Differential effects of phospholamban and Ca\(^{2+}\)/calmodulin-dependent kinase II on [Ca\(^{2+}\)]\(_i\) transients in cardiac myocytes at physiological stimulation frequencies

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Werdich AA, Lima EA, Dzhura I, Singh MV, Li J, Anderson ME, Baudenbacher FJ. Differential effects of phospholamban and Ca\(^{2+}\)/calmodulin-dependent kinase II on [Ca\(^{2+}\)]\(_i\) transients in cardiac myocytes at physiological stimulation frequencies. Am J Physiol Heart Circ Physiol 294: H2352–H2362, 2008. First published March 21, 2008; doi:10.1152/ajpheart.01398.2006.—In cardiac myocytes, the activity of the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is hypothesized to regulate Ca\(^{2+}\) release from and Ca\(^{2+}\) uptake into the sarcoplasmic reticulum via the phosphorylation of the ryanodine receptor 2 and phospholamban (PLN), respectively. We tested the role of CaMKII and PLN on the frequency adaptation of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transients in nearly 500 isolated cardiac myocytes from transgenic mice chronically expressing a specific CaMKII inhibitor, interbred into wild-type or PLN null backgrounds under physiologically relevant pacing conditions (frequencies from 0.2 to 10 Hz and at 37°C). When compared with that of mice lacking PLN only, the combined chronic CaMKII inhibition and PLN ablation decreased the maximum Ca\(^{2+}\) release rate by more than 50% at 10 Hz. Although PLN ablation increased the rate of Ca\(^{2+}\) uptake at all frequencies, its combination with CaMKII inhibition did not prevent a frequency-dependent reduction of the amplitude and the duration of the [Ca\(^{2+}\)]\(_i\) transients. High stimulation frequencies in the physiological range diminished the effects of PLN ablation on the decay time constant and on the maximum decay rate of the [Ca\(^{2+}\)]\(_i\) transient, indicating that the PLN-mediated feedback on [Ca\(^{2+}\)]\(_i\) removal is limited by high stimulation frequencies. Taken together, our results suggest that in isolated mouse ventricular cardiac myocytes, the combined chronic CaMKII inhibition and PLN ablation slowed Ca\(^{2+}\) release at physiological frequencies: the frequency-dependent decay of the amplitude and shortening of the [Ca\(^{2+}\)]\(_i\) transient occurs independent of chronic CaMKII inhibition and PLN ablation, and the PLN-mediated regulation of Ca\(^{2+}\) uptake is diminished at higher stimulation frequencies within the physiological range.

frequency adaptation; frequency-dependent acceleration of relaxation; cytosolic calcium concentration

DURING THE ACTION potential, Ca\(^{2+}\) enters the cell through voltage-gated sarcolemmal ion channels and triggers a massive release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) into the intracellular space, leading to the activation of the contractile machinery (5). For relaxation to occur, the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) must decrease. There are several mechanisms by which this occurs during the cardiac cycle. In the adult mouse ventricle, nearly 90% of the Ca\(^{2+}\) that entered the cytosol during systole is actively transported back into the SR by the sarco(endo)plasmic reticulum Ca\(^{2+}\)/-ATPase type 2 (SERCA2) (21). The rate at which Ca\(^{2+}\) is taken up by the SR is negatively regulated by phospholamban (PLN), a small (52 amino acids) SR protein, which is associated with SERCA2 in its unphosphorylated state (23). The phosphorylation of PLN by cAMP-dependent protein kinase A (PKA) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) relieves this inhibition, leading to an acceleration of Ca\(^{2+}\) uptake and muscle relaxation, which ensures the diastolic filling of the heart at increasing stimulation frequencies (15). Schouten (27) first hypothesized that repeated Ca\(^{2+}\) release activates CaMKII, causing the phosphorylation of PLN in a frequency-dependent manner and leading to the frequency-dependent acceleration of relaxation (27). Three main findings support this hypothesis. First, an increase in stimulation frequency increases the rate of Ca\(^{2+}\) influx, causing a sustained activation of CaMKII (12). Second, the frequency dependence of cytosolic Ca\(^{2+}\) removal and myocardial relaxation are abolished by inhibiting SR Ca\(^{2+}\) uptake (4, 18, 27, 28). Finally, CaMKII is active at the SR membrane and phosphorylates PLN at a specific site [threonine-17 (Thr17)], which is distinct from that for PKA phosphorylation [serine-16 (Ser16)]. Therefore, CaMKII is a strong candidate to control the frequency dependence of cytosolic Ca\(^{2+}\) removal. However, the contributions of PLN and CaMKII to the frequency adaptation of the [Ca\(^{2+}\)]\(_i\) transient, particularly under physiological conditions, have not been measured and are not completely understood.

Some reports confirmed the hypothesis that the frequency-dependent activation of CaMKII occurred in vivo (29) and was correlated with an increase in PLN Thr17 phosphorylation (16, 36). The pharmacological inhibition of CaMKII by 1-[N-O-Bis(5-isoquinolinesulfonyl)-N-methyl-t-tyrosyl]-4-phenylpiperazene or 2-[N-(2-hydroxyethyl)-N-(4methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) suppressed the shortening of the [Ca\(^{2+}\)]\(_i\) transient during steady-state pacing (4, 13). These experiments support the hypothesis that CaMKII and PLN each play a role in modulating SR Ca\(^{2+}\) uptake in a frequency-dependent manner. However, another report did not support a role for PLN phosphorylation in the frequency-dependent shortening of the [Ca\(^{2+}\)]\(_i\) transient (18). The results in transgenic animals with different levels of PLN indicated that the frequency-dependent
acceleration of Ca\(^{2+}\) uptake depends on CaMKII but does not require PLN (13, 21), even though PLN is believed to be a major contributor to the frequency dependence of Ca\(^{2+}\) uptake and myocardial relaxation in the mammalian heart (6, 9, 36).

CaMKII may also regulate Ca\(^{2+}\) release from the SR via the phosphorylation of the SR Ca\(^{2+}\)-release channel ryanodine receptor 2 (RyR2). The CaMKII-dependent phosphorylation of RyR2 at serine-2815 has been found to increase with pacing frequency (29), likely leading to a frequency-dependent increase in the open probability of the isolated channel (17, 31) and an increase in Ca\(^{2+}\) release from the SR in intact cardiac myocytes (11, 22, 24). Most experiments aimed at investigating the frequency dependence of [Ca\(^{2+}\)], regulation in a mouse or rat have been performed at stimulation frequencies in the range of 1–5 Hz, which is well below the physiological range (i.e., 10–12 Hz in mouse). If it is true that CaMKII activity increases with stimulation frequency (12), studies at high-pacing frequencies are necessary to understand the physiological signaling role of CaMKII in the heart. Furthermore, most single cell data were taken at room temperature where the activities of kinases and phosphatases could be significantly different than those at body temperature (28). Pharmacological CaMKII inhibition has been used to elucidate the role of CaMKII in the frequency dependence of the [Ca\(^{2+}\)]\(\text{max}\), transient. However, these drugs are known for their unspecific side effects on ion channels. For example, KN-93 caused a CaMKII-independent steady-state inhibition of a peak L-type Ca\(^{2+}\) current at concentrations required for CaMKII inhibition (1). KN-93, when present in the extracellular space, has been shown to act as an inhibitor of a wide range of potassium channels (25). Our approach using transgenic mice expressing a highly selective CaMKII inhibitory peptide avoids potentially confounding nonspecific effects of currently available CaMKII inhibitory drugs.

The goal of this study was to test the frequency dependence of [Ca\(^{2+}\)], transients in single cardiac myocytes at physiological stimulation frequencies and at body temperature in the presence and absence of PLN and in combination with genetic CaMKII inhibition. We used an established genetic approach of CaMKII inhibition by the cardiomyocyte-delimited expression of a specific CaMKII inhibitory peptide [AC3 inhibitory (I)] (32, 35). A transgenic control mouse was generated with an inactive scrambled version of AC3-I, called AC3 control (C). CaMKII inhibition is chronic in AC3-I hearts, because the expression of both AC3-I and AC3-C begins in the perinatal period and continues through adulthood. Transgenic AC3-I and AC3-C mice were interbred with PLN knockout (PLN\(^{-/-}\)) mice to eliminate PLN-dependent CaMKII contributions to SR Ca\(^{2+}\) release and uptake. We found that the combination of PLN ablation and chronic CaMKII inhibition slowed Ca\(^{2+}\) release at physiological stimulation frequencies but did not eliminate the frequency-dependent shortening of the [Ca\(^{2+}\)]\(\text{max}\). The influence of PLN on the regulation of cytosolic Ca\(^{2+}\) removal decreased exponentially with increasing stimulation frequency, suggesting that the PLN-dependent regulation of Ca\(^{2+}\) uptake is inhibited by high stimulation frequencies within the physiological range.

METHODS

Mice with genetic CaMKII inhibition. The AC3-I and AC3-C mice were generated by the synthesis of a minigene based on the peptide sequence of AC3-I (KKALHRQ2ACDVCL) or AC3-C (KKHALQ2ERVCL) (8) and were described earlier (35). Both AC3-I and AC3-C were fused to an enhanced green fluorescence protein (EGFP) for stabilization and to visualize the cellular tissue distribution of transgene expression.

PLN\(^{-/-}\) mice (19) were interbred with AC3-I and AC3-C mice for more than four generations, and the genetic identity of the PLN\(^{-/-}\) pups with the transgenic expression of AC3-I or AC3-C (named AC3-I \(\times\) PLN\(^{-/-}\) or AC3-C \(\times\) PLN\(^{-/-}\) here) was confirmed using PCR (35). There was no evidence of cellular hypertrophy in isolated ventricular myocytes based on equivalent cell membrane capacitance measurements (34). Mice expressing AC3-I have slightly shorter action potentials and an increased peak L-type Ca\(^{2+}\) current compared with those of wild-type (WT) mice, due to a compensatory local upregulation of PKA signaling at L-type Ca\(^{2+}\) channels, but these changes did not affect myocyte contractility (35). We isolated single cardiac myocytes from 3- to 5-mo-old male and female mice (half of the cells originated from female mice). WT, AC3-C, AC3-C \(\times\) PLN\(^{-/-}\), and PLN\(^{-/-}\) littermates served as controls to discriminate any possible data artifacts resulting from the expression of AC3-I and AC3-C as EGFP fusion proteins.

Isolation of ventricular myocytes. Animals were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. The mice were anesthetized by an intraperitoneal injection of a 25 mg/ml Avertin solution (5 mg Avertin/10 g body wt; T48402; Sigma-Aldrich) containing 3 mg/10 ml heparin (H9399; Sigma-Aldrich). The heart was rapidly excised and placed into ice-cold Ca\(^{2+}\)-free HEPES-buffered normal Tyrode (NT) solution. The aorta was canulated, and the heart was perfused retrograde with Ca\(^{2+}\)-free NT solution at room temperature for 10 min to stop contractions. The NT solution contained (in mM) 140 Na\(^{+}\), 4.5 K\(^{+}\), 1.5 Mg\(^{2+}\), 150 Cl\(^{-}\), 0.4 H\(_2\)PO\(_4\), 10 HCO\(_3\), 10 HEPES, and 10 glucose. Ca\(^{2+}\) was added when specified. After 10 min, the perfusion was switched to NT solution containing 10 \(\mu\)M Ca\(^{2+}\), 178 U/ml collagenase (CLS2; Worthington Biochemical), and 0.64 U/ml protease (P5147; Sigma-Aldrich) for 8–10 min at 37°C. The ventricles and septum were cut away, coarsely minced, and placed into Ca\(^{2+}\)-free Tyrode solution containing 0.5% BSA at room temperature. Myocytes were dispersed by gentle agitation, and the Ca\(^{2+}\) concentration was gradually increased within 30 min from 0 to 0.5 mM to obtain Ca\(^{2+}\)-tolerant cardiac myocytes. The cells were stored in BSA solution until used. All experiments were performed within the first 3 h after isolation.

Immunoblotting and Western blot analysis. Mouse hearts were homogenized in a lysis buffer containing 50 mM HEPEs, 150 mM NaCl, 2 mM EDTA, 1.25% sodium deoxycholate, and 1.25% (vol/vol) Nomidet P-40 at pH 7.6 as well as protease and phosphatase inhibitors. Equal amounts of protein from each heart lysate (20 \(\mu\)g/lane) were loaded onto 4–12% Bis-Tris polyacrylamide gels, and electrophoresis was performed using the NuPAGE electrophoresis system (Invitrogen). For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and were probed using antibodies to total PLN (Cyclacell), PLN-Thr17 (Cyclacell), SERCA2 (Santa Cruz), and RyR2 (Affinity Bioreagents). The same blots were probed with an anti-actin rabbit polyclonal antibody (A2066; Sigma-Aldrich). This served as an internal control for protein loading and was used to normalize RyR2 protein levels. Immunoblot signals were detected using enhanced chemiluminescence (ECL; Roche).

Intracellular Ca\(^{2+}\) measurements. We used the near-infrared emitting single wavelength dye X-Rhod-1 (X14210; Molecular Probes-Invitrogen), which is characterized by long-wavelength absorption.
and emission maxima at ~580 and 602 nm, respectively, and by a ~100-fold increase in fluorescence upon binding to Ca$^{2+}$. Importantly, the excitation and emission spectra of X-Rhod-1 are distinct from those of the EGFP, thereby avoiding contamination of the Ca$^{2+}$ signal by the EGFP background and allowing high-bandwidth recordings of [Ca$^{2+}$_i], transients with high signal-to-noise ratios (7). Using this dye, we avoided low-pass filtering our data, taking advantage of the full bandwidth (~10 kHz) of our amplifier, which allowed an accurate characterization of the time course of the [Ca$^{2+}$_i], transient, in particular of the fast upstroke. After the acquisition of each transient and for every cell, the AC3-I or AC3-C expression was confirmed by EGFP fluorescence observation. The X-Rhod-1 dye was purchased in 50-μg aliquots. Before each experiment, one aliquot of the dye was dissolved in 40 μl DMSO (276855; Sigma-Aldrich). From this solution, 20 μl were added to 5 ml of cell suspension (in NT solution), resulting in a final dye concentration of 4 μM. The cells were kept in the staining solution for 5 min at room temperature and then centrifuged at 27 g for 10 min. The supernatant was removed, and the cells were resuspended in NT solution containing 1.0 mM CaCl$_2$. To minimize phototoxic effects, only 100 μl of stained cells were pipetted into the bath solution, which was completely replaced after each exposure to the excitation light. Only a single [Ca$^{2+}$_i], transient was recorded and analyzed for each cell to minimize photobleaching and phototoxic side effects. Therefore, a large number of cells were used for this study. We recorded [Ca$^{2+}$_i], transients from nearly 500 cardiac myocytes from six different mouse models (AC3-I, AC3-C, WT, AC3-I × PLN$^{+/−}$, AC3-C × PLN$^{+/−}$, and PLN$^{−/−}$) at six different stimulation frequencies (0.2, 1, 2, 4, 8, and 10 Hz). The number of cells (number of animals) analyzed at each frequency and for each genotype is shown in Table 1.

The bath solution consisted of NT solution with 1.0 mM CaCl$_2$, supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, and 6.7 ng/ml sodium selenite (100× medium supplement, 41400; Gibco-Invitrogen), and maintained at 37°C. The medium supplement has been shown to enhance glucose uptake and monovalent cation transport and allowed prolonged steady-state high-speed stimulation in the serum-free bath solution (26). The myocytes were field stimulated via two parallel platinum wires connected to a stimulator (701B; Aurora Scientific). We recorded [Ca$^{2+}$_i] transients at locations near the center of the cells where motion was greatly reduced. Furthermore, contraction occurred with a significant delay from the upstroke of the [Ca$^{2+}$_i], transient in all cardiac myocytes and thus did not affect the dynamics of the [Ca$^{2+}$_i], transients. Therefore, motion artifacts were negligible, and the use of an excitation-contraction uncoupler was not necessary.

We interpreted changes in Ca$^{2+}$ fluorescence as changes in SR Ca$^{2+}$ release and upstroke because the mouse ventricular myocardium relies on SR Ca$^{2+}$ release and upstroke as the major source/sink of Ca$^{2+}$ to activate contraction/relaxation. Other Ca$^{2+}$ transport mechanisms, such as sarcolemmal Na$^+$/Ca$^{2+}$ exchanger (NCX), sarcolemmal Ca$^{2+}$-ATPase, or the mitochondrial Ca$^{2+}$ transport, have been shown to contribute to a much smaller extent to overall [Ca$^{2+}$_i], cycling in the mouse myocardium. Bassani et al. (2, 3) initially evaluated the Ca$^{2+}$ transport mechanisms that compete for cytosolic Ca$^{2+}$ during relaxation in rabbit and in rat ventricular myocytes. These reports show that in WT and (PLN$^{−/−}$) mouse cardiac myocytes, 90% (96%) of Ca$^{2+}$ that entered the cytosol during a twitch is removed by SERCA2, 9% (3.4%) by NCX, and only 0.5% (0.1%) by the slow mechanisms, i.e., sarcolemmal Ca$^{2+}$-ATPase and mitochondrial Ca$^{2+}$ uniporter (21).

**Microscope setup.** We used an inverted microscope (Axiovert 200; Carl Zeiss) equipped with a ×63, 1.4 numerical aperture oil immersion lens (Plan Apochromat; Carl Zeiss). The dye was excited by a monochromator (Optoscan; Cairn Research) at a wavelength of 560/30 nm. The fluorescence emission was directed through a 595-nm cutoff dichroic mirror (XF2029; Omega Optical), a 645/74-nm emission filter (XF3081; Omega Optical), and a 1-mm diameter optical fiber connected to a photomultiplier module (H6780; Hamamatsu). The expression of the EGFP was confirmed using a standard FITC filter set (XF115-2; Omega Optical). Signals were amplified by a high-bandwidth, custom-built amplifier and digitized at a sampling rate of 20 kHz by an analog-to-digital converter board (PCI-6071E; National Instruments) in a personal computer. The signals were further digitally low-pass filtered with separate cutoff frequencies for the fast fluorescence upstroke (5 kHz) and the slow decay (1 kHz).

**SR Ca$^{2+}$ content.** SR Ca$^{2+}$ content was measured as the integrated NCX current after caffeine exposure. Myocytes were perfused during a prolonged voltage command to −80 mV with a modified bath solution containing 20 mM caffeine using a fast solution exchanger, with 10 preconditioning pacing pulses at a frequency of 2 Hz. All recordings were performed at 34°C. The resulting NCX inward current was integrated using pCLAMP 9.2 and normalized for total membrane capacitance versus cell surface area. Na$^+$ and K$^+$ currents were eliminated in the bath solution by adding Cs$^+$ and tetraethylammonium chloride. The pipettes contained (in mM) 120 Cs$^+$, 10 HEPES, 10 tetraethylammonium, 5 phosphocreatine, 1 MgATP, and 1 NaGTP; the pH was adjusted to 7.2 with CsOH. The extracellular bath solution contained (in mM) N-methyl-D-glucamine, 25 Cs$^+$, 10 HEPES, 10 glucose, 1.8 Ca$^{2+}$, and 0.5 Mg$^{2+}$; the pH was adjusted to 7.4 with HCl.

**Data analysis.** For statistical comparison, two-tailed t-tests or ANOVA with Holm-Bonferroni correction for multiple comparisons was used. Unless otherwise noted, data were displayed as means ± SEM. The significance level was set at P < 0.05. Data were processed using Matlab (R14; The MathWorks). Background and autofluorescence were subtracted from the transients before analysis, and relative X-Rhod-I fluorescence emission [fluorescence amplitude (F/F$_0$)] was used as an indicator of [Ca$^{2+}$_i], transient upstroke and decay were fit to mathematical functions for calculating numerical time derivatives as previously described (30). Because derivatives of high-bandwidth fluorescence recordings are extremely susceptible to noise, we used these empirical models to obtain smooth derivatives of the signal. Briefly, the [Ca$^{2+}$_i]-transient upstroke was best fit by [Ca$^{2+}$_i](t) = [Ca$^{2+}$_i]$_{max}$[P(t)/1 − P(t)], with P(t) = 0.5(1 − e$^{-t/\tau}$), where t is time, $\tau$ is the exponential constant, and [Ca$^{2+}$_i]$_{max}$.
is the amplitude of the transient. The parameters $\tau_1$, $n$, and $m$ were used as fit parameters and were not further analyzed. Transient decay was best fit by a sum of two exponentials, $[\text{Ca}^{2+}]_i = Ae^{-\tau_1 t} + Be^{-\tau_2 t}$, with fit parameters $A$, $B$, $\tau_1$, and $\tau_2$. The time constants $\tau_1$ and $\tau_2$ obtained from the fit were used to quantify the fluorescence decay. All fluorescence amplitudes, $F/F_0$, were normalized by the mean fluorescence amplitude $F/F_0$ of the WT group measured at 0.2 Hz. Thus all fluorescence values shown here represent relative changes in fluorescence intensity compared with those of WT cardiac myocytes stimulated at 0.2 Hz.

RESULTS

Combined chronic CaMKII inhibition and PLN ablation slowed the $[\text{Ca}^{2+}]_i$-transient upstroke at high stimulation frequencies. CaMKII activity has been shown to modulate the activity of the SR $\text{Ca}^{2+}$ release channel RyR2 via phosphorylation (29) and indirectly via an increase in the SR $\text{Ca}^{2+}$ load (33). We investigated whether CaMKII inhibition, PLN ablation, or the combination of these interventions could influence $\text{Ca}^{2+}$ release in intact cardiac myocytes. In Fig. 1A, raw data of a typical X-Rhod-1 $[\text{Ca}^{2+}]_i$ transient are shown for a WT cardiac myocyte stimulated in steady state at 1 Hz. Data acquisition rate was 20 kHz. B: magnified view of the $[\text{Ca}^{2+}]_i$ transient in A showing the time courses of the upstroke and the stimulus. Sample fit of the upstroke as described in METHODS is shown (dotted line). $F/F_0$, fluorescence amplitude.

<3% of the signal amplitude (dotted line in Fig. 1B). To characterize the frequency dependence of the $[\text{Ca}^{2+}]_i$-transient in our models, we first measured the maximum fluorescence amplitude $[(F/F_0)_{\text{max}}]$ at increasing stimulation frequencies in the presence (Fig. 2A) and absence (Fig. 2B) of PLN. In the presence of PLN, $(F/F_0)_{\text{max}}$ decreased monotonically with increasing stimulation frequency and with no apparent influence from CaMKII inhibition (Fig. 2A). PLN ablation increased $(F/F_0)_{\text{max}}$ at all frequencies except at 10 Hz (Fig. 2B). In the absence of PLN and for frequencies larger than 4 Hz, maximum $[\text{Ca}^{2+}]_i$-transient amplitudes were significantly smaller after chronic CaMKII inhibition compared with those of cells with PLN ablation alone. At the highest stimulation frequency of 10 Hz and in cells with chronic CaMKII inhibition, maximum fluorescence even reduced after PLN ablation compared with that of cells with normal levels of PLN. We

Fig. 1. X-Rhod-1 cytosolic $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) transient from a single wild-type (WT) cardiac myocyte. A: unfiltered X-Rhod-1 $[\text{Ca}^{2+}]_i$ transient from a single WT cardiac myocyte recorded during steady-state field stimulation at 1 Hz. Data acquisition rate was 20 kHz. B: magnified view of the $[\text{Ca}^{2+}]_i$ transient in A showing the time courses of the upstroke and the stimulus. Sample fit of the upstroke as described in METHODS is shown (dotted line). $F/F_0$, fluorescence amplitude.

Fig. 2. Frequency dependence of the $[\text{Ca}^{2+}]_i$-transient amplitude $[(F/F_0)_{\text{max}}$, maximum $F/F_0$, $A$: $[\text{Ca}^{2+}]_i$-transient amplitude $[(F/F_0)_{\text{max}}$ as a function of stimulation frequency in the presence of phospholamban (PLN) in cells with chronic $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II (CaMKII) inhibition [AC3 inhibitory (AC3-I)] and in control [AC3 control (AC3-C; WT)]. B: $[\text{Ca}^{2+}]_i$-transient amplitude $[(F/F_0)_{\text{max}}$ as a function of stimulation frequency in the absence of PLN in cells with chronic CaMKII inhibition (AC3-I × PLN−/−) and in control (AC3-C × PLN−/− and PLN−/−). All $(F/F_0)_{\text{max}}$ amplitudes were normalized to the mean fluorescence amplitude of the WT cells stimulated at 0.2 Hz. $T$, time. *$P < 0.05$.
further investigated whether the smaller peak [Ca\(^{2+}\)]-transient amplitude that we observed in cells with combined chronic CaMKII inhibition and PLN ablation would lead to an earlier termination of Ca\(^{2+}\) release in these cells at high frequencies compared with cells with PLN ablation alone. We measured the rise time of the [Ca\(^{2+}\)] transient from 10% to 90% of the maximum fluorescence at increasing stimulation frequencies (Fig. 3). In the presence of PLN, mean fluorescence rise time decreased with increasing frequency and with no apparent influence from chronic CaMKII inhibition, which was confirmed by the negative slope of a linear fit to the time-frequency relationship (Fig. 3, A and C). In contrast, cells with combined chronic CaMKII inhibition and PLN ablation showed a later termination of the Ca\(^{2+}\) release at higher frequencies than that of cells with PLN ablation alone, resulting in a reversal of the time-frequency relationship (Fig. 3, B and C). Examples of the linear fits used in the analysis are shown in Fig. 3A for AC3-I and in Fig. 3B for AC3-I × PLN\(^{-/-}\) and AC3-C × PLN\(^{-/-}\) cardiac myocytes.

The reduced amplitude (Fig. 2B) and the later termination (Fig. 3B) of the [Ca\(^{2+}\)] transient in AC3-I × PLN\(^{-/-}\) cardiac myocytes are expected to produce a smaller maximum rate of Ca\(^{2+}\) release in AC3-I × PLN\(^{-/-}\) compared with AC3-C × PLN\(^{-/-}\) cells. To confirm this hypothesis, we calculated the maximum upstroke velocity \((dF/dt)_{max}\), of the [Ca\(^{2+}\)] transient as an indicator of the maximum rate of increase in [Ca\(^{2+}\)]. In the presence of PLN, \((dF/dt)_{max}\) decreased with increasing stimulation frequencies in all cells and independent of CaMKII inhibition (Fig. 4A). In cells with combined chronic CaMKII inhibition and PLN ablation and at frequencies higher than 4 Hz, \((dF/dt)_{max}\) was significantly smaller compared with that of cells with PLN ablation alone (Fig. 4B).

Taken together, our results presented in Figs. 3 and 4 suggest that the combination of chronic CaMKII inhibition and PLN ablation slowed the [Ca\(^{2+}\)]-transient upstroke at high stimulation frequencies.

Combined CaMKII inhibition and PLN ablation did not prevent the frequency-dependent shortening of the [Ca\(^{2+}\)] transient. We analyzed the [Ca\(^{2+}\)]-transient decay as a function of stimulation frequency to characterize the influence of CaMKII inhibition and PLN ablation on [Ca\(^{2+}\)] removal. Consistent with previous studies (14, 20), the shortening of the [Ca\(^{2+}\)] transient occurred with increasing stimulation frequency as shown in Fig. 5A for WT cardiac myocytes. To describe the decay phase of the [Ca\(^{2+}\)] transient, we fitted the sum of two exponential functions, with time constants \(\tau_1\) and \(\tau_2\), to the falling phase of each transient. A representative fit is shown in Fig. 5B for a single WT cell stimulated at 1 Hz. We found that for all stimulation frequencies and for all cells, one of the time constants, \(\tau_1\), was much smaller than the other one. \(\tau_2\), contributing <15% to the total transient duration (measured from 20% to 90% of the maximum) and thus represented the curvature near the maximum of the [Ca\(^{2+}\)] transient. The value of the larger time constant, \(\tau_2\), was consistently on the order of the duration of the [Ca\(^{2+}\)] transient and was therefore used to characterize the transient decay phase (Fig. 5, B and C).

The decay time constant \(\tau_2\) decreased with increasing stimulation frequency in the presence of PLN but independent of CaMKII inhibition (Fig. 6A). Even PLN ablation or its combination with CaMKII inhibition did not abolish the frequency-dependent shortening of the [Ca\(^{2+}\)] transient, although the time constants were significantly smaller in the absence of PLN, presumably due the lack of negative feedback of PLN on SERCA2 (Fig. 6B). Time constants were slightly, at 2 Hz even significantly, smaller in AC3-I compared with AC3-C and WT.
and at 8 Hz in AC3-I \times PLN^{-/-} compared with AC3-C \times PLN^{-/-} and PLN^{-/-} myocytes. We confirmed this result by a complementary measurement of the 20–90\% of maximum intensity decay time (Fig. 7A, inset). The \([Ca^{2+}]_i\)-transient decay time measurements confirmed that \([Ca^{2+}]_i\) transients were shortened both in the presence and in the absence of PLN and, in both cases, were independent of CaMKII inhibition (Fig. 7A and B). Combined CaMKII inhibition and PLN ablation did not prevent a significant shortening of the \([Ca^{2+}]_i\) transient.

**PLN-dependent cytosolic \([Ca^{2+}]_i\) removal is limited by the stimulation frequency.** PLN ablation shortened \([Ca^{2+}]_i\) transients by almost 80\% at 1 Hz but by merely 15\% at 10 Hz (Fig. 7). We hypothesized that for the mouse, high stimulation rates within the physiological range may limit the ability of PLN to modulate \([Ca^{2+}]_i\) uptake. To test this hypothesis, we calculated the differences in the time constant \(\tau_2\) and in the 20–90\% 

**Fig. 4. Frequency dependence of the maximum \([Ca^{2+}]_i\)-transient upstroke velocity \((df/dt)_{max}\).** A: \((df/dt)_{max}\) as a function of stimulation frequency in cardiac myocytes in the presence of PLN with chronic CaMKII inhibition (AC3-I) and in control (AC3-C and WT). B: \((df/dt)_{max}\) as a function of stimulation frequency after PLN ablation with chronic CaMKII inhibition (AC3-I \times PLN^{-/-}) and in control (AC3-C \times PLN^{-/-} and PLN^{-/-}). \(t\), time. *P < 0.05.
fluorescence decay time from cells with normal levels of PLN and from cells lacking PLN. We subtracted the results shown in Fig. 6A from those shown in Fig. 6B and the results shown in Fig. 7A from those shown in Fig. 7B and plotted these differences as a function of stimulation frequency in Fig. 8, A and B, respectively. The difference in the time constant (Fig. 8A) and in the transient decay duration (Fig. 8B) decreased with increasing stimulation frequency in a monoexponential fashion. The exponential decay constants were the same for all cells and independent of CaMKII inhibition. The difference in the time constant \( \tau_2 \) decreased with a decay constant of 2.3 ± 0.9 Hz in AC3-I, with 2.0 ± 0.2 Hz in AC3-C, and with 2.7 ± 1.0 Hz in WT cardiac myocytes, respectively (Fig. 8A). The difference in the transient decay time (20% to 90% of maximum fluorescence) decreased with a decay constant of 2.5 ± 1.0 Hz in AC3-I, 2.6 ± 0.5 Hz in AC3-C, and 2.9 ± 0.9 Hz in WT cardiac myocytes, respectively (Fig. 8B). Given the reduced shortening and the smaller amplitude of the \([\text{Ca}^{2+}]_i\) transient at high stimulation frequencies in the AC3-I × PLN-/- cardiac myocytes, we tested the hypothesis that high stimulation frequencies actually diminish the increase in the maximum \([\text{Ca}^{2+}]_i\) uptake rate that is expected to occur after PLN ablation. We calculated the maximum \([\text{Ca}^{2+}]_i\)-transient decay rate, \((dF/dt)_{\text{max}}\), in the absence and presence of PLN and plotted the relative difference as a function of stimulation frequency (Fig. 8C). PLN ablation led to a seven- to eightfold increase in the maximum \([\text{Ca}^{2+}]_i\)-
uptake rate at 0.2 and 1 Hz. In contrast, at 10 Hz, PLN ablation increased the maximum [Ca\(^{2+}\)]\(_i\)-transient decay rate by merely 0.7- and 1.3-fold in WT and in AC3-C cardiac myocytes, respectively. Importantly, in cells with chronic CaMKII inhibition and at 10 Hz, PLN ablation failed to increase the maximum [Ca\(^{2+}\)]\(_i\)-transient decay rate.

**PLN ablation eliminated differences in SR Ca\(^{2+}\) content resulting from chronic CaMKII inhibition.** It is possible that a combined CaMKII inhibition and PLN ablation decreased the amount of Ca\(^{2+}\) stored in the SR, thereby causing the observed decrease in the [Ca\(^{2+}\)]\(_i\)-transient amplitude and upstroke velocity. We tested this hypothesis by measuring the SR Ca\(^{2+}\) content as the caffeine-evoked integrated inward NCX current adjusted for cell membrane at a stimulation frequency of 2 Hz. Figure 9 shows SR Ca\(^{2+}\) content in AC3-I (n = 5 cells), AC3-C (n = 12), AC3-I × PLN\(^{-/}\) (n = 10), AC3-C × PLN\(^{-/}\) (n = 9), and in PLN\(^{-/}\) (n = 14) cardiac myocytes. We also measured SR Ca\(^{2+}\) load for WT cells at room temperature to compare with previously published data and found that it was the same as for AC3-C at body temperature (not shown). Chronic CaMKII inhibition alone reduced the SR Ca\(^{2+}\) load slightly, but this difference was eliminated by PLN ablation, suggesting that chronic CaMKII inhibition per se does not affect SR Ca\(^{2+}\) content in the absence of PLN.

RyR2, SERCA2, and PLN protein levels. To determine whether the inhibition of CaMKII activity in the absence of PLN affects the levels of Ca\(^{2+}\) handling proteins in the SR, we performed immunoblot analyses of total PLN, CaMKII-specific phospho-epitope of PLN (PLN-Thr17), SERCA2, and RyR2 on the lysates of three mouse hearts from each genotype (n = 3; Fig. 10). We first confirmed equal PLN protein levels in AC3-C and AC3-I hearts using specific antibodies (Fig. 10A, lanes 1–6) and its absence in the PLN\(^{-/}\) mice hearts (lanes 7–12). Using a phosphospecific antibody, we also confirmed the loss of CaMKII-specific PLN-Thr17 phosphorylation owing to AC3-I peptide-dependent CaMKII inhibition (Fig. 10A, lanes 4–6) compared with that of AC3-C (Fig. 10A, lanes 1–3). The PLN-Thr17 signal was essentially lost in the AC3-I compared with the AC3-C cardiac myocytes, showing that CaMKII activity was significantly reduced in AC3-I cells (Fig. 10B).

Upon the probing of the immunoblots with anti-RyR2 antibody and normalizing the resulting RyR2 signal to the actin signal (not shown), our results further show that mean RyR2 protein levels were significantly reduced after PLN ablation in the

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**Fig. 8. PLN-dependent [Ca\(^{2+}\)]\(_i\)-transient decay.** A: absolute difference in the [Ca\(^{2+}\)]\(_i\)-transient decay time constant, \(\tau_2\), of cells with normal levels of PLN and cells lacking PLN as a function of stimulation frequency. B: difference in the duration of the [Ca\(^{2+}\)]\(_i\)-transient decay (20–90%) of cells with normal levels of PLN and cells lacking PLN as a function of stimulation frequency. C: relative increase in the maximum [Ca\(^{2+}\)]\(_i\)-transient decay rate \([-(dF/dt)_{max}]\), for WT, AC3-C, and AC3-I cardiac myocytes after PLN ablation.

**Fig. 9. Sarcoplasmic reticulum (SR) Ca\(^{2+}\) content caffeine-evoked Na\(^+/Ca\(^{2+}\) exchanger current from a PLN\(^{-/}\)ventricular cardiac myocyte (inset).** SR Ca\(^{2+}\) content in AC3-I (n = 5 cells), AC3-C (n = 12), AC3-I × PLN\(^{-/}\) (n = 10), AC3-C × PLN\(^{-/}\) (n = 9), and in PLN\(^{-/}\) (n = 14) cardiac myocytes stimulated at 2 Hz. C/F, charge per capacitance in units of coulombs per farad. *P < 0.05.
AC3-C hearts (AC3-C compared with AC3-C × PLN⁻⁻⁻; Fig. 10C). No significant differences in RyR2 protein content were found between AC3-I and AC3-C and between AC3-I × PLN⁻⁻⁻ and AC3-C × PLN⁻⁻⁻ hearts. In the same lysates, SERCA2 protein levels appeared to be unaffected (Fig. 10A).

**DISCUSSION**

Combined chronic CaMKII inhibition and PLN ablation slowed Ca²⁺ release at physiological stimulation frequencies. It has been suggested that CaMKII activity affects the SR Ca²⁺ release channel RyR2 (29). We hypothesized that this interaction may result in a change of the [Ca²⁺]ₐ-transient upstroke, possibly in a frequency-dependent fashion. At high stimulation frequencies, the combination of chronic CaMKII inhibition and PLN ablation resulted in a decrease in the amplitude (Fig. 2), an increase in the duration (Fig. 3), and a decrease in the maximum upstroke velocity (Fig. 4) of the [Ca²⁺]ₐ transient, indicating that SR Ca²⁺ release was slowed at high stimulation frequencies compared with that of cells with PLN ablation alone. A significant reduction in RyR2 phosphorylation had been reported previously in the AC3-I hearts (32), but given our results, it is not clear whether this would directly translate into the observed slowing of the [Ca²⁺]ₐ-transient upstroke. Although the spark frequency, a commonly used indicator of RyR2 open probability in intact myocytes, has been shown to be unaffected by chronic CaMKII inhibition (35), it is still possible that high-frequency stimulation affected RyR2 opening probability. This is because spark frequency is usually measured in resting myocytes. We further tested RyR2 protein levels in heart homogenates and found that they were not significantly different in AC3-I × PLN⁻⁻⁻ compared with AC3-C × PLN⁻⁻⁻ hearts (Fig. 10, A and C). Therefore, the slowing of Ca²⁺ release at high stimulation frequencies in the AC3-I × PLN⁻⁻⁻ compared with the AC3-C × PLN⁻⁻⁻ and PLN⁻⁻⁻ hearts may unlikely be a result of a decrease in the amount of the RyR2 protein. We found, however, that there was a significant reduction in RyR2 levels in the AC3-C × PLN⁻⁻⁻ compared with the AC3-C hearts (Fig. 10C), consistent with a previous report showing a reduction in RyR2 protein levels after PLN ablation (10). The reduced RyR2 protein levels might have contributed to an increase in the mean rise time across all frequencies observed after PLN ablation, as evident in Fig. 3B, although it is likely that this was merely a consequence of the larger [Ca²⁺]ₐ-transient amplitudes (Fig. 2), which may result from the significantly higher SR Ca²⁺ load in these cells (Fig. 9).

We further considered an effect of CaMKII inhibition on the SR Ca²⁺ content that could be independent of PLN ablation and would explain the reduced [Ca²⁺]ₐ-transient amplitude in the AC3-I × PLN⁻⁻⁻ cells. We quantified SR Ca²⁺ content in our models and found that it was slightly lower in the AC3-I cells, but this difference was abolished by PLN ablation (Fig. 9), consistent with previous reports (32, 35). Our experimental techniques did not allow assessing SR Ca²⁺ content at frequencies higher than 2 Hz (see METHODS), so that we cannot exclude different SR Ca²⁺ loading conditions at higher frequencies. However, because the smaller SR Ca²⁺ content in the AC3-I cardiac myocytes failed to reduce the [Ca²⁺]ₐ-transient amplitude compared with that of the AC3-C and WT at any given frequency (Fig. 2A), and because CaMKII inhibition did not affect the SR Ca²⁺ content in the absence of PLN neither at 0.5 Hz (32) nor at 2 Hz (Fig. 9), we suggest that possible changes in SR Ca²⁺ content caused by CaMKII inhibition may not solely be responsible for the pronounced slowing of the [Ca²⁺]ₐ-transient upstroke in the AC3-I × PLN⁻⁻⁻ cells at higher frequencies.

**Frequency-dependent shortening of the [Ca²⁺]ₐ transient occurs independent of chronic CaMKII inhibition and PLN ablation.** Combined CaMKII inhibition and PLN ablation did not abolish the frequency-dependent shortening of the [Ca²⁺]ₐ transient (Figs. 6 and 7). In fact, in AC3-I × PLN⁻⁻⁻ cells, the transient shortened in average by about 30% when the stimulation frequency was raised from 1 to 10 Hz (Fig. 7B). This result was surprising and may argue against previous reports showing that the frequency dependence of the [Ca²⁺]ₐ transient decline was abolished and the [Ca²⁺]ₐ transient decay prolonged in cardiac myocytes from PLN⁻⁻⁻ hearts after the pharmacological CaMKII inhibition (13, 21). We hypothesize that it was due to our broad range of stimulation frequencies that we were able to detect the frequency dependence of the [Ca²⁺]ₐ transient decay in the PLN⁻⁻⁻ cells. Our data show that after PLN ablation, differences in the [Ca²⁺]ₐ transient decay are difficult to find when small frequency intervals are used (Figs. 6B and 7B).

Because the frequency-dependent shortening of the [Ca²⁺]ₐ transient occurred in intact cardiac myocytes lacking PLN and
expressing the highly specific CaMKII inhibitor AC3-I, we suggest that there is a component of the frequency-dependent acceleration of cytosolic [Ca\(^{2+}\)] removal that is neither PLN nor CaMKII dependent. Because diastolic [Ca\(^{2+}\)], rises with increasing stimulation frequency (30), we speculate that frequency-dependent changes in Ca\(^{2+}\) cycling between intracellular compartments may be the underlying cause of the frequency dependence of the time course of the [Ca\(^{2+}\)], transient. Clearly, more experiments are needed to test the hypothesis that frequency-dependent changes in absolute Ca\(^{2+}\) fluxes between intracellular compartments and their effects on Ca\(^{2+}\) handling proteins (e.g., SERCA2) can cause a frequency-dependent acceleration of [Ca\(^{2+}\)], removal, independent of PLN and CaMKII.

We also observed that [Ca\(^{2+}\)] transients were slightly shorter in cells with CaMKII inhibition (AC3-I and AC3-I \(\times\) PLN\(^{-/-}\); Figs. 6 and 7), although this difference was only significant at two frequencies. For the AC3-I \(\times\) PLN\(^{-/-}\) cells, this may be a result of the smaller [Ca\(^{2+}\)],-transient amplitude (Fig. 2B). We also considered a possible increase in the amount of SR Ca\(^{2+}\) pump molecules that could explain the shortening of the [Ca\(^{2+}\)]] transients in cells with CaMKII inhibition. However, we found that SERCA2 protein levels were the same in all hearts (Fig. 1A). Possibly, there are other unanticipated cellular compensations for AC3-I expression that could cause a slightly faster [Ca\(^{2+}\)],-transient decay in these cells. For example, Wu et al. (32) found that PLN phosphorylation at the PKA site, Ser16, was increased in the AC3-I compared with the AC3-C hearts, indicating increased PKA activity (32). Additional experiments are needed to investigate these effects.

In mouse ventricular cardiac myocytes, PLN-mediated regulation of cytosolic Ca\(^{2+}\) removal is limited by the stimulation frequency within the physiological range. The effects of PLN ablation on the [Ca\(^{2+}\)], transient were most evident at small frequencies, causing large increases in the amplitude/decay rate and large decreases in the time constant/duration. For example, in WT cells at 0.2 Hz, PLN ablation caused a twofold increase in the amplitude (Fig. 2B), an eightfold increase in the maximum decay rate (Fig. 8C), and a 75% shortening (Fig. 7B) of the [Ca\(^{2+}\)], transient. At 10 Hz in the same cells, PLN ablation resulted in a 0.5-fold increase in the amplitude, a 0.7-fold increase in the maximum [Ca\(^{2+}\)],-transient decay rate, and a merely 16% shortening of the [Ca\(^{2+}\)], transient. These results suggest that the PLN-mediated regulation of Ca\(^{2+}\) uptake is limited by the stimulation frequency. We estimated the frequency limit at which PLN ablation did not result in a measurable impact on the time course of the [Ca\(^{2+}\)], transient (Fig. 8). The differences in the Ca\(^{2+}\) decay time constant and the duration between cells with normal levels of PLN and cells lacking PLN decreased in a monoeponential fashion and independent of chronic CaMKII inhibition (Fig. 8). The exponential decay constants were the same for all animal models. Using the measured decay constants, we calculated the frequency limit at which PLN ablation would not result in a measurable shortening of the [Ca\(^{2+}\)], transient. With the assumption that a difference in the time constant or in the transient duration of <1 ms would be within the standard error interval of the measurement, the time constant/duration of the fluorescence decay of a cell from a PLN\(^{-/-}\) and a WT mouse would be the same at a stimulation frequency of 12 Hz (720 beats/min), which is still within the physiological range. Because [Ca\(^{2+}\)],-transient amplitudes were depressed in cells with chronic CaMKII inhibition and PLN ablation at high frequencies, the maximum [Ca\(^{2+}\)],-transient decay rates were reduced. The frequency limit at which PLN ablation did not result in a measurable increase in the maximum [Ca\(^{2+}\)],-transient decay rate was smaller in the AC3-I than in the AC3-C or WT cells (Fig. 8C). In AC3-I cardiac myocytes, PLN ablation failed to increase the maximum [Ca\(^{2+}\)],-transient decay rate at 10 Hz (Fig. 8C, inset).

Taken together, our results show that in mouse ventricular cardiac myocytes, the PLN-dependent regulation of [Ca\(^{2+}\)], removal is limited by high stimulation frequencies within the physiological range.

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