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

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Abstract

Rationale—Phospholamban (PLN) is an inhibitor of cardiac sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2a). PLN knockout (PLN-KO) enhances sarcoplasmic reticulum (SR) Ca^{2+} load and Ca^{2+} leak. Conversely, PLN-KO accelerates Ca^{2+} sequestration and aborts arrhythmogenic spontaneous Ca^{2+} waves (SCWs). An important question is whether these seemingly paradoxical effects of PLN-KO exacerbate or protect against Ca^{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 ventricular tachycardia (CPVT).

Methods and Results—We generated a PLN-deficient, RyR2 mutant mouse model ($PLN^{-/-}$/ RyR2-R4496C$^{+/-}$) by crossbreeding PLN-KO mice with CPVT-associated RyR2-R4496C mutant mice. Ca^{2+} imaging and patch-clamp recording revealed cell-wide propagating SCWs and triggered activities in RyR2-R4496C$^{+/-}$ ventricular myocytes during SR Ca^{2+} overload. PLN-KO fragmented these cell-wide SCWs into mini-waves and Ca^{2+} sparks, and suppressed triggered activities evoked by SR Ca^{2+} overload. Importantly, these effects of PLN-KO were reverted by partially inhibiting SERCA2a with 2,5-Di-tert-butylhydroquinone (tBHQ). However, Bay K, caffeine, or Li^+ failed to convert mini-waves to cell-wide SCWs in $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ ventricular myocytes. Furthermore, ECG analysis showed that PLN-KO mice are not susceptible

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DISCLOSURES
None.

to stress-induced VTs. On the contrary, PLN-KO protected RyR2-R4496C mutant mice from stress-induced VTs.

Conclusions—Our results demonstrate that despite severe SR Ca^{2+} leak, PLN-KO suppresses triggered activities and stress-induced VTs in a mouse model of CPVT. These data suggest that breaking up cell-wide propagating SCWs by enhancing Ca^{2+} sequestration represents an effective approach for suppressing Ca^{2+}-triggered arrhythmias.

Keywords

Sarcoplasmic reticulum; ryanodine receptor; phospholamban; Ca^{2+} waves; Ca^{2+} leak; Ca^{2+}-triggered arrhythmias

INTRODUCTION

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

Since RyR2 mediates SCWs, inhibiting the RyR2 channel would be effective in suppressing SCWs. Indeed, reducing the RyR2 activity by tetracaine has been shown to inhibit spontaneous Ca^{2+} release[16]. Further, it has recently been shown that flecainide, a Na^+ channel blocker, suppresses SCWs in cardiac cells and CPVT in both mice and humans by modifying the gating of the RyR2 channel[17–19]. Flecainide reduces the duration and increases the frequency of openings of the RyR2 channel. Similarly, we have recently shown that carvedilol, a non-selective beta-blocker, also reduces the duration and increases the frequency of RyR2 openings, and suppresses SCWs and CPVT in mice[20]. Interestingly, by modifying the gating of RyR2, flecainide increases the frequency and reduces the mass of Ca^{2+} sparks without affecting the SR Ca^{2+} content[18]. These actions of flecainide effectively break up cell-wide propagating SCWs into non-propagating spontaneous Ca^{2+} release events (mini-waves or Ca^{2+} sparks)[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[19].

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) in the heart also plays a critical role in determining the initiation and propagation of SCWs[21–25]. It has been shown

that increasing the activity of SERCA2a by removing phospholamban (PLN), an inhibitor of SERCA2a, elevated SR Ca^{2+} load and markedly enhanced the frequency and amplitude of Ca^{2+} sparks[26–28]. Interestingly, despite severe SR Ca^{2+} leak, no spontaneous cardiac arrhythmias in PLN knockout (PLN-KO) mice have been reported. Further, cell-wide propagating SCWs were hardly observed or frequently aborted in PLN-KO cardiomyocytes[29]. These observations raise an important question as to whether accelerating SR Ca^{2+} uptake by removing PLN is pro-arrhythmic or anti-arrhythmic. On one hand, PLN-KO elevates SR Ca^{2+} content and increases SR Ca^{2+} leak, which would enhance the propensity for Ca^{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 employed the PLN-KO mice along with the CPVT RyR2-R4496C mutant mice that are prone to SCWs and DAD-evoked VTs[20, 26]. 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, and diminishes 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^{2+} uptake is effective in suppressing Ca^{2+}-triggered arrhythmias.

METHODS

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$^{-/-}$/RyR2-R4496C$^{+/-}$). Detailed methods are provided in the Online Supplement.

RESULTS

PLN-KO breaks cell-wide propagating spontaneous Ca^{2+} waves in isolated RyR2-R4496C$^{+/-}$ mutant ventricular myocytes

It is well known that cardiomyocytes display spontaneous Ca^{2+} waves (SCWs) propagating throughout the entire cell under the conditions of SR Ca^{2+} overload[4–8]. 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[29]. To determine whether PLN-KO is also able to break up cell-wide propagating SCWs in ventricular myocytes harbouring a CPVT-causing RyR2 mutation R4496C that is prone to 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$^{+/-}$. Ventricular myocytes were isolated from the RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ mice, loaded with fluo-4 AM, and perfused with elevated extracellular Ca^{2+} (6 mM) to induce SR Ca^{2+} overload and SCWs. Intracellular Ca^{2+} dynamics were monitored using line-scan confocal Ca^{2+} imaging. As shown in Fig.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[20, 30–32]. 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 (Fig. 1B). Similar spontaneous Ca^{2+} release events were also detected in ventricular myocytes from PLN$^{-/-}$ mouse hearts (Fig. 1C), consistent with those shown previously[29]. Further, 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 Fig. I). Taken 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[33]. Rhod-2 AM loaded hearts from the RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$, and PLN$^{-/-}$ mice were Langendorff-perfused with elevated extracellular Ca^{2+} (6 mM) and paced at 6 Hz to induce SR Ca^{2+} overload and subsequent SCWs. As seen in Fig. 2A (top panel), after interruption of electrical 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 revealed well-separated spontaneous Ca^{2+} release events with amplitudes similar to that of stimulated Ca^{2+} transients (Fig. 2A, bottom panel). On the other hand, spontaneous Ca^{2+} release in ventricular myocytes in intact PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ (Fig. 2B, top panel) or PLN$^{-/-}$ (Online Fig. II, top panel) 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 that of the stimulated Ca^{2+} transients (Fig. 2B, Online Fig. II, bottom panels). 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 (Fig. 1). Thus, the distinct features of spontaneous Ca^{2+} release in isolated PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ myocytes reflect the intrinsic properties of intracellular Ca^{2+} 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 Ca^{2+} release in ventricular myocytes in intact RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$ hearts, we analyzed all spontaneous Ca^{2+} release events (Figs. 2A, 2B, Online Fig. II, middle panels, and Online Fig. III) and classified them into three categories: waves, mini-waves, and sparks, based on their total fluorescence/event. As seen in Fig. 3, RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$, and PLN$^{-/-}$ ventricular myocytes displayed very different distributions of spontaneous Ca^{2+} release events. In RyR2-R4496C$^{+/-}$ ventricular myocytes, 93% of the total spontaneously released Ca^{2+} was released in the form of Ca^{2+} waves, while mini-waves and Ca^{2+} sparks together consisted of only 7% of the total spontaneously released Ca^{2+} (Fig. 3A,D). In contrast, a majority of the spontaneously released Ca^{2+} in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ cells was released as mini waves (77–74%), while Ca^{2+} waves and sparks consisted of 20–25% and 3-2% of the total released Ca^{2+}, respectively (Fig. 3B,C,D). Furthermore, the occurrence of Ca^{2+} waves was significantly greater in RyR2-R4496C$^{+/-}$ cells than in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ cells (Fig. 3D). On the other hand, the occurrence of mini-waves and Ca^{2+} sparks was significantly greater in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ cells than in RyR2-R4496C$^{+/-}$ cells (Fig. 3E,F,G). In other words, RyR2-R4496C$^{+/-}$ ventricular myocytes displayed primarily Ca^{2+} waves, whereas PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ ventricular myocytes exhibited predominantly mini-waves and Ca^{2+} sparks with few Ca^{2+} waves (Fig. 3A,B,C).

We next determined and compared the properties of Ca^{2+} waves, mini waves, and Ca^{2+} sparks in ventricular myocytes in intact RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$hearts. We found that the amplitude, full duration at half maximum (FDHM), and rate of rise of Ca^{2+} waves or mini waves are significantly greater in RyR2-R4496C$^{+/-}$ cells than in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ cells (Fig. 4A,B). On the other hand, the

amplitude and duration of Ca^{2+} sparks are significantly smaller in RyR2-R4496C$^{+/-}$ cells than in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$ cells. Consistent with previously reported data[27], PLN-KO increased the amplitude and decreased the FDHM of stimulated Ca^{2+} transients (Fig. 2, Online Fig. IV). Taken together, our single cell and intact heart Ca^{2+} 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 Ca^{2+} sparks and reducing the amplitude, duration, and rate of rise of SCWs.

PLN-KO suppresses triggered activities in RyR2-R4496C$^{+/-}$ ventricular myocytes

Spontaneous SR Ca^{2+} 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 Ca^{2+} release can generate DADs with amplitudes that are sufficient to trigger APs depends on the amplitude and rate of rise of the spontaneous Ca^{2+} release[10, 34]. The substantially different spatial and temporal properties of spontaneous Ca^{2+} release in RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$/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$^{-/-}$/RyR2-R4496C$^{+/-}$ mice with 6 mM 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 Fig. 5, RyR2-R4496C$^{+/-}$ ventricular myocytes displayed frequent DADs and spontaneously triggered APs (Figs. 5Aa, C and D), which is consistent with those reported previously[31]. Interestingly, under the same conditions, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ ventricular myocytes exhibited a large number of small DADs, but little or no triggered APs (Figs. 5Ba, C and D). Thus, these observations indicate that PLN-KO suppresses the occurrence of triggered APs in RyR2-R4496C$^{+/-}$ ventricular myocytes.

Given the close link between SCWs and triggered activities[10, 34], the lack of triggered APs in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ cells is likely 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 μM), a SERCA2a inhibitor. As shown in Fig. 5E, partial inhibition of SERCA2a by tBHQ in PLN$^{-/-}$/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 (Figs. 5Bb, C,D) in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ ventricular myocytes. On the other hand, the tBHQ treatment did not markedly affect the occurrence of DADs or triggered APs in RyR2-R4496C$^{+/-}$ cells (Figs. 5Ab,C,D). 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$^{-/-}$/RyR2-R4496C$^{+/-}$ ventricular myocytes

The conversion of mini-waves to cell-wide SCWs by tBHQ in PLN$^{-/-}$/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, it is possible that 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$^{-/-}$/RyR2-R4496C$^{+/-}$ cells. To test this possibility, we assessed the expression level of RyR2, LTCC, SERCA2a, and NCX proteins in the RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ hearts using immunoblotting analysis. As shown in Fig. 6A, there were no significant differences in their expression levels except for RyR2 that exhibited a slightly higher (~10%, $P<0.05$) expression in PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ hearts than in RyR2-R4496C$^{+/-}$ hearts.

It is also possible that PLN-KO may break SCWs by altering the activity of LTCC, RyR2, or NCX in addition to 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 the propagation of Ca^{2+} waves. Further, mini-waves could also result from increased activity of NCX, which would enhance Ca^{2+} removal, and thus reduce SR Ca^{2+} content and SR Ca^{2+} release. To test these possibilities, we assessed the impact of Bay K 8644 (a LTCC agonist), caffeine (a RyR2 agonist), 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 mini-waves into cell-wide SCWs in $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ cells (Fig. 6B,C,D).

The SR Ca^{2+} content is also a critical determinant of spontaneous Ca^{2+} waves[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 (Fig. 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 break-up of cell-wide SCWs.

PLN-KO protects the RyR2-R4496C$^{+/-}$ mice from stress-induced VTs

It has been shown that 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 upon SR Ca^{2+} overload raises the possibility that PLN-KO may also suppress CPVT. To directly test this possibility, we recorded ECG in WT 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 those reported previously[20], caffeine and epinephrine induced long-lasting ventricular tachyarrhythmias (VTs) in RyR2-R4496C$^{+/-}$ mice, but not in their WT littermates (Fig. 7). The RyR2-R4496C$^{+/+}$ homozygous mice are especially vulnerable to stress-induced VTs, displaying sustained VTs for the entire 30 min-period of recording after the injection of the triggers[20]. Remarkably, caffeine and epinephrine induced little or no VTs in the $PLN^{-/-}$ mice or $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ mice, and only short-lasting VTs in the $PLN^{-/-}$/RyR2-R4496C$^{+/+}$ mice (Fig.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 WT, 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 those reported previously[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 enhanced spontaneous Ca^{2+} leak (mini waves and Ca^{2+} sparks) in the $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ or $PLN^{-/-}$ cardiomyocytes. This is consistent with previous observation 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 WT mice and between $PLN^{-/-}$ and WT mice (Online Table I). Thus, as with $PLN^{-/-}$ hearts, $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ hearts show no severe defects in cardiac structure.

DISCUSSION

A novel and surprising finding of the present study is that, despite severe SR Ca^{2+} leak, PLN-KO mice are not susceptible to stress-induced VTs. In fact, on the contrary, PLN-KO protects a mouse model harbouring the CPVT-causing RyR2-R4496C mutation from stress-induced VTs. Single cell and intact heart Ca^{2+} imaging reveal that PLN-KO effectively breaks the cell-wide propagating SCWs into mini-waves and Ca^{2+} sparks. Furthermore, PLN-KO markedly suppresses SCW-evoked triggered activities in RyR2-R4496C mutant ventricular myocytes. These observations indicate that spontaneous SR Ca^{2+} leak in the forms of mini-waves and Ca^{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^{2+} sparks is protective against Ca^{2+} triggered arrhythmias.

An important question is how PLN-KO rescues the CPVT phenotype of the RyR2-R4496C mutant mice in the face of severe diastolic SR Ca^{2+} leak? Increased SR Ca^{2+} leak is often observed in cardiomyocytes from heart failure and is thought to be a major cause of Ca^{2+} triggered arrhythmias[12–14]. This is because diastolic SR Ca^{2+} leak can alter the membrane potential via the activation of the electrogenic Na^+/Ca^{2+} exchanger (NCX), resulting in DADs. These DADs can potentially trigger ectopic APs that in turn can lead to triggered arrhythmia[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^+ channels. Furthermore, the amplitude of DADs is dependent on the amplitude and rate of rise of spontaneous SR Ca^{2+} release[10, 34]. It has been estimated that a total SR Ca^{2+} release of 50–70% of the SR Ca^{2+} load is required to generate DADs with amplitudes sufficient to produce an AP[10]. Therefore, the small diastolic SR Ca^{2+} leak in the form of brief, localized Ca^{2+} sparks or even mini-waves themselves are unlikely to produce DADs with amplitudes that are high enough to cause triggered activities. It is the SR Ca^{2+} overload induced cell-wide propagating SCWs that are 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 $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes that displayed severe SR Ca^{2+} leak in the form of Ca^{2+} sparks and mini-waves. On the other hand, we observed a number of triggered APs in RyR2-$R4496C^{+/-}$ ventricular myocytes that exhibited cell-wide propagating SCWs. Interestingly, triggered APs were readily detected in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes after transforming mini-waves to cell-wide propagating SCWs by partially inhibiting SERCA2a with tBHQ. On the other hand, increasing the activity of LTCC with Bay K or the activity of RyR2 with caffeine or decreasing the activity of NCX with Li^+ failed to convert mini-waves to cell-wide SCWs in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes. Further, we found that the SR Ca^{2+} content was elevated in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes compared to that in RyR2-$R4496C^{+/-}$ cells. Thus, enhanced SERCA2a activity as a result of PLN-KO likely contributes to the break-up of cell-wide SCWs in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes, rather than reduced SR Ca^{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 re-sequestration of the released Ca^{2+} into the SR. This would effectively buffer or reduce the cytosolic Ca^{2+} level that is important for the propagation of Ca^{2+} waves via Ca^{2+} induced Ca^{2+} release, thus limiting the spatial spread of Ca^{2+} waves[29]. This effect on SCWs would reduce the amplitude of DADs and thus decrease the propensity for triggered APs and triggered arrhythmias. It is of interest to note that Davia et al.[41] have shown that adenovirus-mediated overexpression of SERCA2a in adult rabbit ventricular myocytes reduced the occurrence of aftercontractions. Our present findings are consistent with those of Davia et al. and further demonstrate 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. Albeit not specifically on stress-induced arrhythmias, a number of studies have investigated the impact of PLN-KO on heart failure and cardiomyopathies[42–44]. For example, it has been shown that PLN-KO rescues the heart failure and dilated cardiomyopathy phenotypes in a mouse model in which the cytoskeletal, muscle specific LIM protein (MLP) is ablated[42]. PLN-KO has also been shown to reverse the cardiac hypertrophy phenotype in a mouse model with calsequestrin overexpression[43]. However, PLN-KO does not rescue cardiac dysfunction in all mouse models of heart failure and cardiomyopathies tested[45–47]. For instance, it has recently been shown that despite the rescue of SR Ca^{2+} handling, PLN-KO exaggerates heart failure and mortality in CaMKIIδc overexpressing mice[46]. It was suggested that PLN deficiency in the CaMKIIδc overexpressing mice resulted in markedly increased SR Ca^{2+} load in the face of enhanced diastolic SR Ca^{2+} leak due to CaMKIIδc-dependent hyperphosphorylation of RyR2. The combination of increased SR Ca^{2+} load and enhanced SR Ca^{2+} leak predisposes cardiomyocytes to cell death and other Ca^{2+}-mediated abnormalities. Similarly, the combination of enhanced SR Ca^{2+} load as a result of overexpression of the skeletal muscle SR Ca^{2+} ATPase (SERCA1a) or PLN-KO and increased SR Ca^{2+} leak as a consequence of CASQ2-KO led to myocyte apoptosis, dilated cardiomyopathy, and early mortality[48]. On the other hand, we found that the PLN-KO RyR2-R4496C mutant mice show no severe structural and functional defects. Thus, unlike that seen in the CaMKIIδc overexpressing mice or CASQ2-KO mice, PLN-KO does not lead to cardiac dysfunction in the $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ mice even in the face of enhanced spontaneous SR Ca^{2+} release. The exact reasons for this discrepancy are not clear. Spontaneous SR Ca^{2+} release in the CaMKIIδc-overexpressing or CASQ2-KO mice may be much more severe than that in the RyR2-$R4496C^{+/-}$ mice. Consistent with this view, both CaMKIIδc-overexpressing and CASQ2-KO mice, but not RyR2-$R4496C^{+/-}$ mice, exhibit dilated cardiomyopathy, heart failure or hypertrophy[38, 49]. Thus, it is possible that the enhanced SERCA2a activity as a result of PLN-KO may not be able to fully compensate for the much more severe SR Ca^{2+} leak caused by CaMKIIδc overexpression or CASQ2-KO, leading to chronic diastolic SR Ca^{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. It is also important to note that, opposite to those observed in PLN-KO mice, PLN deficiency in humans as a result of nonsense mutations is associated with severe dilated cardiomyopathy and heart failure[50]. 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^{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-$R4496C^{+/-}$ mutant mice against CPVT. Thus, as with inhibiting RyR2 activity, breaking up SCWs by enhancing SERCA2a activity represents an effective means in suppressing Ca^{2+} triggered arrhythmias.

Limitations

In this study, we used confocal linescan imaging to estimate and compare the SR Ca^{2+} contents in cardiomyocytes with different genotypes by measuring the amplitude of caffeine evoked Ca^{2+} transients. Although this approach yielded useful information on the relative SR Ca^{2+} contents of different groups of cells, it did not provide a quantitative assessment of the SR Ca^{2+} content. Further, the amplitude of caffeine evoked Ca^{2+} transients could be influenced by various factors such as cytosolic Ca^{2+} buffering. Since increased SERCA2a activity as a result of PLN ablation would enhance the removal of cytosolic Ca^{2+} (equivalent

to increased cytosolic Ca^{2+} buffering), the increase in the relative SR Ca^{2+} content detected in $PLN^{-/-}$/$R4496C^{+/-}$ cells would have been underestimated due to this increased Ca^{2+} removal/buffering. However, since PLN ablation increases the SR Ca^{2+} content in $PLN^{-/-}$/$R4496C^{+/-}$ cardiomyocytes, the lack of cell-wide propagating SCWs in these cells is unlikely to be due to a reduced SR Ca^{2+} content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

RyR2	cardiac ryanodine receptor
CPVT	catecholaminergic polymorphic ventricular tachycardia
DAD	delayed afterdepolarization
PLN	phospholamban
SR	sarcoplasmic recticulum
SCWs	spontaneous Ca^{2+} waves
SERCA2a	cardiac sarco(endo)plasmic recticulum Ca^{2+}-ATPase
AP	action potential
tBHQ	2,5-Di-tert-butylhydroquinone
CASQ2	cardiac calsequestrin
LTCC	L-type Ca^{2+} Channel
Na^{+}/Ca^{2+}	sodium/calcium exchange

References

1. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol. 1983; 245:C1–C14. [PubMed: 6346892]
2. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002; 415:198–205. [PubMed: 11805843]
3. Fill M, Copello JA. Ryanodine receptor calcium release channels. Physiol Rev. 2002; 82:893–922. [PubMed: 12270947]
4. Kass RS, Tsien RW. Fluctuations in membrane current driven by intracellular calcium in cardiac purkinje fibers. Biophys J. 1982; 38:259–269. [PubMed: 6809065]

5. Stern M, Kort A, Bhatnagar G, Lakatta E. Scattered-light intensity fluctuations in diastolic rat cardiac muscle caused by spontaneous Ca^{2+}-dependent cellular mechanical oscillations. J Gen Physiol. 1983; 82:119–153. [PubMed: 6886671]
6. Orchard C, Eisner D, Allen D. Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. Nature. 1983; 304:735–738. [PubMed: 6888540]
7. Marban E, Robinson SW, Wier WG. Mechanisms of arrhythmogenic delayed and early afterdepolarizations in ferret ventricular muscle. J Clin Invest. 1986; 78:1185–1192. [PubMed: 3771791]
8. Lakatta EG. Functional implications of spontaneous sarcoplasmic reticulum Ca^{2+} release in the heart. Cardiovasc Res. 1992; 26:193–214. [PubMed: 1423412]
9. Lakatta EG, Guarnieri T. Spontaneous myocardial calcium oscillations: Are they linked to ventricular fibrillation? J Cardiovasc Electrophysiol. 1993; 4:473–489. [PubMed: 8269314]
10. Schlotthauer K, Bers DM. Sarcoplasmic reticulum Ca^{2+} release causes myocyte depolarization. underlying mechanism and threshold for triggered action potentials. Circ Res. 2000; 87:774–780. [PubMed: 11055981]
11. Bers DM. Calcium and cardiac rhythms: Physiological and pathophysiological. Circ Res. 2002; 90:14–17. [PubMed: 11786512]
12. Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. Trends Cardiovasc Med. 2004; 14:61–66. [PubMed: 15030791]
13. Nuss HB, Kaab S, Kass DA, Tomaselli GF, Marban E. Cellular basis of ventricular arrhythmias and abnormal automaticity in heart failure. Am J Physiol Heart Circ Physiol. 1999; 277:H80–H91.
14. Zaugg C, Buser P. When calcium turns arrhythmogenic: Intracellular calcium handling during the development of hypertrophy and heart failure. Croat Med J. 2001; 42:24–32. [PubMed: 11172652]
15. Priori SG, Chen SR. Inherited dysfunction of sarcoplasmic reticulum Ca^{2+} handling and arrhythmogenesis. Circ Res. 2011; 108:871–883. [PubMed: 21454795]
16. Venetucci LA, Trafford AW, Diaz ME, O'Neill SC, Eisner DA. Reducing ryanodine receptor open probability as a means to abolish spontaneous Ca^{2+} release and increase Ca^{2+} transient amplitude in adult ventricular myocytes. Circ Res. 2006; 98:1299–1305. [PubMed: 16614307]
17. Watanabe H, Chopra N, Laver D, Hwang HS, Davies SS, Roach DE, Duff HJ, Roden DM, Wilde AA, Knollmann BC. Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans. Nat Med. 2009; 15:380–383. [PubMed: 19330009]
18. Hilliard FA, Steele DS, Laver D, Yang Z, Le Marchand SJ, Chopra N, Piston DW, Huke S, Knollmann BC. Flecainide inhibits arrhythmogenic Ca^{2+} waves by open state block of ryanodine receptor Ca^{2+} release channels and reduction of Ca^{2+} spark mass. J Mol Cell Cardiol. 2010; 48:293–301. [PubMed: 19835880]
19. Galimberti ES, Knollmann BC. Efficacy and potency of class I antiarrhythmic drugs for suppression of Ca^{2+} waves in permeabilized myocytes lacking calsequestrin. J Mol Cell Cardiol. 2011; 51:760–768. [PubMed: 21798265]
20. Zhou Q, Xiao J, Jiang D, Wang R, Vembaiyan K, Wang A, Smith CD, Xie C, Chen W, Zhang J, Tian X, Jones PP, Zhong X, Guo A, Chen H, Zhang L, Zhu W, Yang D, Li X, Chen J, Gillis AM, Duff HJ, Cheng H, Feldman AM, Song LS, Fill M, Back TG, Chen SR. Carvedilol and its new analogs suppress arrhythmogenic store overload-induced Ca^{2+} release. Nat Med. 2011; 17:1003–1009. [PubMed: 21743453]
21. Lukyanenko V, Subramanian S, Gyorke I, Wiesner TF, Gyorke S. The role of luminal Ca^{2+} in the generation of Ca^{2+} waves in rat ventricular myocytes. J Physiol. 1999; 518(Pt 1):173–186. [PubMed: 10373699]
22. Landgraf G, Gellerich FN, Wussling MH. Inhibitors of SERCA and mitochondrial ca-uniporter decrease velocity of calcium waves in rat cardiomyocytes. Mol Cell Biochem. 2004; 256:257–379. [PubMed: 14977186]
23. O'Neill SC, Miller L, Hinch R, Eisner DA. Interplay between SERCA and sarcolemmal Ca^{2+} efflux pathways controls spontaneous release of Ca^{2+} from the sarcoplasmic reticulum in rat ventricular myocytes. J Physiol. 2004; 559:121–128. [PubMed: 15194743]
24. Keller M, Kao JP, Egger M, Niggli E. Calcium waves driven by "sensitization" wave-fronts. Cardiovasc Res. 2007; 74:39–45. [PubMed: 17336953]

25. Maxwell JT, Blatter LA. Facilitation of cytosolic calcium wave propagation by local calcium uptake into the sarcoplasmic reticulum in cardiac myocytes. J Physiol. 2012; 590(Pt 23):6037–6045. [PubMed: 22988145]
26. Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. Circ Res. 1994; 75:401–409. [PubMed: 8062415]
27. Santana LF, Kranias EG, Lederer WJ. Calcium sparks and excitation-contraction coupling in phospholamban-deficient mouse ventricular myocytes. J Physiol. 1997; 503(Pt 1):21–29. [PubMed: 9288671]
28. Li Y, Kranias EG, Mignery GA, Bers DM. Protein kinase A phosphorylation of the ryanodine receptor does not affect calcium sparks in mouse ventricular myocytes. Circ Res. 2002; 90:309–316. [PubMed: 11861420]
29. Huser J, Bers DM, Blatter LA. Subcellular properties of [Ca^{2+}]i transients in phospholamban-deficient mouse ventricular cells. Am J Physiol. 1998; 274:H1800–H1811. [PubMed: 9612393]
30. Cerrone M, Colombi B, Santoro M, di Barletta MR, Scelsi M, Villani L, Napolitano C, Priori SG. Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. Circ Res. 2005; 96:e77–e82. [PubMed: 15890976]
31. Liu N, Colombi B, Memmi M, Zissimopoulos S, Rizzi N, Negri S, Imbriani M, Napolitano C, Lai FA, Priori SG. Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: Insights from a RyR2 R4496C knock-in mouse model. Circ Res. 2006; 99:292–298. [PubMed: 16825580]
32. Sedej S, Heinzel FR, Walther S, Dybkova N, Wakula P, Groborz J, Gronau P, Maier LS, Vos MA, Anthony Lai F, Napolitano C, Priori SG, Kockskamper J, Pieske B. Na+-dependent SR Ca^{2+} overload induces arrhythmogenic events in mouse cardiomyocytes with a human CPVT mutation. Cardiovasc Res. 2010; 87(1):50–59. [PubMed: 20080988]
33. Chen B, Guo A, Gao Z, Wei S, Xie YP, Chen SR, Anderson ME, Song LS. In situ confocal imaging in intact heart reveals stress-induced Ca^{2+} release variability in a murine catecholaminergic polymorphic ventricular tachycardia model of type 2 ryanodine receptor (R4496C+/-) mutation. Circ Arrhythm Electrophysiol. 2012; 5:841–849. [PubMed: 22722659]
34. Hoeker GS, Katra RP, Wilson LD, Plummer BN, Laurita KR. Spontaneous calcium release in tissue from the failing canine heart. Am J Physiol Heart Circ Physiol. 2009; 297:H1235–H1242. [PubMed: 19648256]
35. Venetucci LA, Trafford AW, Eisner DA. Increasing ryanodine receptor open probability alone does not produce arrhythmogenic calcium waves: Threshold sarcoplasmic reticulum calcium content is required. Circ Res. 2007; 100:105–111. [PubMed: 17110597]
36. Kashimura T, Briston SJ, Trafford AW, Napolitano C, Priori SG, Eisner DA, Venetucci LA. In the RyR2(R4496C) mouse model of CPVT, beta-adrenergic stimulation induces ca waves by increasing SR ca content and not by decreasing the threshold for ca waves. Circ Res. 2010; 107:1483–1489. [PubMed: 20966392]
37. Maier LS, Zhang T, Chen L, DeSantiago J, Brown JH, Bers DM. Transgenic CaMKII{delta}C overexpression uniquely alters cardiac myocyte Ca^{2+} handling: Reduced SR Ca^{2+} load and activated SR Ca^{2+} release. Circ Res. 2003; 92:904–911. [PubMed: 12676813]
38. Zhang T, Maier LS, Dalton ND, Miyamoto S, Ross J Jr, Bers DM, Brown JH. The {delta}C isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. Circ Res. 2003; 92:912–919. [PubMed: 12676814]
39. Hoit BD, Khoury SF, Kranias EG, Ball N, Walsh RA. In vivo echocardiographic detection of enhanced left ventricular function in gene-targeted mice with phospholamban deficiency. Circ Res. 1995; 77:632–637. [PubMed: 7641333]
40. Chu G, Ferguson DG, Edes I, Kiss E, Sato Y, Kranias EG. Phospholamban ablation and compensatory responses in the mammalian heart. Ann N Y Acad Sci. 1998; 853:49–62. [PubMed: 10603936]
41. Davia K, Bernobich E, Ranu HK, del Monte F, Terracciano CM, MacLeod KT, Adamson DL, Chaudhri B, Hajjar RJ, Harding SE. SERCA2A overexpression decreases the incidence of

aftercontractions in adult rabbit ventricular myocytes. J Mol Cell Cardiol. 2001; 33:1005–1015. [PubMed: 11343422]

42. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J Jr, Kranias EG, Giles WR, Chien KR. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell. 1999; 99:313–322. [PubMed: 10555147]
43. Sato Y, Kiriazis H, Yatani A, Schmidt AG, Hahn H, Ferguson DG, Sako H, Mitarai S, Honda R, Mesnard-Rouiller L, Frank KF, Beyermann B, Wu G, Fujimori K, Dorn GW 2nd, Kranias EG. Rescue of contractile parameters and myocyte hypertrophy in calsequestrin overexpressing myocardium by phospholamban ablation. J Biol Chem. 2001; 276:9392–9399. [PubMed: 11115498]
44. Engelhardt S, Hein L, Dyachenkow V, Kranias EG, Isenberg G, Lohse MJ. Altered calcium handling is critically involved in the cardiotoxic effects of chronic beta-adrenergic stimulation. Circulation. 2004; 109:1154–1160. [PubMed: 14967726]
45. Song Q, Schmidt AG, Hahn HS, Carr AN, Frank B, Pater L, Gerst M, Young K, Hoit BD, McConnell BK, Haghighi K, Seidman CE, Seidman JG, Dorn GW 2nd, Kranias EG. Rescue of cardiomyocyte dysfunction by phospholamban ablation does not prevent ventricular failure in genetic hypertrophy. J Clin Invest. 2003; 111:859–867. [PubMed: 12639992]
46. Zhang T, Guo T, Mishra S, Dalton ND, Kranias EG, Peterson KL, Bers DM, Brown JH. Phospholamban ablation rescues sarcoplasmic reticulum ca($^{2+}$) handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. Circ Res. 2010; 106:354–362. [PubMed: 19959778]
47. Shanmugam M, Gao S, Hong C, Fefelova N, Nowycky MC, Xie LH, Periasamy M, Babu GJ. Ablation of phospholamban and sarcolipin results in cardiac hypertrophy and decreased cardiac contractility. Cardiovasc Res. 2011; 89:353–361. [PubMed: 20833651]
48. Kalyanasundaram A, Lacombe VA, Belevych AE, Brunello L, Carnes CA, Janssen PM, Knollmann BC, Periasamy M, Gyorke S. Upregulation of sarcoplasmic reticulum Ca^{2+} uptake leads to cardiac hypertrophy, contractile dysfunction and early mortality in mice deficient in CASQ2. Cardiovasc Res. 2012
49. Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C, Pfeifer K. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca^{2+} release, and catecholaminergic polymorphic ventricular tachycardia. J Clin Invest. 2006; 116:2510–2520. [PubMed: 16932808]
50. Haghighi K, Kolokathis F, Pater L, Lynch RA, Asahi M, Gramolini AO, Fan GC, Tsiapras D, Hahn HS, Adamopoulos S, Liggett SB, Dorn GW 2nd, MacLennan DH, Kremastinos DT, Kranias EG. Human phospholamban null results in lethal dilated cardiomyopathy revealing a critical difference between mouse and human. J Clin Invest. 2003; 111:869–876. [PubMed: 12639993]

Novelty and Significance

What Is Known?

- Spontaneous Ca^{2+} waves (SCWs) are a major cause of Ca^{2+}-mediated arrhythmias.
- Mutations in the cardiac ryanodine receptor (RyR2) associated with catecholaminergic polymorphic ventricular tachycardia (CPVT)- enhance the propensity for SCWs.
- Ablation of phospholamban (PLN), an inhibitor of sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2a), increases sarcoplasmic reticulum (SR) Ca^{2+} leak, but aborts SCWs.

What New Information Does This Article Contribute?

- PLN ablation breaks cell-wide propagating SCWs into mini-waves and Ca^{2+} sparks in intact CPVT RyR2 mutant hearts.
- Despite markedly increased SR Ca^{2+} leak, PLN ablation protects against CPVT in RyR2 mutant mice.
- Breaking up SCWs by enhancing Ca^{2+} uptake represents an effective means for suppressing Ca^{2+}-mediated arrhythmias.

Enhanced SR Ca^{2+} sequestration as a result of PLN ablation has been shown, on one hand, to increase potentially arrhythmogenic SR Ca^{2+} leak, but, on the other hand, to abort SCWs that could lead to triggered activity. These seemingly paradoxical actions raise an important question as to whether enhanced SR Ca^{2+} sequestration is pro-arrhythmic or anti-arrhythmic. To this end, we assessed the effect of PLN ablation on SR Ca^{2+} leak, SCWs, triggered activity, and stress-induced ventricular tachycardia's (VTs) in a mouse model of CPVT. We found that enhanced SR Ca^{2+} uptake as a consequence of PLN ablation fragmented cell-wide propagating SCWs, but increased SR Ca^{2+} leak in the forms of mini-waves and Ca^{2+} sparks. Despite the presence of severe SR Ca^{2+} leak, PLN ablation suppressed triggered activity evoked by SR Ca^{2+} overload and protected CPVT-associated RyR2 mutant mice from stress-induced VTs. Our data show that enhancing SR Ca^{2+} sequestration suppresses CPVT in mice. These observations suggest that breaking up cell-wide propagating SCWs either by reducing the duration of RyR2 openings or by enhancing SR Ca^{2+} sequestration represents a promising strategy for suppressing Ca^{2+} mediated cardiac arrhythmias.

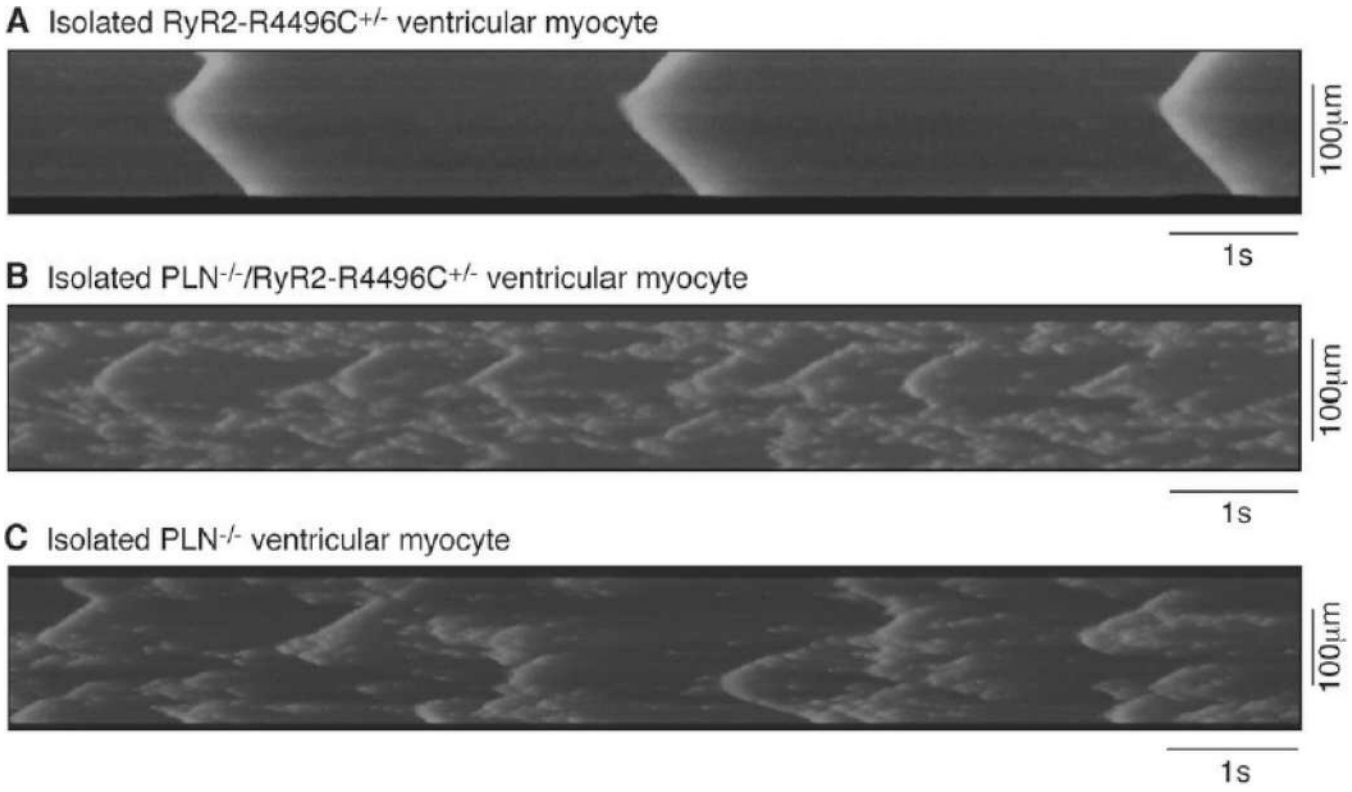

Figure 1. PLN-KO breaks cell-wide propagating spontaneous Ca^{2+} waves in isolated ventricular myocytes

Ventricular myocytes were isolated from RyR2-R4496C$^{+/-}$ mutant mice, the PLN deficient, RyR2-R4496C$^{+/-}$ mice (PLN$^{-/-}$/RyR2-R4496C$^{+/-}$) or the 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 6 mM extracellular Ca^{2+} to induce SR Ca^{2+} overload. Store overload induced spontaneous SR Ca^{2+} release events were detected by line-scan confocal Ca^{2+} imaging.

Representative line-scan images of spontaneous Ca^{2+} release in isolated RyR2-R4496C$^{+/-}$ (n=39) (A), PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ (n=43) (B), and PLN$^{-/-}$ (n=9) (C) ventricular myocytes are shown.

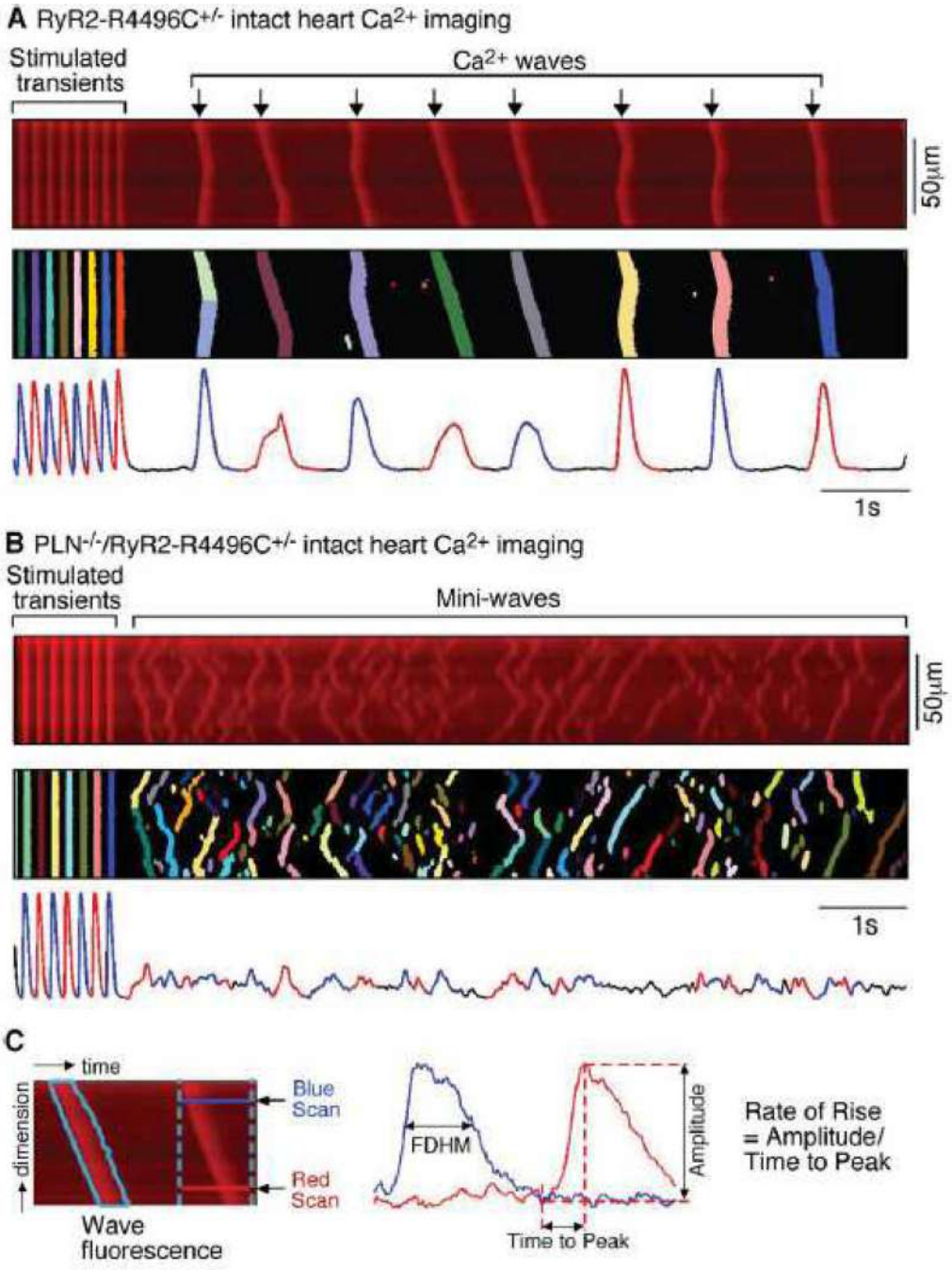

Figure 2. PLN-KO fragments cell-wide propagating spontaneous Ca^{2+} waves in intact hearts

Intact hearts isolated from RyR2-R4496C$^{+/-}$ or PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ mice were loaded with Rhod-2-AM and Langendorff-perfused with 6 mM extracellular Ca^{2+} and paced at 6 Hz to induce SR Ca^{2+} overload. Spontaneous SR Ca^{2+} release in epicardial ventricular myocytes in intact hearts was monitored by line-scan confocal Ca^{2+} imaging. Representative line-scan images (top) and the corresponding digitized images (middle) of cell-wide propagating spontaneous Ca^{2+} waves in intact RyR2-R4496C$^{+/-}$ hearts (A) and of mini-waves and Ca^{2+} sparks in intact PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ hearts (B) are shown. Panels A and B (bottom) show the spatial average of fluorescence signal along the scan-line. (C)

Definition of wave fluorescence, full duration at half maximum (FDHM), time to peak, amplitude, and rate of rise.

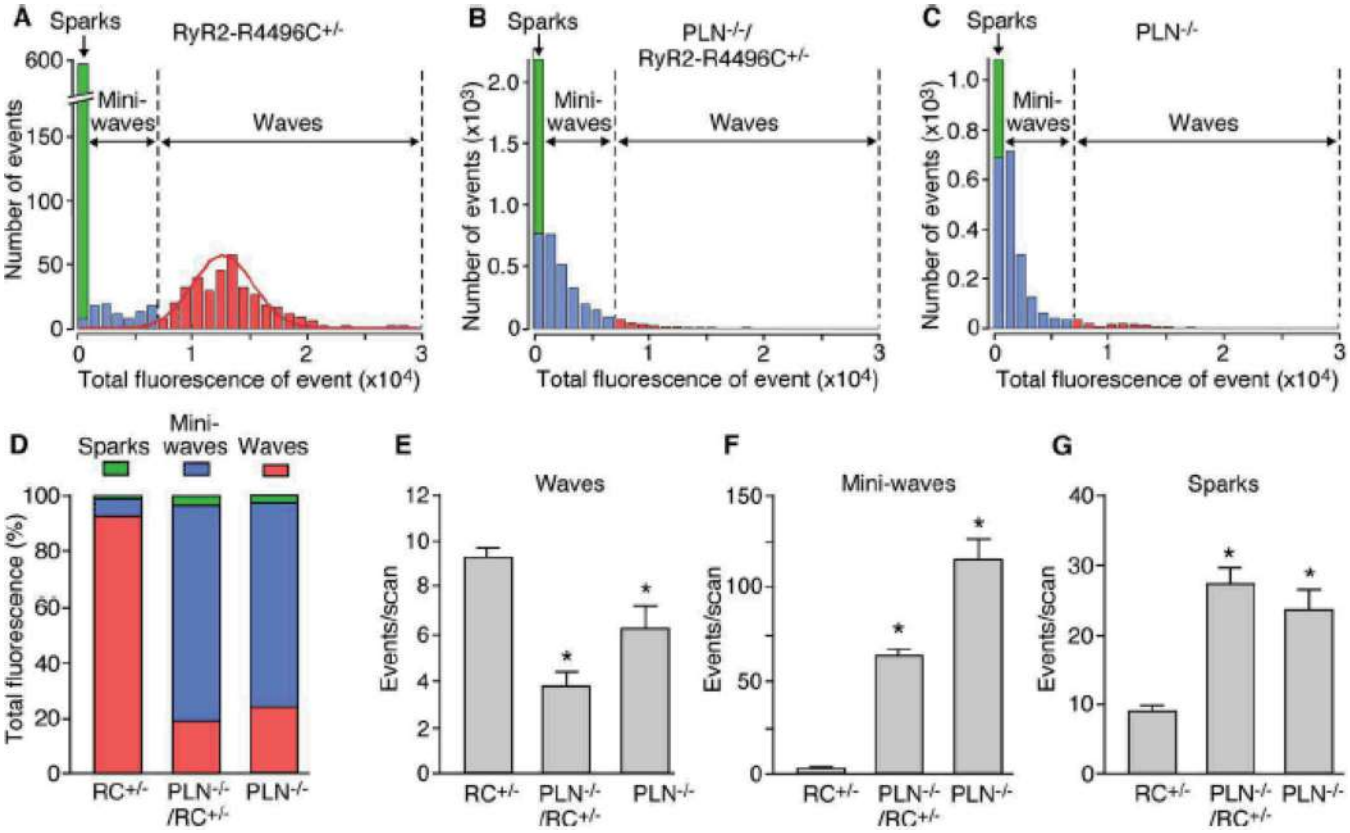

Figure 3. Distribution of spontaneous Ca^{2+} release events in intact RyR2-R4496C mutant hearts with or without PLN

Line-scan confocal images were digitized and spontaneous Ca^{2+} release events were detected and classified using a custom-made program as described in the supplementary methods. (A, B, C) Distribution of spontaneous Ca^{2+} release events in RyR2-R4496C$^{+/-}$ mutant (A), PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ (B), and PLN$^{-/-}$ (C) hearts according to their total fluorescence. Three types of spontaneous Ca^{2+} release events (Ca^{2+} sparks, mini-waves, and waves) were classified based on the size of the total fluorescence (see Supplementary Methods). The red line represents a Gaussian fit of the distribution of Ca^{2+} waves in RyR2-R4496C$^{+/-}$ hearts. (D) The overall contribution (%) of sparks, mini-waves, and waves in RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$, and PLN$^{-/-}$ hearts. (E, F, G) The occurrence (events/scan) of Ca^{2+} waves (E), mini-waves (F), and Ca^{2+} 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).

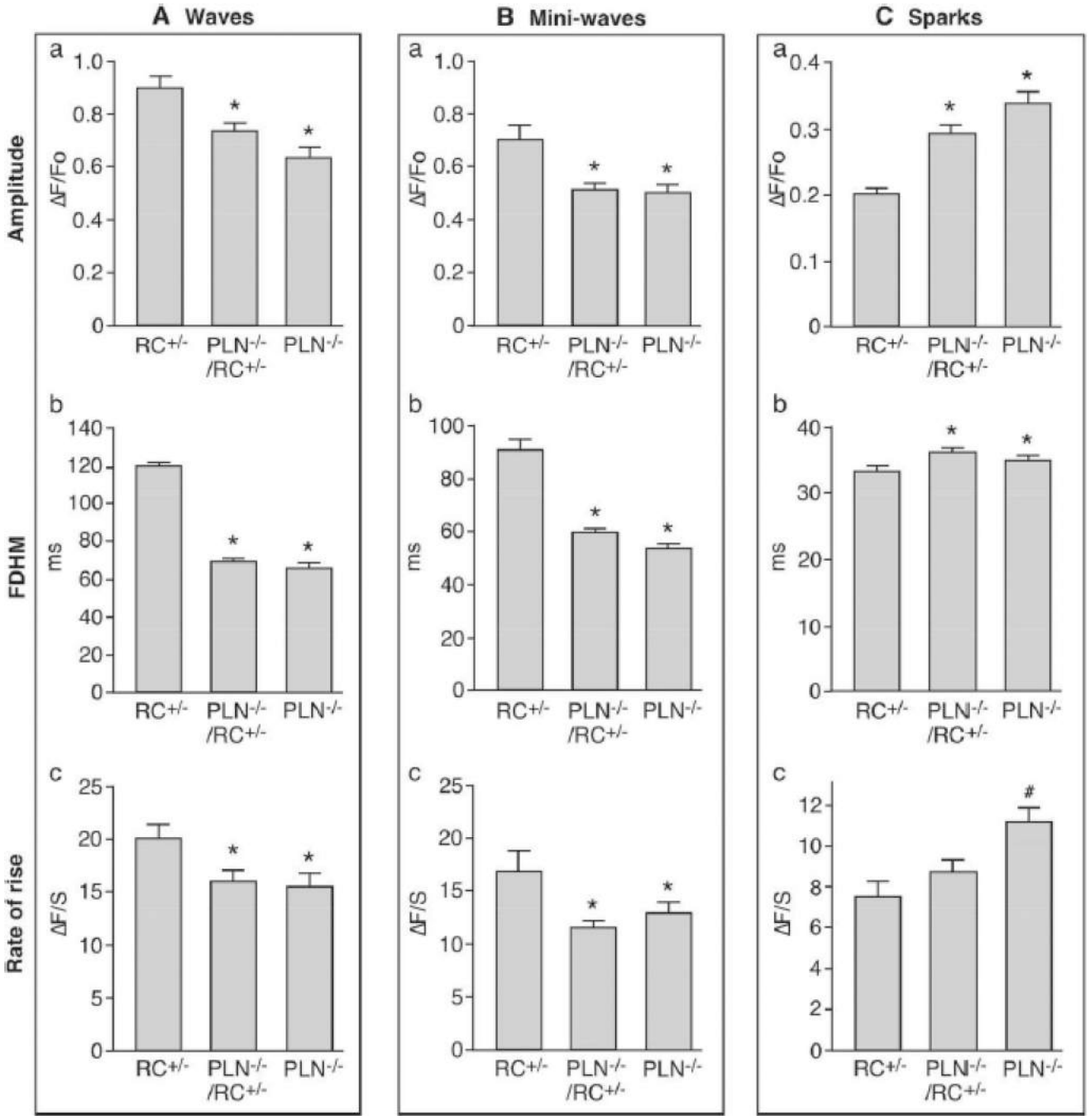

Figure 4. Effect of PLN-KO on spontaneous Ca^{2+} release in intact RyR2-R4496C mutant hearts

Spontaneous Ca^{2+} release events in intact RyR2-R4496C$^{+/-}$, PLN$^{-/-}$/RyR2-R4496C$^{+/-}$, and PLN$^{-/-}$ hearts were divided into Ca^{2+} waves (A), mini-waves (B), and Ca^{2+} sparks (C) as described in the legend of Fig. 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 19–43 line-scan images ($^{\#}P < 0.01$, $^{*}P < 0.001$, vs RC$^{+/-}$).

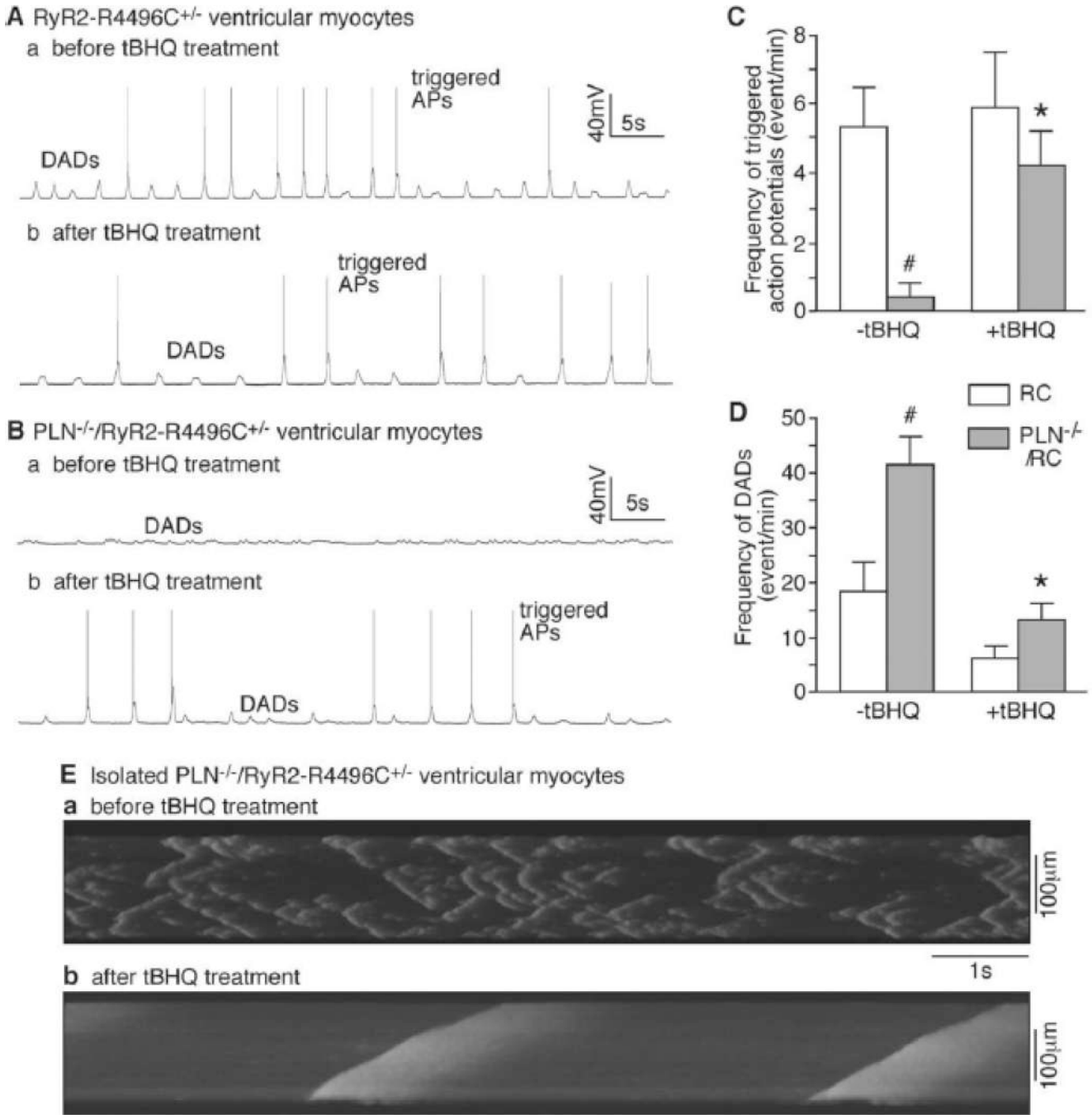

Figure 5. Effect of PLN-KO on delayed afterdepolarizations and triggered activities

Ventricular myocytes were isolated from RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ hearts and perfused with 6 mM extracellular Ca^{2+} to induce spontaneous Ca^{2+} release. Membrane potentials in RyR2-R4496C$^{+/-}$ (A) or PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ (B) myocytes before (a) and after (b) the treatment with tBHQ, a SERCA2a inhibitor, were recorded using the perforated patch current clamp technique. (C, D) The frequency of spontaneously triggered APs (C) and delayed afterdepolarizations (DADs) (D) in RyR2-R4496C$^{+/-}$ and PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ ventricular myocytes before (a) and after (b) tBHQ treatment. Data shown are mean ± SEM from 8–12 cells ($^{\#}P < 0.05$, vs RC mice; $^{*}P < 0.05$, vs -

tBHQ). (E) Representative line-scan images of spontaneous Ca^{2+} release in isolated $PLN^{-/-}$/RyR2-R4496$C^{+/-}$ ventricular myocytes before (a) and after (b) the treatment with 5 μM tBHQ (n = 21–22 cells) are shown.

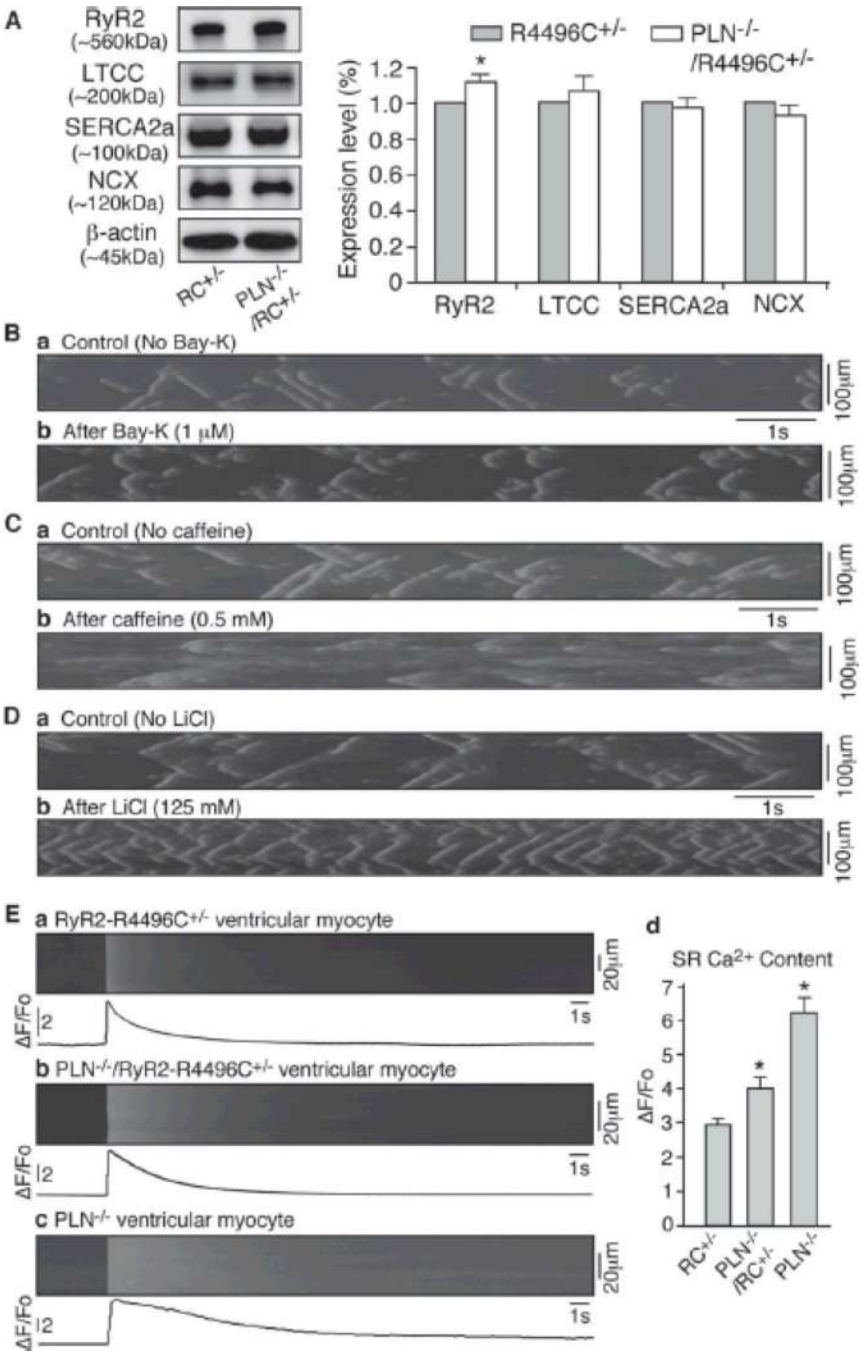

Figure 6. Role of RyR2, LTCC, NCX, and SR Ca^{2+} load in the generation of mini-waves in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ ventricular myocytes

(A) Whole heart homogenates were prepared from RyR2-$R4496C^{+/-}$ and $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ mutant mice and used for immunoblotting analysis using antibodies against RyR2, LTCC, SERCA2a, NCX or β-actin. Data shown are mean ± SEM (n=3, $*P < 0.05$, vs RyR2-$R4496C^{+/-}$). (B, C, D) Ventricular myocytes were isolated from $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ hearts and loaded with the fluorescent Ca^{2+} indicator dye fluo-4-AM. Representative line-scan images of spontaneous Ca^{2+} release induced by elevated extracellular Ca^{2+} (6 mM) in $PLN^{-/-}$/RyR2-$R4496C^{+/-}$ cells before (a) and after (b) the treatment with Bay K (B), caffeine (C), and LiCl (replacing NaCl in KRH) (D) (n = 14–18

cells) are shown. Note that none of these treatments converted mini-waves to cell-wide SCWs. (E) SR Ca^{2+} contents in fluo-4-AM loaded RyR2-R4496C$^{+/-}$ (a), PLN$^{-/-}$/RyR2-R4496C$^{+/-}$ (b), and PLN$^{-/-}$ (c) ventricular myocytes were estimated by measuring the amplitude of caffeine (20mM) induced Ca^{2+} transients (d). Data shown are mean ± SEM (n=12–18) (*$P < 0.05$, vs RyR2-R4496C$^{+/-}$).

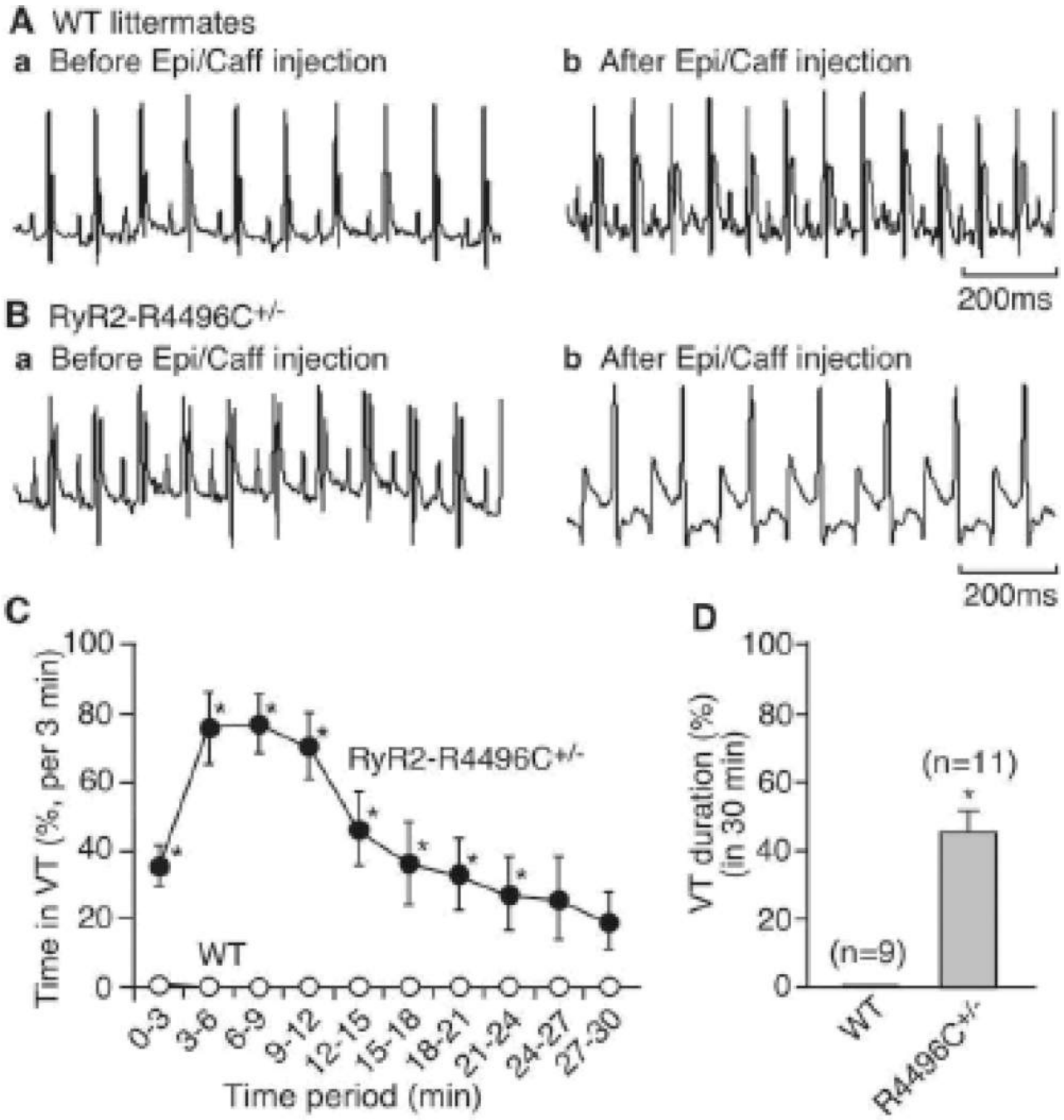

Figure 7. RyR2-R4496C$^{+/-}$ mice are susceptible to CPVT

Representative ECG recordings of WT littermates (A) and RyR2-R4496C$^{+/-}$ (B) mice before (a) and after (b) the injection of epinephrine (1.6 mg/kg) and caffeine (120 mg/kg). VT duration (%) in WT littermates and RyR2-R4496C$^{+/-}$ mutant mice within each 3-min (C) or 30-min (D) period of ECG recordings. Data shown are mean ± SEM from 9–11 mice (*P < 0.05, vs WT).

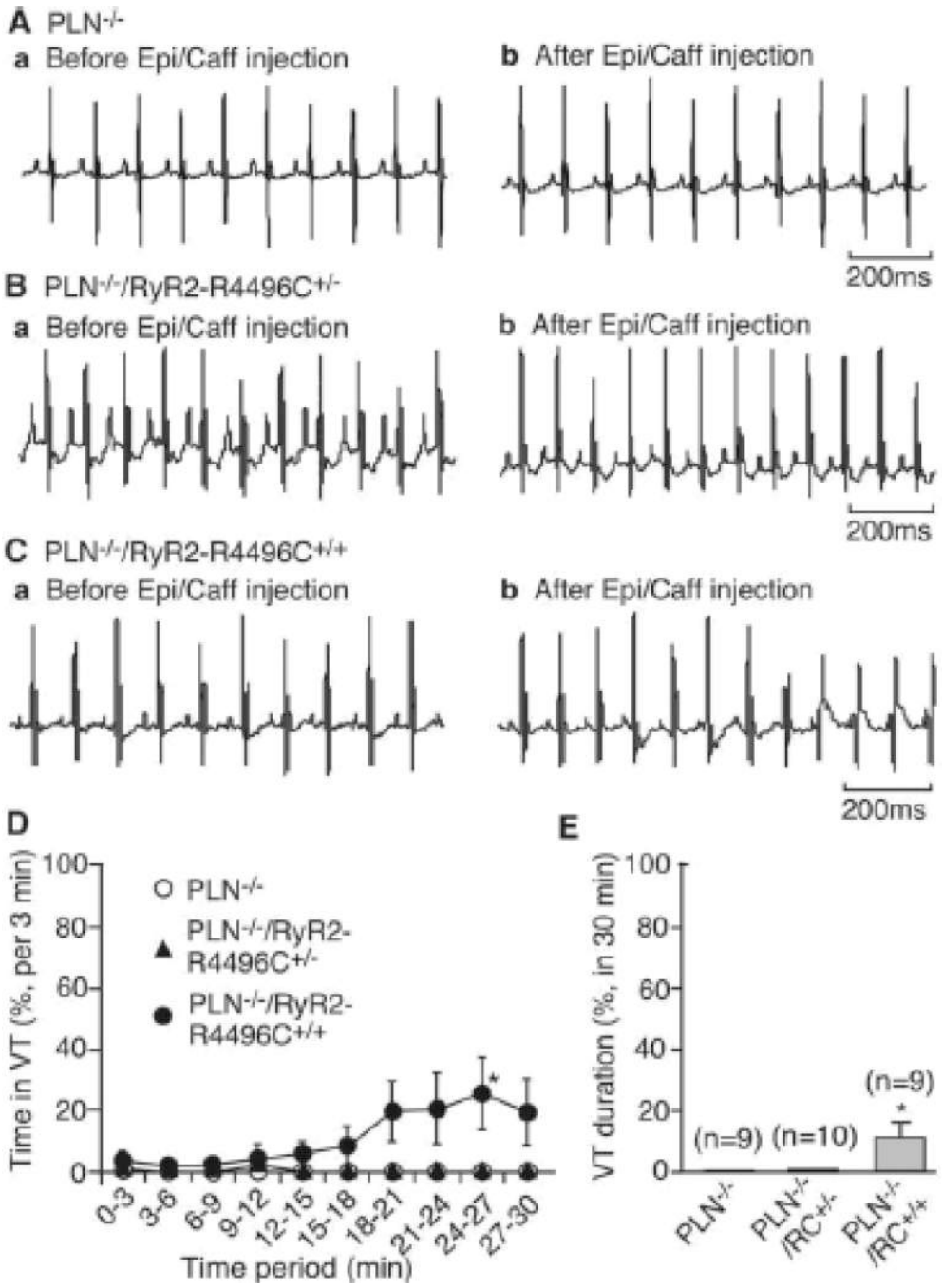

Figure 8. PLN-KO protects against stress-induced VTs in mice

Representative ECG recordings of $PLN^{-/-}$ (A), $PLN^{-/-}$/RyR2-R4496C$^{+/-}$ (B), and $PLN^{-/-}$/RyR2-R4496C$^{+/+}$ (C) mice before (a) and after (b) the injection of epinephrine (1.6 mg/kg) and caffeine (120 mg/kg). VT duration (%) in $PLN^{-/-}$, $PLN^{-/-}$/RyR2-R4496C$^{+/-}$, and $PLN^{-/-}$/RyR2-R4496C$^{+/+}$ mice within each 3-min (D) or 30-min (E) period of ECG recordings. Data shown are mean ± SEM from 9–10 mice (*$P < 0.05$, vs $PLN^{-/-}$).