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## A Single Protein Kinase A or Calmodulin Kinase II Site Does Not Control the Cardiac Pacemaker Ca<sup>2+</sup> Clock

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### Abstract

**Background**—Fight or flight heart rate (HR) increases depend on protein kinase A (PKA) and calmodulin kinase II (CaMKII) mediated enhancement of Ca<sup>2+</sup> uptake and release from sarcoplasmic reticulum (SR) in sinoatrial nodal cells (SANC). However, the impact of specific PKA and CaMKII phosphorylation sites on HR is unknown.

**Methods and Results**—We systematically evaluated validated PKA and CaMKII target sites on phospholamban (PLN) and the ryanodine receptor (RyR2) using genetically modified mice. We found that knockin alanine replacement of RyR2 PKA (S2808) or CaMKII (S2814) target sites failed to affect HR responses to isoproterenol or spontaneous activity in vivo or in SANC. Similarly, selective mutation of PLN amino acids critical for enhancing SR Ca<sup>2+</sup> uptake by PKA (S16) or CaMKII (T17) to alanines did not affect HR in vivo or in SANC. In contrast, CaMKII inhibition by expression of AC3-I has been shown to slow SANC rate responses to isoproterenol and decrease SR Ca<sup>2+</sup> content. PLN deficiency rescued SR Ca<sup>2+</sup> content and SANC rate responses to isoproterenol in mice with AC3-I expression, suggesting CaMKII affects HR by modulation of SR Ca<sup>2+</sup> content. Consistent with this, mice expressing a superinhibitory PLN mutant had low SR Ca<sup>2+</sup> content and slow HR in vivo and in SANC.

**Conclusions**—SR Ca<sup>2+</sup> depletion reduces HR and SR Ca<sup>2+</sup> repletion restores physiological SANC rate responses despite CaMKII inhibition. PKA and CaMKII do not affect HR by a unique target site governing SR Ca<sup>2+</sup> uptake or release. HR acceleration may require an SR Ca<sup>2+</sup> content threshold.

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## Keywords

sinoatrial node; heart rate; calcium/calmodulin-dependent protein kinase II; phospholamban; ryanodine receptor; protein kinase A

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## Introduction

Cardiac pacemaker cells control heart rate by generating spontaneous and repetitive action potentials. Action potential acceleration drives heart rate increases by a mechanism that relies on an intracellular  $\text{Ca}^{2+}$  release and uptake process that is coupled to inward cell membrane ionic currents. These cellular mechanisms are often described separately as a  $\text{Ca}^{2+}$  clock and a cell membrane clock <sup>1</sup>. Cardiac pacemaker cells can accelerate heart rates in response to  $\beta$  adrenergic receptor agonist stimulation as part of the physiological fight-or-flight response.  $\beta$  adrenergic receptor agonists increase the activity of protein kinase A (PKA) and calmodulin kinase II (CaMKII), serine-threonine kinases, in cardiac pacemaker cells. Both CaMKII <sup>2</sup> and PKA <sup>3</sup> contribute to fight or flight heart rate increases in response to isoproterenol injection or spontaneous ambulatory activity by catalyzing phosphorylation of proteins important for regulating intracellular  $\text{Ca}^{2+}$  uptake and release from the sarcoplasmic reticulum (SR). However, to our knowledge, a systematic study of the effects of PKA and CaMKII phosphorylation on individual proteins that govern SR  $\text{Ca}^{2+}$  uptake and release in sinoatrial node (SAN) pacemaker cells is lacking.

SR  $\text{Ca}^{2+}$  release in SAN cells occurs through type 2 ryanodine receptors (RyR2). RyR2  $\text{Ca}^{2+}$  release is enhanced by phosphorylation of S2808 (by CaMKII and PKA) <sup>4,5</sup> and by S2814 (by CaMKII) <sup>6</sup>. The increased RyR2  $\text{Ca}^{2+}$  leak drives SAN cell membrane depolarizing inward current through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. The  $\text{Na}^+/\text{Ca}^{2+}$  exchanger current ( $I_{\text{NCX}}$ ) increases heart rate by hastening the late diastolic SAN cell membrane depolarization rate, causing higher frequency action potential triggering in SAN pacemaking cells <sup>7</sup>. We measured heart rates and SAN automaticity in mice with RyR2 knockin mutations that disable PKA (S2808A) and CaMKII (S2814A) catalyzed phosphorylation at these sites.

SR  $\text{Ca}^{2+}$  uptake in SAN cells is governed by a protein complex that includes phospholamban (PLN) and the sarcoplasmic endoplasmic reticulum ATPase (SERCA2a) <sup>1-3</sup>. PLN is a negative regulator of SERCA2a under basal conditions <sup>8</sup>. PLN phosphorylation by PKA (at S16) or CaMKII (at T17) leads to loss of SERCA2a inhibition and increased  $\text{Ca}^{2+}$  transport from the cytoplasm to the SR. We studied mice and isolated SAN cells lacking PLN (*Pln*<sup>-/-</sup>), expressing mutant PKA-resistant PLN (S16A), CaMKII-resistant PLN (T17A) and mice with a superinhibitory PLN mutant, N27A, which constitutively suppresses SERCA2a even after PKA and CaMKII catalyzed phosphorylation <sup>9-11</sup>.

Despite the important roles of PKA and CaMKII in activating the  $\text{Ca}^{2+}$  clock and promoting fight or flight heart rate increases, the relative importance of PKA and CaMKII sites on RyR2 and PLN are unknown; furthermore, the potential for any particular site to exert a controlling influence over fight or flight physiology in vivo and in vitro is untested. In order

to address this knowledge gap, we used a cohort of genetically modified mouse models in which CaMKII and PKA phosphorylation sites were specifically ablated to interrogate the role of each site and determine if any of these SR protein sites exercises a decisive influence on heart rate responses to isoproterenol or activity.

Here we show that none of four well validated PKA or CaMKII phosphorylation sites on PLN and RyR2 are individually required for physiological rate increases in isolated SAN cells or in vivo in response to spontaneous activity or isoproterenol injection. Although no single phosphorylation site in our models had a controlling role in heart rate acceleration, we found that superinhibitory PLN mutants significantly lowered SR Ca<sup>2+</sup> content and limited heart rate acceleration while PLN deficiency prevented reduction in SR Ca<sup>2+</sup> content and normalized heart rate acceleration during CaMKII inhibition. Taken together, our data show that loss of any single site failed to affect heart rates and suggest that fight-or-flight heart rate increases are built from redundant PKA and CaMKII catalyzed phosphorylation target proteins. Our data are consistent with a concept where CaMKII inhibition slows heart rate by lowering the pool of SR Ca<sup>2+</sup> in cardiac pacemaker cells.

## Methods

### Genetically modified mice

The cohort of mice used in this study is shown in Table 1. *Pln*<sup>-/-</sup>, S16A, T17A, N27A, and WT-PLN mice were obtained from MMRRRC (Columbia, MO), but were originally developed by the Kranias group<sup>9-11</sup>. All of these mice were engineered by transgenic expression of wild type (WT) and mutant PLN on a *Pln*<sup>-/-</sup> background. The lines were selected for similar transgenic expression levels of S16A, T17A, N27A and WT-PLN<sup>10, 11</sup>. The littermate controls S16A(-), T17A(-) are *Pln*<sup>-/-</sup>. WT mice in the C57 background are designated (WT), and WT-*Pln* indicates controls where a WT *Pln* transgene was expressed in a *Pln*<sup>-/-</sup> background. The S2808A and S2814A mice were developed by knockin replacement of the designated alleles, as described<sup>12, 13</sup>. *Pln*<sup>-/-</sup> mice were interbred with mice overexpressing CaMKII inhibitor peptide AC3-I<sup>2</sup> (AC3-Ix*Pln*<sup>-/-</sup>) and mice overexpressing scrambled control peptide AC3-C (AC3-Cx*Pln*<sup>-/-</sup>) for > 10 generations and the genetic identity of *Pln*<sup>-/-</sup> pups with transgenic expression of AC3-I or AC3-C was confirmed using PCR, as described<sup>14</sup>.

### Telemetry ECG and spontaneous activity recording

Surgical implantation of ECG and ambulatory activity-sensing and recording telemeters was conducted as described<sup>15</sup>. (see supplemental methods for detail).

### SAN cell isolation, action potential, I<sub>f</sub>, I<sub>NCX</sub> and SR Ca<sup>2+</sup> content recordings

Isolation of single SAN cells from mice, action potential recordings, I<sub>f</sub> and I<sub>NCX</sub> current measurements from single SAN cell were performed as previously published<sup>2, 16, 17</sup>. (see supplemental methods for detail).

Briefly, SAN cells were identified by their characteristic morphology (spindle or spider shape) and spontaneous activity. SAN cells were also identified electrophysiologically by

typical spontaneous action potentials with slow depolarizing phase 4 and the hyperpolarization-activated current ( $I_f$ ).

Spontaneous action potentials were recorded in current clamp ( $I=0$ ) mode using the perforated (amphotericin B) patch-clamp technique on single SAN cell at  $36\pm 0.5$  °C in Tyrode's solution. The pipette was filled with (mM): 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 CaCl<sub>2</sub>, amphotericin B 240 µg/ml.  $I_f$  was recorded in voltage clamp mode. Membrane potential was held at  $-35$  mV, the voltage steps were applied for 5 s ranging from  $-125$  mV to  $-45$  mV in 10 mV increments, or vice versa.

$I_{NCX}$  was recorded using whole-cell patch clamp technique at  $36\pm 0.5$  °C. The external solution contained the following (mM): 140 NaCl, 1.0 MgCl<sub>2</sub>, 5.0 Hepes, 2.5 CaCl<sub>2</sub>, 1.0 BaCl<sub>2</sub>, 10 glucose, and 10 µM nitrendipine, and 10 µM strophanthidin. The internal solution contained the following (mM): 110 CsCl, 10 NaCl, 5.0 glucose, 1.0 CaCl<sub>2</sub>, 0.4 MgCl<sub>2</sub>, 10 Hepes, and 20 TEA chloride, 5.0 EGTA. Membrane currents were elicited by a command ramp of 2 s from  $+80$  mV to  $-120$  mV. Holding potential was  $-80$  mV. The protocol was applied every 10 s. Recordings were taken when five consecutive ramp recordings showed nearly identical currents.  $I_{NCX}$  was measured as the Ni-sensitive current. NiCl<sub>2</sub> (5 mM) was added to define the fraction of current from NCX (the difference currents between total currents and post-Ni<sup>2+</sup> currents from the same cell).

SR Ca<sup>2+</sup> content was measured by integrating the  $I_{NCX}$  in response to a 'spritz' of caffeine, as described<sup>2</sup>. Briefly, cells were loaded with Ca<sup>2+</sup> by repetitive voltage command steps (300 ms, 0.5 Hz) from  $-80$  to  $0$  mV at  $36\pm 0.5$  °C. The bath solution contained (mM): 137 NaCl, 10 HEPES, 10 glucose, 1.8 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 25 CsCl. The intracellular pipette solution contained (mM): 120 CsCl, 10 TEA, 1.0 MgATP, 1.0 NaGTP, 5.0 phosphocreatine, 10 HEPES, Indo-1 0.2. After > 15 conditioning voltage command steps the SAN cell membrane was held at  $-80$  mV and a caffeine (20 mM) spritz was locally applied. The resultant  $I_{NCX}$  was integrated and normalized to cell size.

### Data analysis and statistics

Spontaneous action potential rates were analyzed using Clampfit 10. Arbitrary activity counts from the telemetry ECG recording system (Data Sciences International) were binned for analysis, with a bin width of 5 counts (see supplemental methods for detail). The mean heart rate was determined separately for each mouse and activity bin. One way ANOVA combined with Holm-Sidak test was used when more than two groups were compared. Student's t-test was used when appropriate, paired t-test was used when comparing before and after treatment data on the same cells; the unpaired t-test was used for comparing data from two groups. Data were tested for normal distribution (Shapiro-Wilk (normality test) or Brown-Forsythe (equal variance test), as appropriate). A Signed Rank Test was used to generate a P value for non-parametric data. We rejected the null hypothesis for  $P < 0.05$ .

## Results

### Loss of RyR2 S2808 or S2814 failed to affect heart rates in vivo

We studied mice with knockin mutations that selectively eliminated a validated PKA (S2808A)<sup>5, 12</sup> or CaMKII phosphorylation site (S2814A)<sup>6, 13</sup>. The S2808A and S2814A mice had normal heart rate acceleration compared to WT control in response to isoproterenol injection (Fig 1A and B) or spontaneous activity (Fig 1C and D). These findings suggest that loss of RyR2 S2808 or S2814 alone are insufficient to impair the in vivo heart rate response to catecholamine or physiological stress related to spontaneous ambulatory activity.

### Loss of RyR2 S2808 or S2814 did not affect SAN cell rates

Heart rate is a complex integrated response where multiple inputs converge on SAN cells to drive basal and fight or flight heart rate responses<sup>18</sup>. We found that SAN cells isolated from S2808A and S2814A mice had similar action potential rates at baseline, in the absence of catecholamine agonist stimulation, and after isoproterenol compared to SAN cells isolated from WT littermates (Fig 2A and B). These results showed that neither loss of S2808 nor S2814 on RyR2 was sufficient to disrupt SAN cell rate responses, suggesting that the observed preservation of in vivo heart rate responses in S2808A and S2814A was not the result of compensatory changes in SAN cell innervation. The results so far, using S2808A and S2814A knockin mice, showed that loss of these RyR2 target sites individually was inadequate to affect heart rate, suggesting that PKA and CaMKII cannot modulate heart rate by affecting SR Ca<sup>2+</sup> release through post-translational modification of a single RyR2 site.

### Loss of RyR2 S2814 increases SAN cell SR Ca<sup>2+</sup> content

RyR2 phosphorylation by PKA and CaMKII can increase late diastolic SR Ca<sup>2+</sup> leak<sup>6, 12, 19, 20</sup>, potentially reducing SR Ca<sup>2+</sup> content. We measured SAN cell SR Ca<sup>2+</sup> content by integrating the I<sub>NCX</sub> following caffeine-triggered SR Ca<sup>2+</sup> release and found that S2814A SAN cells had relatively increased basal SR Ca<sup>2+</sup> content compared to S2808A and controls (Fig 2 C and D). SAN cells isolated from all backgrounds exhibited a significant increase in SR Ca<sup>2+</sup> content after isoproterenol (Fig 2 D). These data showed that loss of a PKA site (S2808) was insufficient to affect basal SAN cell SR Ca<sup>2+</sup> content or the physiological increase in SR Ca<sup>2+</sup> content after isoproterenol, but loss of the S2814 CaMKII site did result in a modest but significant increase in basal SAN cell SR Ca<sup>2+</sup> content.

### Loss of PLN maximized SR Ca<sup>2+</sup> content without affecting heart rate

We next turned our attention to processes affecting SR Ca<sup>2+</sup> uptake. PKA and CaMKII catalyze phosphorylation of PLN at single, distinct, extensively validated sites, leading to loss of negative regulation of SERCA2a by PLN and increased SR Ca<sup>2+</sup> uptake<sup>2, 3, 8</sup>.

As a first step, we assessed whether loss of PLN affects heart rate or SR Ca<sup>2+</sup> content in SAN cells. We found that *Pln*<sup>-/-</sup> mice had heart rates that were similar to WT controls at rest and in response to isoproterenol (Fig 3A and B), similar to earlier descriptions<sup>9</sup>, or in response to spontaneous activity (Fig 3C). SAN cells isolated from *Pln*<sup>-/-</sup> and WT-PLn mice also exhibited similar resting and isoproterenol-stimulated rates (Fig 3D), mirroring the in

vivo heart rate responses to isoproterenol or activity. We next measured SR Ca<sup>2+</sup> content in isolated SAN cells at baseline and after isoproterenol. The SR Ca<sup>2+</sup> content in WT SAN cells was significantly increased over baseline by isoproterenol exposure. In contrast, SR Ca<sup>2+</sup> content in *Pln*<sup>-/-</sup> SAN cells was replete under basal conditions and not further increased by isoproterenol (Fig 3E and F). We interpreted these findings as confirming that loss of PLN expression did not affect heart rate responses in vivo nor in isolated SAN cells but effectively released SERCA2a from inhibition, increasing basal SR Ca<sup>2+</sup> content in SAN cells, similar to established actions in ventricular myocytes<sup>21</sup>.

### Expression of S16A or T17A did not impair heart or SAN cell rates

We next turned our attention to extensively characterized mice where *Pln* was transgenically replaced by WT *Pln* or mutant *Pln* lacking either the PKA (S16A) or CaMKII (T17A) sites<sup>10</sup>. We found that mice transgenically expressing PLN S16A or T17A had normal heart rate acceleration in response to isoproterenol injection (Fig 4A and B); mice with transgenic expression of WT, S16A and T17A had equivalent rate responses to spontaneous activity (Fig 4C and D). SAN cells isolated from S16A and T17A mice had similar action potential rates at baseline and after isoproterenol compared to SAN cells isolated from mice with transgenic WT PLN expression (Fig 5A and B). We next checked SR Ca<sup>2+</sup> content at baseline and in response to ISO in SAN cells isolated from S16A and T17A mice (Fig 5C and D). Neither PLN mutation affected SR Ca<sup>2+</sup> content at baseline, but each mutation abolished the physiological increase in SR Ca<sup>2+</sup> content after ISO (Fig 5D). These data suggest that PLN and PKA and CaMKII sites on PLN are individually dispensable for fight or flight heart rate responses in vivo and in isolated pacemaker cells.

### SAN rate slowing by CaMKII inhibition required PLN

SAN cell CaMKII inhibition by expression of a CaMKII inhibitory peptide, AC3-I, compresses the dynamic range of heart rate acceleration by approximately 50%<sup>2</sup>, however the mechanism of action for AC3-I expression in SAN cells to slow heart rate is incompletely understood. AC3-I expression in isolated SAN cells<sup>2</sup> and ventricular myocytes<sup>22</sup> reduces SR Ca<sup>2+</sup> filling in response to isoproterenol (Fig 6A), suggesting the possibility that SAN SR Ca<sup>2+</sup> repletion, above a threshold, is required for physiological rate acceleration and that the heart rate slowing actions of CaMKII inhibition are related, at least in part, to reduction in SR Ca<sup>2+</sup> content below a critical threshold required for physiological heart rate increases.

Based on the hypothesized association between SR Ca<sup>2+</sup> content and SAN rate responses to isoproterenol, we next isolated SAN cells from *Pln*<sup>-/-</sup> mice interbred with AC3-I transgenic mice (AC3-Ix*Pln*<sup>-/-</sup>) or interbred with mice transgenically expressing an inactive control peptide, AC3-C (AC3-Cx*Pln*<sup>-/-</sup>)<sup>14, 22</sup> to determine if CaMKII inhibition affected SR Ca<sup>2+</sup> content in the absence of *Pln*, potentially under conditions of replete SR Ca<sup>2+</sup> content. The SR Ca<sup>2+</sup> content was replete at baseline and not further increased by isoproterenol in SAN cells isolated from AC3-Ix*Pln*<sup>-/-</sup> and AC3-Cx*Pln*<sup>-/-</sup> mice (Fig 6A), indicating that reduced SR Ca<sup>2+</sup> content in SAN cells expressing AC3-I was repaired by loss of PLN expression.

These interbred mice provided a new model to test if the relationship between AC3-I expression and reduced SR Ca<sup>2+</sup> content was essential for slower SAN rates identified in AC3-I transgenic mice<sup>2, 22, 23</sup>. We found that all SAN cells had similar resting and isoproterenol stimulated rates compared to WT and *Pln*<sup>-/-</sup> SAN cells (Fig 6B and C, Fig 3D). We compared these SAN cell rates with published rates from AC3-I SAN cells (Fig 6C), to demonstrate the ISO rate recovery response to *Pln* knock out. The interbred, AC3-Ix*Pln*<sup>-/-</sup> mice unexpectedly exhibited atrioventricular nodal conduction block that prevented a meaningful assessment of in vivo heart rates (Supplemental Fig 1). These data show that CaMKII inhibition does not slow isoproterenol-stimulated SAN cell rates in the absence of PLN and are consistent with the idea that CaMKII inhibition hinders heart rate acceleration by reducing SR Ca<sup>2+</sup> content.

### Heart rate slowing in mice with super inhibitory PLN

Based on our findings that the heart rate slowing from AC3-I expression was rescued in mice with a *Pln*<sup>-/-</sup> background, we next asked if a superinhibitory PLN mutant (N27A) that inhibits SERCA2a despite phosphorylation<sup>11</sup> would depress heart rate. We found that N27A mice had significantly reduced heart rates at baseline and in response to isoproterenol or spontaneous activity (Fig 7A-C). We next isolated SAN cells from N27A and *Pln*<sup>-/-</sup> littermate mice as well as WT-*Pln* mice to test if defective chronotropic performance was present in SAN cells. SAN cells isolated from N27A mice had significantly reduced resting rates and impaired isoproterenol rate responses compared to WT-*Pln* control and littermate *Pln*<sup>-/-</sup> SAN cells (Fig 5A, 6B, 7D and E). Finally, we found that SR Ca<sup>2+</sup> content was reduced at baseline and was not significantly increased by isoproterenol in N27A SAN cells (Fig. 7F). We interpreted these findings to show that enhanced PLN-mediated SERCA2a inhibition could constrain SAN cell SR Ca<sup>2+</sup> filling and reduce physiological heart rate responses. We summarized the SR Ca<sup>2+</sup> content findings for all models, before and after ISO, to facilitate comparisons (supplementary Table).

### HCN (I<sub>f</sub>) currents and NCX currents were not altered by PLN deletion or mutation

We recorded I<sub>f</sub> currents from SAN cells isolated from WT-*Pln*, *Pln*<sup>-/-</sup> and N27A mice (Fig 8A and B). There were no difference in I<sub>f</sub> density between those groups. Similarly, we did not find significant differences in I<sub>NCX</sub> current density between those groups (Fig 8C and D).

## Discussion

### Heart rate is protected by partially redundant mechanisms

Given the importance of heart rate to survival it is not surprising that the SAN cellular mechanisms for maintaining and accelerating heart rate are reinforced by redundancy. However, certain components of the pacemaking machinery exert clear and quantifiable contributions to heart rate maintenance and/or acceleration because their removal or inactivation causes loss of basal heart rate or impairs heart rate increases. The drug ivabradine slows heart rate, at least in part, by antagonist actions on HCN4<sup>24</sup>. Loss of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger<sup>25-27</sup>, inhibition of the mitochondrial Ca<sup>2+</sup> uniporter<sup>17</sup> and application of toxins that prevent SR Ca<sup>2+</sup> uptake (thapsigargin)<sup>16, 28</sup> and release (ryanodine)<sup>16, 28</sup>

reduce or eliminate heart rate acceleration. These latter examples comprise some of the evidence in support of the  $\text{Ca}^{2+}$  clock concept for pacemaker cell automaticity<sup>1</sup>. Mouse models of PKA<sup>29</sup> and CaMKII<sup>2</sup> inhibition by  $\alpha$  myosin heavy chain promoter driven transgenic expression of kinase-inhibitory peptides maintain normal resting heart rates but exhibit selective loss of heart rate acceleration after isoproterenol injection. Based on the now established role of SR  $\text{Ca}^{2+}$  uptake and release to affect SAN rates and the role of PKA and CaMKII in regulating SR  $\text{Ca}^{2+}$ , we determined whether individual loss of one of four established PKA or CaMKII sites on RyR2 or PLN could impact basal heart rate or heart rate acceleration.

### RyR2 phosphorylation sites do not control heart rate individually

We found that loss of either RyR2 phosphorylation site was insufficient to affect basal heart rate or heart rate acceleration in response to isoproterenol injection or spontaneous activity. Furthermore, the behaviors of isolated SAN cells mirrored in vivo responses in that resting and isoproterenol accelerated automaticity was similar between mutant and control preparations. Our findings are partially confirmed by one study using the same S2808A mice<sup>30</sup> but different than another report showing S2808A mice have defective heart rate acceleration<sup>31</sup>. While we are uncertain about the basis for the discrepant findings, we note that our study and the work by Shan et al.<sup>31</sup> used the same isoproterenol dosing for in vivo studies, although heart rates in our mice, implanted with ECG and activity telemeters, were measured in the absence of anesthesia. It is also possible that the lack of alignment between our results and the findings of Shan et al.<sup>31</sup> are due to undetermined differences in the mouse models. Taken together, we believe our new data constitute strong evidence that RyR2 S2808 and S2814 are individually dispensable for normal resting heart rates and for physiological heart rate responses. Furthermore, additional RyR2 phosphorylation sites, already known (e.g. S2030<sup>32</sup>) or yet to be identified, may contribute to heart rate control. In contrast to the lack of effect on heart rate, our data suggest that the S2814 site is more important than S2808 for determining basal SR  $\text{Ca}^{2+}$  leak because S2814A SAN cells show elevated SR  $\text{Ca}^{2+}$  content compared to S2808A and WT counterparts.

### A role for PLN in heart rate?

We found that *Pln*<sup>-/-</sup> mice and SAN cells isolated from *Pln*<sup>-/-</sup> mice have similar resting heart and automaticity rates compared to WT and transgenic controls. These findings are partially consistent with earlier studies showing *Pln*<sup>-/-</sup> and WT mice had equivalent baseline heart rates and responses to isoproterenol<sup>9</sup>. We interpret these data to suggest that PLN plays a permissive role, perhaps to set SR  $\text{Ca}^{2+}$  content to a physiological set point, but is not required for basal or physiological heart rate responses. We hypothesize that phosphorylation at either S16 or T17 is not required to disinhibit SERCA2a sufficiently to sustain such a set point. In contrast, mice with superinhibitory PLN (N27A) had severely impaired resting, isoproterenol- and activity-stimulated heart rates, consistent with earlier reports<sup>11, 16</sup>. These data show that extreme inhibition of SERCA2a activity does impact heart rate.



## CaMKII inhibition does not affect heart rate in mice lacking PLN

SAN CaMKII activity is critical for sustaining the full dynamic range of heart rate under physiological stress but is dispensable for maintaining resting heart or SAN rates <sup>2</sup>. We tested the idea that CaMKII actions to affect heart rate in SAN cells operated by a PLN or PLN-associated pathway by interbreeding AC3-I and *Pln*<sup>-/-</sup> mice. Surprisingly, loss of *Pln* allowed AC3-I expressing hearts and SAN cells to achieve a heart rate similar to WT. We interpret this result to suggest lowering of SR Ca<sup>2+</sup> content in AC3-I transgenic mice may be a key component of the mechanism of heart rate slowing by CaMKII inhibition. We originally reported that SR Ca<sup>2+</sup> content is reduced in AC3-I SAN cells <sup>2</sup> and here we show that AC3-I expression is not capable of reducing SAN cell SR Ca<sup>2+</sup> content in the absence of PLN. We speculate that AC3-I expression reduces SR Ca<sup>2+</sup> content below a set point required to sustain physiological rate increases and that loss of PLN supersedes the SR Ca<sup>2+</sup> lowering consequences of AC3-I expression. Based on our published (Wu et al. <sup>2</sup>, see Fig 6C) and new data (Fig 2D, Fig 3F, Fig 5D, Fig 6A and Fig 7F) we expect that SAN cell SR Ca<sup>2+</sup> content less than 1.0 C/F, assessed as a caffeine releasable store and interrogated by integration of INCX, is insufficient to support physiological heart rate responses (Supplemental Table). It is also possible that CaMKII contributes to SR Ca<sup>2+</sup> filling by actions at extra-PLN targets, such as mitochondria or novel sites on RyR2, PLN and/or RyR2 associated proteins.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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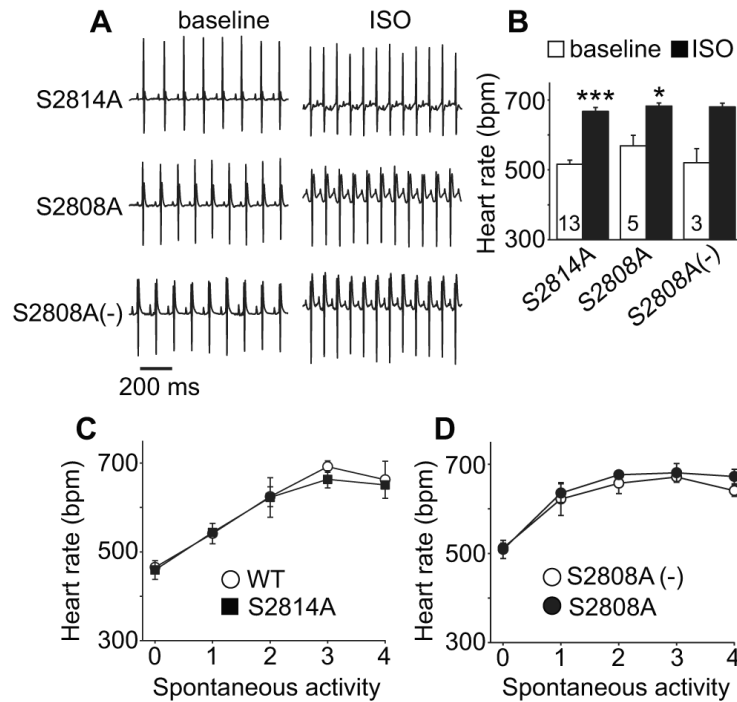
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**WHAT IS KNOWN**

- Fight or flight heart rate increases depend on protein kinase A (PKA) and Ca<sup>2+</sup>/calmodulin kinase II (CaMKII) mediated enhancement of Ca<sup>2+</sup> uptake and release from sarcoplasmic reticulum (SR) in sinoatrial nodal pacemaker cells (SANC).
- CaMKII inhibition slows fight or flight heart rate increases.
- The impact of individual PKA and CaMKII phosphorylation sites regulating SR Ca<sup>2+</sup> uptake and release on heart rate modulation is unknown.

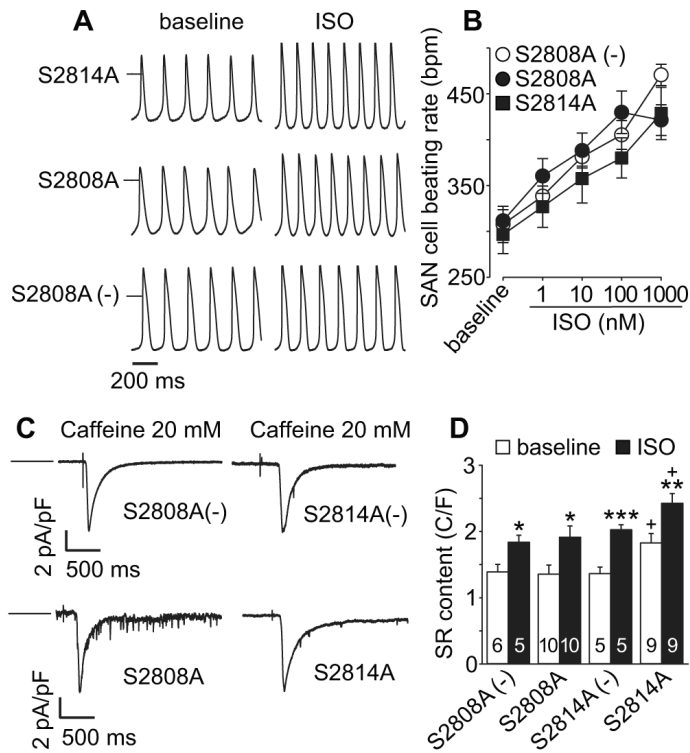
**WHAT THE STUDY ADDS**

- We used a suite of genetically modified mice to evaluate PKA and CaMKII target sites on phospholamban (PLN), which modulates SR Ca<sup>2+</sup> uptake, and the ryanodine receptor (RyR2), which mediates SR Ca<sup>2+</sup> release, in SANC.
- We found that ablation of individual RyR2 or PLN PKA or CaMKII target sites failed to affect heart rate in vivo or in SANC.
- Our data suggest CaMKII inhibition slows heart rate by reducing SR Ca<sup>2+</sup> content and that a minimum SR Ca<sup>2+</sup> content is required in SANC for physiological fight or flight heart rate increases.



**Figure 1.**

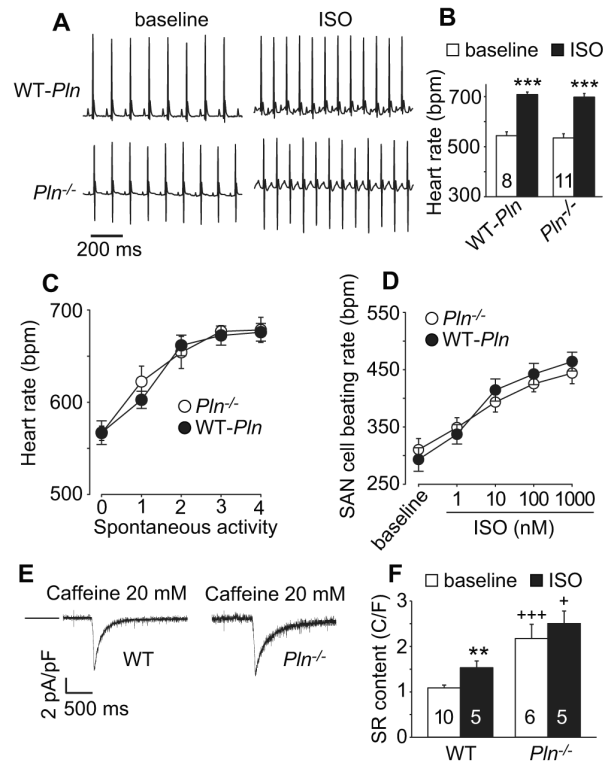
In vivo basal heart rate and heart rate responses to ISO or spontaneous activity were not altered in Ryr2 mutant mice implanted with ECG and activity-sensing telemeters. **A.** Representative telemetry ECG traces (at baseline and after ISO injection) from S2814A, S2808A and littermate WT (S2808A (-)) mice, scale bar 200 ms. **B.** Summary of telemetry-recorded heart rates at baseline and in response to ISO stimulation in S2814A, S2808A and littermate control mice. The numerals indicate the number of mice in each experimental group here and in other figures. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , paired Student's t-test, Error bars indicate s.e.m. **C.** Activity related heart rate change in S2814A and WT (C57) control mice,  $n = 3-6$ . **D.** Activity related heart rate changes (see Methods) in S2808A and S2808A (-) littermate control mice,  $n = 4-7$ . The error bars indicate s.e.m. in all figures.



**Figure 2.**

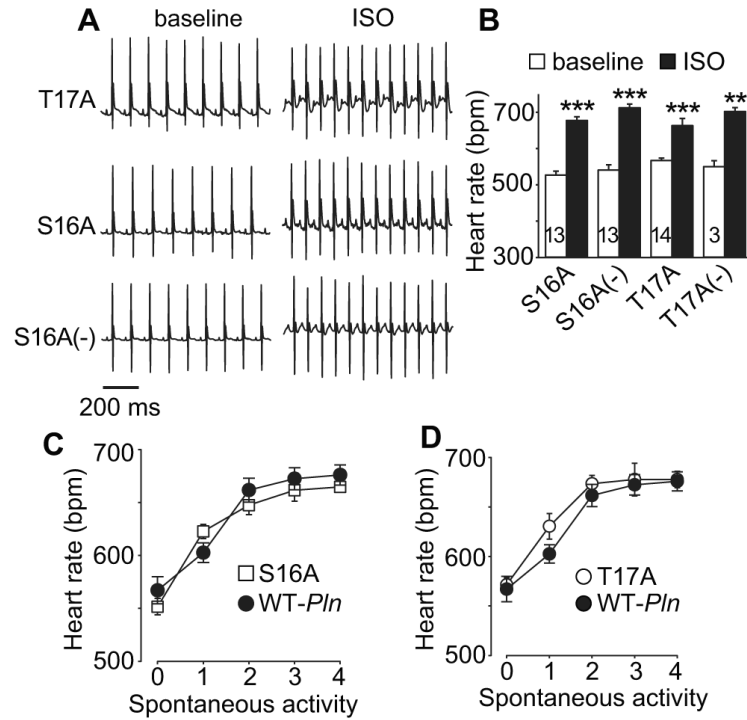
SAN cell baseline beating rate and response to ISO were not altered in RyR2 mutant mice.

**A.** Representative action potential recording traces (at baseline and after ISO application) from SAN cells isolated from S2814A (upper), S2808A (middle) and S2808A (-) littermate control (lower) mice. The short horizontal bars mark 0 mV. **B.** Summary data of SAN cell beating rates at baseline and response to increasing ISO concentration. n=6-14 cells/data point. **C.** Representative  $I_{NCX}$  current traces induced by caffeine triggered (20 mM) SR  $Ca^{2+}$  release (indicated by perfusion mark) in SAN cells isolated from the mice as indicated. The horizontal bars indicate 0 current level. **D.** SR  $Ca^{2+}$  content was increased in SAN cells isolated from the RYR mutation mice compared to SAN cells from littermate WT mice (C/F: Coulombs/Farad). Numbers of experimental SAN cells is indicated by numerals. \*p < 0.05, \*\* p < 0.01 \*\*\* p < 0.001 baseline vs ISO, unpaired Student's t-test. + p < 0.05, S2814A vs the other groups, One way ANOVA combined with Holm-Sidak test.



**Figure 3.**

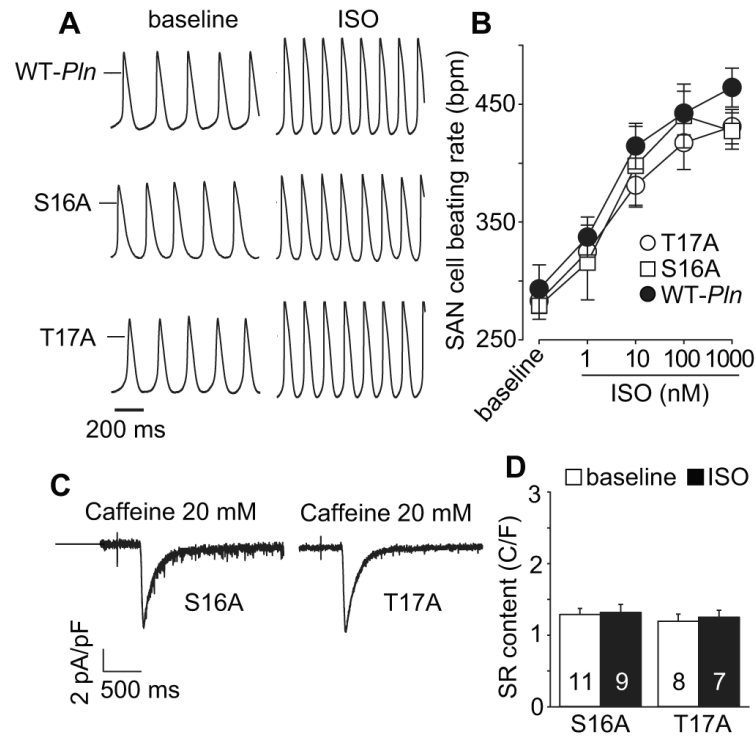
In vivo heart rate responses to ISO and activity were not affected by *Pln* knock out or transgenic replacement with WT *Pln*. **A.** Representative telemetry ECG recording traces (at baseline and after ISO injection) from *Pln*<sup>-/-</sup> and WT-*Pln* mice. **B.** Summary ECG telemetry data at baseline and in response to ISO stimulation in WT-*Pln* and *Pln*<sup>-/-</sup> mice. \*\*\* p<0.001, paired Student's t-test. **C.** Activity related heart rate changes in *Pln*<sup>-/-</sup> and WT-*Pln* mice, n=4-20. **D.** SAN cell beating rates at baseline and in response to increasing ISO concentrations. n=8-14 cells/data point. **E.** Representative  $I_{NCX}$  current traces induced by caffeine triggered (20 mM) SR  $Ca^{2+}$  release (indicated by arrow) in SAN cells isolated from WT (C57) (left) or *Pln*<sup>-/-</sup> (right) mice. The horizontal bars indicate 0 current level. **F.** SR  $Ca^{2+}$  content was increased in SAN cells isolated from *Pln*<sup>-/-</sup> mice compared to SAN cells from WT (C57) mice (C/F: Coulombs/Farad). Numbers of experimental SAN cells is indicated by numerals. \*\* p<0.01 baseline vs ISO, + p<0.05, \*\*\* p<0.001, *Pln*<sup>-/-</sup> vs WT (C57), unpaired Student's t-test.



**Figure 4.**

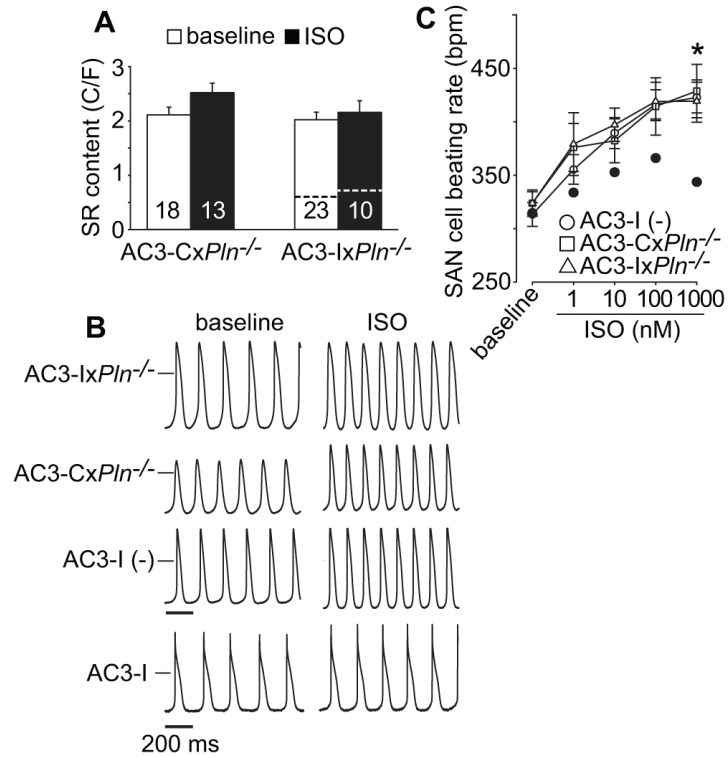
In vivo basal heart rate and response to ISO or activity were not altered in S16A or T17A mice. **A.** Representative ECG telemetry recording traces (at baseline and after ISO injection) from T17A (upper), S16A (middle) and S16A (-) (lower) mice. **B.** Summary data of ECG telemetry recorded heart rate at baseline and in response to ISO stimulation in S16A, T17A and their littermate control *Pln*<sup>-/-</sup> background mice (S16A(-) and T17A(-)). The number of mice used in the summary data is indicated by numerals in the corresponding bars. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , paired Student's t-test, Wilcoxon Signed Rank Test was used for T17A group data. **C.** Activity related heart rate changes in S16A and WT-*Pln* control mice,  $n = 3-20$ . **D.** Activity related heart rate changes (see Methods) in T17A and WT-*Pln* control mice,  $n = 4-20$ .



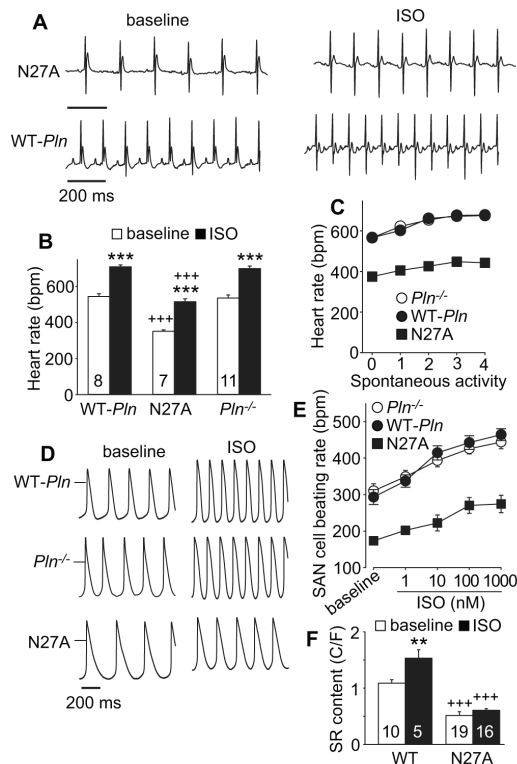


**Figure 5.**

SAN cell baseline and ISO response rates were not altered in *Pln* mutant mice. **A.** Example action potential traces (at baseline and after ISO application) from SAN cells isolated from WT-*Pln* (upper), S16A (middle) and T17A (lower) mice. The horizontal bars mark 0 mV. **B.** Summary data for beating rates at baseline and in response to increasing ISO concentrations in SAN cells isolated from *Pln* mutant mice. n=5-8 cells/data point. **C.** Representative  $I_{NCX}$  current traces induced by caffeine (20 mM) triggered SR  $Ca^{2+}$  release (indicated by perfusion mark) in SAN cells isolated from the mice as indicated. The horizontal bars indicate 0 current level. **D.** SR  $Ca^{2+}$  content was not increased in SAN cells isolated from the PLN mutation mice. Numbers of experimental SAN cells are indicated by numerals.

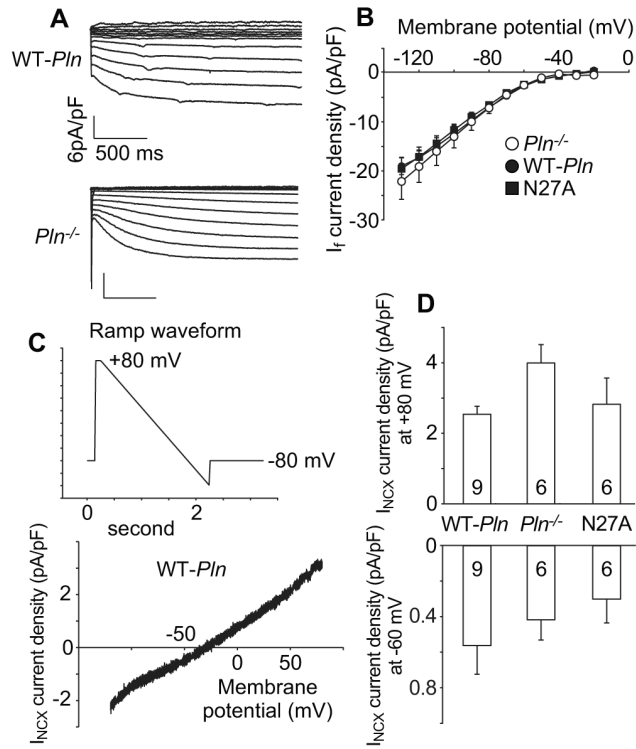
**Figure 6.**

Increased SR Ca<sup>2+</sup> content and restored ISO stimulated rate responses in *Pln*<sup>-/-</sup> mice with transgenic AC3-I expression. **A.** SR Ca<sup>2+</sup> content in *Pln*<sup>-/-</sup> mice with transgenic expression of AC3-I and AC3-C. The number of SAN cells studied is indicated by numerals in the corresponding bars. Dotted line in right group of bars indicated SR Ca<sup>2+</sup> content level and response to ISO in SAN cells isolated from AC3-I mice (Data re-draw of Figure 6C data from Wu et al., PNAS 2009). **B.** Example action potential recording traces (at baseline and after ISO application) from SAN cells isolated from AC3-Ix*Pln*<sup>-/-</sup>, AC3-Cx*Pln*<sup>-/-</sup>, AC3-I (-) and AC3-I mice as indicated. The horizontal bars mark 0 mV and the scale bar indicates 200ms. **C.** Summary data for SAN cell rates at baseline and in response to increasing ISO concentrations, n=7-10 cells/data point. Dotted curve (black filled circle) indicated ISO response in SAN cells isolated from AC3-I mice. (Data of AC3-I mice are re-draw of Figure 2B data from Wu et al., PNAS 2009). \* p<0.05, One way ANOVA combined with Holm-Sidak test.



**Figure 7.**

Reduced in vivo and SAN cell rates in N27A mutant mice. **A.** Example telemetry ECG recordings (at baseline and after ISO injection) from N27A mutant and WT-*Pln* mice, scale bar indicates 200 ms. **B.** Summary data for ECG telemetry recorded heart rates at baseline and in response to ISO stimulation in WT-*Pln*, N27A and littermate control *Pln*<sup>-/-</sup> mice. The number of mice used is indicated in the corresponding bars. \*\*\* p<0.001, baseline vs. after ISO, paired Student's t-test. +++ p<0.001, N27A vs. WT-*Pln* or *Pln*<sup>-/-</sup>, ANOVA, holm-sidak test. **C.** Activity related heart rate changes in N27A and *Pln*<sup>-/-</sup> and WT-*Pln* control mice, n=4-20. **D.** SAN cells rates at baseline and in response to ISO were significantly reduced in SAN cells isolated from N27A mice. Example action potential recording traces (at baseline and after ISO application) from SAN cells isolated from WT-*Pln* (upper), *Pln*<sup>-/-</sup> (middle) and N27A (lower) mice. The horizontal bars mark 0 mV. **E.** Summary data for rates at baseline and in response to increasing ISO concentrations from SAN cells isolated from N27A, *Pln*<sup>-/-</sup> and WT-*Pln* mice. n=6-14 cells/data point. **F.** Reduced SR Ca<sup>2+</sup> content at baseline and after ISO stimulation in SAN cells isolated from N27A mice. The summary SR Ca<sup>2+</sup> content data at baseline and after ISO stimulation in SAN cells from WT (C57) and N27A mice. The number of SAN cells studied is indicated by numerals in the corresponding bars. The data for WT (C57) presented here are the same data in shown in Figure 3F. \*\* p<0.01 baseline vs ISO, +++ p<0.001, N27A vs WT, unpaired Student's t-test.

**Figure 8.**

HCN currents ( $I_f$ ) and NCX currents ( $I_{NCX}$ ) were not altered by PLN mutation. **A.** Representative  $I_f$  currents traces recorded from an SAN cell isolated from WT-*Pln* (upper) and *Pln*<sup>-/-</sup> mice (lower). **B.** Summary I-V relationship of  $I_f$  density from WT-*Pln* (n=6), *Pln*<sup>-/-</sup> (n=13) and N27A mice (n=6). **C.** Ramp voltage clamp protocol for recording  $I_{NCX}$  (upper) and an example  $I_{NCX}$  trace (lower). **D.** Summary data of  $I_{NCX}$  density at +80 mV (upper) and at -60 mV (lower) from SAN cells isolated from WT-*Pln*, *Pln*<sup>-/-</sup> and N27A mice.

Table 1

## Genetically modified mice

| MICE                              | MUTATIONS                         | NOTES  | CONTROL                              | SOURCE  |
|-----------------------------------|-----------------------------------|--|--------------------------------------|---------|
| <i>Pln</i> <sup>-/-</sup>         | <i>Pln</i> <sup>-/-</sup>         | Background for all PLN mutant mice                                     | WT*, WT- <i>Pln</i>                  | MMRRC   |
| S16A                              | PLN S16A                          | TG replacement of S16A in the <i>Pln</i> <sup>-/-</sup> background     | S16A(-), WT- <i>Pln</i>              | MMRRC   |
| T17A                              | PLN T17A                          | TG replacement of T17A in the <i>Pln</i> <sup>-/-</sup> background     | T17A(-), WT- <i>Pln</i>              | MMRRC   |
| S16A(-)                           | <i>Pln</i> <sup>-/-</sup>         | Littermates of S16A lacking TG S16A                                    |                                      | MMRRC   |
| T17A(-)                           | <i>Pln</i> <sup>-/-</sup>         | Littermates of T17A lacking TG T17A                                    |                                      | MMRRC   |
| WT- <i>Pln</i>                    | WT-PLN                            | TG replacement of WT PLN in the <i>Pln</i> <sup>-/-</sup> background   |                                      | MMRRC   |
| N27A                              | PLN N27A                          | TG replacement of N27A PLN in the <i>Pln</i> <sup>-/-</sup> background | WT*, N27A(-), WT- <i>Pln</i>         | MMRRC   |
| N27A(-)                           | <i>Pln</i> <sup>-/-</sup>         |  | WT- <i>Pln</i>                       | MMRRC   |
| S2808A                            | S2808A                            |  | S2808A(-)                            | HV lab  |
| S2814A                            | S2814A                            |  | WT*, S2814A(-)                       | XW lab  |
| S2808A(-)                         |                                   | Littermates of S2808A with WT RyR2                                     |                                      | HV lab  |
| S2814A(-)                         |                                   | Littermates of S2814A with WT RyR2                                     |                                      | XW lab  |
| AC3-IX <i>PLN</i> <sup>-/-</sup>  | AC3-I & <i>PLN</i> <sup>-/-</sup> |  | AC3-I(-) & <i>PLN</i> <sup>-/-</sup> | MEA lab |
| AC3-C X <i>PLN</i> <sup>-/-</sup> | AC3-C & <i>PLN</i> <sup>-/-</sup> |  | AC3-C(-) & <i>PLN</i> <sup>-/-</sup> | MEA lab |
| AC3-I(-)                          |                                   | littermate control lacking tg AC3-I                                    |                                      | MEA lab |
| AC3-C(-)                          |                                   | littermate control lacking tg AC3-C                                    |                                      | MEA lab |

\* WT mice in C57 background