneficial effects of PLN-KO may also be species dependent.

In summary, we show that PLN-KO effectively breaks SCWs into mini-waves and Ca\textsuperscript{2+} sparks in mouse ventricular myocytes expressing the SCW-prone, CPVT-causing RyR2-R4496C mutant. We further show that PLN-KO markedly suppresses SCW-evoked triggered activity and completely protects the RyR2-R4496C\textsuperscript{−/−} mutant mice against CPVT. Thus, as with inhibition of RyR2 activity, breaking up SCWs by enhancing SERCA2a activity represents an effective means for suppressing Ca\textsuperscript{2+}-triggered arrhythmias.

Limitations

In this study, we used confocal line-scan imaging to estimate and compare the SR Ca\textsuperscript{2+} contents in cardiomyocytes with different genotypes by measuring the amplitude of caffeine-evoked Ca\textsuperscript{2+} transients. Although this approach yielded useful information on the relative SR Ca\textsuperscript{2+} contents of different groups of cells, it did not provide a quantitative assessment of the SR Ca\textsuperscript{2+} content. Furthermore, the amplitude of caffeine-evoked Ca\textsuperscript{2+} transients could be influenced by various factors, such as cytosolic Ca\textsuperscript{2+} buffering. Because increased SERCA2a activity as a result of PLN ablation would enhance the removal of cytosolic Ca\textsuperscript{2+} (equivalent to increased cytosolic Ca\textsuperscript{2+} buffering), the increase in the relative SR Ca\textsuperscript{2+} content detected in PLN\textsuperscript{−/−}/R4496C\textsuperscript{−/−} cells would have been underestimated due to this increased Ca\textsuperscript{2+} removal/buffering. However, because PLN ablation increases the SR Ca\textsuperscript{2+} content in PLN\textsuperscript{−/−}/R4496C\textsuperscript{−/−} cardiomyocytes, the lack of cell-wide propagating SCWs in these cells is unlikely to be due to a reduced SR Ca\textsuperscript{2+} content.

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Disclosures

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References

1. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol. 1983;245:C1–C14.
18. Hilliard FA, Steele DS, Laver D, Yang Z, Le Marchand SJ, Chopra N, Piston DW, Huke S, Knollmann BC. Flecainide inhibits arrhythmogenic Ca\textsuperscript{2+} waves by open state block of ryanodine receptor Ca\textsuperscript{2+} release channels and reduction of Ca\textsuperscript{2+} spark mass. J Mol Cell Cardiol. 2010;48:293–301.


Phospholamban Knockout Breaks Arrhythmogenic Ca\textsuperscript{2+} Waves and Suppresses Catecholaminergic Polymorphic Ventricular Tachycardia in Mice


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SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

Generation of RyR2-R4496C and PLN-KO/RyR2-R4496C mutant mice  RyR2-R4496C mutant mice were generated as previously described \(^1\). To determine whether removal of PLN alters the spatial and temporal profiles of intracellular Ca\(^{2+}\) signalling in RyR2 R4496C\(^{+/−}\) mutant ventricular myocytes, we crossbred the RyR2-R4496C\(^{+/−}\) mutant mice with the PLN knockout (PLN-KO) mice (PLN\(^{−/−}\)) to produce a PLN deficient mouse line expressing the RyR2 R4496C\(^{+/−}\) mutation (PLN\(^{−/−}/\text{RyR2-R4496C}\(^{+/−}\)).

Isolation of ventricular myocytes  Ventricular myocytes were isolated using retrograde aortic perfusion as described previously \(^2\). Isolated cells were kept at room temperature in Krebs-Ringers-HEPES (KRH) buffer (in mM: 125 NaCl, 12.5 KCl, 25 HEPES, 6 glucose, and 1.2 MgCl\(_2\), pH7.4) containing 20 mM taurine, 20 mM 2,3-butanedione monoxime (BDM), 5 mg/ml albumin, and 1 mM free Ca\(^{2+}\) until use.

Single cell Ca\(^{2+}\) imaging of isolated ventricular myocytes  Freshly isolated mouse ventricular myocytes were added to glass coverslips pre-coated with 50\(\mu\)g/ml laminin, and loaded with 5 \(\mu\)M fluo-4, AM (Molecular Probes, USA) in KRH buffer containing 1 mM Ca\(^{2+}\) for 20 min at room temperature as described previously \(^1\). The glass coverslip pre-mounted to a recording chamber was then placed onto an inverted microscope (Nikon ECLIPSE Ti) equipped with a Nikon CFI Plan Apo VC 60xWI objective. The fluo-4 loaded cells were perfused with KRH buffer. The extracellular Ca\(^{2+}\) concentration was then increased stepwise from 1 to 3, and 6 mM to induce spontaneous Ca\(^{2+}\) release, followed by the addition of 5\(\mu\)M 2,5-Di-tert-butylhydroquinone (tBHQ), 1\(\mu\)M Bay K 8644, 0.5 mM caffeine, or 125 mM LiCl (replacing NaCl in the KRH buffer). In the case of isoproterenol treatment, the fluo-4 loaded cells were perfused with KRH buffer containing 2 mM extracellular Ca\(^{2+}\) plus 100 nM isoproterenol. Confocal line-scanning (512 pixels and 1.9 ms per line) were performed along the longitudinal axis of cells for 10 seconds using the Nikon A1R confocal system. The fluo-4 loaded myocytes were excited using the 488 nm argon laser and the fluorescence emission at 500-550 nm was recorded. The line-scan images were processed with the NIS-Elements AR 4.0 and analyzed with a custom-made wavelet-based
detection method applied to each time-column in a linescan image in order to detect and separate spontaneous Ca\textsuperscript{2+} release events into Ca\textsuperscript{2+} sparks, mini-waves and cell-wide propagating Ca\textsuperscript{2+} waves.

**In situ confocal Ca\textsuperscript{2+} imaging of intact hearts** Excised hearts were perfused with Tyrode solution (in mM: 120 NaCl, 5.4 KCl, 25 NaHCO\textsubscript{3}, 1 MgCl\textsubscript{2}, 0.42 NaH\textsubscript{2}PO\textsubscript{4}, 10 glucose, 10 taurine, 5 creatine, pH 7.4) containing 5\(\mu\)M Rhod-2, AM (Biotium, San Francisco, CA, USA) for 45 min via retrograde Langendorff perfusion system at 25 °C as previously described \textsuperscript{3}. The extracellular Ca\textsuperscript{2+} concentration was then increased stepwise from Ca\textsuperscript{2+} free to 0.125, 0.25, 0.5, 1.0, and 2 mM. The Langendorff-perfused hearts were placed on a recording chamber mounted onto the Nikon A1R microscope for in situ confocal imaging (line-scan) of Ca\textsuperscript{2+} signals from epicardial ventricular myocytes at 33 °C. To avoid motion artifacts during Ca\textsuperscript{2+} imaging, (s)-(−)-blebbistatin (10 \(\mu\)M, Toronto Research Chemical Inc., Toronto, Canada) was added to the perfusion solution. The extracellular Ca\textsuperscript{2+} concentration was then increased stepwise from 2 to 3 and 6 mM to induce SR Ca\textsuperscript{2+} overload. The sinoatrial node was ablated using the gemini cautery system (Harvard Apparatus, MA, USA). The hearts were stimulated using electrodes placed at the right atrium at 6Hz for at least 30 seconds. Pacing was then interrupted and the post-pacing induced spontaneous Ca\textsuperscript{2+} release was monitored by line scanning (512 pixels and 1.9 ms per line) along the longitudinal axis of a cell using the Nikon A1R confocal system. The Rhod-2 loaded hearts were excited using the diode laser (561 nm) and fluorescence emission was detected at 570-620 nm. The line-scan images were processed with the Nikon NIS-Elements AR 4.0 and analyzed with a custom-made wavelet-based detection method in order to detect and separate spontaneous Ca\textsuperscript{2+} release events into Ca\textsuperscript{2+} sparks, mini-waves and cell-wide propagating Ca\textsuperscript{2+} waves.

**Immunoblotting analysis** Mouse hearts were crushed by a Wollenberger clamp precooled in liquid nitrogen. The crushed heart tissues were stored at -80 °C until use. Frozen cardiac tissues were pulverized in liquid nitrogen and homogenized immediately with a Brinkmann Polytron PT 15 homogenizer (setting 8, 4 bursts for 15 s each) in 6 volumes of 30 mM KH\textsubscript{2}PO\textsubscript{4} (pH 7.0), 40 mM NaF, 5 mM EDTA, 300 mM sucrose, 4 \(\mu\)M leupeptin, 1 mM benzamidine, 100 \(\mu\)M PMSF and 0.5 mM DTT. Aliquots of homogenates were solubilized in 50 mM Tris-HCl (pH 7.4) plus 3% SDS for 1 h at room temperature. The insoluble materials were then removed by centrifugation at
4000 x g for 10 min. The protein concentration of the supernatant was determined using a BioRad detergent-compatible protein assay kit. Solubilized proteins (20 - 30 µg) were used for SDS-PAGE. SDS-PAGE resolved proteins were transferred to nitrocellulose membranes at 45 V for 18 - 20 h at 4 °C in the presence of 0.01% SDS according to the method of Towbin et al. The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween-20 and 5% skim milk powder. The blocked membrane was incubated with anti-RyR2, anti-LTCC, anti-SERCA2a, anti-NCX, or anti-β-actin antibodies, and washed with PBS containing 0.5% Tween-20 for 15 min three times. The membrane was then incubated with the secondary anti-mouse or anti-rabbit IgG (H&L) antibodies conjugated to horseradish peroxidase (1:20,000) for 30 min. After washing for 15 min three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce. The band intensities were analyzed using Image-J software, and normalized to that of the β-actin band.

**Image analysis:** All image processing and detection methods were implemented using MATLAB (The Mathworks Inc., Boston, MA).

*Image preprocessing:* RBG images were converted to grayscale intensity images by using the green channel in the case of single cells experiments and the red channel for intact heart experiments. The noise of each image was robustly estimated by computing the median absolute deviation of the image pixels. Each image was then denoised by iteratively applying a 10x10 median filter from 1-4 times depending on its noise content determined by the noise estimate. In experiments in which the cell did not cover the full width of the linescan, pixels presenting a persistently high fluorescence activity were identified as cell pixels. Cell boundaries were detected as regions presenting a large spatial derivative of the time integral of the fluorescence. In cases where cell contraction results in a movement of the cell boundaries, cell limits are defined by the minimum width of the cell during the experiment thus avoiding the use of fluorescence data from regions outside the cell. Images were normalized using a time-dependent basal fluorescence $b_i(t)$ at each spatial location $i$ inside the cell. The reason for using a time-dependent baseline is twofold: First, to correctly account for consecutive events between which fluorescence does not return to the basal level. Second, to correct for temporal drifts in the basal fluorescence due to experimental factors such as photobleaching. We refer to $s_i(t)$ as the time-dependent fluorescence signal at cell location $i$. At each time $t$, the baseline $b_i(t)$ is estimated as the first decile (lower 10%) of $s_i(t)$.
computed in a time window centered at sample $t$. In order to avoid overestimation of the basal fluorescence due to the occurrence of large events within the temporal window, the window size is suitably chosen as the maximum number of consecutive time samples in which the fluorescence exceeds the mean fluorescence of the experiment. Normalized linescan at each pixel $i$ is then defined as $z_i(t) = s_i(t)/b_i(t)$, so that the normalized fluorescence of release events is measured relative to its local baseline in both space and time. In order to ensure a common global contrast of all images, the contrast of each image was adjusted so that pixel values range from 1 to the average maximum fluorescence of all the images in the study.

**Event detection**: For each cell location, a wavelet-based detection method was applied to the normalized time-dependent fluorescence signal $z_i(t)$ in order to detect Ca$^{2+}$ release events. The method uses the continuous wavelet transform (CWT), which measures the similarity between the local shape of the signal $z_i(t)$ and the shape of a reference template function (the wavelet). In particular, we use a bell-shaped Gaussian wavelet function with a duration of 65ms, which allows the localization of Ca$^{2+}$ release events with a duration ranging from 20-40ms (Ca$^{2+}$ sparks) to 300ms (mini-waves). Events with longer durations were detected using an amplitude threshold of 1.3, corresponding to events with a maximum fluorescence 20% over the baseline fluorescence. Further filtering of the detected events was performed by removing those that did not present a statistically significant increase in fluorescence with respect to the local activity in the surrounding region. Specifically, a two sample Student’s t-test with significance level $p=0.01$ was performed to compare the average event signal and the average signal in a surrounding region with equal pixel area. In order to avoid erroneous grouping of close events in a single detected region, all the events are skeletonized and eventually divided using a probabilistic clustering method based on orientation, occurrence time and spatial overlap. Indeed, these features allow distinguishing cases in which Ca$^{2+}$ waves with different origins converge or Ca$^{2+}$ waves with a common origin that propagate in opposite directions. Online Fig. III shows an example of wavelet detection and skeletonization (panel a-c).

**Event characterization and classification**: Each detected event is characterized by the following measures: total time duration (ms), total spatial extension ($\mu$m), area ($\mu$m*ms), total fluorescence ($\mu$m*ms*F/F0), propagation velocity ($\mu$m/ms), Full Duration at Half Maximum (ms, FDHM, spatially averaged along event width), time to peak (ms, spatially averaged along event
width) and rate of rise (s-1, spatially averaged along event width). Both stimulated and spontaneous Ca$^{2+}$ transients are identified as events presenting a total spatial extension covering the full linescan width and propagation velocity above 2 mm/s (yellow events in supplementary figure 1e). Such events are removed from the set of analyzed events. The remaining detected events are classified in three groups using the total emitted fluorescence. Classification boundaries are heuristically set by visual inspection of the detected events in the linescan images and result in the following groups: (a) Spontaneous Ca$^{2+}$ waves: Events presenting a total fluorescence above 7000 µm*ms*ΔF/F0. This corresponds to spontaneous Ca$^{2+}$ release events that fall within the Gaussian fit in Fig. 3A and that are at least 23 µm wide (red events in Online Fig. IIIe). (b) Spontaneous mini-waves: Events presenting a total fluorescence between 400 and 7000 µm*ms*ΔF/F0. This corresponds to Ca$^{2+}$ release events wider than 4 µm with a mean width of 21 µm (blue events in Online Fig. IIIe). (c) Ca$^{2+}$ sparks: Events presenting a total fluorescence between 50 and 400 µm*ms*ΔF/F0. This corresponds to non-propagating Ca$^{2+}$ release events wider than 0.8 µm with a mean width of 4 µm (green events in Online Fig. IIIe). Events presenting a total fluorescence under 50 µm*ms*ΔF/F0 are considered as false alarms of the detection method and removed from the analyzed set of events. The mean width of these events was 1.2 µm.

**Perforated patch current clamp recordings of isolated ventricular myocytes**  
Freshly isolated mouse ventricular myocytes were placed on glass coverslips pre-coated with 20 mg/ml laminin and superfused with normal Tyrode solution (in mM: 140 NaCl, 5.4 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 1 Na$_2$HPO$_4$, 5 HEPES, 5 glucose, pH 7.4) at room temperature (22 °C). Measurements of resting membrane potential were made using the amphotericin-perforated patch-clamp method. Amphotericin-b (final concentration 200 µg/ml) was added to the pipette solution from a 20 mg/ml stock solution in dimethyl sulfoxide. Patch pipettes were fabricated from borosilicate capillaries, and were lightly fire-polished before use. The d.c. resistance of the pipettes was in the range of 3-5 MΩ when filled with intracellular solution (in mM: 110 K-aspartate, 10 KCl, 5 MgCl$_2$, 5 Na$_2$ATP, 1 CaCl$_2$, 10 EGTA, 10 HEPES, pH 7.2). Recordings of membrane potential were carried out with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in current-clamp mode (I=0). Current signals were digitized using a 1200 Digi-Data acquisition system (Axon Instruments, Foster City, CA, USA), stored on a PC and analyzed off-line with pClamp 8. Plots and statistical analysis of the data were generated with SigmaPlot (Systat Software; San Jose, CA,
Note that one of the 10 PLN<sup>-/-</sup>/RyR2-R4496C<sup>+/+</sup> cells patched displayed 54-fold higher frequency of spontaneously triggered APs compared to the mean frequency from the rest of the 9 cells in the absence of tBHQ. This cell is considered an outlier and was excluded from the final analysis shown in panels C and D. Membrane potentials recorded by the patch-clamp amplifier were adjusted by -10mV to compensate for the liquid junction potential between the patch-pipette and bath solutions.

**ECG recordings**

All studies with mice were approved by the Animal Care Committee of the University of Calgary and complied with Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (Publication no. 85-23, revised 1996). Mice (3-4 month-old) were anesthetized with 5% isoflurane and restrained back down on a heating pad at 27°C. ECG electrode leads were placed in the left hindlimb and right forelimb subcutaneously. The anesthetic was reduced to 0.4% to minimize its impact on heart rate. When the heart rate returned to a physiological level (600 - 650bpm) recording was started. The ECG was recorded for 10 minutes before the mice were injected intraperitoneally with epinephrine (1.6 mg/kg) and caffeine (120 mg/kg) to trigger CPVT. The ECG was then recorded for a further 30 minutes. The electric signals were recorded and analyzed with MP100 system (BIOPAC).

**Assessment of cardiac function by echocardiography**

Echocardiograms (M-mode measurements) to assess systolic function were obtained from conscious mice (3-4 month-old), as described previously<sup>7</sup>. Septal wall thickness (SWT), posterior wall thickness (PWT), left ventricular (LV) internal dimensions in systole (LVIDs) and diastole (LVIDd) were determined from LV M-mode scans by use of a Hewlett-Packard Sonus 5500 ultrasound machine with a 15-MHz linear transducer. Values for heart rate (HR) were obtained from Doppler measurements of LV outflow tract velocities. Fractional shortening (FS) and the velocity of circumferential fiber shortening (Vcf) were calculated as indexes of systolic function: \[ FS(\%) = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100; \] \[ Vcf = \frac{FS}{ET}. \] LV mass was calculated from the following equation: \[ LV\ mass\ (mg) = \left[ (LVIDd + SWT + PWT)^{3} - LVIDd^{3} \right] \times 1.055, \] where 1.055 is the density of the myocardium.
**Statistical analysis**  All values shown are mean ± SEM unless indicated otherwise. To test for difference between groups, we used Student’s t test (2-tailed) or one-way ANOVA with post hoc test. A P value < 0.05 was considered to be statistically significant.
Online Figure I

A Isolated RyR2-R4496C+/− ventricular myocyte (100nM ISO)

B Isolated PLN−/−/RyR2-R4496C+/− ventricular myocyte (100nM ISO)

C Isolated PLN−/− ventricular myocyte (100nM ISO)

Online Figure I. PLN-KO breaks cell-wide propagating spontaneous Ca^{2+} waves induced by isoproterenol in ventricular myocytes  Ventricular myocytes were isolated from RyR2-R4496C+/−, PLN−/−/RyR2-R4496C+/− or PLN−/− mice, and loaded with the fluorescent Ca^{2+} indicator dye fluo-4, AM. The fluo-4 loaded cells were perfused with KRH buffer containing 2 mM extracellular Ca^{2+} and 100 nM isoproterenol (ISO). Spontaneous SR Ca^{2+} release events were detected by linescan confocal Ca^{2+} imaging. Representative line-scan images of spontaneous Ca^{2+} release in RyR2-R4496C+/− (A), PLN−/−/RyR2-R4496C+/− (B), and PLN−/− (C) ventricular myocytes (n = 15-18 cells) are shown. (D) SR Ca^{2+} contents in RyR2-R4496C+/− (a), PLN−/−/RyR2-R4496C+/−
(b), and PLN\(^{-/-}\) (c) ventricular myocytes treated with 100 nM ISO were estimated by measuring the amplitude of caffeine (20mM) induced Ca\(^{2+}\) transients (d). Data shown are mean ± SEM (n=13) (*P < 0.01, vs RyR2-R4496C\(^{+/+}\)). Note that the SR Ca\(^{2+}\) contents in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/+}\) and PLN\(^{-/-}\) ventricular myocytes are significantly higher than that in RyR2-R4496C\(^{+/+}\) cells. Thus, the break-up of cell-wide SCWs in PLN\(^{-/-}\)/RyR2-R4496C\(^{+/+}\) and PLN\(^{-/-}\) ventricular myocytes is unlikely to be due to reduced SR Ca\(^{2+}\) content.
Online Figure II

**PLN<sup>−/−</sup> intact heart Ca<sup>2+</sup> imaging**

Stimulated transients

Mini-waves

Representative line-scan images (top) and the corresponding digitized images (middle) of mini-waves and Ca<sup>2+</sup> sparks in intact PLN<sup>−/−</sup> hearts are shown. Bottom panel shows the spatial average of fluorescence signal along the scan-line.

**Online Figure II. Spontaneous Ca<sup>2+</sup> release in intact PLN<sup>−/−</sup> hearts** Intact hearts isolated from PLN<sup>−/−</sup> mice were loaded with Rhod-2-AM and Langendorff-perfused with 6 mM extracellular Ca<sup>2+</sup> and paced at 6 Hz to induce SR Ca<sup>2+</sup> overload. Spontaneous SR Ca<sup>2+</sup> release in epicardial ventricular myocytes in intact hearts was monitored by line-scan confocal Ca<sup>2+</sup> imaging.
Online Figure III. Detection and classification of Ca^{2+} release events. (a) Preprocessed normalized linescan image. (b) Wavelet-based event detection (white regions) and division of large events using a skeletonization method (red lines). (c) Detected events after event division. Different colors are used for visual distinction of different events. The two large events in orange and purple have been divided as different waves. (d) Linescan image from an intact PLN^{+/−}/RyR2-R4496C^{+/−} heart. (e) Classification of events. Stimulated Ca^{2+} transients are yellow, Ca^{2+} waves are red, Ca^{2+} mini-waves are blue and Ca^{2+} sparks are green.
Online Figure IV. Comparison of stimulated Ca^{2+} transients in RyR2-R4496C^{+/−} hearts with or without PLN-KO

Intact hearts isolated from RyR2-R4496C^{+/−} (a) or PLN^{−/−}/RyR2-R4496C^{+/−} (b) mice were loaded with Rhod-2-AM and Langendorff-perfused with 6 mM extracellular Ca^{2+} and paced at 6 Hz. Stimulated Ca^{2+} transients in epicardial ventricular myocytes in intact hearts were monitored by line-scan confocal Ca^{2+} imaging. Representative line-scan images (a, b), the profile of line-integrated fluorescence (c), amplitude (d), and FDHM (e) of stimulated Ca^{2+} transients in RyR2-R4496C^{+/−} or PLN^{−/−}/RyR2-R4496C^{+/−} hearts are shown. Data shown are mean ± SEM from 40-43 line-scan images (*P < 0.05).
Online Table I. RyR2-R4496C+/−, PLN+/−/RyR2-R4496C+/−, or PLN+/− hearts display no severe structural defects Echocardiograms were used to assess the heart structure and function of conscious wild type and mutant mice. Data shown are means ± SEM (n=5-8). IVSd, interventricular septal thickness at diastole (mm), LVIDd, diastolic left ventricular (LV) internal dimension (mm), LVPWd, diastolic LV posterior wall(mm), LVIDs, systolic LV internal dimension (mm), LVET, LV ejection time (ms), FS, fractional shortening (%), EF, ejection fraction (%), LVMASS, LV mass (mg), Vcf, mean circumferential fiber shortening velocity (circs/s), RR int, R-to-R interval time (ms).

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* p<0.05, vs WT

References


