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Phospholamban phosphorylation sites enhance the recovery of intracellular Ca^{2+} after perfusion arrest in isolated, perfused mouse heart

Carlos A. Valverde [a,*], Cecilia Mundiña-Weilenmann [a], Mariano Reyes [b], Evangelia G. Kranias [c], Ariel L. Escobar [b], Alicia Mattiazzi [a]

[a] *Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, 60 y 120, (1900) La Plata, Argentina*
[b] *Department of Physiology, Texas Tech University, Health Science Center, Lubbock, TX 79430, USA*
[c] *Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0575, USA*

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Abstract

Objective: To investigate the importance of the phosphorylation of Ser^{16} and Thr^{17} sites of phospholamban (PLN) on intracellular Ca^{2+} (Ca_i^{2+}) handling and contractile recovery of the stunned myocardium.
Methods: Ca_i^{2+} (Rhod-2, pulsed local-field fluorescence microscopy) and contractility (isovolumic left ventricular developed pressure, LVDP) were simultaneously measured in Langendorff perfused hearts from transgenic mice expressing either intact PLN (PLN-WT) or PLN with both phosphorylation sites mutated to Ala (PLN-DM), subjected to 12 min of global ischemia followed by a reperfusion period of 30 min.
Results: Pre-ischemic values of Ca_i^{2+} and LVDP were similar in both groups. In PLN-WT, a transient increase in Thr^{17} phosphorylation at early reperfusion preceded a recovery of Ca^{2+} transient amplitude, virtually completed by the end of reperfusion. LVDP at 30 min reperfusion was 67.9 ± 7.6% of pre-ischemic values, $n = 14$. In contrast, in PLN-DM, there was a poor recovery of Ca_i^{2+} transient amplitude and LVDP was significantly lower (28.3 ± 6.7%, $n = 11$, 30 min reperfusion) than in PLN-WT hearts. Although myofilament Ca^{2+} responsiveness and troponin I (TnI) degradation did not differ between groups, the episodes of mechanical alternans, typical of Ca_i^{2+} overload, were significantly prolonged in PLN-DM vs. PLN-WT hearts.
Conclusions: PLN phosphorylation appears to be crucial for the mechanical and Ca_i^{2+} recovery during stunning and protective against the mechanical abnormalities typical of Ca_i^{2+} overload. The importance of PLN phosphorylation would primarily reside in the Thr^{17} residue, which is phosphorylated during the critical early phase of reperfusion. Our results emphasize that, although ablation of PLN phosphorylation does not affect basal contractility, it does alter Ca^{2+} handling and mechanical performance under stress situations.

Keywords: Phospholamban phosphorylation residues; Phospholamban mutants; Intracellular calcium; Ischemia–reperfusion; Myofibrillar proteins

1. Introduction

Myocardial stunning describes the sustained contractile dysfunction that follows a brief ischemic insult, clinically manifested as sluggish recovery of pump function after revascularization. This post-ischemic dysfunction occurs in the absence of irreversible damage and despite restoration of normal coronary flow, and evolves towards complete recovery within hours, days or weeks [1,2]. Substantial evidence, mainly stemming from experiments in rodents, supports the idea that Ca^{2+} overload during reflow triggers myofilament dysfunction, which effectively

* Corresponding author. Tel./fax: +54 221 483 4833.
E-mail address: valverdeca@atlas.med.unlp.edu.ar (C.A. Valverde).

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uncouples excitation from contraction so that, at any given Ca^{2+} level, the force generated by the myocardium is diminished [2]. In this sense, myocardial stunning can be considered as an alteration of myofilament function. In spite of the considerable evidence accumulated in favor of a decrease in Ca^{2+} myofilament responsiveness as the cause of stunning in rodents, there is also evidence indicating that Ca^{2+}-handling proteins may also play a role during stunning in this species. Although Ca^{2+} was found to be normal at the end of reperfusion [3,4], sarcoplasmic reticulum (SR) function has been shown to be altered in stunned rat hearts [5]. Moreover, we have previously shown that the level of phosphorylation of phospholamban (PLN), the main regulatory protein of SR Ca^{2+} pump, increased during both ischemia and reperfusion and that this increase appears to contribute to the mechanical recovery of the stunned heart [6,7]. Further experiments in transgenic mice, in which PLN-phosphorylation sites were alternatively replaced by the non-phosphorylatable residue, Ala, indicated that both PLN sites, Ser^{16} (phosphorylated by PKA) and Thr^{17} (phosphorylated by CaMKII), seem to be necessary for the mechanical recovery [7], suggesting that Ca^{2+} handling proteins might be involved in the recovery of the stunned rodent heart. Taken together, these results allowed us to hypothesize that the presence of PLN phosphorylation sites would help to limit Ca_i^{2+} overload and ameliorate Ca^{2+} handling. Two corollary hypotheses are that the decrease in Ca^{2+} myofilament responsiveness and the propensity to mechanical alterations and arrhythmias, typical of stunning [8,9], would occur to a lesser extent in the presence than in the absence of PLN phosphorylation sites. To test these hypotheses, we performed experiments in perfused hearts from mice, in which both PLN phosphorylation sites were mutated to Ala (PLN-DM). In these hearts, mechanical parameters were measured during ischemia and reperfusion simultaneously with intracellular Ca^{2+}, assessed by pulsed local-field fluorescence microscopy [10].

2. Methods

2.1. Animals

Experiments were performed in phospholamban (PLN) transgenic mice (28–32 g body weight) expressing either wild type PLN (PLN-WT) or a mutant PLN in which both phosphorylation residues (Ser^{16} and Thr^{17}) were replaced by Ala (PLN-DM) into the PLN null background (SvJ129/CF1). The transgenic mouse models were developed as previously described [11,12]. Animals used in this study were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

2.2. Langendorff isolated heart preparations. Mechanical and intracellular Ca^{2+} measurements

Isolated mouse hearts (wet weight 0.20–0.30 g) were perfused according to the Langendorff technique at constant temperature (37 °C) and perfusion pressure (80 mm Hg). Hearts were paced at 5 Hz. The composition of the Tyrode solution was (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 0.33 KH_2PO_4, 1 $MgCl_2$, 10 glucose, and 10 HEPES; pH 7.4; equilibrated with 100% O_2. Mechanical activity of the hearts was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer (Motorola MPX5050, Co., USA). The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure (LVEDP) of approximately 18 mm Hg to compare with previous results [7]. Contractile performance of the left ventricle was evaluated by the developed pressure (LVDP) and relaxation was assessed by half relaxation time (t_{50}).

Intracellular Ca^{2+} was estimated by loading the hearts with Rhod-2 (Molecular Probes, Eugene, OR, USA), as previously described [10]. The hearts were perfused with dye-containing Tyrode solution for a period that ranged between 15 and 20 min at 2 Hz and at room temperature. After loading, the temperature was increased to 37 °C. Pulsed light from a Nd–Yag laser (532 nm) was focused into a multimode optical fiber (400 μm) for transmission of the exciting light to the heart surface. Light pulses of short duration allow the use of high peak power, increasing the signal to noise ratio. Because the fluorescence lifetime ($\tau = 5.6$ ns) exceeds the duration of the exciting light pulse, the amount of photobleaching is considerably reduced. Emitted light from the loaded fluorophore was carried back through the same fiber optic, filtered with a 590 nm Longpass Glass Color Filter (Edmund Scientific USA) and focused on an avalanche photodiode (EG & G, Canada) connected to an integrating current-to-voltage converter, controlled by a PIC microcontroller. The fiber optic was positioned lateral to the left ventricle, touching the epicardium. Motion artifacts were reduced by inserting one end of the fiber optic, into a borosilicate patch-clamp pipette, and applying negative pressure to hold the pipette to the tissue surface. Signals were digitized (PCI 6110, National Instruments, TX, USA) at a sampling frequency of 500 Hz, a bandwidth of 125 kHz and the photocurrent was evaluated by digital integration. Left ventricular pressure and temperature were acquired with a different analog-to-digital converter (PCI 6014, National Instruments, TX), at a sampling frequency of 50 kHz. Both acquisition systems were controlled by an Athlon MP PC, running a custom-designed, G-based software program (LabVIEW, National Instruments, TX, USA).

Because the amplitude of Rhod-2 fluorescence transient depends on free $[Ca^{2+}]$, the estimation of its value allows to continuously monitor the intracellular $[Ca^{2+}]$ changes

over time. Ca^{2+} performance was evaluated by the diastolic levels of fluorescence compared to pre-ischemic values, and the amplitude of Ca^{2+} transients, expressed as ratio between emitted ($F-F_0$) and basal (F_0) fluorescence ($\Delta F/F_0$).

2.3. Experimental protocol

After stabilization (pre-ischemia), hearts were subjected to 12 min normothermic global ischemia (interruption of the coronary flow) and coronary perfusion was then restored for 30 min (reperfusion). At the end of the experimental period, hearts were freeze-clamped and stored at −80 °C until biochemical assays. Myocardial contractility and Ca^{2+} transients were not altered in control experiments ($n=4$) in which all conditions of the ischemia/reperfusion protocol [dye loading, shutter openings at the time of acquisition], except for the interruption of coronary flow, were reproduced.

2.4. Preparation of mouse heart homogenates

The pulverized ventricular tissue from mouse hearts was homogenized as previously described [7]. The homogenate was centrifuged at 16,000×g for 20 min. Protein in the supernatant was measured by the method of Bradford.

2.5. Electrophoresis and Western blot analysis

25 and 15 μg of mouse homogenate proteins were electrophoresed per gel lane for PLN and TnI, respectively, in 12% SDS-polyacrylamide gels as previously described [6]. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and immunoblotted with antibodies raised against a PLN peptide (residues 9–19) phosphorylated at Ser^{16} or at Thr^{17} (1:5000) (Badrilla, UK) and anti-TnI (1:2000) (mAb 8I-7, Spectral Diagnostics). Signals were visualized by peroxidase-conjugated antibodies using

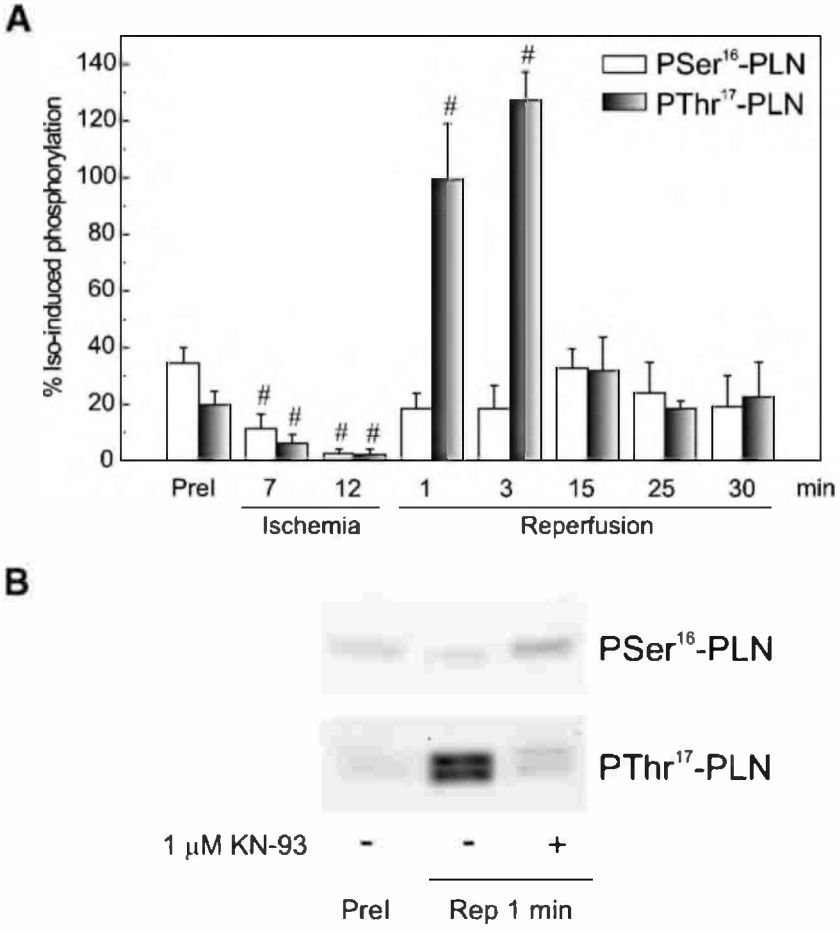

Fig. 1. Time course of PLN site-specific phosphorylation during ischemia and reperfusion in mice. Effect of KN-93. (A) Homogenates from mouse hearts freeze-clamped at the end of the pre-ischemic period (PreI) and at different times during ischemia (I) and reperfusion (R), were resolved in SDS-polyacrylamide gel electrophoresis. Proteins were blotted and assayed with anti $PSer^{16}$-PLN and $PThr^{17}$-PLN antibodies (upper panel). Overall results ($n=4-20$) are expressed as percentage of the phosphorylation of PLN sites induced by 30 nM isoproterenol (Iso) in non-ischemic hearts. #$P<0.05$ with respect to PreI. (B) Representative inmunoblots showing that reperfusion-induced increase in Thr^{17} phosphorylation in the absence of KN-93 (Rep 1 min, −) was abolished by CaMKII inhibition with KN-93 (Rep 1 min , +).

a chemiluminescence detection kit (ECL, Amersham). The signal intensity of the bands was quantified using Scion Image software. PLN site-specific phosphorylation was expressed as percentage of Ser^{16} and Thr^{17} phosphorylation induced by 30 nM isoproterenol in non-ischemic-reperfused hearts. TnI degradation was expressed as a % of the total immunoreactivity: i.e. density of the degradation products/ $\sum$(density of intact band + density of degradation products) × 100.

2.6. Statistics

Data are expressed as means ± S.E. Statistical significance was determined by Student's t-test for unpaired

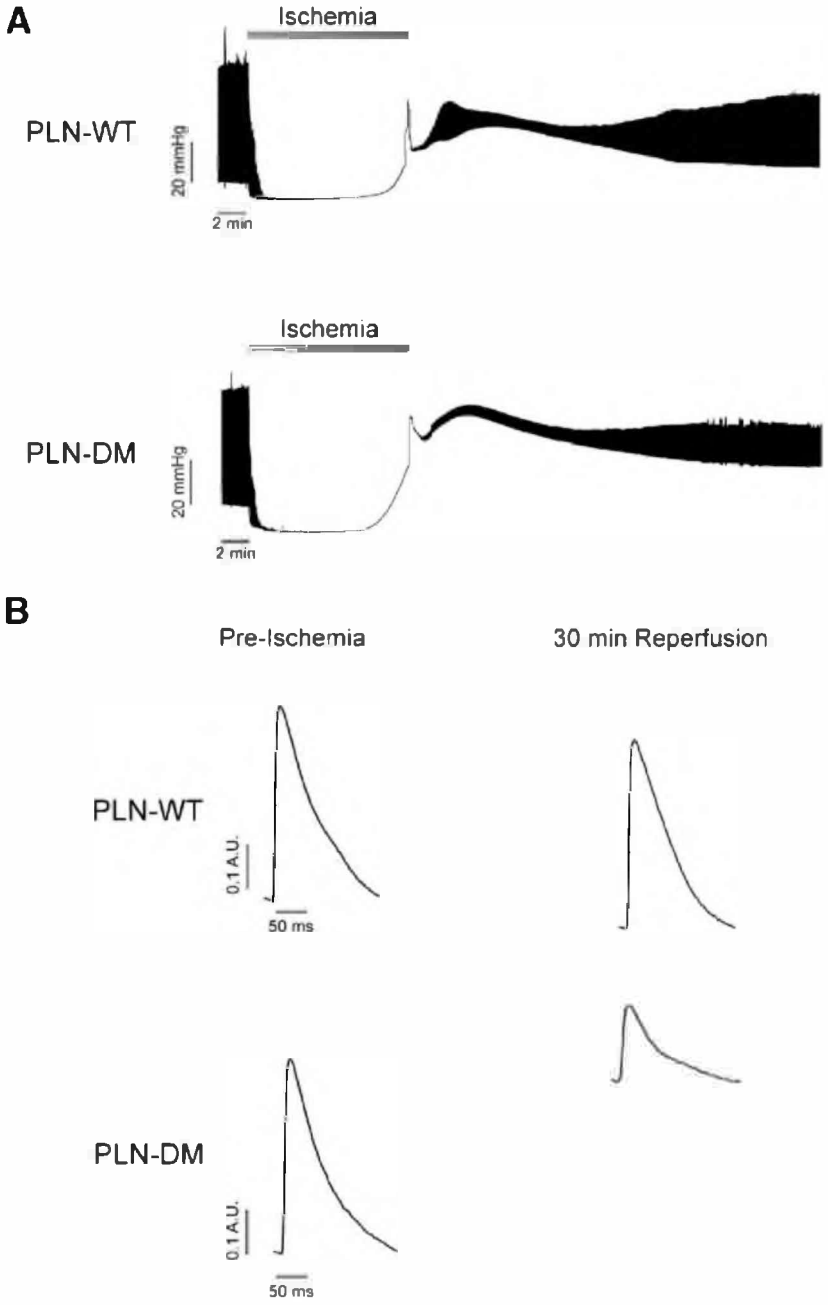

Fig. 2. Representative records of left ventricular pressure and intracellular Ca^{2+} transients of hearts from PLN-WT and PLN-DM mice during ischemia and reperfusion. (A) Continuous records of left ventricular pressure of PLN-WT and PLN-DM hearts. Ischemia reduced contractility to non-detectable levels and then contractility recovered during reperfusion. This recovery was lower is PLN-DM than in PLN-WT. (B) Ca^{2+} transients obtained before ischemia (pre-ischemia) and after 30 min of reperfusion. At the end of reperfusion, there is a decrease in the amplitude of the Ca^{2+} transient and an increase in diastolic Ca^{2+} in PLN-DM hearts with respect to pre-ischemic values and to the corresponding values of hearts from PLN-WT animals. Scale on vertical axis represents arbitrary units (AU) of Rhod-2 fluorescence, expressed as ratio between emitted ($F-F_0$) and basal (F_0) fluorescence ($\Delta F/F_0$).

observations. A P value <0.05 was considered statistically significant.

3. Results

Fig. 1A shows overall results depicting the time course of phosphorylation of Ser^{16} and Thr^{17} of PLN in hearts from WT mice during ischemia and reperfusion. The phosphorylation of both sites decreased by the end of ischemia. Whereas phosphorylation of Ser^{16} remained at basal levels all along the reperfusion period, phosphorylation of Thr^{17} transiently increased at the beginning of reperfusion (1 and 3 min), and then returned to basal values, in agreement with previous findings [7]. Perfusion with 1 μM of the CaMKII inhibitor, KN-93, administered 10 min previous to the ischemia/reperfusion protocol, abolished the reperfusion-induced Thr^{17}-phosphorylation (Fig. 1B). These results confirmed the participation of CaMKII in the phosphorylation of Thr^{17} at the beginning of reperfusion, in agreement with our previous findings in the rat heart [6,7]. To investigate whether the status of phosphorylation of PLN residues influenced Ca_i^{2+} recovery in the stunned heart, we simultaneously evaluated the mechanical and Ca_i^{2+} changes of hearts from PLN-WT and PLN-DM mice. Fig. 2A illustrates continuous recordings of left ventricular developed pressure (LVDP) at baseline and during ischemia and reperfusion, in hearts from PLN-WT and PLN-DM mice. Fig. 2B shows representative Ca^{2+} transients, before ischemia and after 30 min of reperfusion. Basal contractility and Ca_i^{2+} showed no differences between PLN-WT and PLN-DM mice, in agreement with previous findings in

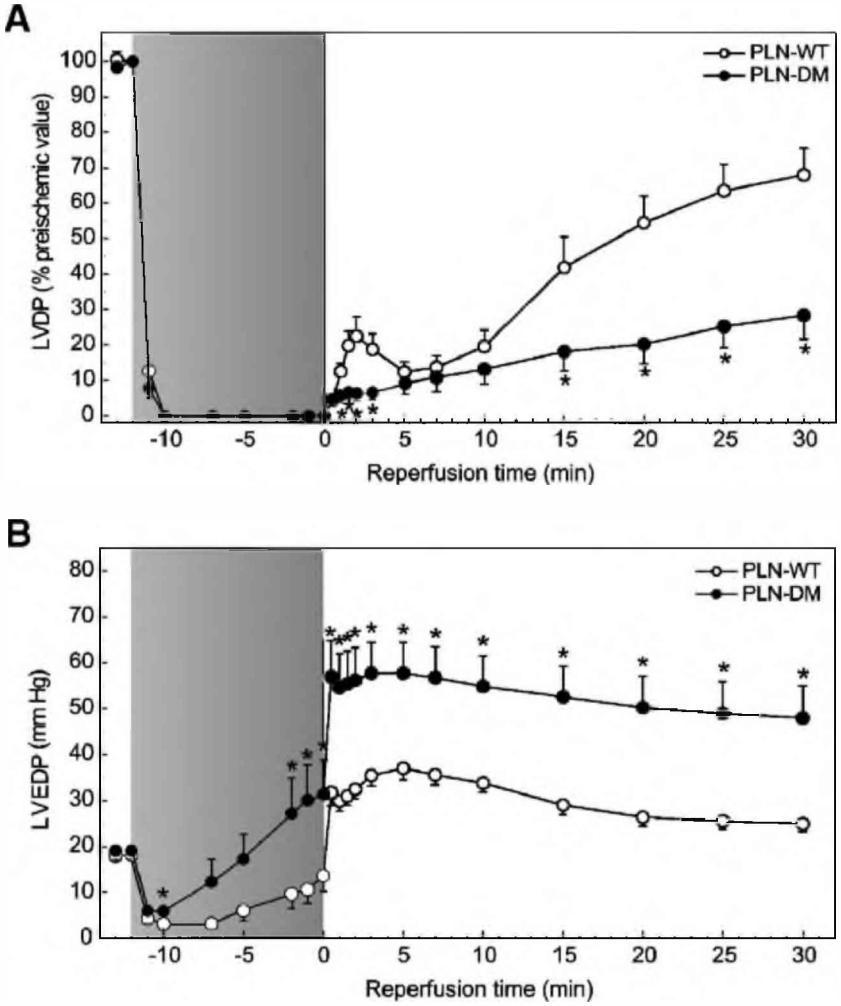

Fig. 3. Time course of left ventricular function of hearts from PLN-WT and PLN-DM mice during ischemia and reperfusion. (A) Overall results of the time course of left ventricular developed pressure (LVDP). LVDP decreased to virtually non-detectable levels after cessation of flow. Upon reperfusion contractility recovered, reaching 67.9±7.6% (PLN-WT) and 28.3±6.7% (PLN-DM) of pre-ischemic values, after 30 min of reperfusion. Absolute pre-ischemic values for LVDP for PLN-WT and PLN-DM were: 70.9±12.6 and 78.4±11.1 mm Hg, respectively. (B) Overall results of the time course of end diastolic pressure (LVEDP). LVEDP decreased immediately after cessation of flow and then increased slowly in PLN-WT and more abruptly in PLN-DM hearts during ischemia. Upon reperfusion, LVEDP increased dramatically in both groups, remaining at this high level throughout reperfusion. LVEDP levels were significantly higher in PLN-DM vs. PLN-WT hearts, all along reperfusion. Points represent mean±S.E.M. of data from 13 to 16 hearts (for WT mice) and 11 hearts (for DM mice). *$P<0.05$ with respect to PLN-WT hearts.

isolated myocytes [13]. When coronary flow was interrupted, LVDP fall abruptly in hearts of both groups of mice, remaining at virtually non-detectable levels during the ischemic period. Upon reperfusion, LVDP recovered gradually, without reaching pre-ischemic values. In PLN-DM hearts, the recovery of contractility was lower than in PLN-WT hearts and it was associated with a markedly reduced Ca^{2+} transient amplitude with respect to pre-ischemic levels. In contrast, in PLN-WT mice, Ca^{2+} transient amplitude recovered to values close to pre-ischemic levels at 30 min of reperfusion. Both the contracture developed during ischemia and the elevation of diastolic pressure in early reperfusion ("hypercontracture", [14]) were higher in PLN-DM than in PLN-WT mice. At the same time, diastolic pressure tended to recover to pre-ischemic levels in PLN-WT mice, while it remained markedly elevated in PLN-DM mice throughout reperfusion. These experiments, performed at constant perfusion pressure, showed a similar mechanical pattern to that observed in previous experiments performed at constant coronary flow in PLN-WT and transgenic mice with either one of both phosphorylation sites mutated to Ala [7]. Diastolic Ca^{2+} recovered towards pre-ischemic levels by the end of reperfusion in PLN-WT mice, while it remained significantly increased in PLN-DM mice. Figs. 3 and 4 illustrate the overall results of the mechanical and intracellular Ca^{2+} measurements. The average data showed that after 30 min of reperfusion, contractility (LVDP) recovered to 67.9±7.6% of pre-ischemic values in PLN-WT hearts, while in PLN-DM hearts the recovery was significantly lower (28.3±6.7%) (Fig. 3A). Similar results were obtained with $+dP/dt$ (data not shown). Moreover, the increase in LVEDP at the end of ischemia and throughout reperfusion was significantly higher in PLN-DM mice vs. PLN-WT mice (Fig. 3B) and the time to the onset of ischemic

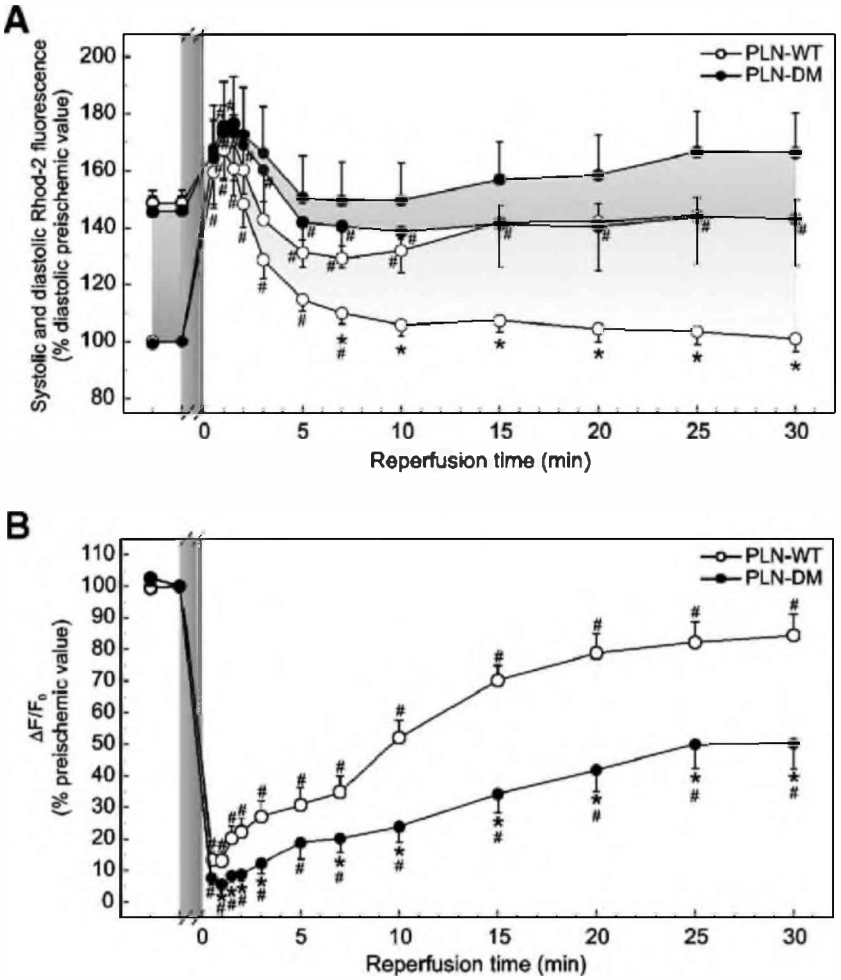

Fig. 4. Time course of intracellular Ca^{2+} and Ca^{2+} transient amplitude during reperfusion. (A) Systolic Ca^{2+} increased upon reperfusion and then remained near pre-ischemic levels all along the reperfusion in PLN-WT hearts and slightly above pre-ischemic values in PLN-DM hearts. In both, PLN-WT and PLN-DM hearts, diastolic Ca^{2+} increased immediately after reperfusion. However, it recovered to pre-ischemic values in PLN-WT hearts, but it remains at high levels in PLN-DM hearts. (B) Overall results of the time course of the recovery of the Ca^{2+} transient amplitude (shadow area in A), after the ischemic insult. Ca^{2+} transient amplitude reached 84.6±6.7% (PLN-WT) and 50.2±8.0% (PLN-DM) of pre-ischemic values at 30 min of reperfusion. Intracellular Ca^{2+} and Ca^{2+} amplitude ($\Delta F/F_0$) were expressed as percentage of pre-ischemic values. Points represent mean±S.E.M. of data from 13 to 16 hearts (for PLN-WT mice) and 17 to 18 hearts (for PLN-DM mice). # indicates $P<0.05$ with respect to pre-ischemic values. * indicates that diastolic Ca^{2+} is significantly ($P<0.05$) different between PLN-DM and PLN-WT.

contracture (an increase in LVEDP of 4 mm Hg from baseline, [15]), was significantly shorter in PLN-DM than in PLN-WT mice (542.7±66.3 vs. 686.6±21.0 s, respectively). Fig. 4A and B shows the time course of diastolic, systolic and Ca^{2+} transient amplitude after the ischemic insult. In PLN-WT hearts, after an initial Ca_i^{2+} overshoot at the beginning of reperfusion, diastolic Ca^{2+} returned to pre-ischemic levels within 10–15 minutes. Ca^{2+} transient amplitude recovered towards pre-ischemic levels during reperfusion, attaining values only slightly, although significantly, lower than pre-ischemic values at 30 min of reperfusion. Thus, the contractile decrease of these hearts should be primarily attributed to a decrease in myofibrillar Ca^{2+} responsiveness, as previously described in rat, guinea pig and ferret hearts [3,4,16]. In contrast, in PLN-DM hearts, diastolic Ca^{2+} remained at high levels during reperfusion and Ca^{2+} transient amplitude was markedly decreased. This decrease may explain the greater impairment of contractility in PLN-DM hearts with respect to PLN-WT hearts, following the ischemia/reperfusion period. In addition, the mishandling of Ca_i^{2+} and the persistency of Ca_i^{2+} overload in PLN-DM hearts could produce a more important alteration in the myofilament Ca^{2+} responsiveness, which would also contribute to the contractile dysfunction observed in these mice. For the same reason, the propensity towards mechanical alterations and arrhythmias exhibited by the stunned heart [8,9] may also be increased in PLN-DM hearts. To investigate these issues, we studied the relationship between developed pressure and Ca^{2+} transient amplitude, as an estimation of myofilament Ca^{2+} responsiveness, and we evaluated the integrity of TnI. TnI was examined because, although somewhat controversial [17], a Ca^{2+}-calpain-dependent TnI degradation has been proposed as the molecular mechanism of myocardial stunning in rodents [2]. Fig. 5A depicts the relationship between LVDP and Ca^{2+} transient normalized to pre-

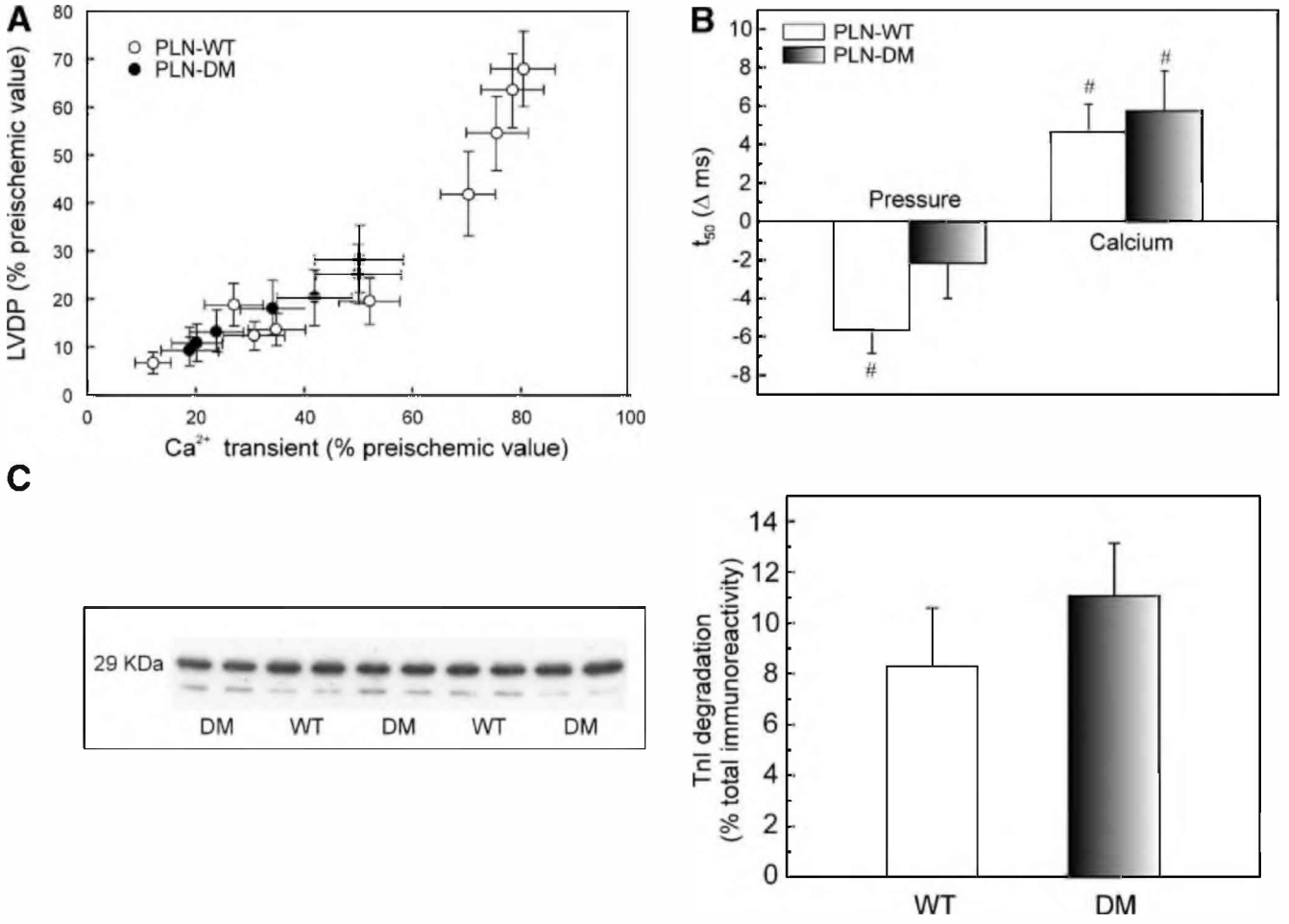

Fig. 5. Ca^{2+} transient and developed pressure relationship in PLN-WT and PLN-DM mice during reperfusion and kinetics of the developed pressure and Ca^{2+} transient at 30 min of reperfusion. (A) Relationship between developed pressure (LVDP) and Ca^{2+} transient amplitude expressed as % of pre-ischemic values, during reperfusion in PLN-WT and PLN-DM mice. (B) t_{50} of the developed pressure and the Ca^{2+} transient decay after 30 min of reperfusion expressed as differences from pre-ischemic values. Only in PLN-WT hearts, the decrease in t_{50} of the pressure decay was significant with respect to pre-ischemic values (relaxant effect). t_{50} of the Ca^{2+} transient decay was prolonged in both mice. Absolute values of t_{50} were: 37.4±2.1 and 37.7±2.5 ms (for pressure) and 44.8±1.8 and 41.3±1.4 ms (for Ca^{2+} transient) for PLN-WT and PLN-DM, respectively. # indicates $P<0.05$ with respect to pre-ischemic values. (C) Left panel. Typical immunoblot showing TnI degradation in two PLN-WT and three PLN-DM hearts, each one in duplicate. Right panel. Overall results of TnI degradation, expressed as % of total TnI, in PLN-WT and PLN-DM hearts. No significant difference could be detected between both groups.

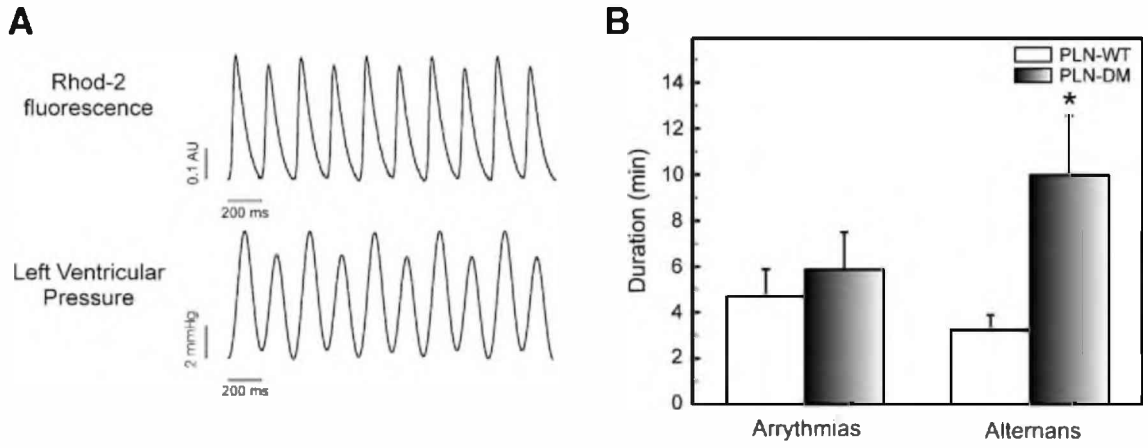

Fig. 6. Mechanical alterations after reperfusion. (A) Typical alterations in the mechanical behavior during reperfusion in PLN-DM hearts. (B) Average duration of the arrhythmic and mechanical alternans episodes in PLN-WT and PLN-DM hearts. * indicates $P<0.05$ with respect to PLN-WT.

ischemic values, at each data point throughout the last 25 min of reperfusion. Points for both PLN-WT and PLN-DM mice followed a similar relationship within the range of LVDP and Ca^{2+} transient common to both groups. Fig. 5B illustrates the behavior of t_{50} of LVDP and Ca^{2+} transient decay in both groups of hearts, expressed as differences with respect to pre-ischemic values (Δ ms). In PLN-WT, there was a significant decrease in the t_{50} of LVDP associated with a significant increase in the t_{50} of the underlying Ca^{2+} transient, consistent with a decrease in Ca^{2+} myofilament responsiveness. PLN-DM hearts exhibit a similar behavior, although the decrease in t_{50} of LVDP did not attain significant levels. This lack of significance observed in the PLN-DM hearts is possible due to the fact that the decrease in Ca^{2+} myofilament responsiveness may be masked by the superimposed abnormalities in Ca^{2+} handling of these hearts. Fig. 5C shows an immunoblot and the overall results depicting TnI degradation in both groups of hearts after 30 min of reperfusion. The results failed to detect any significant difference in the degradation of TnI between PLN-DM and PLN-WT hearts. Additional experiments showed however, that TnI degradation after 12/30 min ischemia/reperfusion in mice with native PLN did not differ, when compared with hearts perfused under control conditions for the same time period, in agreement with some previous findings [17].

Previous studies indicated that the ischemic/reperfusion insult produced mechanical alterations, like mechanical alternans, aftercontractions and cardiac arrhythmias [8,9]. In the present experiments, aftercontractions were not observed in any of the two groups. However, both groups presented episodes of mechanical alternans and cardiac arrhythmias (ventricular tachycardia, VT) at different times during reperfusion. A typical example of an episode of mechanical alternans in a PLN-DM heart is shown in Fig. 6A. The incidence of the VT was similar for both groups of hearts, i.e. 11 out of 15 hearts from PLN-WT animals and 13 out of 18 hearts from PLN-DM. The length of these arrhythmic periods was also similar for both groups (Fig. 6B). Moreover, whereas the incidence of episodes of mechanical alternans was similar in both groups of hearts, the duration of these episodes was significantly higher in PLN-DM hearts (Fig. 6B).

4. Discussion

The main finding of the present study is that the presence of PLN phosphorylation sites enhances the recovery of intracellular Ca^{2+} and contractility in the stunned mouse heart. We showed that in transgenic mice, that expressed intact PLN (PLN-WT), reperfusion produced a significant and transient increase in Thr^{17} phosphorylation, which preceded a rapid and virtually complete recovery of systolic and diastolic intracellular Ca^{2+} to pre-ischemic values. In contrast, in mutant PLN hearts with non-phosphorylatable sites, the recovery of contractility and intracellular Ca^{2+} transient was significantly lower than that of age-matched PLN-WT. The present results provided evidence indicating that the presence of PLN phosphorylation sites is crucial for cardiac performance under stress conditions, like the ischemic–reperfusion insult.

Previous studies from our laboratory demonstrated that phosphorylation of Thr^{17} of PLN transiently increased at the beginning of reperfusion in the mouse and rat heart. This phosphorylation appeared to be important for the mechanical recovery after ischemia and is evoked by CaMKII activation at the beginning of reperfusion [6,7]. Moreover, transgenic mice lacking either phosphorylatable site of PLN showed an impaired mechanical recovery of the stunned heart [7]. It was hypothesized that the presence of these sites is necessary for Ca^{2+} handling during myocardial recovery in the reperfusion period. Thus, their absence would induce a more important impairment of Ca^{2+} transient and would enhance Ca^{2+} overload, with two possible additional consequences: (1) a further decrease in Ca^{2+} myofibrillar responsiveness, which seems to be the hallmark of myocardial stunning in rodents [3,4]; and (2) an increase

in the propensity of the stunned heart to exhibit mechanical alterations and arrhythmias, typical of Ca^{2+} overload and Ca^{2+} mishandling [8,9]. The present results provided evidence supporting that phosphorylation of Thr^{17} of PLN occurs at the beginning of reperfusion by CaMKII activation and further indicate that PLN phosphorylation sites are essential for the recovery of diastolic Ca^{2+} and Ca^{2+} transient during stunning. In addition, although our findings failed to detect any significant difference in myofibrillar Ca^{2+} responsiveness and TnI degradation, between PLN-WT and PLN-DM mice, they did show that the presence of PLN phosphorylation residues is important to reduce the periods of mechanical alternans, one of the consequences of Ca^{2+} overload.

4.1. Ca^{2+} signaling recorded in the beating heart

In the present study, we used a novel technique that allows for simultaneous detection of intraventricular pressure and Ca^{2+} transient at the cellular level but recorded in the beating heart. The measurement of Ca^{2+} signals when the cells are in their natural environment gives important information of the organ function [18,19]. Recordings in the intact beating heart have typically utilized fluorescence spectroscopy [19], but an obstacle, associated with fluorescence approaches, is motion artifacts generated by cardiac contraction. To minimize the effects of contractile motion on the fluorescence signal, these studies have been previously conducted on mechanically [18,20] or chemically immobilized hearts [21]. In any case, "motion-free" signals are then detected with bi-dimensional sensors [20]. Moreover, in these studies, large working distances of the optic limit the spatial resolution. To overcome this problem, optical fibers have been used to conduct epifluorescence measurements [22]. In the present study, we presented a novel way to improve the signal to noise ratio, by combining the pulsed-local field fluorescence illumination with an integrating current to voltage conversion, with a digital evaluation of the integrated photocurrent. This methodology, based on the technique presented by Mejia-Alvarez et al. [10], has both an improved signal to noise ratio and a diminished photobleaching effect. One limitation of this technique is that myoplasmic Ca^{2+} can only be assessed at the epicardium. Differences in Ca^{2+} transients between epicardium and endocardium may arise from the ventricular transmural dispersion of the mechanisms underlying excitation–contraction coupling [23]. This issue requires further research.

Since myoglobin (Mb) strongly absorbs light depending on tissue oxygenation, at 540 and 580 nm (oxy-Mb) and 550 nm (deoxy-Mb) [24], it could be argued that this may affect the Rhod-2-Ca^{2+} measurements. This possibility seems unlikely however, since similar results were obtained in ischemia/reperfusion experiments in which the hearts were simultaneously loaded with Rhod-2 (excited at 532 nm) and Fluo-4 (excited at 452 nm). These experiments indicate that the changes of the Rhod-2 fluorescent signal observed in our experiments reflect actual variations of Ca^{2+} occurring during ischemia/reperfusion.

The results indicated that reperfusion causes an initial dramatic increase in diastolic Ca^{2+} that gradually returned to pre-ischemic levels after 10 min of reperfusion in PLN-WT hearts. Ca^{2+} transient amplitude, that was initially depressed, also returned towards pre-ischemic values by the end of the reperfusion period. In agreement with previous results, this study indicates that in hearts from PLN-WT mice, the availability of activator Ca^{2+} is not the primary cause of the contractile dysfunction of the stunned heart, at least in rodents [3,4]. In contrast, in PLN-DM hearts, diastolic Ca^{2+} remained at levels significantly higher than pre-ischemic values over all the reperfusion period and intracellular Ca^{2+} transients are far from complete recovery at the end of the reperfusion period. The elevated diastolic intracellular Ca^{2+} in PLN-DM hearts would be the result of less Ca^{2+} being reuptaken by the SR because of the lack of PLN phosphorylation sites. As a consequence, Ca^{2+} released by the SR is less and therefore Ca^{2+} transient amplitude in PLN-DM is significantly lower with respect to PLN-WT mice. These results indicate that the decrease in intracellular Ca^{2+} transient is a main component in the contractile alteration of the stunned PLN-DM heart.

4.2. Myofibrillar Ca^{2+} responsiveness

After 30 min of reperfusion, myocardial contractility was significantly lower in PLN-WT hearts. In contrast, intracellular Ca^{2+} transients decreased only slightly, although significantly, at the end of reperfusion, and diastolic Ca^{2+} completely returned to pre-ischemic levels. These findings indicate that myocardial stunning in mice is primarily dependent on a decrease in Ca^{2+} myofibrillar responsiveness, with a minor contribution of intracellular Ca^{2+}, in agreement with previous results obtained in rat, guinea pigs and ferret hearts [3,4,16]. Among the possible mechanisms involved in the decrease of Ca^{2+} myofibrillar responsiveness is a Ca^{2+}-induced proteolytic degradation of contractile proteins [2]. We hypothesized that, if this were the case, the decrease in myofibrillar Ca^{2+} responsiveness might be more important in PLN-DM than in PLN-WT mice, due to the persistent increase in diastolic Ca^{2+} present in PLN-DM hearts during reperfusion. However, our results failed to detect any significant difference in myofibrillar Ca^{2+} responsiveness between the two groups. Moreover, TnI degradation was also similar in both groups of hearts. These results might indicate that the increase in Ca^{2+} overload in PLN-DM hearts was not sufficiently enhanced relative to that in PLN-WT hearts to produce a further decrease in myofibrillar Ca^{2+} responsiveness or to evoke a degradation of TnI, that could not be detected in mice with intact PLN. Interestingly, the results allowed dissociation between the decrease in Ca^{2+} myofilament sensitivity, observed in the stunned hearts of both groups, from the degradation of TnI, which did not occur in either group. Thus, other mecha-

nisms, different from or in addition to intracellular Ca^{2+} overload, are playing a major role in the decrease in myofilament Ca^{2+} responsiveness typical of the stunned heart in rodents [2]. Additional proposed mechanisms are alteration of myofibrillar proteins different from TnI, induced by the production of oxygen-free radicals [2], the persistent elevation of intracellular magnesium [25] or even intracellular edema. Obviously, more work is needed to support these possibilities.

4.3. Mechanical alternans and Ca^{2+} mishandling

Mechanical alternans represents an abnormality of Ca^{2+} handling where large and small contractions follow each other due to alternation in systolic Ca^{2+}. Alternans is not only prominent in heart failure, but is also induced by ischemia and acidosis [9]. In our experimental conditions, both groups of hearts showed the same propensity to mechanical alternans. However, once installed, they persisted for longer periods in PLN-DM than in PLN-WT hearts (Fig. 6). Mechanical alternans have been associated with alternations in the amount of Ca^{2+} release from the SR, due to a beat-to-beat change in either SR Ca^{2+} content or the properties of the Ca^{2+} release process [26]. Although we did not explore the issue, it is reasonable to speculate that the chronic inhibition of SERCA2a in PLN-DM hearts due to the lack of PLN phosphorylation residues is at the basis of the tendency of these hearts to perpetuate the cycle of mechanical alternans, once they started. This propensity of PLN-DM hearts to perpetuate the periods of mechanical abnormalities, coupled with the prominent and persistent increase in diastolic Ca^{2+} and diastolic tone, indicates that these hearts are less tolerant to Ca^{2+} loading than PLN-WT hearts. Our results indicate that the phosphorylation of Thr^{17}, at the onset of reperfusion, only present in PLN-WT hearts, is a crucial factor in the subsequent recovery of Ca^{2+} handling and mechanical performance of the stunned heart. The impairment of Ca^{2+} recovery in the transgenic animals, lacking PLN phosphorylation sites, highlight the role of PLN residues, when they are on site. The important role of PLN phosphorylation during the critical early phase of reperfusion was recently emphasized in a cellular model of ischemia/reperfusion injury [27]. The experiments indicated that cGMP-dependent phosphorylation of Ser^{16} of PLN is protective against reoxygenation injury in isolated myocytes. The increase in SERCA2a activity due to PLN phosphorylation would support an early clearing of excess Ca^{2+} from the cytosol, favoring Ca^{2+} cycling and reducing oscillatory Ca^{2+} rise. This would in turn reduce cardiomyocyte hypercontracture, at the beginning of reperfusion. Previous results from our own laboratory indicated that phosphorylation of Thr^{17} of PLN occurs as a consequence of the increase in cytosolic Ca^{2+} triggered by reperfusion [6]. The present findings also showed that this phosphorylation precedes the recovery of intracellular Ca^{2+} in PLN-WT hearts. This recovery did not occur in animals lacking phosphorylatable sites in PLN. Taken together, the results emphasize the importance of PLN as a target for new strategies of cardioprotection against ischemia/reperfusion injury in the clinical setting.

In summary, our results indicate for the first time that the presence of PLN phosphorylation sites are crucial for the mechanical and intracellular Ca^{2+} recovery in the stunned heart and are protective against the propensity to mechanical abnormalities that occurred during stunning. These results, together with the fact that phosphorylation of Ser^{16} of PLN by cGMP has been also shown to be protective in ischemia/reperfusion injury [27], shed new lights for the search of novel strategies for cardioprotection in the clinical setting. Finally, the present findings also emphasize that although the absence of PLN phosphorylation sites seems not to affect basal contractility, it does alter the handling of Ca^{2+} and the mechanical performance of the heart under stress situations.

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