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# Phospholamban phosphorylation by CaMKII under pathophysiological conditions

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## 1. ABSTRACT

Sarcoplasmic reticulum (SR) $Ca^{2+}$ ATPase (SERCA2a) transports $Ca^{2+}$ into the SR, decreasing the cytosolic $Ca^{2+}$ during relaxation and increasing the SR $Ca^{2+}$ available for contraction. SERCA2a activity is regulated by phosphorylation of another SR protein: Phospholamban (PLN). Dephosphorylated PLN inhibits SERCA2a. Phosphorylation of PLN by either cAMP or cGMP-dependent protein kinase at Ser16 or the $Ca^{2+}$-calmodulin-dependent protein kinase (CaMKII), at Thr17, relieves this inhibition, increasing SR $Ca^{2+}$ uptake and SR $Ca^{2+}$ load. Thus, PLN is a major player in the regulation of myocardial relaxation and contractility. This review will examine the main aspects of the role of CaMKII and Thr17 site of PLN, on different pathophysiological conditions: acidosis, ischemia/reperfusion (I/R) and heart failure (HF). Whereas CaMKII-activation and PLN phosphorylation contribute to the functional recovery during acidosis and stunning, CaMKII results detrimental in the irreversible I/R injury, producing apoptosis and necrosis. Phosphorylation of Thr17 residue of PLN and CaMKII activity vary in the different models of HF. The possible role of these changes in the depressed cardiac function of HF will be discussed.

## 2. PHOSPHOLAMBAN: A CENTRAL PLAYER IN THE EXCITATION-CONTRACTION-COUPLING AND RELAXATION CYCLE

In cardiomyocytes, contraction is coupled to electrical impulse by a $Ca^{2+}$-induced- $Ca^{2+}$-release mechanism (1): Upon depolarization, rapid $Ca^{2+}$ influx through the L-type $Ca^{2+}$ channels triggers the release of a much larger amount of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) into the cytosol, via the $Ca^{2+}$ release channel (ryanodine receptor type 2 (RYR2)), to produce contraction. Uptake of $Ca^{2+}$ by the SR $Ca^{2+}$-ATPase (SERCA2a) and extrusion of $Ca^{2+}$ out of the cell by the forward mode of the Na-$Ca^{2+}$ exchanger (NCX), results in cardiac relaxation. SERCA2a is thus the enzyme responsible for actively transporting $Ca^{2+}$ into the lumen of the SR at the expense of adenosine triphosphate (ATP) hydrolysis (2). By this action, SERCA2a is not only the principal mean by which cytoplasmic $Ca^{2+}$ is lowered during relaxation (in the human heart is responsible for 60-65% of cytoplasmic $Ca^{2+}$ lowering at basal stimulation rate (1-1.5 Hz) (3)), but also the main responsible of the SR $Ca^{2+}$ available for release in the next beat, which is crucial in determining contractility. Figure 1 depicts a scheme of the excitation- contraction-relaxation mechanism.

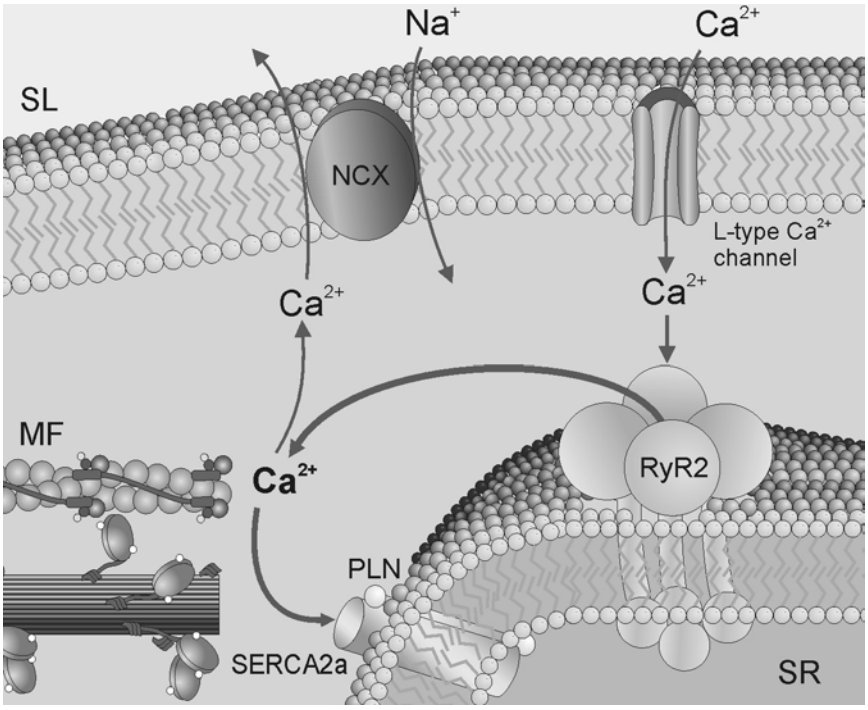


**Figure 1.** Excitation-contraction coupling scheme. A small $Ca^{2+}$ influx through the L-type $Ca^{2+}$ channels initiates the release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) through the SR $Ca^{2+}$-channel (RyR2). The released $Ca^{2+}$ then binds to the myofilaments (MF) which induces myocardial contraction. Relaxation is initiated by dissociation of $Ca^{2+}$ from the myofilaments, followed by its reuptake into the SR through the PLN-regulated $Ca^{2+}$-ATPase (SERCA2a) and the trans-sarcolemmal removal through the $Na^{+}/Ca^{2+}$ exchanger (NCX) operating in the forward mode.

SERCA2a activity is regulated by the phosphorylation state of another SR membrane protein, named phospholamban (PLN). The cloning of PLN revealed that it is a 52-aminoacid SR membrane protein of 6.1 kDa that forms a homopentamer, which accounts for the original observation that PLN has an apparent mass of 22 kDa (4). Experimental evidence suggested that the protein is organized in three different domains: two cytosolic domains: **Ia** (aminoacids 1-20) and **Ib** (aminoacids 21-30), and a transmembrane domain, **II** (aminoacids 31-52). Domain **Ia** contains serine (Ser) 16 residue, phosphorylated by the cAMP and cGMP-dependent protein kinase (PKA and PKG, respectively), and threonine (Thr) 17 residue, phosphorylated by the $Ca^{2+}$-calmodulin-dependent protein kinase (CaMKII). James *et al.* (5) provided the first clear evidence for a physical interaction between PLN and SERCA2a (Figure 2A). In the dephosphorylated state PLN binds to SERCA2a at resting $Ca^{2+}$ concentrations and inhibits $Ca^{2+}$- pump activity by lowering its apparent $Ca^{2+}$ affinity with little effect on Vmax at saturating $Ca^{2+}$ and ATP concentrations (6). Phosphorylation of PLN alters the SERCA2a-PLN interaction, relieving $Ca^{2+}$- pump inhibition and enhancing SR $Ca^{2+}$ uptake, relaxation rate and myocardial contractility. The mechanism by which SERCA2a transports $Ca^{2+}$ into the lumen of the SR has been proposed to have several interconvertible steps (Figure 2B). E1, the high $Ca^{2+}$ affinity state of SERCA2a, binds 2 $Ca^{2+}$ ions and upon binding of ATP, the enzyme is phosphorylated and undergoes a change in conformation so that $Ca^{2+}$ ions are occluded ($E1{\sim}P.Ca_2$). The phosphorylation also causes the transition to $E2{\sim}P.Ca_2$, a state in which the $Ca^{2+}$ binding sites are of low affinity, allowing the release of $Ca^{2+}$ into the lumen of the SR. The pump is then dephosphorylated and recycled into E1. Recent experiments by Chen *et al.*, (7), presented evidence to support the idea that binding of PLN and $Ca^{2+}$ to SERCA2a is mutually exclusive. PLN binds to the E2 conformation of SERCA2a, whereas $Ca^{2+}$ binds to the E1 conformation. These experiments further indicated that binding of PLN to the E2 conformation of the enzyme in the presence of ATP, physically blocks the transition from E2 to E1. For the kinetic cycle to proceed, PLN must dissociate from SERCA2a to allow E1 formation, the high $Ca^{2+}$ affinity conformation that actively hydrolyzes ATP. Therefore, when the enzyme is actively transporting $Ca^{2+}$, this competition between PLN and $Ca^{2+}$ for binding to E2 and E1 respectively, is manifested as a decrease in the apparent affinity of SERCA2a for $Ca^{2+}$, as suggested by earlier kinetics experiments (6). Either the phosphorylation of PLN or the conformational changes in SERCA2a that accompany $Ca^{2+}$ binding and progression from the E2 to the $E1.2Ca^{2+}$ state, dissociate the inhibited PLN-SERCA2a heterodimer, partially restoring $Ca^{2+}$ affinity and SERCA2a activity (7,8) (Figure 2A). It has been shown that although phosphorylation of PLN dissociates *functional* interaction, it is less effective than $Ca^{2+}$ binding to SERCA2a in breaking up physical interactions (9). Figure 2A also shows that in the SR

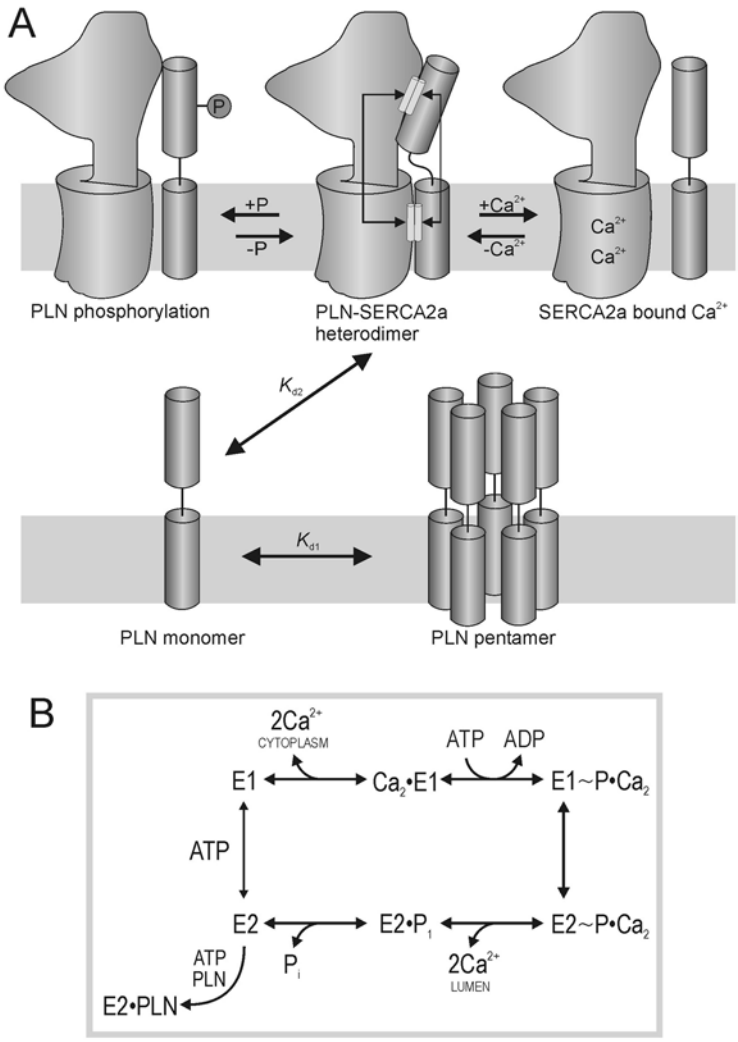


**Figure 2.** A. PLN-SERCA2a Interaction. Regulation of SERCA2a by PLN could be divided into two distinct steps. First (below) the association /dissociation of pentameric PLN ($K_{d1}$) and second (above), the association /dissociation of monomeric PLN and SERCA2a ($K_{d2}$). Monomeric PLN is believed to be the form that produces SERCA2a inhibition. Either the phosphorylation of PLN or the $Ca^{2+}$ binding to SERCA2a, produce the dissociation of PLN-SERCA2a complex, with the consequent SERCA2a activation (Reproduced with permission from, MacLennan and Kranias (8)). B. $Ca^{2+}$ transport by SERCA2a. E1 and E2 represent the high and the low $Ca^{2+}$ affinity conformation of the SERCA2a respectively. Upon $Ca^{2+}$ binding to E1, ATP is hydrolyzed and the phosphoenzyme is formed stimulating the transition from E1 to E2. Subsequently $Ca^{2+}$ is released to the lumen of the SR and the SERCA2a is dephosphorylated and recycled to the E1 conformation. PLN binds to the E2 conformation of the enzyme.

membrane, PLN is in dynamic equilibrium between monomeric and pentameric forms (10), but it is probably the monomeric form the one that interacts with and regulates SERCA2a, whereas the pentameric form may act as an inactive or less active reservoir (8, 11).

The use of gene knockout and transgenic mouse models, in which the expression levels of PLN has been ablated, reduced or increased, constituted a crucial step in the recognition of the role of PLN in the regulation of myocardial contractility and relaxation (12-16). Ablation of PLN produced an enhanced contractility and relaxation. This hypercontractile function of PLN-deficient hearts was associated with increases in the apparent affinity of SERCA2a for $Ca^{2+}$ and in the intraluminal SR $Ca^{2+}$ content (12). In contrast, overexpression of PLN was associated with a decreased apparent affinity of SERCA2a for $Ca^{2+}$ and depressed cardiac contractile performance (15). PLN-heterozygous hearts, expressing reduced protein levels of PLN, further support that the ratio SERCA2a-PLN plays a prominent role in regulating SR function and contractility (16).

In addition to the relationship of SERCA2a-PLN, myocardial contractility and relaxation are also dependent on the degree of PLN phosphorylation. Phosphorylation of PLN by PKA and CaMKII pathways (Ser16 and Thr17 residues, respectively), is the main mediator of the cardiac

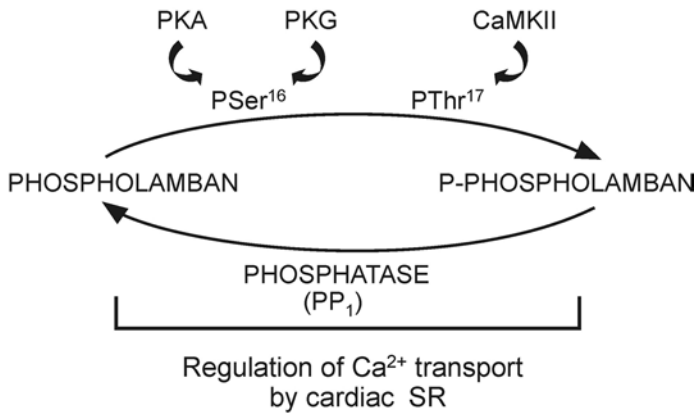


**Figure 3.** PLN phosphorylation. PLN can be phosphorylated *in vivo* by PKA and cGMP-dependent protein kinase (PKG) at the Ser16 site and by CaMKII at the Thr17 site. Phosphorylation of PLN is also dependent on the activity of PP1, the phosphatase that dephosphorylates the protein. The degree of PLN phosphorylation regulates $Ca^{2+}$ transport into the lumen of the SR.

effects of beta-adrenergic stimulation in cardiac muscle (17-27). As mentioned above, phosphorylation of PLN increases SERCA2a activity and $Ca^{2+}$ uptake rate which in turn increase the velocity of relaxation, SR $Ca^{2+}$ load and, as a consequence, SR $Ca^{2+}$ release and myocardial contractility (17-27). The status of phosphorylation of PLN, is also dependent on the activity of the type 1 phosphatase (PP1), the major SR-phosphatase that specifically dephosphorylates PLN (28) (Figure 3). The activity of PP1 is also under the control of different kinases and phosphatases (29, 30). This phosphatase regulatory cascade, frequently overlooked when considering the regulation of PLN phosphorylation sites, is crucial in determining the status of PLN phosphorylation. Supporting the important role of phosphatases, a recent report has stressed the involvement of PP1 in determining the level of phosphorylation of CaMKII and CaMKII substrates (31).

In this article, we will particularly discuss the role of CaMKII and specifically that of Thr17 phosphorylation of PLN under pathophysiological processes.

## 3. CaMKII. STRUCTURE AND REGULATION

CaMKII is a Ser/Thr protein kinase that is a major downstream effector of the $Ca^{2+}$ signaling in the heart as well as in other tissues. CaMKII is encoded by 4 closely related genes, alpha, beta, gamma and delta (32). The delta isoform predominates in the heart (33) and a splice variant, delta c, is localized to the cytosol and phosphorylates several important $Ca^{2+}$ regulatory proteins as it will be described below. All members of the CaMKII family share a similar domain organization: a catalytic domain at the amino-terminal followed by a regulatory region and a region responsible for assembly of the 8-12 subunits that compose the holoenzyme, at the carboxy-terminal (Figure 4 A and B). The regulatory domain interacts and inhibits the catalytic domain, and it is also the site for calmodulin (CaM) binding. A rise in intracellular $Ca^{2+}$ concentration leads to the binding of $Ca^{2+}$ ions to CaM which binds to the CaM binding site and relieves the autoinhibition, allowing substrates and ATP to gain access to the catalytic domain. Moreover, binding of $Ca^{2+}$-CaM also initiates autophosphorylation of CaMKII, providing additional layers of regulation. The first autophosphorylation site identified was Thr287 (Thr286 for the alpha isoform of CaMKII). This phosphorylation occurs as an inter-subunit reaction in the holoenzyme and requires the simultaneous $Ca^{2+}$-CaM binding to adjacent subunits. CaMKII autophosphorylation, although not essential for the activity of the kinase, enhances its avidity for $Ca^{2+}$-CaM and confers the kinase autonomous activity, independent of $Ca^{2+}{}_i$ (32) (Figure 4 C). Thus, transient elevation of $Ca^{2+}{}_i$ can give a prolonged response through the constitutive activity of autophosphorylated CaMKII, conferring the enzyme the unique property of *memory*. The activity of CaMKII is also greatly dependent on various other factors. Particularly, the activity of phosphatases that dephosphorylate CaMKII is of primary importance. Four species of protein phosphatases, PP1, PP2A, PP2C and a novel family of specific CaMKII phosphatases, have been reported to dephosphorylate and negatively regulate CaMKII (34). Recent results by Huke and Bers (31), in intact cardiomyocytes, identified PP1 as the major phosphatase that dephosphorylates CaMKII and were consistent with the idea that phosphatases prevent sustained CaMKII autophosphorylation and thereby severely limit the $Ca^{2+}$/CaM-independent CaMKII activity and CaMKII-dependent protein phosphorylation. The activity of CaMKII is further regulated by targeting to specific compartments. Targeting confers the kinase not only substrate specificity but also allows CaMKII to be in close proximity to the $Ca^{2+}$ signal to which the kinase is intended to respond and determines the type or level of phosphatases to which it is exposed (35). In cardiomyocytes, CaMKII has been found to be enriched in the T tubules, and it colocalizes with the RyR2 and the L-type $Ca^{2+}$ channels, two of its substrates (36-38). In the longitudinal SR, CaMKII may regulate SERCA2a through either direct phosphorylation (39,40) and/or the phosphorylation of its regulatory protein PLN (41). Moreover it has been shown, that the association of CaMKII with different subcellular compartments in neurons may be dynamically regulated, and translocation of the enzyme has been described (42). Finally, CaMKII

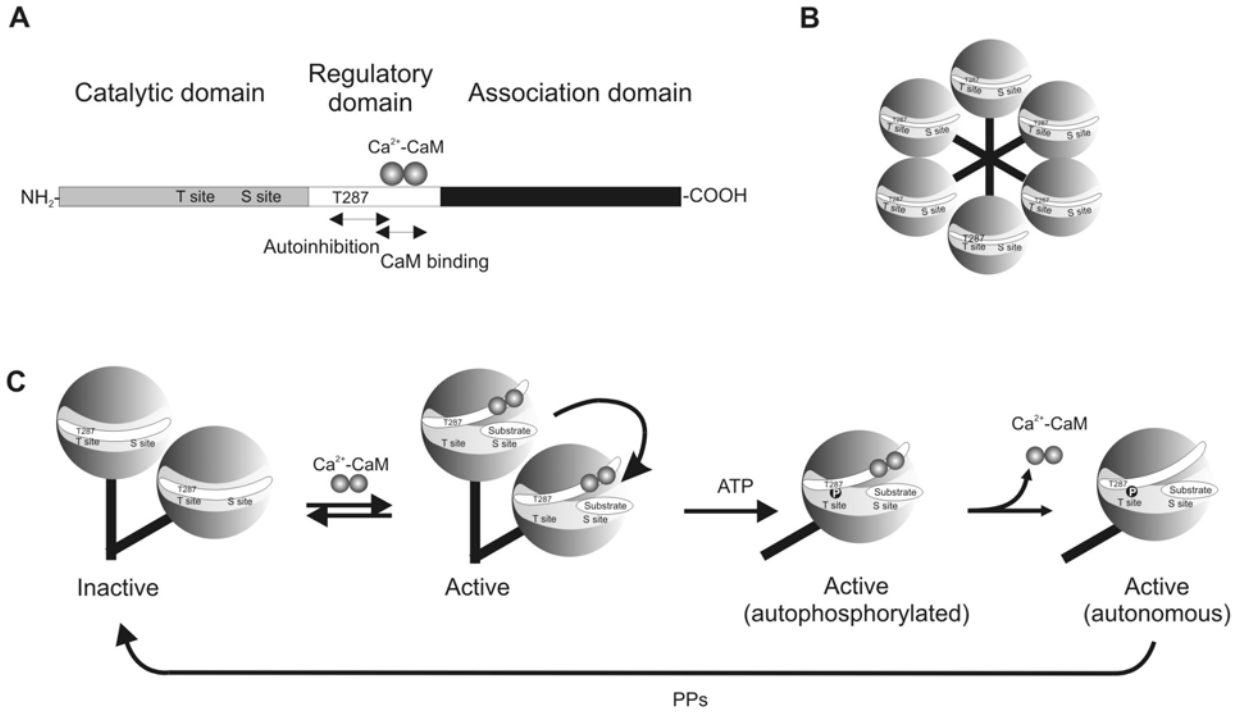


**Figure 4.** A. Schematic diagram of CaMKII domain structure. CaMKII contains a N-terminal catalytic domain, an internal regulatory domain, and a C-terminal association domain. The catalytic domain contains the target binding site (T site) and the substrate binding site (S site). The regulatory domain contains at the proximal end, residues that interact with the catalytic domain (autoinhibitory region) and at the distal portion, the binding site to the $Ca^{2+}$-calmodulin complex ($Ca^{2+}$-CaM). Thr287 (T287), a residue susceptible of being phosphorylated, is within the autoinhibitory region. B. Oligomeric organization of CaMKII monomers. Association domains among monomers assemble into dodecameric holoenzymes by forming the center of a stacked pair of hexameric rings, projecting the regulatory and catalytic domains outward. C. Regulation of CaMKII. In the inactive state, T site and S site of the catalytic domain are blocked by the autoinhibitory domain of the regulatory region. Exposition to $Ca^{2+}$-CaM relieves this inhibition and activates the kinase for substrate phosphorylation. $Ca^{2+}$-CaM binding to adjacent subunits stimulates intersubunit phosphorylation at Thr287. This autophosphorylation resulted in an enzyme that remains active even as $Ca^{2+}$ falls in the cell. Activation of phosphatases (PPs) is required to reset the kinase to its basal state. (Reproduced with permission from, Hudmon and Schulman, (32)).

undergoes association between holoenzymes ("self-association"). This clustering of cytoplasmic CaMKII into compact aggregates has been described in neurons under sustained $Ca^{2+}_i$ elevation and it is favored by decreased levels of ATP and acidic pH (43,44). Self-association between holoenzymes would severely restrict CaMKII targeting and in consequence its access to substrates, offering a cellular defense mechanism that would limit CaMKII activation during $Ca^{2+}$ overload (43,44). It is unknown whether this mechanism is also present in the cardiomyocytes.

## 4. ROLE OF CaMKII-DEPENDENT PHOSPHORYLATION OF PLN UNDER PHYSIOLOGICAL CONDITIONS

### 4.1. Is Thr17 site of PLN phosphorylated under beta-adrenoceptor stimulation?

Although this review is mainly directed to describe the role of CaMKII-dependent phosphorylation of PLN in pathophysiological conditions, it is important to describe first the role of this phosphorylation in physiological conditions, for a better comprehension of the pathological findings.

Cardiac function is regulated on a beat-to-beat basis through the sympathetic nervous system. Beta-agonists (mainly adrenaline and noradrenaline) initiate a signaling cascade in the heart by binding to and activating the beta-receptors at the cell membrane level. The signal proceeds through $G_s$ proteins to stimulate the adenilyl cyclase and the formation of cyclic AMP, which in turn increases the activity of PKA. Activation of PKA phosphorylates several key proteins of the excitation-contraction-coupling-relaxation mechanism, among which is PLN. By phosphoaminoacid analysis and protein sequencing, Wegener *et al.* (19), were the first to present evidence that there were actually two sites in PLN phosphorylated during beta-adrenergic stimulation, Ser16 (phosphorylated by PKA) and Thr17 (phosphorylated by CaMKII). Isoproterenol-induced phosphorylation of PLN by PKA and CaMKII activation was also observed by using

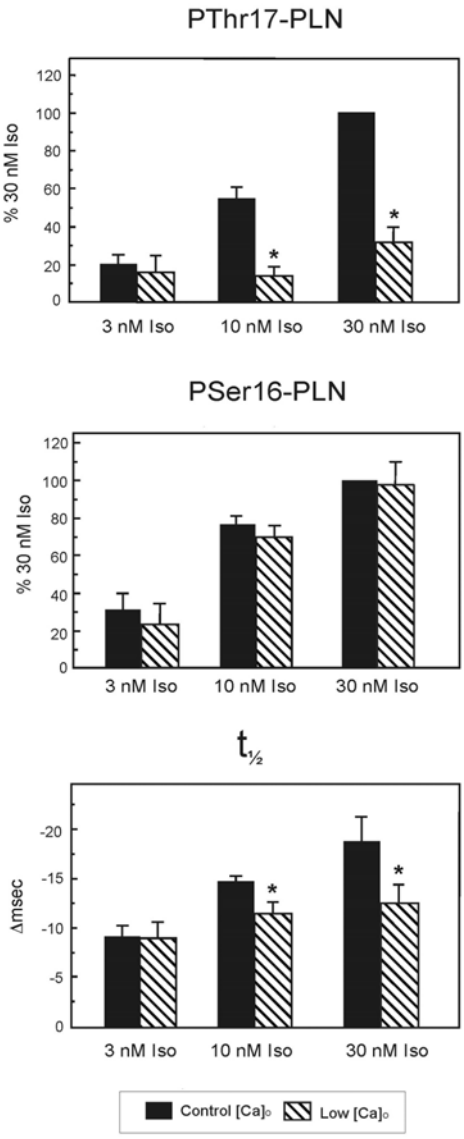


**Figure 5.** Phosphorylation of Ser16 and Thr17 of PLN by beta-adrenoceptor stimulation. Phosphorylation of PLN sites at three different isoproterenol concentrations, in the absence (Control $(Ca)_o$) and the presence of low extracellular $Ca^{2+}$ (Low $(Ca)_o$). It is generally accepted that beta-adrenoceptor stimulation, by increasing PKA activity, produces the phosphorylation of the L-type $Ca^{2+}$channel and Ser16 site of PLN, with the consequent increase in intracellular $Ca^{2+}$ and activation of CaMKII. CaMKII in turn would phosphorylate Thr17 site of PLN. Thus, the maneuver of decreasing extracellular $Ca^{2+}$, allows to dissect the contribution of the phosphorylation of each site to the total phosphorylation of PLN at the different isoproterenol concentrations. At the lowest isoproterenol concentration (3 nM) PLN is exclusively phosphorylated by PKA, whereas at highest isoproterenol concentrations (10 and 30 nM), both sites equally contribute to the total PLN phosphorylation and are both responsible for the relaxant effect of beta-agonists. * $P < 0.05$ with respect to Control $(Ca)_o$ (Reproduced with permission from, Said *et al.*, (27)).

different techniques, i.e. back phosphorylation, $^{32}P$ incorporation into PLN and more recently, specific immunodetection of phosphorylated PLN phosphorylation sites (See below) (18, 21-27). It is generally accepted that the PKA-dependent phosphorylation of different $Ca^{2+}$ handling proteins, like the L-type $Ca^{2+}$ channels and the mentioned Ser16 site of PLN, by increasing intracellular $Ca^{2+}$ would activate CaMKII and provoke CaMKII-dependent phosphorylations, among which is Thr17 site of PLN. Results in transgenic mice expressing either PLN-S16A (in which Ser16 was mutated to Ala), support this idea. These experiments demonstrated that the phosphorylation of Ser16 of PLN is a prerequisite for the phosphorylation of Thr17 (45). The experiments in transgenic animals further show that in PLN-T17A (in which Thr17 site was mutated to Ala), phosphorylation of Ser16 was sufficient for mediating the maximal cardiac responses to beta-adrenergic stimulation (46). These experiments suggested a predominant role of the phosphorylation of Ser16 over that of Thr17, in the mechanical effect produced by beta-adrenergic stimulation. The combination of phosphorylation site specific antibodies with quantification of $^{32}P$ incorporation into PLN, further helped to clarify the relative role of Ser16 and Thr17 phosphorylation (23,27). Perfusion with different isoproterenol concentrations in the presence of extremely low extracellular $Ca^{2+}$ plus nifedipine, to avoid $Ca^{2+}$ entry to the cell, decreased $^{32}P$ incorporation into PLN at the highest but not at the lowest levels of beta-adrenergic stimulation. These results suggested a contribution of the CaMKII pathways to the total PLN phosphorylation at the highest isoproterenol concentrations (22,27). Immunodetection of site-specific phosphorylated PLN fully confirmed this suggestion (Figure 5), further indicating that the phosphorylation of Thr17 accounted for approximately 50 % of the total PLN phosphorylation at the highest isoproterenol concentrations (equal to or higher than 10 nM). This CaMKII–induced phosphorylation was closely associated with an increase in the relaxant effect of beta-agonists (27). In line with these previous findings, Kuschel *et al.* (25) and Bartel *et al.* (26), demonstrated that the dose-response curve of Thr17 phosphorylation to isoproterenol was shifted to the right, compared to that of Ser16 phosphorylation, clearly indicating that Ser16 was the only phosphorylated site at the lowest isoproterenol concentrations. The lack of contribution of Thr17 to the total PLN phosphorylation, at the lowest isoproterenol concentrations, may be attributed to the modest increase in PKA – induced increase in intracellular $Ca^{2+}$, not enough to activate CaMKII and phosphorylate Thr17 site. This small increase in PKA activity would also fail to significantly inhibit the phosphatases that dephosphorylate PLN (30), further favoring the dephosphorylated state of Thr17 residue. Taken together, these findings demonstrated the additive nature of PKA and CaMKII pathways of PLN phosphorylation, in agreement with the *in vitro* results (41). In addition, they indicated that: a) in the absence of a phosphorylatable Thr17 site, Ser16 is sufficient for mediating the maximal cardiac responses to beta-adrenergic stimulation (46); b) when both sites are present, they equally contribute to the total PLN phosphorylation at the

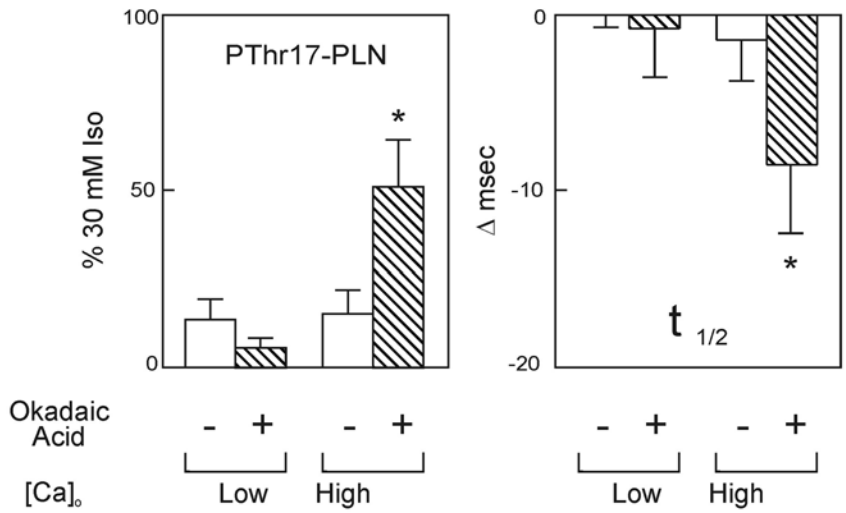


**Figure 6.** Phosphorylation of Thr17 of PLN in the absence of beta-adrenoceptor stimulation. Increasing $(Ca)_o$ in the presence of the specific PP1 inhibitor okadaic acid, enhances Thr17 phosphorylation and accelerates relaxation ($t_{1/2}$) in the absence of beta-adrenoceptor stimulation. * $P < 0.05$ with respect to the absence of okadaic acid. (Reproduced with permission from, Mundiña-Weilenmann *et al.*, (23)).

highest levels of beta-adrenergic stimulation (23), c) at low isoproterenol concentrations (lower than 3 nM), the increase in PLN phosphorylation (and the relaxant effect of isoproterenol), is exclusively determined by the increase in the phosphorylation of Ser16 residue (23,25-27).

**4.2. Can Thr17 of PLN be phosphorylated in the absence of PKA activation under beta-adrenergic stimulation?**

Implicit in the interpretation of the above results is the concept that activation of PKA after beta-adrenoceptor stimulation is necessary for the activation of CaMKII and the phosphorylation of Thr17 site of PLN. This generally accepted view was recently challenged by the demonstration that CaMKII phosphorylation of the RyR2 can occur in the absence of PKA activation. Pereira *et al.* (47), suggest that CaMKII activation after beta-adrenoceptor stimulation could be accomplished through the cAMP binding protein Epac. Epac1 and Epac2 are guanine nucleotide exchange factors which might explain cAMP-dependent activation of the small GTPase Rap1, which was insensitive to PKA inhibition (48). Pereira *et al.* (47), showed that Epac stimulation increased CaMKII activity and CaMKII-dependent phosphorylation of RyR2. Moreover, Curran *et al.*, (49) also showed a CaMKII-dependent phosphorylation of RyR2 which appears to be independent not only on PKA activity but also on the increase in cAMP. In these experiments, stimulation with forskolin to directly stimulate adenilyl cyclase, bypassing the beta-receptor, failed to produce the CaMKII-dependent phosphorylation of RyR2 at Ser2815 site. It is not known whether a pathway similar to that proposed for RyR2 phosphorylation might also work for the CaMKII-dependent phosphorylation of Thr17 of PLN during beta-adrenoceptor stimulation. However, the above mentioned results obtained in PLN-S16A, showing that the PKA-dependent phosphorylation of Ser16 was a prerequisite for the phosphorylation of Thr17 site of PLN (45), argues against this possibility. Supporting a role of cAMP and PKA in the CaMKII-dependent phosphorylations of PLN and RyR2, recent studies indicated that stimulation with forskolin and inhibition of PKA by H-89 during beta-adrenoceptor stimulation, produced an increase and decrease respectively, of the CaMKII-dependent phosphorylation of these two proteins (50). Further experiments are needed to clarify these controversial results.

**4.3. Can Thr17 of PLN be phosphorylated in the absence of beta-adrenergic stimulation?**

The conclusion that CaMKII-dependent PLN phosphorylation can only occur in the intact heart in the presence of beta-adrenergic stimulation, i.e. when the cAMP levels within the cell and PKA activity increase (18), was in sharp contrast with the independence of both pathways of PLN phosphorylation, described in the *in vitro* systems (41). Figure 6 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 Thr17 phosphorylation associated with a relaxant effect, in the absence of any significant increase in cAMP levels and in the phosphorylation of Ser16 of PLN (23). These results indicate that Thr17 residue can be phosphorylated independently of Ser16 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. Therefore, phosphorylation of Thr17 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, is able to account for both effects, i.e. the increase in intracellular $Ca^{2+}$ and the inhibition of PP1.

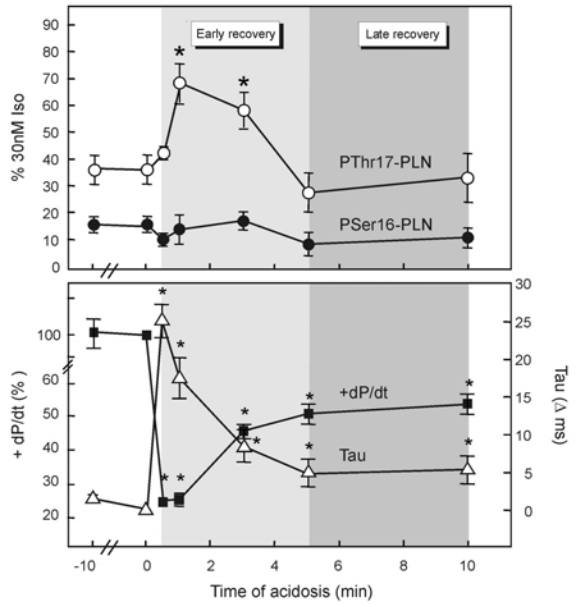


**Figure 7.** Time course of phosphorylation of Ser 16 and Thr17 residues of PLN during *respiratory* acidosis. Increasing the $PCO_2$ of the medium to produce respiratory acidosis, evokes an increase in the phosphorylation of Thr17 site of PLN in the absence of significant changes in the phosphorylation of Ser16 site. The increase in Thr17 phosphorylation is associated with the greatest portion of the contractile (+dP/dt) and relaxation (Tau) recoveries that occur after the initial acidosis-induced depression. * $P < 0.05$ with respect to the basal values (Reproduced with permission from, Mundiña-Weilenmann *et al*, (29).

De Koninck and Schulman (51) elegantly showed that CaMKII can decode the frequency of $Ca^{2+}$ spikes into distinct amounts of kinase activity. Thus, the increase in contraction frequency, one of the fundamental physiological modulators of myocardial performance, would produce a sustained increase in CaMKII, which might lead to the phosphorylation of Thr17 of PLN, without the requisite of phosphatase inhibition. In agreement with this prediction, an increase in Thr17 phosphorylation in the absence of beta-adrenoceptor stimulation was observed in isolated myocytes after increasing stimulation frequency (52,53). Hagemann *et al.* (52) showed that this increase in Thr17 phosphorylation closely correlated with the frequency-dependent acceleration of relaxation (FDAR) (54). Valverde *et al.* (53) also found a good correlation between the phosphorylation of Thr17 and FDAR in isolated cat myocytes. However, they further demonstrated that FDAR actually preceded the increase in the phosphorylation of Thr17 evoked by increasing frequency (53), strongly arguing against a causal link between the phosphorylation of Thr17 and FDAR. Supporting this conclusion are the experiments by DeSantiago *et al.* in PLN-KO mice (55), indicating that FDAR does not require PLN and the finding that the stimulation-frequency induced increase in Thr17 site could not be detected in the perfused heart, in spite of the presence of FDAR (53). Overall, these "stimulation-frequency" results reveal a situation in which the phosphorylation of Thr17 of PLN can occur in the absence of beta-adrenergic stimulation, at least in isolated myocytes. Unexpectedly, this phosphorylation seems not to participate in FDAR.

## 5. ROLE OF CaMKII-DEPENDENT PHOSPHORYLATION OF PLN UNDER PATHOPHYSIOLOGICAL CONDITIONS

### 5.1. Acidosis

It has been known for over a century, that intracellular acidosis is associated with a decrease in the ability of the heart to generate tension (56). Acidosis produces a rapid decrease in the contraction of cardiac muscle, which is largely due to a decrease in myofilament $Ca^{2+}$ responsiveness (57,58), although a decrease in intracellular $Ca^{2+}$ transient due to inhibition of $Ca^{2+}$ release from the SR during this period may also occur (59). Moreover, the decrease in contractility is associated with an impairment of relaxation, that takes place in spite of the decrease in the responsiveness of the contractile proteins (which would tend to produce the opposite effect on relaxation), and appears to be mainly evoked by a direct inhibition of SERCA2a (57,60). This initial impairment of contractility and relaxation is followed by a spontaneous mechanical recovery which occurs despite the persistent acidosis (29,59,61-70). Our understanding of the mechanisms of this recovery remains incomplete. Earlier experiments indicate that the mechanical recovery was mainly produced by an increase in intracellular $Ca^{2+}$. It was proposed that the acidosis-induced activation of the $Na^+$-$H^+$ exchanger (NHE) by leading to high cytosolic $Na^+$ would either slow the forward or activate the reverse mode of the NCX. The increase in intracellular $Ca^{2+}$ evoked by this mechanism would be sufficient to overcome the inhibitory effect of acidosis on SERCA2a (57,60), and increase SR $Ca^{2+}$ load. In recent years various laboratories showed evidence supporting a crucial role of the activation of CaMKII in this recovery (29,69-72), i.e. it was shown that the recovery did not occur in the presence of CaMKII-inhibition (29,69,70,72). These experiments suggested that downstream in the cascade of events involved in the contractile recovery from acidosis must be a phosphorylation dependent on CaMKII activation. The more likely candidate and the first one explored was Thr17 site of PLN. Figure 7 shows that phosphorylation of Thr17 site of PLN transiently increased at the onset of acidosis and is associated with a great 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 (73). Thr17 site became dephosphorylated after 5 min of acidosis (29), which would indicate that the phosphorylation of this site either does not contribute to the contractile and relaxation recoveries observed after 5 min of acidosis or that the phosphorylation of Thr17 residue has *memory*, triggering a mechanism that persist all over the acidotic period. The increase in the phosphorylation of Thr17 site at the beginning of acidosis, might be attributed to the increase in intracellular $Ca^{2+}$ that has been shown to occur during acidosis (73), and the

acidosis-induced phosphatase inhibition (74). These two phenomena provide the necessary conditions to increase the phosphorylation of Thr17 site, as mentioned above (23,75). Dephosphorylation of Thr17 residue would occur after the recovery of phosphatase activity due to the return of intracellular pH towards control values (66). Consistent with the important role of Thr17 phosphorylation in the mechanical recovery from acidosis, experiments by DeSantiago *et al.* (70) showed absence of mechanical recovery in myocytes lacking PLN. The evidence presented about the importance of the phosphorylation of Thr17 of PLN on the mechanical recovery from acidosis does not completely exclude however, that other CaMKII-dependent phosphorylations do not play a role. Further experiments are needed to explore this possibility.

**5.2. Reversible ischemia-reperfusion injury (stunning)**

Myocardial stunning describes the sustained and reversible decrease in myocardial contractility that follows a brief ischemic insult, clinically manifested as sluggish recovery of the pump function after revascularization (76). 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. Although the phenomenon of myocardial stunning was described over 25 years ago (77), the mechanisms responsible for the delayed recovery of contractile function remain not completely clarified (78,79). Numerous studies have documented that a transient $Ca^{2+}$ overload occurs during the early phase of reperfusion and that this $Ca^{2+}$ overload is one of the major mechanisms involved in the pathogenesis of the post-ischemic contractile dysfunction (78,79). A second major mechanism considered as a determinant of cardiac stunning, is the generation of reactive oxygen species (ROS) at the onset of reperfusion (78,79). The final consequences of the cell damage induced by ROS are the modification of the cell membrane structure, due to peroxidation of phospholipids containing unsaturated free fatty acids, and the impaired enzymatic activities, due to oxidation of sulfhydryl proteins (80,81). Eventually, alterations in the $Ca^{2+}$ transport systems in the sarcolemma and the SR, would result in $Ca^{2+}$ overload. Different types of evidence in rodents and human supports the idea that $Ca^{2+}$ overload during reflow triggers myofilament dysfunction, which uncouples excitation from contraction, since the $Ca^{2+}$ transient was not altered although contractility was decreased (78,82,83). This decrease in myofilament $Ca^{2+}$ responsiveness was attributed, at least in rodents, to the degradation of troponin I (TnI) (83), although this is still a matter of controversy (see Canty and Lee (84), for further discussion). Surprisingly, although the $Ca^{2+}$ transient was found to be normal at the end of reperfusion, SR function has been shown to be altered in stunned hearts. A decrease in the activity of SERCA2a and/or in the rate of $Ca^{2+}$ reuptake by the SR have been described in several species, including rats, mice, dogs and humans, submitted to moderate and reversible injury during cardiac surgery (85-87). This contradictory observation raised an obvious question: Why does the intracellular $Ca^{2+}$ transient remain unaltered in species in which the SR function is depressed? A possible explanation to this finding is that compensatory mechanisms can overcome the depressed SERCA2a activity. One possible compensatory mechanism is the phosphorylation of PLN. As stated above, phosphorylated PLN weakens the interaction between PLN and SERCA2a, leading to an increase of SERCA2a activity, SR $Ca^{2+}$ uptake and SR $Ca^{2+}$ load. Experiments in perfused rat hearts demonstrated an increase in the phosphorylation of Thr17 residue of PLN at the beginning of reperfusion (88). Selective inhibition with KB-R7943 of the reverse mode of the NCX, -which appears to be the main source of $Ca^{2+}$ influx on reperfusion-, decreased to basal levels the phosphorylation of PLN at Thr17 residue (88). Blockade of the CaMKII pathway was also achieved by direct inhibition of the kinase with KN-93. The decrease of Thr17 phosphorylation produced by CaMKII inhibition prolonged half relaxation time, which indicates that the phosphorylation of Thr17, when present, attenuates the impaired relaxation that occurs at the beginning of reperfusion (88). Further experiments in transgenic mice, in which Thr17 residue was replaced by the non-phosphorylatable residue Ala (PLN-T17A), indicated that Thr17 site is necessary for the mechanical recovery (89). Figure 8 shows that the presence of Thr17 had a major beneficial impact all along reperfusion: The contractile recovery after the ischemic period was significantly diminished and relaxation was prolonged in PLN-T17A mice when compared to the recovery of mice with intact PLN (89). Moreover, simultaneous measurements of intracellular $Ca^{2+}$ and contractile recovery in perfused hearts from transgenic mice expressing either intact PLN (PLN-WT) or PLN with both phosphorylation sites mutated to Ala (PLN-DM), confirmed and extended these results (90). After ischemia, PLN-DM mice showed a significantly lower recovery of the $Ca^{2+}$ transient amplitude and developed pressure than that of PLN-WT mice. In contrast, myofilament $Ca^{2+}$ responsiveness and TnI degradation did not differ between groups. Moreover, the episodes of mechanical alternans, typical of $Ca^{2+}$ overload, were significantly prolonged in PLN-DM with respect to PLN-WT. The overall findings described above, indicate that the phosphorylation of Thr17, although transient, offers a mechanism that helps to limit intracellular $Ca^{2+}$ overload and interferes with the cascade of events responsible for the contractile dysfunction in myocardial stunning. PLN phosphorylated at Thr17 site, favoring $Ca^{2+}$ re-uptake by the SR, would tend to compensate the depression of SERCA2a and would ameliorate $Ca^{2+}$ overload, typical of the beginning of reflow (78). In accordance to this view, it was reported that intermittent "high-altitude" (IHA) hypoxia mitigated ischemia-reperfusion induced depression of SERCA2a by increasing dual-site phosphorylation of PLN (Ser16 and Thr17 sites) (91). It was also shown that the protection conferred by insuline and by the activation of the cGMP-dependent protein kinase pathway against reoxygenation-induced hypercontracture in cardiomyocytes is due to enhanced SR $Ca^{2+}$ sequestration in association with an increase in the phosphorylation of Ser16 of PLN (92,93).

**5.3. Irreversible ischemia-reperfusion injury**

The role of CaMKII-dependent phosphorylation of PLN in the irreversible ischemia /reperfusion (I/R) injury

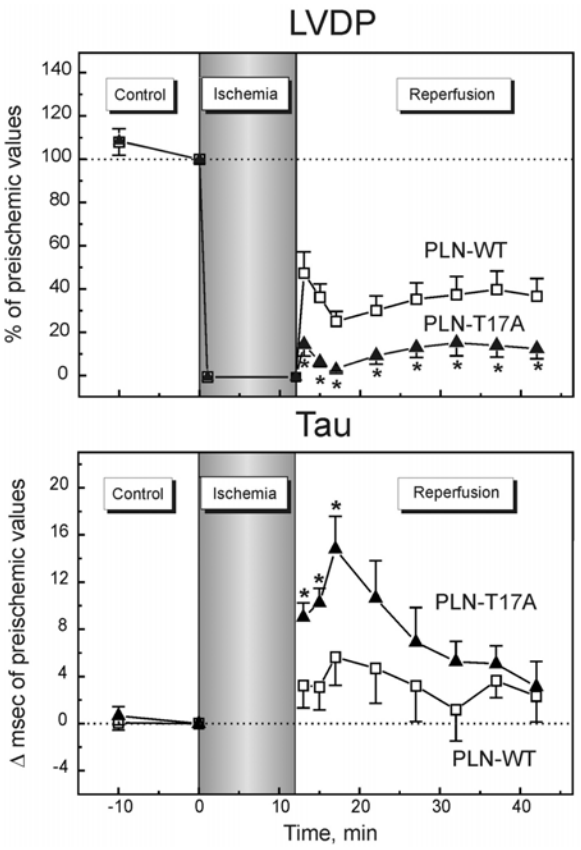


**Figure 8.** Functional role of Thr17 phosphorylation in the ischemia-reperfused mouse heart. Time course of left ventricular developed pressure (LVDP) and the time constant of pressure decay (Tau) during ischemia and reperfusion from wild-type PLN (PLN-WT) and PLN with a Thr17 to Ala mutation (PLN-T17A) mouse hearts. Mutation of Thr17 site is associated with diminished contraction and relaxation recoveries. * $P < 0.05$ with respect to PLN-WT. (Reproduced with permission from, Said *et al,* (89).

is not known. However, recent studies revealed a role of CaMKII on this irreversible process (94,95). In contrast to what one might expect from the protective role of CaMKII and CaMKII -dependent phosphorylation of PLN in the reversible I/R dysfunction (stunning) discussed above, it was found that CaMKII-inhibition was protective in the irreversible I/R injury. In a genetic mouse model of cardiac CaMKII inhibition (AC3-I) developed by Anderson´s group (94), it was shown that CaMKII-inhibition prevented maladaptative remodeling from cathecholamine-induced cardiomyopathy and myocardial infarction. Worsening left ventricular function after myocardial infarction is linked to increased catecholamine activity. Moreover as discussed above, beta-adrenergic stimulation recruits CaMKII-activity and CaMKII activity and expression are increased in myocardial infarction. Finally, the effects of CaMKII inhibition on myocardial dilation and dysfunction mimicked salutary effects of beta-blockers. Taken together these previous results, it was proposed that the resistance of AC3-I mice to cathecholamine-induced cardiomyopathy would correspond to the beneficial effects of CaMKII inhibition on cardiac remodeling after infarction and would indicate that CaMKII is a crucial downstream signal for cardiac hypertrophy, dilation and dysfunction from excessive beta-adrenoceptor stimulation after infarction (96). Studies by Vila-Petroff *et al* (95), further showed the protective role of CaMKII inhibition on the ischemic disease. In isolated rat hearts submitted to a protocol of global irreversible I/R injury, it was shown that CaMKII-inhibition produced a significant improvement of the contractile recovery at the end of reperfusion with respect to control hearts. This recovery was associated with significant decreases in the extent of infarction, lactate dehydrogenase release (necrosis), TUNEL positive cells, caspase-3 activity and increase in the Bcl-2/Bax ratio (apoptosis). Moreover, in isolated myocytes, CaMKII-inhibition produced by the CaMKII inhibitor KN-93 or the more specific one, AIP, prevented simulated I/R-induced spontaneous contractile activity and cell mortality. In contrast, overexpression of CaMKII decreased cell viability after simulated I/R (95). These results indicate that CaMKII is a fundamental component of a cascade of events which promotes cellular apoptosis and necrosis in the irreversible I/R injury. Taken together with the effect of CaMKII on myocardial stunning, the results reveal a dual (and opposite) role of CaMKII in the reversible and irreversible I/R injury: Beneficial and mediated by the CaMKII-phosphorylation of Thr17 of PLN in the stunned heart and detrimental in the irreversible I/R injury (Figures 8 and 9). As in the case of stunning, phosphorylation of Thr17 of PLN was increased at the onset of reperfusion after the prolonged ischemic period. This increase was inhibited by KN-93. On the surface, these results might be taken to indicate that the phosphorylation of Thr17 of PLN is a major player not only in the beneficial effect in the stunned heart but also in the detrimental effect of CaMKII activation in the irreversible I/R injury. However, there is no experimental evidence that could account for this latter possibility. The phosphorylation of Thr17 of PLN described at the beginning of reperfusion after prolonged ischemia (95) might be beneficial as in the case of the stunned heart, but insufficient to oppose to the prevalent detrimental effects (which converge to apoptosis and necrosis), of other putative CaMKII-dependent phosphorylations different from PLN.

### 5.4. Heart failure

There is evidence supporting a decrease in intracellular $Ca^{2+}$-transient and a diminished SR $Ca^{2+}$ load, as a central feature in the altered contractility of the failing heart (HF) (97,98). These abnormalities in intracellular $Ca^{2+}$ have been associated in several HF models, with alterations in the expression and/or activity of different $Ca^{2+}$ regulatory proteins, particularly, a decrease in SERCA2a expression and an increase in NCX expression (99). An increased $Ca^{2+}$ leak, through hyperphosphorylated RyR2, would also contribute to the decrease in SR $Ca^{2+}$content and $Ca^{2+}$ release, typical of HF (100,101). The decrease in SERCA2a expression, (which occurs in many but not all HF models) is associated with either a roughly proportional decrease in PLN expression (which should not alter the $Ca^{2+}$-dependence of SR transport, but would

**Table 1.** Alterations of expression, phosphorylation and function of SERCA2a and PLN in hypertrophy and heart failure

| Model | SERCA2a expression | PLN expression | SERCA2a /PLN | PLN phosphorylation | SR Function | Reference |
|---|---|---|---|---|---|---|
| Rat hypertrophy mild & severe AC | = (mild) ↓ (severe) | ND | | ND | ↓ SR Ca uptake | 117 |
| Human HF | ↓ | ND | | ND | ↓ | 118 |
| Human HF | ↓ | = or ↓ | ↓ | ND | ND | 119 |
| Human HF | = | = | = | ND | ND | 120 |
| Human HF | = | = | = | ND | ↓ | 121 |
| Human HF | = | = | = | ND | ND | 122 |
| Rat β-stimulation-induced hypertrophy | ↓ | ↓ | = | ↓ after acute β-stimulation | ND | 123 |
| Rat hypertrophy AC | ND | ND | | ↑ | ND | 110 |
| Rabbit HF Postinfarction | ↓ | ↓ | ↓ | ↑ | ↓ SR Ca uptake | 124 |
| Human HF | = | = | = | ↓ Ser16 | ↓SERCA2a activity | 102 |
| Rat hypertrophy Postinfarction | = | = | = | ↓ Ser16 ↓ Thr17 | Prolonged relaxation | 106 |
| Human HF | = | = | = | ↓ Ser16 | ↓SERCA2a activity | 125 |
| SHR rats hypertrophy | = | = | = | = Ser 16 ↑ Thr17 | ↑SR Ca uptake | 126 |
| Human HF | ↓ | = | ↓ | ↓ Ser 16 (only males) ↓ Thr17 | ↓SR Ca uptake | 127 |
| Rat HF. Postinfarction | = | = | = | ↓ Ser 16 = Thr17 | ↓SERCA2a activity | 103 |
| Dog HF IC ME | ND | ND | | ↓ Ser 16 ↓ Thr17 | ND | 105 |
| RCat hypertrophy AC | = | ↓ | ↑ | = Ser 16 (↓ after acute β-stimulation) ↑ Thr17 | ND | 128 |
| Rabbit HF Pacing tachycardia | ↓ | ↓ | = | ND | ↓SERCA2a activity | 129 |

The table depicts changes in the expression of SERCA2a and PLN as well as the ration between both proteins (when measured simultaneously), the phosphorylation of either total PLN or the PLN residues, and the functional consequences of these alterations. The table does not pretend to be exhaustive but to exemplify different protein behavior in different models. To show examples of protein abundance, we chose the cases in which protein determinations (and not mRNA measurements) were described. ND: Not determined. AC: Aortic constriction. IC ME: Intracoronary microembolization.

decrease $Ca^{2+}$ transport at all intracellular $Ca^{2+}$), or a smaller decrease or no change in PLN expression (which would produce an increase in the functional stoichiometry of PLN to SERCA, with the consequent increased inhibition of the $Ca^{2+}$ affinity of SERCA2a, decreased SR $Ca^{2+}$ uptake rate and prolongation of relaxation times) (Table 1). As discussed earlier in this review, a second important regulator of the activity of SERCA2a is the degree of phosphorylation of PLN. This phosphorylation has been found to be decreased by some authors, either at Ser16 (102,103), Thr17 (104,105) or both (106). This should be consistent with the beta-adrenergic down-regulation and the increase in PP1 activity, described in some HF models (107,108). Taken together, these results suggest that alterations in the SERCA2a/PLN ratio and the degree of phosphorylation of PLN might contribute to depressed SR $Ca^{2+}$ uptake, leading to an increase in diastolic $Ca^{2+}$, a decrease in SR $Ca^{2+}$ stores and therefore of $Ca^{2+}$ available for contraction. Thus, the strategy of altering SERCA2a and/or PLN levels or activity to restore perturbed $Ca^{2+}$ uptake into the SR are potential therapeutic strategies for HF treatment (109). In this context it should be mention that these maneuvers may cause undesirable effects. Yang *et al.* (94) demonstrated that PLN-/- mice, with PLN ablation, have enhanced vulnerability to cell death by isoproterenol and myocardial infarction. These results suggest that efforts to target repletion of SR $Ca^{2+}$ content to improve mechanical function during HF may increase apoptosis. As regards to the phosphorylation of PLN, it should be mentioned however, that not all the studies found a decrease in PLN phosphorylation levels. Either no changes or even an increase in total PLN phosphorylation or at the Ser16 or Thr17 sites, have been observed (104,110) (See Table 1). The cause for these contradictory results may rest in the different HF models and the different stages at which the phosphorylation of PLN was studied in the development and evolution of HF. Referent in particular to the phosphorylation of Thr17 site, numerous studies indicate that elevation of intracellular $Ca^{2+}$ is implicated in cardiac hypertrophic signaling (111). This elevated $Ca^{2+}$ can transduce signals through various classes of regulated enzymes, among which are CaMKII and the phosphatase PP2B or calcineurin. CaMKII regulates transcriptional gene expression through CaMKII delta b, localized in the nucleus, and $Ca^{2+}$-handling and apoptosis through CaMKII delta c. Among the proteins involved in the excitation-contraction-coupling (ECC) and substrates of CaMKII, PLN, at the Thr17 site, is a possible candidate. However, the increase in $Ca^{2+}$ might also trigger the activation of phosphatases different from PP2B, like PP1, which may lead to a depressed CaMKII activity and diminished Thr17 phosphorylation (112,113). Indeed, although in several models of cardiac hypertrophy and in some HF models, CaMKII have been reported to be

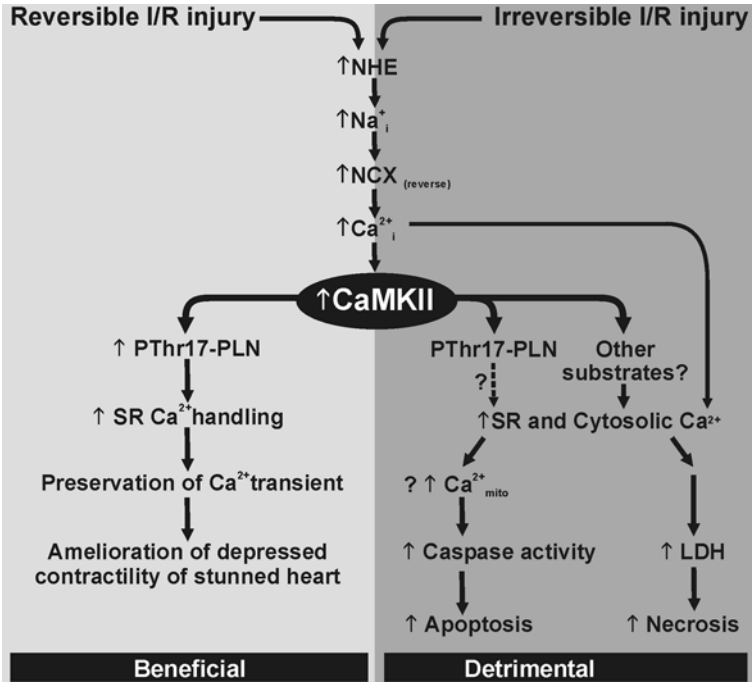


**Figure 9.** Proposed beneficial (right panel) and detrimental (left panel) cascade of events mediated by CaMKII activation on the reversible (stunning) and irreversible ischemia/reperfusion injury. Although the participation of phospholamban (PLN) phosphorylation seems to be clear in the beneficial side of the cascade, it is not known which is the role of PLN phosphorylation on the detrimental action of CaMKII activation during the irreversible ischemia/reperfusion (I/R) injury. NHE: $Na^+/H^+$ exchanger, NCX: $Na^+/Ca^{2+}$ exchanger, SR: sarcoplasmic reticulum, LDH: lactate dehydrogenase, mito: mitochondria.

increased (114-116), it was found to be decreased in others (104,105). As in the case of irreversible I/R, CaMKII-induced apoptosis may also play a role in the diminished cardiac function in HF. Although the signaling cascade involved in this effect is unknown, it might be related, as discussed for the irreversible I/R injury, with the increased phosphorylation, at least when this increase occurs, of proteins involved in the ECC, like PLN and RyR2. It would be important to know the time course of the phosphorylation of Thr17 of PLN (as well as of the other CaMKII-dependent phosphorylations), and the consequences of these putative phosphorylations in the transition and progression from hypertrophy to HF.

## 6. CONCLUSIONS AND PERSPECTIVES

Taken together, these findings suggest that the Thr17 site in PLN is phosphorylated under conditions of beta-adrenoceptor stimulation and contributes to the relaxant effect of the high concentrations of the beta-agonists. Moreover, evidence was presented indicating that the phosphorylation of this residue is also implicated in the mechanical recovery under some pathological conditions, like acidosis and stunning. An interesting point is that, although the phosphorylation experiments reveal that the phosphorylation of Thr17 is transient, -albeit prominent- and occurs only at the beginning of both acidosis and reperfusion, the presence of this residue, (and/or that of intact PLN), seems to be necessary for the mechanical recovery all along the reperfusion or acidotic period. These results shed new lights for the search of novel strategies for cardioprotection in the clinical setting. Phosphorylation of Thr17 of PLN also increased at the beginning of reperfusion after prolonged ischemia (irreversible I/R injury). However, whether this phosphorylation either contributes or opposes to the deleterious action of CaMKII activation, remains to be determined. Finally, it would be important to examine whether the increase in $Ca^{2+}$, involved in the genesis of myocardial hypertrophy, increases CaMKII activity and the phosphorylation of Thr17 of PLN and whether these effects could readily influence the evolution from hypertrophy to HF.

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