Brazilian Journal of Medical and Biological Research (2006) 39: 563-572
ISSN 0100-879X Review

The importance of the Thr[17] residue of phospholamban as a phosphorylation site under physiological and pathological conditions

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Abstract

The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) is under the control of an SR protein named phospholamban (PLN). Dephosphorylated PLN inhibits SERCA2a, whereas phosphorylation of PLN at either the Ser[16] site by PKA or the Thr[17] site by CaMKII reverses this inhibition, thus increasing SERCA2a activity and the rate of Ca^{2+} uptake by the SR. This leads to an increase in the velocity of relaxation, SR Ca^{2+} load and myocardial contractility. In the intact heart, ß-adrenoceptor stimulation results in phosphorylation of PLN at both Ser[16] and Thr[17] residues. Phosphorylation of the Thr[17] residue requires both stimulation of the CaMKII signaling pathways and inhibition of PP1, the major phosphatase that dephosphorylates PLN. These two prerequisites appear to be fulfilled by ß-adrenoceptor stimulation, which as a result of PKA activation, triggers the activation of CaMKII by increasing intracellular Ca^{2+}, and inhibits PP1. Several pathological situations such as ischemia-reperfusion injury or hypercapnic acidosis provide the required conditions for the phosphorylation of the Thr[17] residue of PLN, independently of the increase in PKA activity, i.e., increased intracellular Ca^{2+} and acidosis-induced phosphatase inhibition. Our results indicated that PLN was phosphorylated at Thr[17] at the onset of reflow and immediately after hypercapnia was established, and that this phosphorylation contributes to the mechanical recovery after both the ischemic and acidic insults. Studies on transgenic mice with Thr[17] mutated to Ala (PLN-T17A) are consistent with these results. Thus, phosphorylation of the Thr[17] residue of PLN probably participates in a protective mechanism that favors Ca^{2+} handling and limits intracellular Ca^{2+} overload in pathological situations.

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Presented at the XX Annual Meeting of the Federação de Sociedades de Biologia Experimental, Águas de Lindóia, SP, Brazil, August 24-27, 2004.

Research supported by PICT #08592 (FONCYT), PIP #02256 and 02257 (CONICET) and Fogarty International Research Award Grant #1-R03-TW-06294 (NIH).

Received August 9, 2005
Accepted January 2, 2006

Key words
- Phospholamban
- Thr[17] site phosphorylation
- ß-adrenergic stimulation
- Acidosis
- Ischemia

Introduction

Figure 1 is a schematic illustration of the central players in the excitation-contraction coupling mechanism. Upon depolarization, Ca^{2+} enters the cell through L-type Ca^{2+} channels and triggers the release of more Ca^{2+} from the sarcoplasmic reticulum (SR) through the activation of the ryanodine receptors. This process, known as Ca^{2+}-induced-Ca^{2+} release (1), amplifies and coordinates the Ca^{2+} signal to produce contraction by interacting with myofilament proteins. The decrease in cytosolic Ca^{2+} to produce relaxation is mainly induced by SR Ca^{2+}-ATPase (SERCA2a), which mediates Ca^{2+} uptake into the SR, and, to a lesser extent, by the Na^+/Ca^{2+} exchanger, which transfers Ca^{2+} to the extracellular space (2).

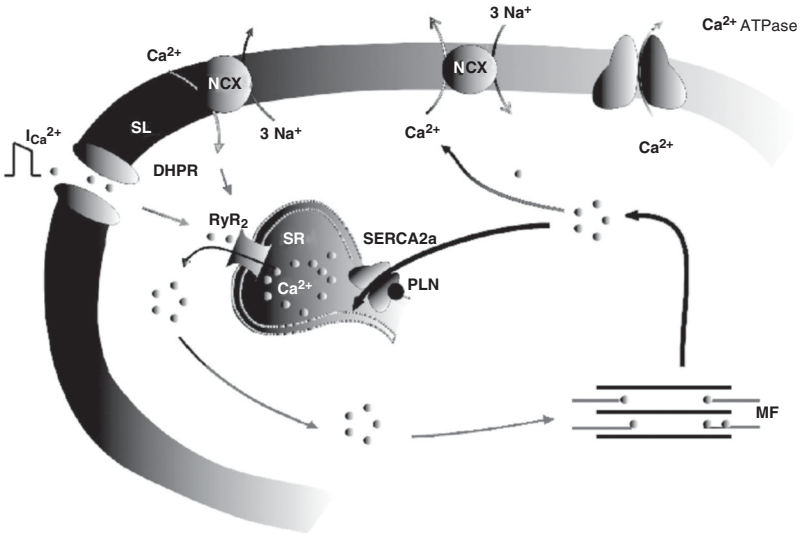

Figure 1. Central players of excitation-contraction coupling (ECC) in cardiac muscle. Scheme of the ECC mechanism in cardiac muscle. Upon depolarization, Ca^{2+} influx through L-type Ca^{2+} channels or dihydropyridine receptor (DHPR) triggers the release of more Ca^{2+} from the sarcoplasmic reticulum (SR). This Ca^{2+} binds to the myofilaments (MF) to produce contraction. Part of the Ca^{2+} is then extruded outside of the cell through the Na^+/Ca^{2+} exchange mechanism (NCX), working in the forward mode, but most of it is re-taken up by SR Ca^{2+}-ATPase (SERCA2a). Phospholamban (PLN) is a small SR protein associated with SERCA2a. The state of PLN phosphorylation regulates the activity of this Ca^{2+} pump. RyR_2 = ryanodine receptor; SL = sarcolemma.

The activity of SERCA2a is under the control of a closely associated SR protein named phospholamban (PLN) (3). In the dephosphorylated form, PLN decreases the apparent affinity of SERCA2a for Ca^{2+} (4,5). Phosphorylation of PLN relieves this inhibition, thus increasing SR Ca^{2+} uptake. Experiments on the intact heart have shown that ß-adrenoceptor stimulation phosphorylates PLN at the Ser^{16} residue by cAMP-dependent protein kinase (PKA) and at the Thr^{17} site by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). Phosphorylation of these sites reverses the inhibition of SERCA2a by PLN, thus increasing the affinity of the enzyme for Ca^{2+} and the rate of Ca^{2+} uptake by the SR. This in turn leads to an increase in the velocity of relaxation, SR Ca^{2+} load and, as a consequence, increased SR Ca^{2+} release and myocardial contractility (6-9) (Figure 2). The status of phosphorylation of PLN also depends on the activity of type 1 phosphatase (PP1), the major SR-phosphatase, which specifically dephosphorylates PLN (10). Although, as stated above, ß-adrenergic stimulation phosphorylates Ser^{16} and Thr^{17} of PLN, the role of the phosphorylation of Thr^{17} has been generally disregarded. The present article focuses on the role of the Thr^{17} site of PLN during ß-adrenergic stimulation and under different pathological conditions such as acidosis and ischemia/reperfusion injury.

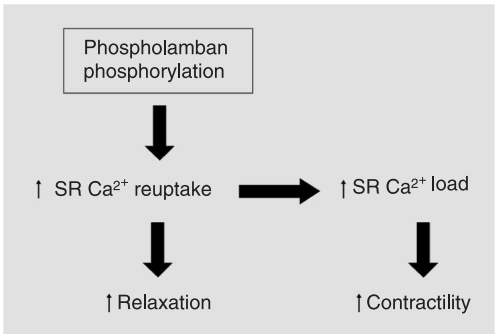

Figure 2. Phospholamban phosphorylation leads to an increase in SR Ca^{2+} uptake which accelerates relaxation and enhances SR Ca^{2+} load. More Ca^{2+} is then available for release from the SR, resulting in increased contractility. Thus, phospholamban is a major regulator of myocardial relaxation and contractility. SR = sarcoplasmic reticulum.

Role of the phosphorylation of Thr^{17} of PLN during ß-adrenergic stimulation

Although *in vitro* studies indicate that PKA and CaMKII phosphorylations are independent of each other (11), earlier at-

tempts to phosphorylate PLN by CaMKII in the intact heart usually failed, unless cellular cAMP levels increased (6,7,12). These findings suggested an interaction between the PKA and CaMKII pathways for PLN phosphorylation. The availability of transgenic models expressing wild-type PLN (PLN-WT), the Ser16→Ala mutant PLN (PLN-S16A) or the Thr17→Ala mutant PLN (PLN-T17A) in the cardiac compartment of PLN knock-out mice, and of phosphorylation site-specific antibodies to PLN, which precisely discriminate between the Ser16 and the Thr17 phosphorylation sites, in combination with the quantification of ^{32}P incorporation into PLN, helped to clarify the relative role of Ser16 and Thr17 phosphorylation (9,13,14). Experiments in transgenic mice expressing either PLN-WT or the PLN-S16A, indicated that the phosphorylation of Ser16 of PLN is a prerequisite for the phosphorylation of Thr17 (13). Other experiments conducted with PLN-T17A hearts further indicated that the Ser16 site can be phosphorylated independently of Thr17 *in vivo* and that phosphorylation of Ser16 was sufficient to evoke the maximal effect of ß-adrenergic stimulation (14).

Figure 3A, shows the increase in total PLN phosphorylation produced by increasing isoproterenol concentration in the cardiac perfusate. When Ca^{2+} influx to cardiac cells was virtually abolished by the presence of low extracellular Ca^{2+} $(Ca^{2+})_o$ and the Ca^{2+} channel blocker nifedipine to prevent CaMKII activation, total PLN phosphorylation decreased at the highest isoproterenol concentrations (10, 30, and 300 nM), but did not significantly change at the lowest levels of ß-adrenoceptor stimulation (0.3 and 3 nM). These results suggested that there was no contribution of the CaMKII pathways to the total phosphorylation of PLN at the lowest isoproterenol concentrations. Immunodetection of site-specific phosphorylated PLN revealed an isoproterenol concentration-dependent increase in both Ser16 and Thr17 residues of PLN at normal $(Ca^{2+})_o$

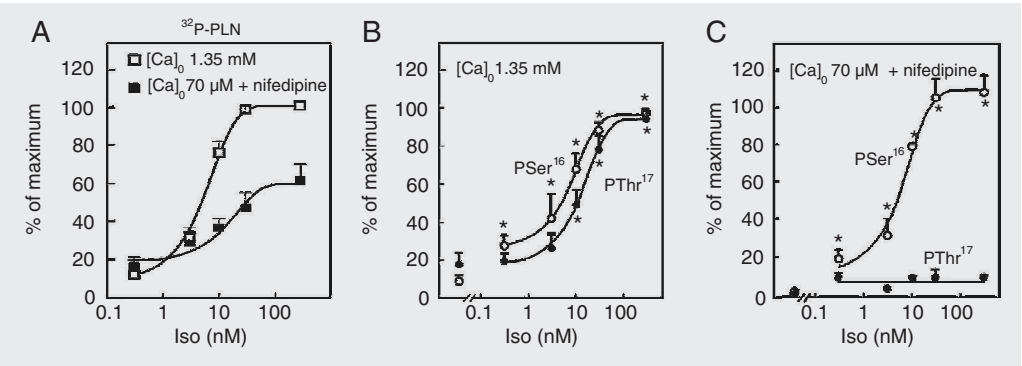

Figure 3. Isoproterenol (Iso) concentration-dependent increase in ^{32}P incorporation into phospholamban (PLN) and in the phosphorylation of Ser16 and Thr17 residues at two different levels of extracellular Ca^{2+}. *A*, Rat hearts were perfused with ^{32}P and then with different isoproterenol concentrations at two different levels of Ca^{2+}. The virtual suppression of Ca^{2+} supply to the heart (70 µM $(Ca)_o$ + 0.4 µM nifedipine) significantly decreased ^{32}P incorporation into PLN (^{32}P-PLN), at 10, 30 and 300 nM Iso, but failed to affect it at 0.3 and 3 nM Iso. Data are reported as percent maximal ^{32}P incorporation into PLN. *B* and *C*, Densitometric analysis of the signal of immunoblots of sarcoplasmic reticulum membrane vesicles isolated from rat hearts perfused with different Iso concentrations at two $(Ca^{2+})_o$, and probed with anti-PSer16-PLN and anti-PThr17-PLN. At 1.35 mM $(Ca)_o$, Iso increased the phosphorylation of both Ser16 and Thr17 (B). Phosphorylation of Thr17 was shifted to the right relative to the increase in the phosphorylation of Ser16. The decrease in $(Ca^{2+})_o$, as shown in panel A, did not significantly affect the phosphorylation of Ser16 but inhibited the increase in the phosphorylation of Thr17 at all Iso concentrations (C). Data are reported as percent of the maximal signal achieved in each experimental series. *P < 0.05 compared to no drug values at normal $(Ca^{2+})_o$ (B) and at low $(Ca^{2+})_o$ (C) (Student *t*-test for unpaired samples). Modified from Ref. 15, with permission.

(Figure 3B). However, when isoproterenol was perfused in the presence of low $(Ca^{2+})_o$, Ser^{16} was the only site to show a concentration-dependent increase in phosphorylation (Figure 3C). At each isoproterenol concentration, Ser^{16} was phosphorylated to a similar extent, independently of the phosphorylation of Thr^{17}. In order to correlate the changes observed at the specific phosphorylation sites of PLN with the mechanical activity of the heart during ß-adrenergic stimulation, Ca^{2+} supply to the cell was decreased so as to cancel the positive inotropic effect of each isoproterenol concentration. Figure 4 shows that this maneuver decreased the phosphorylation of Thr^{17} in association with a significant reduction of the relaxant effect of the ß-agonist at 10 and 30 nM isoproterenol (9,15). However, at 3 nM isoproterenol, decreasing $(Ca^{2+})_o$ had no significant effect on Thr^{17} or Ser^{16} phosphorylation, nor did it modify the relaxant effect of isoproterenol, supporting earlier experiments in which total PLN phosphorylation was evaluated with ^{32}P (15,16). The lack of a contribution by Thr^{17} to the total PLN phosphorylation at the lowest isoproterenol concentrations might be attributed to a modest PKA activity, unable to cause an increase in intracellular Ca^{2+}, sufficient to activate CaMKII, as well as a significant phosphatase inhibition necessary to detect the phosphorylation of the Thr^{17} residue (15), as it will be discussed below.

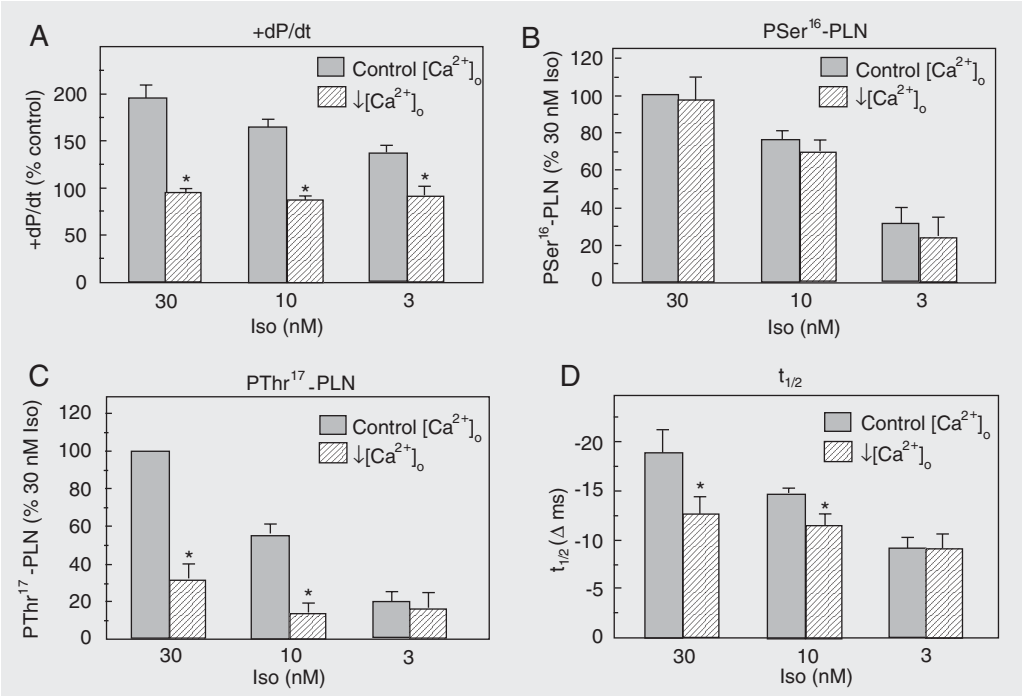

Figure 4. Effects of decreasing $(Ca^{2+})_o$ on mechanical parameters and phospholamban (PLN) phosphorylation sites at different isoproterenol (Iso) concentrations. *A*, Ca^{2+} supply to the heart was decreased so as to abolish the Iso-induced positive inotropic effect at the different concentrations. $(Ca)_o$ was 0.25 mM at 30 and 10 nM Iso and 0.50 mM at 3 nM Iso. Contractility was evaluated by the maximal rate of rise in pressure (+dP/dt). Phosphorylation of Ser^{16} of PLN ($PSer^{16}$-PLN), induced by the different concentrations of Iso, was not affected by the decrease in $(Ca^{2+})_o$ (B); however, this decrease, reduced the phosphorylation of Thr^{17} (C) as well as the isoproterenol-induced relaxant effect (decrease in half relaxation time, $t_{1/2}$) (D), at 30 and 10 nM Iso. At 3 nM Iso the decrease in $(Ca^{2+})_o$ did not affect either the Iso-induced phosphorylation of Thr^{17} or the relaxant effect. +dP/dt is expressed as percentage of control values. $t_{1/2}$ is expressed as difference from control values (Δ ms). Site-specific phosphorylation of PLN is expressed as percent of the signal obtained with 30 nM Iso at 1.35 mM $(Ca)_o$. *P < 0.05 compared to the corresponding Iso concentration at control $(Ca^{2+})_o$ (Student *t*-test for unpaired observations). Modified from Ref. 15, with permission.

Taken together, these findings demonstrate: a) the additive nature of the PKA and CaMKII pathways of PLN phosphorylation, in agreement with *in vitro* results (11); b) that both sites equally contribute to the total PLN phosphorylation at the highest levels of ß-adrenergic stimulation; c) that at low isoproterenol concentrations (≤3 nM), the increase in PLN phosphorylation and the relaxant effect of isoproterenol appear to be exclusively determined by the increase in the phosphorylation of the Ser^{16} residue.

Phosphorylation of Thr^{17} of PLN in the absence of ß-adrenergic stimulation

The conclusion that CaMKII-dependent PLN phosphorylation can only occur in the intact heart in the presence of ß-adrenergic stimulation, i.e., when the cAMP levels within the cell and PKA activity increase (7,12), was in sharp contrast with the independence of both pathways of PLN phosphorylation described in *in vitro* systems (11). Figure 5 shows that the increase in contractility (intracellular Ca^{2+}) produced by increasing extracellular Ca^{2+}, in the presence of the phosphatase inhibitor, okadaic acid, evoked a significant increase in Thr^{17} phosphorylation associated with a relaxant effect, in the absence of any significant increase in cAMP levels or in the phosphorylation of Ser^{16} of PLN (9). These results indicate that the Thr^{17} residue can be phosphorylated independently of Ser^{16} phosphorylation, as was described *in vitro* and further emphasize that phosphatases are as important as kinases in determining the level of phosphorylation of any protein. Accordingly, phosphorylation of the Thr^{17} residue in the intact heart could be detected in two situations: a) in the presence of high extracellular Ca^{2+} (to activate CaMKII) and of okadaic acid (to inhibit PP1), and b) at high intracellular cAMP, which, by activation of PKA, accounts for both effects, i.e., the increase in intracellular Ca^{2+} and the inhibition of PP1.

In the context of the experiments decribed above, it is important to discuss the apparent contradiction between some of the results of the experiments on transgenic mice (13,14) and of phosphorylation experiments conducted on animals with an intact PLN (7-9,15). The failure to find PLN phosphorylation (i.e., Thr^{17} phosphorylation) in transgenic mice in which the Ser^{16} site was mutated to Ala (13) - which would apparently contradict the results of the phosphorylation experiments showing that Thr^{17} can be phosphorylated independently of Ser^{16} phosphorylation (9,22) - must be attributed to the fact that the lack of phosphorylation of the Ser^{16} site under conditions of ß-stimulation precludes the increase in intracellular Ca^{2+} necessary to phosphorylate the Thr^{17} residue. Moreover, the experiments showing that in transgenic mice in which the Thr^{17} site was mutated to Ala, Ser^{16} phosphorylation was sufficient to mediate the maximal response to isoproterenol (14), do not necessarily contradict the results of the phosphorylation experiments which indicate that, when both

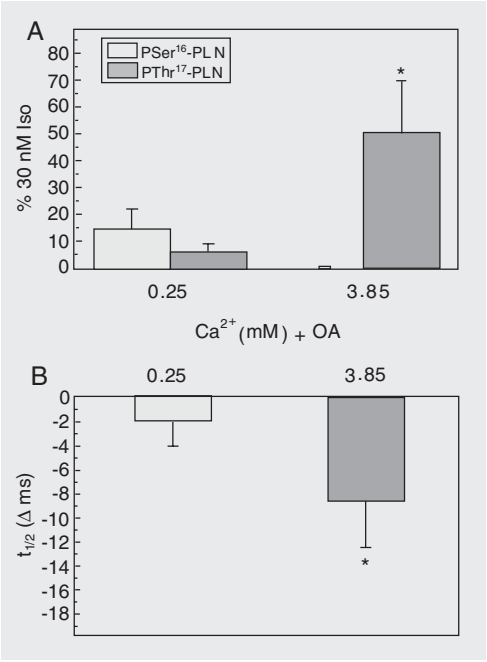

Figure 5. Effects of increasing $(Ca^{2+})_o$ and inhibition of phosphatases on the phosphorylation of Thr^{17} of phospholamban (PLN) and mechanical relaxation. *A*, The increase in $(Ca^{2+})_o$ in the presence of okadaic acid (OA) increased the phosphorylation of Thr^{17} site of PLN. *B*, The increased phosphorylation of the Thr^{17} site was associated with a decrease in half relaxation time ($t_{1/2}$). *P < 0.05 compared to 0.25 mM Ca^{2+} + OA. (Student *t*-test for paired (mechanical) or unpaired (biochemical) data). Modified from Ref. 9, with permission.

sites are present, they both contribute to the total phosphorylation of PLN and to the mechanical effect of ß-adrenoceptor stimulation (7,9,15).

Role of the phosphorylation of Thr^{17} of PLN in pathological conditions

Acidosis

Intracellular acidosis is associated with a decrease in the ability of the heart to generate tension (17). Acidosis produces a rapid decrease in the contraction of cardiac muscle mainly due to a decrease in myofilament Ca^{2+} responsiveness (18) and an impairment of relaxation, which occurs in spite of the decrease in the responsiveness of the contractile proteins and appears to be mainly produced by a direct inhibition of SERCA2a (19). This initial impairment of contractility and relaxation is followed by a spontaneous mechanical recovery. The mechanisms underlying this recovery are poorly understood. Figure 6 shows that phosphorylation of the Thr^{17} site of PLN transiently increases at the onset of acidosis and is associated with a large part of the contractile and relaxation recoveries, most of which occurred within the first 3 min of acidosis. This phosphorylation would provide a mechanism to overcome the direct depressant effect of acidosis on SERCA2a (19). Whereas Ser^{16} was not phosphorylated during the period of acidosis, the Thr^{17} site became dephosphorylated after 5 min of acidosis (20), which would indicate that the phosphorylation of these sites does not contribute to the contractile and relaxation recoveries observed after 5 min of acidosis or that the phosphorylation of the Thr^{17} residue has "memory", triggering a mechanism that persists throughout the period of acidosis. The increase in the phosphorylation of the Thr^{17} site at the beginning of acidosis might be attributed to the increase in intracellular Ca^{2+} that has been shown to occur during acidosis (19), and to the acidosis-induced phosphatase inhibition (21). These two phenomena provide the necessary conditions to increase the phosphorylation of the Thr^{17} site (22). Dephosphorylation of the Thr^{17} residue would occur after the recovery of phosphatase activity due to the return of intracellular pH to values close to control (23). Consistent with the important role of Thr^{17} phosphorylation in the mechanical recovery from acidosis, experiments by DeSantiago et al. (24) showed absence of mechanical recovery in myo-

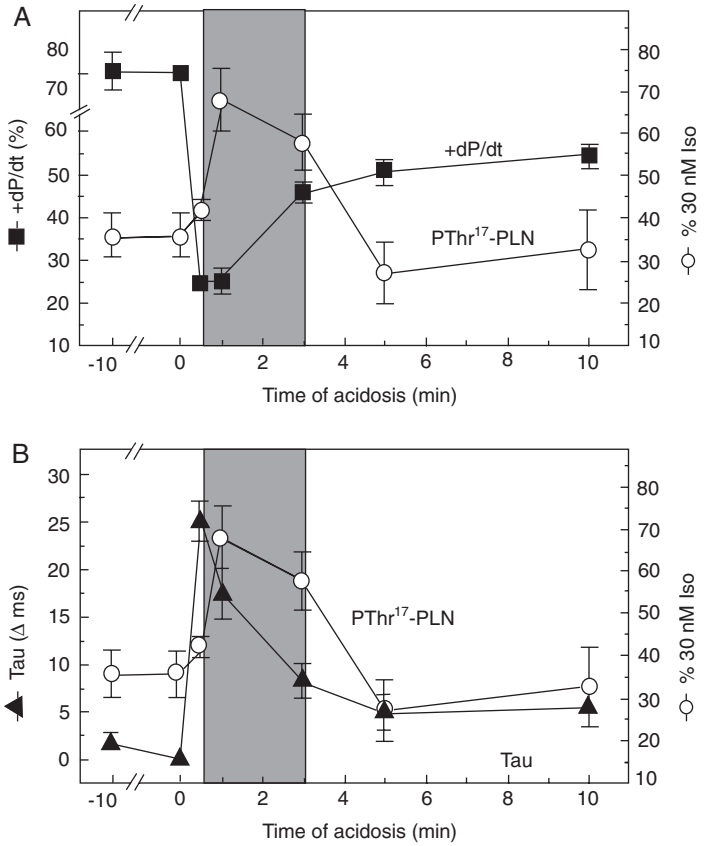

Figure 6. Effect of acidosis on left ventricular function and on the phosphorylation of Thr^{17} of phospholamban (PLN). Hypercapnic acidosis produced an initial (30 s) decrease in contractility (maximal rate of rise in pressure, +dP/dt) (A) and an antirelaxant effect (prolongation of the time constant of left ventricular developed pressure decay, Tau) (B), followed by mechanical recovery, most of which occurred during the first 3 min of acidosis. Phosphorylation of the Thr^{17} site of PLN increased simultaneously with the early recovery of contractility and relaxation (A and B) and then returned to basal levels. Phosphorylation of Ser^{16} remained at basal levels throughout the acidosis period (not shown). Phosphorylation of Thr^{17} is reported as indicated in Figure 4C. +dP/dt is reported as percent of basal values. Tau is reported as Δ ms of basal values. *P < 0.05 compared to basal values (Student *t*-test for paired (mechanical) or unpaired (biochemical) data). Modified from Ref. 20, with permission.

cytes lacking PLN. Finally, a more prominent role of the phosphorylation of PLN residues might be expected *in vivo*. Systemic acidosis is known to increase sympathetic nerve activity (25). This increased sympathetic tone may produce a more persistent phosphorylation of both the Thr17 and Ser16 residues of PLN, with a further contribution to mechanical recovery.

Stunning

Ischemic heart diseases still constitute the leading cause of mortality and morbidity in industrialized countries. Myocardial stunning is recognized as the reversible decrease in myocardial contractility that follows a brief ischemic episode, clinically manifested as sluggish recovery of the pump function

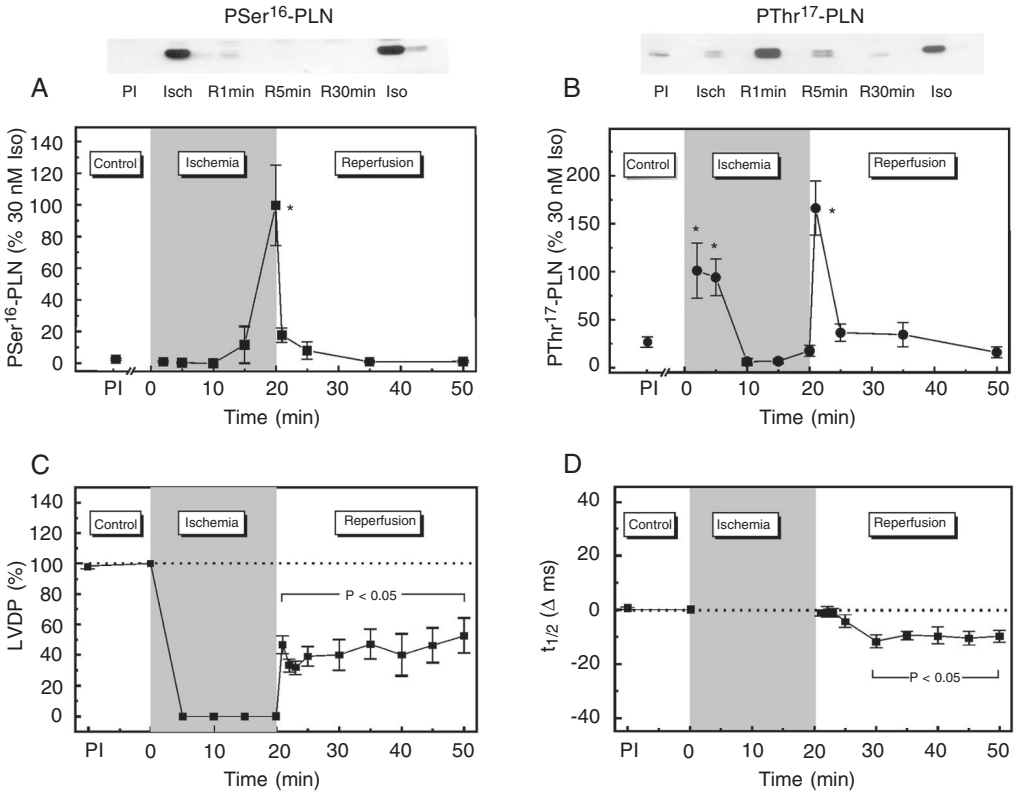

Figure 7. Time course of phospholamban (PLN) Ser16 and Thr17 phosphorylation and of left ventricular function during ischemia and reperfusion. Representative immunoblots (A and B, above) and overall results of densitometric analysis of the signals of inmunoblots (A and B, below) of sarcoplasmic reticulum membrane vesicles isolated from hearts freeze-clamped after 20 min of global ischemia (Isch) and at 1, 5, and 30 min during reperfusion (R1-R30 min). The blots were probed with anti PSer16-PLN (A) and anti PThr17-PLN (B). Phosphorylation of PLN sites induced by 30 nM isoproterenol (Iso) in non-ischemic hearts is shown in the blots for reference. Ser16 phosphorylation significantly increased after 20 min of ischemia and Thr17 phosphorylation transiently increased at 2-5 min of ischemia and at 1 min of reperfusion. Left ventricular developed pressure (LVDP) (C) decreased to virtually non-detectable levels after cessation of flow and reperfusion resulted in a partial recovery of contractility. In spite of the impairment in contractility, $t_{1/2}$ (D) recovered immediately after ischemia and then significantly decreased below pre-ischemic values (PI), indicating an acceleration of ventricular relaxation. Points represent the mean ± SEM of data from 51 hearts freeze-clamped at 20 min of ischemia and 1, 2, 3, 5, 15, and 30 min of reperfusion. LVDP is reported as percent PI and $t_{1/2}$ as Δ ms of PI. Site-specific PLN phosphorylation is reported as shown in previous figures. *P < 0.05 compared to PI (Student *t*-test for paired (mechanical) or unpaired (biochemical) data). Modified from Ref. 35, with permission.

after revascularization (26,27). Although the phenomenon of myocardial stunning was described over 25 years ago (28), the mechanisms responsible for the delayed recovery of contractile function are not completely clear (29). Different types of evidence obtained in rodents and humans indicate that the ultimate cause of the alteration of myocardial contractility is a decrease in myofilament Ca^{2+} responsiveness, since the Ca^{2+} transient is not altered, although contractility is decreased (29). On the other hand, experimental evidence indicates that the function of the SR is altered both in reversible and irreversible ischemia-reperfusion injury (30-34). In particular, in the case of myocardial stunning, a decrease in the activity of SERCA2a and/or in the rate of Ca^{2+} reuptake by the SR has been described in several species (31-34). Thus, an obvious question is: why does the intracellular Ca^{2+} transient remain unaltered in species in which SR function is depressed? A possible explanation for this apparent contradiction is that compensatory mechanisms can overcome the depressed SERCA2a activity. One possible compensatory mechanism is the phosphorylation of PLN. To test this hypothesis, we studied the time course of phosphorylation of PLN residues at different times during ischemia/reperfusion (35). Figure 7 shows the time course of left ventricular developed pressure (LVDP), half relaxation time ($t_{1/2}$) and the phosphorylation of Ser^{16} and Thr^{17} residues of PLN during ischemia and reperfusion. LVDP decreased to virtually undetectable levels after cessation of flow. Reperfusion resulted in a partial recovery of LVDP. In spite of the reduction of contractility, $t_{1/2}$ recovered immediately after ischemia and then significantly decreased below pre-ischemic values, indicating an acceleration of ventricular relaxation. Phosphorylation of Ser^{16} was significantly increased at the end of ischemia, but returned to basal values during the reperfusion period. Phosphorylation of the Thr^{17} residue of PLN transiently increased both at the beginning of ischemia (2-5 min) and at the beginning of reperfusion (1 min), remaining at pre-ischemic values for the rest of the reperfusion period. The decrease in Thr^{17} phosphorylation by CaMKII inhibition, prolonged $t_{1/2}$, which indicates that the phosphorylation of Thr^{17}, when present, attenuates the impaired relaxation that occurs at the beginning of reperfusion (36). Figure 8 shows the time course of contractile and relaxation parameters during ischemia and reperfusion in PLN-WT and PLN-T17A mutant hearts. The recovery of LVDP was significantly lower in PLN-T17A mutant hearts than in PLN-WT hearts throughout the reperfusion period, whereas the time constant of pressure decay, Tau, was significantly prolonged at the beginning of reperfusion and then recovered to values close to control. Additional experiments also showed that the contractile recovery was depressed in PLN-S16A mutant hearts compared to the heart of PLN-WT mice. However, this depression only occurred at the beginning of reperfusion (36). These results indicate that phosphorylation of

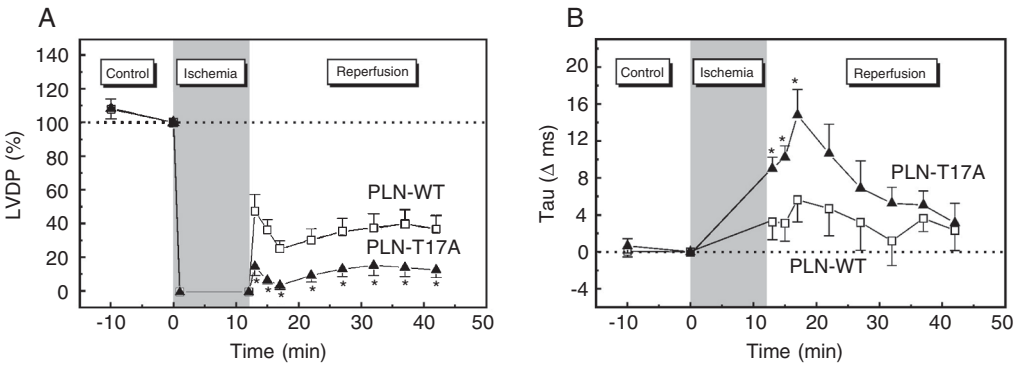

Figure 8. Functional role of Thr^{17} phosphorylation in the ischemia-reperfused mouse heart. Time course of left ventricular developed pressure (LVDP; A) and Tau (B) during ischemia and reperfusion in wild-type phospholamban (PLN-WT) and PLN with a Thr^{17} to Ala mutation (PLN-T17A) mouse hearts. Mutation of Thr^{17} to Ala is associated with diminished contraction and relaxation recoveries after the ischemic insult compared to PLN-WT mouse hearts. *P < 0.05 compared to PLN-WT (Student *t*-test for unpaired observations). Modified from Ref. 36, with permission.

Thr^{17}, although transient, may play a significant role in the critical phase of initial reperfusion, favoring Ca^{2+} re-uptake by the SR. This would tend to compensate for the depression of SERCA2a and would ameliorate Ca^{2+} overload, typical of the beginning of reflow (29). Moreover, these experiments indicate that activation of SERCA2a by any other means, at this crucial moment of reperfusion, may contribute significantly to Ca^{2+} handling. In agreement with this view, recent experiments suggested that the cardioprotection produced by cGMP-mediated stimuli in reoxygenated cells is associated with an increase in the phosphorylation of Ser^{16} of PLN (37).

Conclusions

Taken together, these findings suggest that the Thr^{17} site of PLN has an important role, not only under physiological conditions, such as ß-adrenoceptor stimulation, but also in the mechanical recovery under some pathological conditions such as acidosis and stunning. An interesting point is that, although the phosphorylation experiments reveal that the phosphorylation of Thr^{17} is transient and occurs only at the beginning of both acidosis and reperfusion, the presence of this residue and/or of an intact PLN seems to be necessary for mechanical recovery during the reperfusion or acidosis period.

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