Cardiac myocyte calcium transport in phospholamban knockout mouse: relaxation and endogenous CaMKII effects

Li, Li, Guoxiang Chu, Evangelia G. Kranias, and Donald M. Bers. Cardiac myocyte calcium transport in phospholamban knockout mouse: relaxation and endogenous CaMKII effects. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1335–H1347, 1998.—Increases in heart rate are accompanied by acceleration of relaxation. This effect is apparent at the single myocyte level and depends on sarcoplasmic reticulum (SR) Ca transport and Ca/calmodulin dependent protein kinase [CaMKII; see R. A. Bassani, A. Mattiazzi, and D. M. Bers, Am. J. Physiol. 268 (Heart Circ. Physiol. 37): H703–H712, 1995]. Because phosphorylation of phospholamban (PLB) by CaMKII can stimulate SR Ca transport, it is a plausible candidate mechanism. We examined this issue using ventricular myocytes isolated from wild-type (WT) mice and those in which the PLB gene was ablated by gene targeting (PLB-KO). During steady-state (SS) stimulation, twitch relaxation and intracellular Ca concentration ([Ca]i) decline were significantly faster than after a rest in both WT and PLB-KO myocytes. Furthermore, the CaMKII inhibitor KN-93 (1 µM) abolished the stimulation-dependent acceleration of twitch [Ca]i decline in PLB-KO. This indicates that neither PLB nor its phosphorylation are required for the CaMKII-dependent acceleration of the SS twitch [Ca]i decline and relaxation. Other quantitative aspects of Ca transport in WT and PLB-KO myocytes were also examined. As expected, time constant (τ) of [Ca]i decline during the SS twitch is much faster in PLB-KO than in WT myocytes (112 ± 6 vs. 188 ± 14 ms, P < 0.0001). There was also an increase in SS SR Ca load, based on the change of [Ca]i during rapid caffeine-induced contractures (CafC) with Na/Ca exchange blocked (565 ± 74 nM for WT, 1118 ± 133 nM for PLB-KO, P < 0.01). Accounting for cytosolic Ca buffering, this implies a 37% increase in SS SR Ca content. The τ for [Ca]i decline of the CafC with Na present indicated slower extrusion by Na/Ca exchange in the PLB-KO mouse (2.2 ± 0.2 s in WT vs. 3.2 ± 0.2 s in PLB-KO, P < 0.01), although exchanger protein expression was unchanged. Integrated Ca flux analysis in WT and PLB-KO myocytes, respectively, shows that 90 and 96% of Ca during twitch relaxation is removed by the SR Ca-ATPase, 9 and 3.4% by Na/Ca exchange, and 0.5 and 0.1% by slow mechanisms (mitochondria Ca uniporter and sarcoplasmic Ca-ATPase). We conclude that the PLB-KO myocytes retain a CaMKII-dependent acceleration of SS twitch [Ca]i decline. The PLB-KO vs. (WT) myocytes also have higher SR Ca pump activity, higher SS Ca load, and reduced Na/Ca exchange activity.

Sarcoplasmic reticulum calcium-adenosinetriphosphatase; sodium-calcium exchange; calcium flux

Phospholamban (PLB) is a key regulator of the sarcoplasmic reticulum (SR) Ca-ATPase in ventricular myocytes. Dephosphorylated PLB is closely associated with the SR Ca-ATPase and acts as an inhibitor of the Ca pump (21, 22, 35, 36). Phosphorylation by cAMP-dependent protein kinase (PKA) and Ca/calmodulin-dependent protein kinase II (CaMKII) can relieve this inhibition (25, 41), allowing greater Ca transport at a given intracellular Ca concentration ([Ca)]i). The adjacent residues Ser-16 and Thr-17 of PLB were identified as the unique sites phosphorylated by PKA and CaMKII, respectively (38). However, most of the information about PLB has come from in vitro studies, since multiple proteins are involved in Ca handling in vivo, and it is difficult to isolate the effects of PLB. The recently generated PLB knockout (PLB-KO) mouse (27) allows unique insights into the functional consequences of PLB ablation in either the intact animal or in relatively intact preparations such as isolated myocytes.

In permeabilized ventricular myocyte, CaMKII inhibitors (KN-62 and specific peptides) decreased SR Ca pump function, and this effect could be prevented by an antibody that interferes with the PLB-Ca pump interaction (30). In intact rat myocytes, [Ca]i decline is slower postrest (PR) than during steady-state (SS) twitches (6). The CaMKII inhibitor KN-62 prevents the frequency-dependent acceleration of [Ca]i decline in SS (vs. PR), whereas phosphatase inhibitors prevent the slowing of [Ca]i decline at the PR twitch (vs. SS; see Ref. 6). This suggests that the faster [Ca]i decline during SS twitch is due to activation of CaMKII, and PLB was a possible target for such CaMKII regulation. This frequency-dependent acceleration of relaxation may be physiologically important during alteration in cardiac frequency, and the PLB-KO mouse provides a unique opportunity to directly test whether PLB is required for this effect.

Normal activation of cardiac cells involves Ca-induced Ca release, and the increased [Ca]i and contraction are only transient because Ca is removed from the cytosol by Ca transport systems (7). A great deal is known about Ca transport systems in ventricular myocytes from several mammalian species, such as rat (1, 31), rabbit (1, 9) and ferret (2). In all of these species, the SR Ca-ATPase plays the dominant role in rapid removal of Ca from the cytosol during relaxation, although the percentage of contribution varies widely among species. The sarcoplasmic Na/Ca exchange is the next most important Ca transport system during relaxation, with the sarcoplasmic Ca-ATPase and mitochondrial Ca uniporter generally playing a small role in removing Ca from the cytosol (1, 31).

This type of detailed quantitative information about the balance of Ca fluxes is not available for mouse ventricular myocytes, and we address this in the present study for both wild-type (WT) and PLB-KO mice. Given the emergence of transgenic and knockout mouse...
models in cardiovascular research (10), this fundamental quantitative information may be of broad importance and utility. Many different genetically altered mice have been generated, and the partially characterized PLB-KO mouse has been a valuable one (12, 15, 16, 27–29, 43).

In the present study, we address two major issues. First, we directly test the hypothesis that PLB is required for the frequency- and CaMKI-dependent regulation of twitch relaxation and [Ca]i decline. Second, we evaluate for the first time the quantitative balance of Ca fluxes in WT mouse myocytes during relaxation and excitation-contraction (E-C) coupling and extend this to compare how they are altered in the PLB-KO model. A major finding is that the acceleration of SS twitch relaxation and [Ca]i decline is still observed in the PLB-KO mouse and is still abolished by the CaMKI inhibitor KN-93. We conclude that the frequency-dependent acceleration of relaxation and [Ca]i decline cannot be attributed solely to PLB phosphorylation. We also find that Ca transport in the WT mouse is quantitatively similar to that reported in rats (1, 31), whereas in the PLB-KO mouse the SR Ca-ATPase is even more dominant over the Na/Ca exchange. Furthermore, in the PLB-KO, we find increased SR Ca content, reduced fractional SR Ca release during E-C coupling (at a given SR Ca load), and reduced ability of the Na/Ca exchange to extrude Ca (even without competition from SR transport).

METHODS

Cardiac myocyte preparation. Isolation of ventricular myocytes from WT and PLB-KO mice was carried out as previously described (18). Briefly, hearts were excised from adult male mice (species-matched WT and PLB-KO, 35–45 g) anesthetized with pentobarbital sodium (70 mg/kg i.p.). Hearts were mounted in a Langendorff perfusion apparatus and perfused with nominally Ca-free Tyrode solution for 6 min at 37°C. Perfusion was then switched to the same solution containing 0.8 mg/ml collagenase (type B; Boehringer-Mannheim, Indianapolis, IN) and 0.03 mg/ml pronase (Boehringer-Mannheim), with perfusion continuing until the heart became flaccid (~7–12 min). The ventricular tissue was then dispersed and filtered. The cell suspension was rinsed several times, with a gradual increase in the Ca concentration ([Ca]o), from 0.2 to 1 mM. Before experimental use, the myocytes were plated onto Plexiglas superfusion chambers, with the glass bottoms of the chambers treated with laminin (GIBCO, Grand Island, NY) to increase cell adhesion. Although our yield of viable cells was lower with mouse (~40%) than with other species, no systematic difference was seen between PLB-KO and WT.

Measurement of cell shortening. Myocyte shortening was measured as previously described (5). The cells were superfused with normal Tyrode (NT) solution at room temperature (22–23°C) and field stimulated (square waves, 0.5 and 1 Hz). Cells were transilluminated by a red light source (to avoid interference with indo 1 epifluorescence measurement), and shortening was measured using a video-edge detection system (Crescent Electronics, Sandy, UT).

Measurement of intracellular Ca. To obtain [Ca]i measurements while allowing myocytes to control their own intracellular environment (including Na concentration) and action potential, cells were loaded with indo 1 by incubation with the acetoxyethyl ester form of the dye (indo 1-AM, 10 µM; Molecular Probes, Eugene, OR) for 20 min at room temperature. After loading, the cells were superfused with NT solution for at least 30 min to wash out excess indicator and allow deesterification (5). The excitation source was a 150-W xenon lamp (Oriel, Stratford, CT) with a 355 ± 5 nm interference filter (Chroma Technology, Brattleboro, VT). Within the microscope, a 380-nm dichroic mirror reflected the ultraviolet light toward a fluorescence objective (Nikon CF Fluor ×40). The field illuminated was restricted to a circular spot of 100 µm in diameter. The fluorescence emitted by the cells was transmitted by a dichroic mirror (600 nm) onto another dichroic mirror (440 nm) such that 405- and 485-nm light could be selected by interference filters (40-nm bandwidth; Chroma Technology) placed in front of two photomultiplier tubes (Hamamatsu, Bridgewater, NJ). The microscope emission field was restricted to a single cell with the aid of an aperture in the emission path. The average background fluorescence recorded at both wavelengths from cells not loaded with indo 1 was subtracted before the fluorescence ratio was calculated. Cell fluorescence signals were digitized at 120–133 Hz (filtered at 60 Hz) and stored on a computer.

In mouse myocytes, we found that these indo 1 loading conditions resulted in substantial intracellular compartmentalization of indo 1, which contrasts with other mammalian ventricular myocytes (1, 2). Digitonin permeabilization indicated that ~40% of indo 1 was compartmentalized (and shorter loading time did not reduce this fraction). This greatly complicates calibration, and use of the traditional Grynkiewicz et al. (14) equation was not practical. As a compromise, fluorescence ratios (R = fluorescence at 405 nm/fluorescence at 485 nm) were converted to [Ca]i by a modified “pseudo-ratio” method (11), that is, [Ca]i = Kd (R/Rrest)/(Kd/[Ca]i)rest + 1 – R/Rrest, where Kd is the dissociation constant for indo 1 (450 nM), R is the ratio of the free to bound indo 1 fluorescence at 485 nm (3.3), Rrest is the fluorescence ratio minus the minimum R (Rmin), and [Ca]i is [Ca]i at rest. Because we did not observe significant differences of resting fluorescence ratio in WT and PLB-KO myocytes, it was assumed that [Ca]i was 150 nM (3). Rmin was determined in vivo in indo 1-AM-loaded cells superfused with solutions containing 5 mM EGTA/nominally zero Ca in the presence of the nonfluorescent Ca ionophore A-23187 (10 µM; Calbiochem, La Jolla, CA). Although the resulting [Ca]i values seem reasonable in the context of our previous experience, the absolute values should be taken as practical approximations. Most data were calculated with both traditional and pseudo-ratio methods, and there were no differences in the qualitative conclusions. Thus we feel that our practical assumptions are justified.

Assessment of SR Ca load, Na/Ca exchange, and slow mechanisms. Rapid application of 10 mM caffeine was used to induce release of SR Ca and assess the SR Ca load as well as the participation of Na/Ca exchange and slow transport systems (mitochondrial Ca unipporter and sarcolemmal Ca-ATPase) during [Ca]i decline. Cells were superfused with NT solution and stimulated at 0.5 Hz until twitch characteristics stabilized before each caffeine application. The time between the last stimulation and the start of caffeine was normally 2 s. The amplitude of the caffeine-induced Ca transient (CafC) can be used as an index of SR Ca content (5, 24, 39). Because the amplitude of the CafC in NT can be affected by Ca extrusion via Na/Ca exchange (5), CafCs were also evoked in Na- and Ca-free solution (0 Na, 0 Ca). In this case, [Ca]i decline was attributable to the mitochondrial Ca unipporter and sarcolemmal Ca-ATPase (referred to as slow mechanisms). Decline of [Ca]i during a CafC in NT (140 mM Na) was attributable to Na/Ca exchange and slow mechanisms. Caffeine
feine solution was introduced into the chamber via a quickswitching device (5) and was continued for 20 s to study the kinetics of cell relaxation and \([Ca]\) decline.

SS vs. PR twitch contractions. Cells were superfused with NT solution and stimulated at 0.5 or 1 Hz until twitch stabilization (SS twitch). Electrical stimulation was then stopped for 15, 30, or 60 s before stimulation was resumed to assess the first PR twitch. In experiments using the CaMKII inhibitor KN-93, cells were exposed to 1 \(\mu M\) KN-93 for 5 min after the control series was completed. The stimulation was then resumed to reach a new SS.

Quantitative immunoblotting of the Na/Ca exchange. The protein levels of the Na/Ca exchange in the PLB-KO and WT mouse hearts were determined using quantitative immunoblotting in conjunction with the BioMax chemiluminescent detection system (Scientific Imaging Systems; Eastman Kodak, Rochester, NY). Mouse hearts were excised, rinsed with ice-cold phosphate-buffered saline, and frozen rapidly in liquid N2. The hearts were then homogenized in buffer (pH 7.0) containing (in mM) 10 imidazole, 30 sucrose, 1 dithiothreitol, 10 sodium metabisulfite, and 0.3 phenylmethylsulfonyl fluoride. Cardiac homogenates from six PLB-KO mice or six WT mice were pooled together and subsequently used for Western blot analysis of the Na/Ca exchanger. Aliquots of the pooled homogenates of WT and PLB-KO mice were applied to parallel lanes at different concentrations on each of eight separate SDS gels and processed in parallel. The cardiac homogenates (5–20 mg) were separated on a 13% SDS-polyacrylamide gel and blotted onto a 0.22-mm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 200 mA and 4°C for 3 h. After transfer, the membrane was incubated in blocking solution containing 5% nonfat dried milk at 4°C overnight, washed with Tris-buffered saline (TBS: 10 mM Tris·HCl, pH 7.8, and 150 mM NaCl), and then incubated with a mouse monoclonal anti-Na/Ca exchange antibody (Affinity Bioreagents, Golden, CO) diluted 1:1,000 in 0.5% milk/TBS for 30 min with gentle agitation at room temperature. After the membrane was thoroughly washed with TBS and reincubated with a goat anti-mouse antibody conjugate (1:25,000 dilution; Kodak Scientific Imaging Systems) in 1% milk/TBS for 30 min, the membrane was immersed in the Kodak BioMax 1×CDS buffer to ensure optimal pH for the chemiluminescent reaction. The wet membrane was then placed right side up in a reaction folder, and a piece of chemiluminescent matrix was overlaid on this membrane with the emulsion side down. The reaction folder was exposed to a Kodak X-OMAT AR film for 5–10 min when the light output remained stable. The signals were analyzed by laser densitometry using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To verify the amount of protein loaded for SDS-PAGE and the efficiency of protein transfer onto the nitrocellulose membranes, three of the membranes were also probed with a mouse anti-actin monoclonal antibody (1:500 dilution; Accurate Chemical & Scientific), and these signals were also quantified using the chemiluminescent method.

Reagents and solutions. Unless otherwise stated, experimental reagents used were of analytical grade and were supplied by Sigma (St. Louis, MO). 2-(N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl))-amino-N-(4-chlorodimlanyl)-N-methybenzylamine (KN-93) was from Seikagaku America (Rockville, MD). A 10 mM stock solution was made up in water and was stored at 4°C. Aliquots of the solution were added to the perfusate immediately before use. The NT solution contained (in mM) 140 NaCl, 6 KCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 5 MMPHES, with the pH adjusted to 7.4 with NaOH at 22°C. In 0 Na, 0 Ca solution, NaCl in NT was replaced by LiCl, CaCl2 was omitted, and 1 mM EGTA was added.

**RESULTS**

Before addressing the issue of frequency-dependent effects on relaxation and \([Ca]\) decline, we will first describe quantitative results concerning the fundamental properties of cellular Ca transport in ventricular myocytes isolated from WT and PLB-KO mice.

Ca transients and SR Ca load. Figure 1 shows examples of Ca transients during twitches and CaCFS under SS conditions and compares PLB-KO with WT myocytes (with pooled data in Fig. 2). Figure 1A shows superimposed Ca transients during SS twitches (0.5

**Fig. 1.** Intracellular Ca concentration (\([Ca]\)) transients in wild-type (WT) and phospholamban (PLB) knockout (KO) mouse myocytes stimulated at 0.5 Hz. A: during steady-state (SS) twitch in normal Tyrode (NT); B: during caffeine (Caff) application in NT solution; and C: during application of caffeine in 0 Na, 0 Ca. Data shown were from different cells, and SS twitch was the average of three traces in both WT and PLB-KO mice. Time constant (\(\tau\)) was obtained by monoexponential curve fit to \([Ca]\) decline.
H1338  CARDIAC [Ca], CAMKII, AND RELAXATION IN PLB KNOCKOUT

Fig. 2. Amplitude and time course of twitch and caffeine (Caff)-induced Ca transients and contractions in myocytes from WT (filled bars) and PLB-KO (KO, open bars) mice. A: pooled data of 0.5-Hz SS twitch contraction amplitude (left) as percentage of resting cell length (RCL, n = 45 and 42) and change in [Ca], (Δ[Ca], right, n = 16 and 19 for WT and PLB-KO, respectively). Unpaired t-test showed no significant difference (ns) for either contraction or Δ[Ca], B: τ of cell relaxation (left) and Δ[Ca], decline (right) during SS twitch at 0.5 Hz were significantly longer in WT (τ = 116 ± 9 ms for twitch, n = 45; and 188 ± 13.6 ms for [Ca], decline, n = 17) than in PLB-KO (31.7 ± 1.3 ms for twitch, n = 42; and 112 ± 6 ms for [Ca], decline, n = 14, P < 0.0001 for both). Contraction data in A and B were collected from cells without indo 1-acetoxymethyl ester (AM) loading. C: mean value of sarcoplasmic reticulum (SR) Ca load evaluated by caffeine application in NT (left) and 0 Na, 0 Ca solution (right). SR Ca load was significantly higher in both cases in PLB-KO mouse (n = 10–17, P < 0.01). D: mean ± values of [Ca], decline were slower in PLB-KO during both caffeine application in NT (left, n = 14, P < 0.01) and in 0 Na, 0 Ca (right, n = 12 and 8).

Hz) from representative WT and PLB-KO myocytes. Each trace is the average of three SS twitch Ca transients. Time to peak [Ca], is shorter in the PLB-KO mouse (142 ± 4 vs. 162 ± 6 ms, P < 0.05). The time constant (τ) for [Ca], decline during the SS twitch is also significantly faster in the PLB-KO mouse (τ = 188 ± 14 ms in WT vs. 112 ± 6 ms in PLB-KO; n = 17 and 14, respectively; P < 0.001, Fig. 2B). The faster [Ca], decline in PLB-KO is not surprising, since the absence of PLB is expected to relieve inhibition of the SR Ca-ATPase and increase the Ca transport rate at a given [Ca]. This acceleration of SR Ca-ATPase may also curtail the peak [Ca], in PLB-KO and thereby contribute to the shorter time to peak (also see DISCUSSION). The amplitude of the SS twitch Ca transient was slightly higher in the PLB-KO, although not significantly (change in [Ca], (Δ[Ca], in nM): 167 ± 21 for WT, n = 16, and 177 ± 19 for PLB-KO, n = 19, Fig. 2A). Given the increase in apparent SR Ca content (Figs. 1B, 1C, and 2C), a larger increase in SS twitch Ca transient and contraction might have been expected (15, 43).

Figure 1, B and C, is representative of traces during Caff in NT and Caff in 0 Na, 0 Ca solution, respectively. The peak [Ca], during Caffs are higher in the PLB-KO mouse both in NT (Δ[Ca], = 456 ± 58 nM in WT, n = 16, vs. 918 ± 89 nM in PLB-KO, n = 17, P < 0.01) and in 0 Na, 0 Ca solution (Δ[Ca], = 565 ± 74 nM for WT, n = 11, vs. 1118 ± 133 nM for KO, n = 10, P < 0.01, Fig. 2C). These results indicate a higher SS SR Ca content in the PLB-KO compared with the WT myocytes. Translating these values to total SR Ca content using the passive intracellular Ca buffering measured in rabbit ventricular myocytes by Hove-Madsen and Bers (17) gives SR Ca contents of 102 and 140 µmol/l cytosol for WT and PLB-KO myocytes, respectively.

During Caffs, [Ca], declines more slowly in myocytes from the PLB-KO mice. The τ of [Ca], decline for the Caff in NT (Caff NT) is 2.19 ± 0.24 s in WT vs. 3.22 ± 0.21 s in PLB-KO (P < 0.01, Fig. 2D). During Caff in 0 Na, 0 Ca (Caff 0 Na, 0 Ca), the [Ca], transient in the PLB-KO is almost flat, as shown in Fig. 1C. About one-half of the PLB-KO cells showed such a Caff in 0 Na, 0 Ca, making estimation of τ impractical (i.e., τ approaches infinity). Thus, for Caff in 0 Na, 0 Ca, we use the rate constant (λ) of [Ca], decline (1/τ) for initial pooled comparisons (so when [Ca], decline is extremely slow, λ approaches 0). The mean λ for Caff in 0 Na, 0 Ca is lower in PLB-KO than in WT mice but is not statistically different (0.0106 ± 0.0088 vs. 0.0284 ± 0.0088 s⁻¹, respectively). The mean λ values are converted back to τ values for comparison with other τ values in Fig. 2D (τ was 35.2 s in WT vs. 94.5 s in PLB-KO).

The τ of [Ca], decline during Caff in 0 Na, 0 Ca is much slower than the value of 12 s reported for rat and rabbit ventricular myocytes by Bassani et al. (1), even for the WT mouse. Because we found significant indo 1 compartmentalization in mouse ventricular myocytes, the extremely slow decay of [Ca], could be an artifact.
due to this compartmentalization. Because ~50% of the Ca released during a CafC in 0 Na, 0 Ca is transported into mitochondria [based on results in rabbit by Bassani et al. (5)], the apparently slow [Ca] decline could be confounded by a gradually increasing mitochondrial [Ca]. This would cause τ to be overestimated for CafC in 0 Na, 0 Ca. The impact of this potential problem will be significantly less for the cases of the twitch and the CafC in NT, since <7% of Ca is taken up by mitochondria in these situations (1). When cell contraction is simultaneously recorded in WT mouse myocytes during CafC in 0 Na, 0 Ca, the τ of cell relaxation (8.19 ± 1.15 s, n = 5) is similar to that reported in both rabbit and rat myocytes (where relaxation and [Ca], decline are roughly parallel; see Ref. 1) and much faster than the τ of [Ca], decline in mouse myocytes. This result further suggests that indo 1 mitochondrial compartmentalization complicates our measurement of τ in CafC 0 Na, 0 Ca.

We also recorded cell contractions from cells without indo 1 loading, thus preventing any disturbance of intracellular Ca buffering (Fig. 2, A and B, left). Although SS twitch contractions are, on average, 21% larger in PLB-KO mice, the difference is not significant (as % of resting cell length: 5.96 ± 0.72 for WT, n = 45, and 7.22 ± 0.62 for PLB-KO, n = 42). However, Fig. 2B shows that relaxation τ is much faster in the PLB-KO mouse (WT, 116 ± 9 ms, n = 45, KO, 31.7 ± 1.3 ms, n = 42, P < 0.0001). Both of these results are consistent with data for [Ca], decline. Cells loaded with indo 1 also showed ν twitch of value twitch relaxation comparable to those without indo 1 loading (106 ± 10 ms in WT and 34.6 ± 2.7 ms for PLB-KO, n = 7–10, P < 0.001). This indicates that the degree of indo 1 loading used did not appreciably alter contraction.

We also measured cell relaxation during CafC in NT using cells without indo 1-AM loading. These were done explicitly to evaluate whether the prolonged τ of [Ca], decline during CafC in PLB-KO in Fig. 2D, left, might be complicated by mitochondrial compartmentalization of indo 1. As for the Ca transients, the τ of relaxation of CafC in NT is significantly slower in the PLB-KO (2.12 ± 0.15 s in PLB-KO vs. 1.21 ± 0.28 s for WT; P = 0.019). The 75% slowing of Ca relaxation in the PLB-KO (without indo 1) is consistent with the 50% slowing of [Ca], decline during CafC in NT (Fig. 2D, left) and with slowing of Na/Ca exchange in PLB-KO.

Overall, the results in Figs. 1A and 2, A and B, are consistent with an increase in SR Ca pump function in the PLB-KO mouse. This may be the factor responsible for the shorter time to peak and faster relaxation of contraction and [Ca], as well as the higher SR Ca load. In addition, Ca extrusion via Na/Ca exchange may also be slower in the PLB-KO mouse (based on slow decline of CafC in NT). Although there might also be slower Ca transport by the slow Ca removal mechanisms (sarcolemmal Ca-ATPase and mitochondrial Ca uniporter), this is complicated by dye compartmentalization.

SR Ca load is an important factor regulating SR Ca release during E-C coupling. Figure 3 shows the amplitude of the SS twitch [Ca] as a function of the SR Ca content (CafC in NT) for individual WT and PLB-KO myocytes. Linear regressions shown in Fig. 3 are significantly different between WT and PLB-KO myocytes (P < 0.05). It can be seen that, for a given SR Ca content, there is a relatively larger twitch [Ca], in the WT mouse. This could be due to either decreased fractional SR Ca release for a given SR Ca load or faster SR Ca uptake in the PLB-KO mouse (see DISCUSSION).

Analysis of Ca fluxes during relaxation. The relative contributions of the different Ca removal systems (SR Ca-ATPase, Na/Ca exchange, and the slow mechanisms) to [Ca], decline during the SS twitch in WT and PLB-KO mouse myocytes are calculated in a manner similar to that described by Bassani et al. (1) for rat and rabbit myocytes (Figs. 4 and 5). Mean values for the τ of [Ca], decline and Ca transient amplitudes for twitches and CafCs from Fig. 2 are used, creating noise-free curves that matched the mean experimental data. Free [Ca], is first converted to total [Ca], ([Ca],) using cellular passive cytosolic Ca-buffering measurements by Hove-Madsen and Bers (17). We assume that there are no major differences in the intracellular Ca buffering or indo 1 Ca affinity between PLB-KO and WT myocytes. Ca buffering differences are unknown, but if different they could complicate our comparisons. The rate of change in [Ca], (d[Ca]/dt) is then attributed to the sum of fluxes by the individual Ca transport systems (J, + J, Na/CaX slow, where J, is flux of the SR, J, Na/CaX is flux of Na/Ca exchange, and J, slow is flux through the slow mechanisms). During the CafC in 0 Na, 0 Ca, it is assumed that only the slow mechanisms were functional and could be lumped into a Hill relationship describing Ca flux as follows: J, slow = V, min + (V, max – V, min)/(1 + (K, n[Ca],)), where V, max is the maximum transport velocity, K, n is a Michaelis constant, and n is the Hill coefficient. V, min was introduced to allow the J value to approach zero at nonzero values of [Ca],.

Figure 4A shows that the transformed flux data for CafC in 0 Na, 0 Ca is well described by the J, slow equations over the relevant [Ca], range. It should be noted that this provides an adequate empirical descrip-
Fig. 4. Rate of Ca removal by SR Ca-ATPase, Na/Ca exchange, and slow mechanisms in WT and PLB-KO mouse myocytes. We assumed that total myoplasmic Ca concentration ([Ca]t) is [Ca]t = 215/[1 + (420/[Ca]i)] + 702/[1 + (79,000/[Ca]i)] + ([indo]/[1 + ([indo]/450)]), where [indo], is intracellular indo 1 concentration. The last term reflects Ca binding to intracellular indo 1. Two classes of binding sites with maximal binding (215 and 702 µmol/L cytosol) and dissociation constant (420 and 79,000 nM) were used to describe endogenous cytosolic Ca buffering (8, 17).

(A) Caff 0Na 0Ca  
(B) Caff NT  
(C) SS Twitch

Meet Figure

Our ability to make realistic predictions of the [Ca]i dependence of Ca transport by the slow systems. It does not require that the equations have strict mechanistic interpretations, and the same is true for Na/Ca exchange and SR Ca-ATPase as shown in Fig. 4, B and C. However, the Jslow transport appears to show a maximum of only 0.5-1.6 µM/s and is one-half of this value at 200–300 nM [Ca]i.

Figure 4B is obtained by transforming the Ca flux data from the CafC in NT and is fit by the sum of Jslow at each [Ca]i (taken exactly as obtained in Fig. 4A) plus a curve fit for JNaCaX. The Ca flux data then should be attributable to JNaCaX + Jslow. Figure 4B shows that the d[Ca]i/dt data as a function of [Ca]i are again well described by JNaCaX + Jslow and that Na/Ca exchange fluxes are about 10 times larger than those by the combined slow mechanisms (repeated from Fig. 4A). Next it is assumed that all Ca removal systems are functional during the twitch. Thus the twitch Ca flux in Fig. 4C was fit by JSR + JNaCaX + Jslow, where JNaCaX and Jslow parameters are held exactly as obtained in Fig. 4, A and B, allowing a fit for JSR parameters. The upper curve fits in Fig. 4C are the overall fits for JSR + JNaCaX + Jslow, and they describe the d[Ca]i/dt flux data quite well. The data curves in Fig. 4C do not appear to approach a clear Vmax value because [Ca]i does not rise as high during the twitch as during a CafC. This limits our ability to make realistic predictions of Vmax or Km for the SR Ca-ATPase. Nevertheless, it is clear that the SR Ca-ATPase flux is much higher at all [Ca]i in the PLB-KO myocytes. Furthermore, for WT and PLB-KO, respectively, the SR Ca-ATPase fluxes are ~10 and 30 times higher than the sum of JNaCaX + Jslow (replotted from Fig. 4B).

On the basis of the [Ca]i dependence determined in Fig. 4 for the different Ca transport systems, we then calculate the cumulative Ca flux through each transporter during a normal twitch in Fig. 5. In this case, we use twitch [Ca]i as the driving function and the flux values for each system as derived in Fig. 4. The fraction of activating Ca transported out of the cytosol by the SR, Na/Ca exchange, and the slow mechanisms in the WT mouse are 90.3%, 9.2%, and 0.5%, respectively. This is similar to the values obtained in rat ventricular myocytes by Bassani et al. (1) and Negretti et al. (31). Thus, as in rat ventricular myocytes, the SR Ca-ATPase plays a dominant role in removal of Ca from the cytosol during the normal twitch. In the PLB-KO mouse, the SR Ca-ATPase is even more dominant such that the SR Ca-ATPase accounts for 96.4% of Ca removal during relaxation and the Na/Ca exchange only 3.4%. Although Sr uptake is about two times faster in the PLB-KO mouse (Figs. 1, 2, and 4), it will not increase the fractional SR contribution twofold, since it is already >90% in WT. Looking at it another way, ~10% of activating Ca is removed by mechanisms other than the
SR Ca pump in WT, but this drops to 3.5% in the PLB-KO mouse.

As we mentioned above, indo 1 compartmentalization in mouse myocytes probably causes an underestimation of $J_{\text{slow}}$. To test if this underestimation will bias our conclusion about Ca fluxes, we also repeated the entire analysis above (Figs. 4 and 5) using the faster $J_{\text{slow}}$ parameters previously derived for rat ventricular myocytes (1) and consistent with the mouse myocyte relaxation data, but we kept the $\tau$ and peak data of CafC NT and SS twitch [Ca] measured from the WT mouse. This analysis makes only a modest difference in the overall flux contribution. For WT mouse myocytes, the integrated Ca flux attributed to the SR Ca-ATPase, Na/Ca exchange, and slow systems by this new analysis, compared with Fig. 5, was 90.3, 7.2, and 2.5%, respectively. Although Ca transport by the slow systems is higher, the overall impact on the balance between the SR Ca-ATPase and Na/Ca exchange is not altered much.

Frequency dependence and postrest cell contractions. Figure 6A shows superimposed SS twitch contractions in WT and PLB-KO myocytes stimulated at 0.5 and 1 Hz. The strikingly faster twitch kinetics in the PLB-KO myocyte are apparent. WT mouse myocytes exhibit a flat to modestly negative force-frequency relationship over this range of frequencies (Fig. 6B), similar to our observations with rat ventricular myocytes (not shown). The PLB-KO mouse myocytes show a significantly more negative force-frequency relationship. Figure 6C shows that both WT and PLB-KO myocytes exhibit marked and comparable PR potentiation. PR twitch potentiation is also a normal feature in rat ventricular myocytes (7).

Figure 6D shows that both WT and PLB-KO myocytes also show a slowing of relaxation at the first PR twitch after 60 s of rest compared with that at 1 Hz SS. In the WT relaxation, $\tau$ increases from $117 \pm 17$ ms for the SS twitch to $151 \pm 19$ ms for the PR twitch ($n = 8$, $P < 0.05$). In the PLB-KO, twitch relaxation was overall much faster, and 60 s of rest increases the $\tau$ of relaxation by almost a factor of two ($26.9 \pm 2$ ms).
47.4 ± 6.8 ms at the PR twitch, n = 6, P < 0.05). Such slowing down of relaxation at PR twitches has been previously described in rat ventricular myocytes (37) and is attributed to slow dissipation of CaMKII-dependent phosphorylation during rest (6). Because PLB phosphorylation is considered to be a likely target for this CaMKII effect, the PLB-KO mice provide a unique system to test this possibility. The fact that slowed relaxation at PR twitches is still observed in the PLB-KO mouse myocytes indicates that this effect cannot be solely due to PLB phosphorylation.

We further studied the frequency-dependent acceleration of [Ca]i decline in PLB-KO myocytes loaded with indo 1-AM to test whether this acceleration of SS twitch relaxation in PLB-KO is still sensitive to CaMKII inhibition. Figure 7 shows that the slowing of the PR (vs. SS) Ca transient decline in these indo 1-loaded PLB-KO cells in Fig. 7C is not as great as observed for unloaded shortening in Fig. 6D. Nevertheless, there is still a significant difference such that τ of [Ca]i decline is 92.9 ± 4.7 ms for SS and 111 ± 5.5 ms for PR (n = 19, P < 0.01). Furthermore, Fig. 7C shows that this difference in τ is completely abolished by pretreatment of the PLB-KO myocytes for 5 min with the CaMKII inhibitor KN-93 (1 µM). After KN-93, τ for both SS and PR [Ca]i decline is the same as for the slower control PR τ, that is, after KN-93, τ is 120 ± 4.6 ms for SS and 115 ± 6.5 ms for PR (n = 19, not significant).

The amplitude of the Ca transient can have intrinsic effects on the τ of [Ca]i decline (8). However, the larger PR twitch would be expected to decrease τ rather than increase τ so that the observed slowing of PR τ of [Ca]i decline might be an underestimate of the functional effect. We also measured the amplitude of the SS and PR twitch Ca transients before and after KN-93 exposure (Fig. 7D). In both cases, the PR twitch Δ[Ca]i is potentiated (as for contraction in Fig. 6C), and the degree of potentiation is comparable before and after KN-93 treatment. Thus there seems to be a CaMKII-dependent process that accelerates [Ca]i decline during SS stimulation, but this process still occurs in the complete absence of PLB.

Immunoblots of the Na/Ca exchange. We have previously shown that the transcript levels of the sarclemmal Na/Ca exchange mRNA were not altered in PLB-KO hearts compared with WT (12). Because the Ca transient and contraction data (e.g., Figs. 1B and 2D) imply slower Na/Ca exchange function in the PLB-KO myocytes, we also assessed the protein levels of the Na/Ca exchange in six PLB-KO and six WT mouse hearts using quantitative immunoblotting in conjunction with the chemiluminescent detection system (Fig. 8). On parallel lanes of each of eight polyacrylamide gels, various concentrations of homogenates from WT and PLB-KO hearts were loaded. The densitometric signals obtained were linear functions of protein concentration over the range of 10–20 µg protein loaded onto gel lanes for both actin and the Na/Ca exchanger. The signals from PLB-KO mouse hearts were compared with those of WT mouse hearts, which were set as 100%. The amount of protein loaded on polyacrylamide gels and the efficiency of protein transfer onto the membrane were verified by probing three of the same blots with a mouse monoclonal antibody to actin. Actin served as an internal control, since its protein levels were similar between WT and PLB-KO mouse hearts (12). Our
results indicate no significant alteration in the expression levels of the Na/Ca exchange protein in the PLB-KO mouse hearts (97 ± 4 vs. 100% in WT).

**DISCUSSION**

In the present study, we have provided new information with respect to 1) Ca regulation and contraction in normal mouse ventricular myocytes, 2) how Ca regulation is altered in the PLB-KO mouse, and 3) the mechanism of frequency-dependent acceleration of relaxation. In addition to confirming the faster kinetics of relaxation and [Ca]i decline in PLB-KO (15, 27, 28, 43), we show the first quantitative analysis of cellular Ca balance in WT (and PLB-KO) mouse myocytes. Competition between the SR Ca-ATPase and Na/Ca exchange in WT mouse myocytes is quantitatively similar to that reported in rats (1, 31), whereas in the PLB-KO myocytes the SR Ca-ATPase is even more dominant over the Na/Ca exchange. Additional new results show that, in the PLB-KO myocytes (vs. WT), there is increased SS SR Ca content, reduced fractional SR Ca release during E-C coupling (at a given SR Ca load), and reduced ability of the Na/Ca exchange to extrude Ca (even without competition by SR transport). A key finding here is also proof that PLB is not required for the physiologically relevant frequency- and CaMKII-dependent acceleration of SS twitch relaxation and [Ca]i decline.

Altered E-C coupling. In the present study, SR Ca load during SS twitches is 37% higher in the PLB-KO vs. WT mouse myocytes (based on a 98% increase in Δ[Ca]i during CaC). This agrees reasonably with the 86% increase in SR Ca content measured by Chu et al. (12) using electron microprobe analysis (EPMA). If all other things were constant, the increased SR Ca load would be expected to produce a greater SR Ca release (4). Indeed, because increased SR Ca load was also reported to increase the fraction of SR Ca release, it would have been reasonable to expect that the SS twitch Ca transient in the PLB-KO mouse would be increased by >37% compared with WT. However, our results indicate that both SS cell contraction and Ca transient are increased only 21 and 6%, respectively, in PLB-KO myocytes (not significant). Indeed, the twitch Ca transient for a given SR Ca load was actually somewhat smaller in the PLB-KO vs. WT myocytes (see Fig. 3).

Masaki et al. (29) reported no alteration in L-type Ca current density in PLB-KO vs. WT myocytes, suggesting that the trigger density for E-C coupling is not changed. The apparently smaller fractional SR Ca release [for a given Ica (Ca current) trigger and SR Ca load] could be due in part to the 25% reduction in the number of ryanodine receptors in the PLB-KO mouse (12). However, the more rapid Ca resequestration by the faster SR Ca-ATPase in the PLB-KO mouse may also curtail the peak of the twitch Ca transient. This could also contribute to the shorter time to peak [Ca]i (Fig. 1A). This effect of the SR Ca-ATPase to curtail the normal twitch Ca transient was reported in control rat and rabbit ventricular myocytes (1).

To assess how reduced fractional SR Ca release and accelerated reuptake might interact, we used a simple model like that developed in Fig. 5 (including a simple SR Ca release flux during the time to peak of the Ca transients; 160 and 140 ms in WT and PLB-KO myocytes, respectively). In WT myocytes, an SR Ca release of 56.3 µmol/l cytosol is sufficient to produce the measured peak free Δ[Ca]i of 167 nM using the Ca removal parameters from the Ca flux analysis in Figs. 4 and 5. If we increase SR Ca load by 37%, as measured in the PLB-KO mouse, and assume the same fractional release (55%), the SR Ca release would be 77 µmol/l cytosol, and SS twitch Δ[Ca]i would be expected to be 256 nM [even with the faster SR Ca pump flux in the PLB-KO mouse (from Figs. 4 and 5)]. This 53% increase in predicted Δ[Ca]i is much higher than the Δ[Ca]i that we measured in PLB-KO myocytes here (177 nM), suggesting that the increased SR Ca-ATPase activity alone is not sufficient to explain the lack of significant increase in twitch Δ[Ca]i and contraction in the PLB-KO mouse. To obtain the observed 177 nM Δ[Ca]i in PLB-KO myocytes with the 37% increase in SR Ca load, we have to reduce the fraction of SR Ca released during the twitch by 16% (from 55 to 46% of the SR Ca load). This possible reduction seems plausible, based on the 25% lower number of ryanodine receptors reported in the PLB-KO compared with the WT heart (12). Thus the reduced number of ryanodine receptors may explain the apparent decrease in fractional SR Ca release in the PLB-KO myocytes (at a given SR Ca load). Put another way, the expected increase in SS twitch Ca transients in PLB-KO myocytes (vs. WT) for a similar Ca current and larger SR Ca load may be limited by an offsetting reduction in the number of ryanodine receptors (which could reduce the fraction of SR Ca released in the PLB-KO myocytes).

Another possible explanation for the limited cellular inotropy is a spatial dropout or inhomogeneity of SR Ca release during the twitch observed by Hüser et al. (19) in the PLB-KO mouse. They suggested that local SR Ca release at certain sites might be prevented by the
uniquely strong negative feedback created by the strong SR Ca pump in the PLB-KO mouse. This could limit the ability of SR Ca release at one locus to activate Ca release from a neighboring region.

Other reports have shown larger increases in SS twitch $\Delta [Ca]$, and contraction in the PLB-KO vs. WT myocytes than the 6 and 21%, respectively, that we found here (15, 43). It is not clear why our results differ in this regard (particularly as conditions were similar to the study by Wolska et al. (43)). It is quite possible that these studies, which showed larger increases in contraction and $\Delta [Ca]$, actually had a higher SR Ca load in the PLB-KO mouse [e.g., 86% higher than in WT (12) compared with the 37% we reported here]. Indeed, SS SR Ca load is very sensitive to numerous factors, such as frequency, temperature, mean [Ca], intracellular Na concentration, extracellular K concentration, action potential configuration, and duration. Plugging the 86% increase in SR Ca load into our model above (with a 25% decrease in fractional release due to fewer ryanodine receptors) would give a 60% increase in the SS twitch $\Delta [Ca]$. Thus higher SR Ca load could better offset the reduced number of ryanodine receptors and produce the stronger inotropic effect seen by some others. Indeed, because increased SR Ca load strongly increases the fraction of SR Ca release (4), such an effect could easily explain the quantitative discrepancy. Given the dramatic hyperdynamic state of the PLB-KO heart in the intact animal (27), it seems possible that our results here with respect to SS twitch $\Delta [Ca]$, and contraction underestimate the in vivo situation. On the other hand, having intracellular Ca transients of more comparable amplitude is functionally advantageous for our main goals here concerning comparison of kinetic parameters (8). In any event, the results here do agree with others with respect to the more rapid kinetics of twitches and Ca transient in the PLB-KO mouse (15, 27, 28, 43).

Balance of Ca fluxes during relaxation. It is particularly important to understand basic cellular Ca handling in mouse myocytes because of the increasing number of studies using mouse myocytes, especially from genetically altered mouse phenotypes. This is the first report analyzing Ca fluxes during relaxation in mouse myocytes. On the basis of the strategy of Bassani et al. (1), we obtain the relative contribution by SR Ca-ATPase, sarcolemmal Na/Ca exchange, and slow Ca removal mechanisms (sarcolemmal Ca-ATPase and mitochondrial Ca uniporter) during relaxation of a single twitch. The results in the WT mouse (90.3; 9.2, and 0.5%, respectively) are very similar to those in rat ventricular myocytes (92, 7, and 1%, respectively; see Ref. 1) but differ greatly from those in rabbit, ferret, and guinea pig ventricular myocytes (1, 2, 7) in which the Na/Ca exchange is a much stronger competitor with the SR Ca-ATPase. It is not surprising that in the PLB-KO mouse the contribution of SR Ca-ATPase is increased (96.4%), with a decreased contribution of Na/Ca exchange (3.4%) and slow mechanisms (0.1%). This is because dephosphorylated PLB normally works as an inhibitor of SR Ca-ATPase. Thus, without PLB, the knockout mouse SR Ca-ATPase becomes an even more dominant competitor with Na/Ca exchange and other Ca transport mechanisms. Because relaxation in other species (e.g., rabbit and guinea pig) normally depends less on SR Ca-ATPase and more on Na/Ca exchange (7), the effect of PLB ablation in these species would be expected to be greater with respect to the percentage of Ca flux via the SR Ca-ATPase during relaxation.

If the Na/Ca exchange in mouse myocytes removes only ~9% of the activating Ca during SS twitch relaxation, it may also be anticipated that only 9% of the activating Ca enters the cell via Ca current during the action potential. Voltage-clamp results in rat ventricular myocytes support this sort of SS conclusion (i.e., the amount of Ca influx during action potential is about the same as Ca efflux for each cardiac cycle; see Ref. 13), and the short action potential in rat limits the amount of Ca influx via $I_{Ca}$ (45). Similar voltage-clamp studies have not been carried out in mouse myocytes, but the results might be expected to resemble those in rat. In the PLB-KO mouse, the Ca extrusion via Na/Ca exchange was even smaller, which might imply a smaller Ca influx during the action potential. Although Masaki et al. (29) found no difference in peak L-type Ca channel density in WT and PLB-KO mice, they did observe a greater increase in $I_{Ca}$ inactivation in PLB-KO myocytes. This more rapid $I_{Ca}$ inactivation may well be secondary to a faster rise in [Ca] in the PLB-KO (43) and would also result in lower net Ca influx during the action potential.

Changes in slow Ca transport mechanisms. When both the SR Ca-ATPase and Na/Ca exchange were inhibited during CafC 0 Na, 0 Ca, the decline of the Ca transient was very slow in both WT and PLB-KO myocytes ($\tau \sim 34$ and 90 s, respectively). This is much longer than previous observations in rat and rabbit ($\tau \sim 12$ s; see Ref. 1). The problem of mitochondrial ino1 compartmentalization in mouse myocytes (see METHODS and RESULTS) makes it impossible to draw any clear conclusion from these results. However, given the energetic changes observed in the PLB-KO mouse myocytes, including an increase in the fraction of pyruvate dehydrogenase, which is in the active form (12), it may be of interest to determine if mitochondrial Ca transport is altered. Initial estimates of mitochondrial Ca content (12) showed no difference between WT and PLB-KO using EPMA, but it is difficult to detect small Ca changes with this approach.

Depressed Na/Ca exchange. The $\tau$ of [Ca] decline and relaxation during a CafC in NT was faster in WT than in PLB-KO mice. With SR net Ca uptake blocked, the main mechanism for Ca removal from the myoplasm is Na/Ca exchange (5, 24). These results suggest that the Na/Ca exchange system is slower at extruding Ca in the PLB-KO mouse. Measurements of [Ca] decline during CafC in NT should be much less affected by mitochondrial compartmentalization of ino1. This is because very little Ca is expected to enter mitochondria during relaxation (1). Furthermore, we found 75% slowing of CafC relaxation in NT for PLB-KO vs. WT cells not loaded with indo 1. Thus the 50% slowing of [Ca],
decline in the PLB-KO mouse probably reflects a true
decrease in the ability of Na/Ca exchange to remove Ca
from the cytosol in PLB-KO mice.

Chu et al. (12) reported that, in PLB-KO mouse
heart, there was no change in Na/Ca exchange mRNA
levels, and here we extend this to show that there is no
change in Na/Ca exchange protein levels. Thus the
decreased Na/Ca exchange function in the PLB-KO
mouse might be attributable to changes in exchanger
regulation. One possibility would be elevated intracel-
lular Na concentration in the PLB-KO mouse, but EPMA
did not reveal any difference in Na content in the
myocyte A-band (12).

Enhanced SR Ca load. We found a 37% increase in
the SR Ca content in the PLB-KO mouse (based on a
98% increase in caffeine-induced Ca transient ampli-
tude in 0 Na, 0 Ca). This agrees qualitatively with
EPMA measurements of 86% increase in SR Ca content
(12). Three factors in our experiments could contribute
to the increased SR Ca load. First, the absence of PLB is
expected to increase the affinity of the SR Ca pump for
Ca in a similar manner to the physiological phosphory-
lation of PLB (23, 26, 41). Second, there is a functional
decrease of Ca transport by Na/Ca exchange as dis-
cussed above. Any inhibition of the Na/Ca exchange
will tend to increase cellular Ca load and result in an
ever larger fraction of the activating Ca being taken up
by the SR. Third, the apparent reduction of fractional
SR Ca release in the PLB-KO mouse (see above) would
also tend to increase SR Ca load. Thus a combination of
faster SR Ca-ATPase, depressed Na/Ca exchange, and
reduced fractional SR Ca release may all contribute to
the increased SR Ca load in the PLB-KO mouse.

No difference was found in calsequestrin mRNA or
protein levels in the PLB-KO mouse (12). Thus the SR
Ca buffer capacity may not be changed. Unless there
was a large increase in SR volume or other SR Ca
buffers, the increased SR Ca load must increase free
intra-SR [Ca] and more fully saturate existing SR
calsequestrin. This indicates that, in the WT mouse,
there was still room to increase intra-SR [Ca] before
reaching the thermodynamic limit that the SR Ca
pump can generate.

CaMKII-dependent acceleration of relaxation. The
frequency- and CaMKII-dependent acceleration of relax-
ation in cardiac muscle is likely to be an important
physiological mechanism of autoregulation (6, 20, 37).
The SR Ca-ATPase has been implicated in this autoreg-
ulation and is also the major Ca transport system
involved in relaxation in mouse ventricular myocytes
(Figs. 4 and 5) responsible for >90% of Ca removal
during a SS twitch. As such, changes in SR Ca uptake
rate can dramatically modify the rate of [Ca] decline
during a twitch in these cells. Indeed, both SS twitch
relaxation and [Ca] decline are much faster in PLB-KO
mouse myocytes, consistent with stimulation of the SR
Ca-ATPase.

In rat ventricle, relaxation of the first PR twitch after
a 1-min rest is slower than during SS (6, 37). Because
this rest-dependent effect was suppressed or reversed
by thapsigargin, ryanodine, caffeine, or by replacement
of Ca by Sr (6, 37), it seems clear that the effect depends
on SR Ca uptake and on [Ca]. Schouten (37) hypothe-
sized that the abbreviation of SS twitches was due to
enhanced SR Ca uptake secondary to PLB phosphoryla-
tion by CaMKII, which was activated by the cyclic
increase in [Ca]. Rest would allow PLB dephosphoryla-
tion, reversing the frequency-dependent stimulation
of SR Ca-ATPase and slowing the time course of the SR
Ca uptake. Mattiazzi et al. (30) found that inhibition of
endogenous CaMKII-dependent PLB phosphorylation
increased the SR Ca uptake rate in permeabilized rat
myocytes. Furthermore, Bassani et al. (6) showed that
phosphatase inhibition prevents the slowing of [Ca],
decline at the PR twitch and that CaMKII inhibition
can prevent the activation-dependent acceleration of
[Ca], decline. Thus SR Ca transport and CaMKII are
involved in the acceleration of relaxation during SS
twitches. However, the involvement of PLB phosphory-
lation has not been proven.

If PLB was the only target of CaMKII and caused the
acceleration of relaxation during SS twitch, this phe-
nomenon should be abolished in the PLB-KO mouse.
However, as shown in Figs. 6 and 7, the PR slowing of
[Ca], decline is still present in the PLB-KO mouse.
Indeed, relaxation of the PR twitch was slowed by 76%
compared with the SS twitch. This proves that PLB is
not required for this effect. Recently, Hussain et al. (20)
also reported that the stimulation rate-dependent
changes in Ca transient duration in rat are not associ-
ated with PLB phosphorylation. On the other hand,
they suggested that SR Ca-ATPase might still be
involved, since the rate-dependent abbreviation of
Ca transient depends on a functional SR.

The accelerating effect of SS stimulation of the τ of
[Ca], decline could also be abolished by the CaMKII
inhibitor KN-93 at 1 µM (a concentration at which this
agent is expected to be quite selective for CaMKII; see
Ref. 40). Although this result strongly implicates
CaMKII in mediation of the acceleration of [Ca], decline
during SS, this conclusion does rely on the ability and
selectivity of 1 µM KN-93 to inhibit CaMKII. It might
be speculated that a CaMKII-dependent phosphoryla-
tion of the SR Ca-ATPase could be responsible, since
Toyofuku et al. (42) and Xu and colleagues (44) have
reported direct CaMKII-dependent phosphorylation
of the cardiac SR Ca-ATPase, which results in increased
V max for Ca transport. However, this result has been
challenged (32, 34), so the identity of the CaMKII
target involved in accelerating SR Ca transport during
SS twitches in rat and mouse myocytes is not yet
definitely identified.

We thank Christina Zakavec Hovance for careful work in isolating
cardiac myocytes.

This work was supported by National Heart, Lung, and Blood
Institute Grants HL-30077, HL-26057, HL-22619, and HL-52318.
Address for reprint requests: D. M. Bers, Dept. of Physiology,
Stritch School of Medicine, Loyola Univ. Chicago, 2160 South First
Ave., Maywood, IL 60153.

Received 24 October 1997; accepted in final form 18 December 1997.
REFERENCES


42. Toyofuku, T., K. Kurzydowski, N. Narayanan, and Mac Lennan. Identification of Ser\(^{38}\) as the site in cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase that is phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinase. J. Biol. Chem. 269: 26492–26496, 1994.

