The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) is under the control of a closely associated SR protein named phospholamban (PLN). Dephosphorylated PLN inhibits the SR Ca^{2+} pump, whereas phosphorylation of PLN, at either Ser^{16} by PKA or Thr^{17} by calmodulin-dependent protein kinase II (CaMKII), reverses this inhibition, thus increasing SERCA2a activity and the rate of Ca^{2+} uptake by the SR. This would in turn lead to an increase in the velocity of relaxation, SR Ca^{2+} load, and myocardial contractility. Thus, PLN is a major determinant of cardiac contractility and relaxation. Although in the intact heart, β-adrenoceptor stimulation results in phosphorylation of PLN at both Ser^{16} and Thr^{17} residues, the role of Thr^{17} site has long remained equivocal. In this review, we attempt to highlight the signaling cascade and the physiological relevance of the phosphorylation of this residue in the heart under both physiological and pathological situations.

Keywords: Phospholamban; Thr^{17} site phosphorylation; β-adrenergic stimulation; Acidosis; Ischemia; Heart failure

1. Introduction

During cardiac action potential, Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channels) and subsequently binds and activates the ryanodine receptors (RyR2) on the sarcoplasmic reticulum (SR), to trigger further Ca^{2+} release. This process, termed Ca^{2+-}induced-Ca^{2+-}release [1], amplifies and coordinates the Ca^{2+} signal, which, by interacting with myofilament proteins, produces contraction. To allow for muscle relaxation between contractions, cytosolic Ca^{2+} must be decreased rapidly. This is mainly accomplished by the SR Ca^{2+}-ATPase (SERCA2a), which mediates Ca^{2+} uptake into the SR, and in less proportion by the Na^{+}/Ca^{2+} exchanger (NCX), which removes Ca^{2+} from the extracellular space [2].

The activity of SERCA2a is under the control of a closely associated SR protein, named phospholamban (PLN) [3]. PLN is a 52 amino acid phosphoprotein, which, in the dephosphorylated form, decreases the apparent Ca^{2+}-affinity of SERCA2a [4]. 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 [5–9]. 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 [5]. In contrast, overexpression of PLN was associated with a decreased apparent affinity of SERCA2a for Ca^{2+} and depressed cardiac contractile performance [8]. PLN-heterozygous hearts, expressing reduced protein levels of PLN, further support that the ratio PLN/SERCA2a plays a prominent role in regulating SR function and contractility [9].
In addition to the relationship of PLN/SERCA2a, myocardial contractility and relaxation are also dependent on the degree of PLN phosphorylation. In vitro experiments have shown that PLN can be phosphorylated at three distinct sites: Ser\(^{16}\) by cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), Thr\(^{17}\) by Ca\(^{2+}\)–calmodulin-dependent protein kinase II (CaMKII), and Ser\(^{10}\) by protein kinase C (PKC) [10–13]. Phosphorylation of these sites reverses the inhibition of PLN upon SERCA2a, thus increasing the affinity of the enzyme for Ca\(^{2+}\) and the rate of SR Ca\(^{2+}\) uptake. Experimental evidence indicated that phosphorylation of Ser\(^{10}\) by PKC is not physiologically relevant [14]. Phosphorylation of Ser\(^{16}\) by PKG seems to play a role in the modulation of smooth muscle contraction [15] and has been also associated with a positive inotropic and lusitropic effect in mammalian heart [16]. Finally, phosphorylation of PLN by PKA and CaMKII pathways (Ser\(^{16}\) and Thr\(^{17}\) residues, respectively), is the main mediator of the positive inotropic and relaxant effect of \(\beta\)-adrenergic stimulation in cardiac muscle [17–27]. The increase in SERCA2a activity and Ca\(^{2+}\) uptake rate produced by these phosphorylations would lead to an increase in the velocity of relaxation, SR Ca\(^{2+}\) load and, as a consequence, SR Ca\(^{2+}\) release and myocardial contractility [17,18,20–27]. 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]. The activity of PP1 is also under the control of different kinases and phosphatases. As will be discussed later, this phosphatase regulatory cascade, frequently overlooked when considering the regulation of PLN phosphorylation sites, is crucial in determining the status of PLN phosphorylation.

In this article, we will particularly discuss the role of Thr\(^{17}\) phosphorylation of PLN under physiological and pathological processes.

2. Phosphorylation of Thr\(^{17}\) of PLN under physiological conditions

2.1. \(\beta\)-Adrenergic stimulation

2.1.1. PLN vs. other regulatory proteins

It is well established that \(\beta\)-adrenergic stimulation phosphorylates several proteins in the cardiac myocytes, among which are PLN and the RyR2 at the SR level, the L-type Ca\(^{2+}\) channels, the NCX and phospholemman at the sarcoslemma, and troponin I (TnI), myosin-binding protein C and myosin light chain at the level of the myofibrils [2,17–27]. Different studies indicated a fundamental role of PLN phosphorylation in the contractile and relaxant effects of \(\beta\)-adrenergic agents [17,18,20–27]. It was further demonstrated that PLN phosphorylation is predominant in determining the mechanical effects of \(\beta\)-agonists vs. the phosphorylation of other proteins, also involved in the excitation–contraction-coupling. Two different experimental approaches are particularly eloquent: 1. Dialysis of ventricular myocytes with a monoclonal antibody against PLN virtually suppressed the mechanical effects of the \(\beta\)-agonists [29]; 2. Studies in different cardiac preparations from PLN-deficient mice indicated a significant attenuation of the inotropic and lusitropic effects of isoproterenol, compared with wild type preparations [5]. The effects of \(\beta\)-adrenergic stimulation appeared also greatly attenuated in in vivo echocardiographic studies in PLN-ablated hearts [6]. This experimental evidence indicated that PLN is a major mediator of the \(\beta\)-adrenergic response in the mammalian heart. However, a role of other proteins different from PLN could not be excluded. In particular, recent experiments reconfirmed a previously questioned role of TnI phosphorylation in the relaxant effect of \(\beta\)-agonists [30].

2.1.2. Dual site PLN phosphorylation

Wegener et al. [19] first localized the amino acid residues of PLN phosphorylated in the intact heart in response to \(\beta\)-adrenoceptor stimulation, by using monoclonal antibody affinity purification of the \(^{32}\)P-labeled protein from perfused hearts, followed by phosphoaminoacid analysis and protein sequencing. These experiments showed that there were only two sites phosphorylated during \(\beta\)-adrenergic stimulation, Ser\(^{16}\) and Thr\(^{17}\), indicating that under these conditions, PKA and CaMKII pathways were phosphorylating PLN. Moreover, phosphorylation at Ser\(^{16}\) precedes that at Thr\(^{17}\), although at steady state, both sites were phosphorylated in approximately equimolar amounts [19]. Isoproterenol-induced phosphorylation of PLN by PKA and CaMKII activation was also observed by using different techniques, like back phosphorylation, \(^{32}\)P incorporation into PLN and more recently, specific immunodetection of PLN phosphorylation sites (see below) [18,21–27]. All these studies indicated that dual phosphorylation of PLN does occur in vivo as it was the case of in vitro experiments (Fig. 1).

2.1.3. Independence and interdependence of PLN phosphorylation pathways. Functional role of Thr\(^{17}\) phosphorylation

Although in vitro studies indicate that PKA and CaMKII phosphorylations are independent of each other [31], earlier attempts to phosphorylate PLN by CaMKII in the intact heart had systematically failed, unless cAMP levels within the cell increase [17,18,22,32]. These findings suggested an interaction between PLN phosphorylation pathways. The availability of transgenic models, expressing either PLN-wild type (PLN-WT), the Ser\(^{16}\)→Ala mutant PLN (PLN-S16A) or the Thr\(^{17}\)→Ala mutant PLN (PLN-T17A) in the cardiac compartment of the PLN knockout mice (PLN-KO), and of phosphorylation site-specific antibodies to PLN, which precisely discriminate between phosphorylated Ser\(^{16}\) and Thr\(^{17}\) phosphorylation sites, allowed a more complete comprehension of the independence and/or interdependence, as well as of the relative functional role of dual site PLN phosphorylation in the intact heart. Experiments in transgenic mice, expressing either PLN-WT or PLN-S16A,
demonstrated that the phosphorylation of Ser16 of PLN is a prerequisite for the phosphorylation of Thr17 [33], in line with earlier findings which indicated the dependence of the phosphorylation of Thr17 on intracellular cAMP levels [18,22,32]. Experiments in PLN-T17A hearts further showed that Ser16 can be phosphorylated independently of Thr17 in vivo and that phosphorylation of Ser16 was sufficient for mediating the maximal cardiac responses to \(\beta\)-adrenergic stimulation [34]. 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 that there was no contribution of the CaMKII pathways to the total PLN phosphorylation at the lowest isoproterenol concentrations [22,27]. Immunodetection of site-specific phosphorylated PLN fully confirmed this suggestion, further indicating that the phosphorylation of Thr17 accounted for approximately 50% of the total PLN phosphorylation at the highest isoproterenol concentrations (\(\geq 10\) nM). This CaMKII-induced phosphorylation was closely associated with an increase in the relaxant effect of \(\beta\)-agonists [27]. In line with these 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 activity produced by the low \(\beta\)-adrenergic stimulation, which would produce only a small increase in intracellular Ca\(^{2+}\), not enough to activate CaMKII and phosphorylate Thr17 site. As will be discussed below, the modest increase in PKA activity would also fail to significantly inhibit the phosphatases that dephosphorylate PLN, further favoring the dephosphorylated state of Thr17 residue (Fig. 1).

A similar modest or null contribution of Thr17 phosphorylation to the total PLN phosphorylation evoked by \(\beta\)-adrenergic agents occurred, even at the highest isoproterenol concentrations, when the drug was administrated under conditions that either preclude or produce only a moderate increase in intracellular Ca\(^{2+}\) or that inhibited CaMKII activity, as was the case of the experiments in which isoproterenol was administrated at low external Ca\(^{2+}\) and the presence of nifedipine, or in the presence of ryanodine or thapsigargin to block the function of SR and SERCA2a, respectively, or with the CaMKII inhibitor KN-93. In all these cases, Ser16 was the only PLN site showing an

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**Fig. 1.** Cascade of events leading to phosphorylation of PLN during \(\beta\)-adrenergic stimulation. Binding of a \(\beta\)-agonist to its receptor activates the heterotrimeric G protein, which through the dissociation of its \(\alpha\) subunit enhances adenylate cyclase (AC) activity. AC catalyzes cAMP formation, which binds to the regulatory subunits of PKA (R), causing the activation of the catalytic subunit C. The C subunit phosphorylates L-type Ca\(^{2+}\) channel, increasing the Ca\(^{2+}\) influx, Ser16 residue of PLN and PP1. PP1 is inhibited by PKA, acidosis or by the phosphatase inhibitor, okadaic acid (OA). The increase in intracellular Ca\(^{2+}\) causes the activation of CaMKII, a multimeric protein that undergoes autophosphorylation, a process that allows the enzyme to retain enzymatic activity in the absence of Ca\(^{2+}\). This autophosphorylation state is also controlled by PP1. CaMKII in turn phosphorylates PLN at the Thr17 residue.
increase in phosphorylation [23,26]. These results provide an explanation for the low level of Thr$^{17}$ phosphorylation observed with maximal isoproterenol concentrations in adult rat myocytes by Calaghan et al. [24]. In these experiments, the contribution of Thr$^{17}$ to the total PLN phosphorylation was much lower than that observed in isolated rat hearts labeled with $^{32}$P [23,27]. The reason for this discrepancy might lay in the lower extracellular Ca$^{2+}$ used in the experiments in isolated myocytes [24], compared with that used in the perfused rat heart (1.0 vs. 1.34 mM). This difference, although small, is not negligible, since it is sufficient to evoke a significant increase in contractility in isolated rat myocytes [35]. These experimental conditions may lead to the erroneous conclusion that the Thr$^{17}$ site is poorly phosphorylated and not important under β-adrenergic stimulation. Similarly, the failure to find PLN phosphorylation in transgenic mice in which Ser$^{16}$ site was mutated to Ala must be attributed to the fact that the lack of phosphorylation of the Ser$^{16}$ site precludes the increase in intracellular Ca$^{2+}$ necessary to phosphorlylate Thr$^{17}$ [33].

Taken together, these findings demonstrated the additive nature of PKA and CaMKII pathways of PLN phosphorylation, in agreement with the in vitro results [31]. In addition, they indicated that: a) in the absence of a phosphorylatable Thr$^{17}$ site, Ser$^{16}$ is sufficient for mediating the maximal cardiac responses to β-adrenergic stimulation [34]; b) when both sites are present, they both equally contribute to the total PLN phosphorylation at the highest levels of β-adrenergic stimulation [23]; c) at low isoproterenol concentrations (≤3 nM), the increase in PLN phosphorylation (and the relaxant effect of isoproterenol) is exclusively determined by the increase in the phosphorylation of Ser$^{16}$ residue [23,25–27].

2.2. Phosphorylation of Thr$^{17}$ of PLN in the absence of β-adrenergic stimulation

The observation that CaMKII-dependent PLN phosphorylation can only occur in the presence of β-adrenergic stimulation, i.e. when the cAMP levels within the cell and PKA activity increase [17,18,20–27,32], was in sharp contrast with the independence of both pathways of PLN phosphorylation, described in vitro [31]. A clue for understanding this apparent discrepancy was given by experiments in which the activation of the CaMKII pathway and the phosphorylation of Thr$^{17}$ residue were studied in the presence of the phosphatase inhibitor, okadaic acid. Under these conditions, the increase in contractility (intracellular Ca$^{2+}$), produced by increasing extracellular Ca$^{2+}$, evoked a significant increase in Thr$^{15}$ phosphorylation associated with a relaxant effect, in the absence of any significant increase in cAMP levels and in the phosphorylation of Ser$^{16}$ of PLN [23]. These results indicate that Thr$^{17}$ residue can be phosphorylated independently of Ser$^{16}$ phosphorylation, as was described in vitro. Thus, the nature of the interaction between PKA and CaMKII cascades lies in a basic mechanism underlying any phosphorylation process, i.e., the relative degree of kinases and phosphatases activity. It is important to note in this context that PP1 also dephosphorylates CaMKII [36]. Thus, inhibition of PP1 would contribute to sustained phosphorylation of CaMKII, which allows the enzyme to maintain its activity, independently of the Ca$^{2+}$ level (Fig. 1).

Taken together the results indicate that phosphorylation of Thr$^{17}$ residue could be detected in intact preparations in the following situations (Fig. 1): a) in the presence of high extracellular Ca$^{2+}$ (to activate CaMKII) and under conditions that inhibit PP1; 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 [37].

2.2.1 FDAR and Thr$^{17}$ phosphorylation

A still controversial issue is the role of Thr$^{17}$ phosphorylation on the frequency-dependent acceleration of relaxation (FDAR) [2]. Frequency is one of the fundamental physiological modulators of myocardial performance. In most of the species, the increase in contraction frequency produces an increase in contractility (positive treppe phenomenon). However, rats and mice as well as the failing myocardium commonly show a flat or even negative force–frequency relationship. Regardless of whether the force–frequency relationship is positive or negative, an increase in stimulation frequency is always associated with a relaxant effect, the mechanism of which is unclear. De Konink and Schulman elegantly showed that CaMKII can decode the frequency of Ca$^{2+}$ spikes into distinct amounts of kinase activity [38]. Thus, the increase in contraction frequency would produce a sustained increase in CaMKII, which might lead to the phosphorylation of Thr$^{17}$ of PLN, without the necessity of phosphatase inhibition. In agreement with this attractive hypothesis, Hagemann et al. did show an increase in the phosphorylation of Thr$^{17}$ with the increase in stimulation frequency and a good correlation between this increase and FDAR in rat myocytes [39]. Moreover, experiments in transgenic mice demonstrated that myocytes from Thr$^{17}$→Ala mutant PLN mice have a significantly diminished FDAR with respect to myocytes from Ser$^{16}$→Ala mutant PLN and WT mice [40]. Whereas these results indicate that Thr$^{17}$ is indeed involved in FDAR, experiments by DeSantiago et al. in PLN-KO mice indicated that FDAR does not require PLN [41]. Moreover, although experiments in cat myocytes did show a good correlation between the phosphorylation of Thr$^{17}$ and FDAR, they further demonstrated that FDAR actually preceded the increase in the phosphorylation of Thr$^{17}$ evoked by increasing frequency [42]. Finally, the stimulation-frequency induced increase in Thr$^{17}$ site could not be detected in the perfused heart, in spite of the presence of FDAR [42]. Overall, it is difficult to reconcile the outcomes of the different experimental approaches used to study the role of the phosphorylation of Thr$^{17}$ of PLN on FDAR, and the
underlying mechanism of this phenomenon still remains an open question.

2.3. Interaction of PKA and Ca$^{2+}$ signaling pathways at the level of PP1

PP1 is a serine/threonine phosphatase composed of two subunits, PP1G~M~ and PP1C. PP1G~M~, the regulatory subunit, is responsible for the localization of the catalytic subunit, PP1C, to the SR. The binding of PP1C to PP1G~M~ enhances the activity of the phosphatase towards its substrates, like PLN. Fig. 2 depicts some central players of the PP1 regulatory cascade. PKA phosphorylates PP1G~M~ on Ser$^{48}$ and Ser$^{67}$ residues, both in vitro and in vivo [43,37]. Phosphorylation of Ser$^{67}$ site triggers dissociation of PP1C from PP1GM, thereby inactivating its phosphatase activity [43]. In contrast, the phosphorylation of Ser$^{48}$ increases the activity of PP1C, but this activation is overridden by the phosphorylation of Ser$^{67}$ and the consequent dissociation of PP1C from PP1G~M~ [44]. Thus, the net result of PKA phosphorylation is a PP1 inhibition. Ser$^{48}$ and Ser$^{67}$ are dephosphorylated by the serine/threonine protein phosphatases type 2A and 2B (PP2A and PP2B). PP2B is a Ca$^{2+}$-calmodulin dependent enzyme, also named calcineurin [45]. Furthermore, PP1 is regulated by two heat and acid-stable proteins, inhibitor 1 (INH-1) and INH-2. INH-1 becomes active upon phosphorylation on Thr$^{35}$ site by PKA [45,46]. In turn, the INH-1 is also dephosphorylated by PP2A and PP2B [45]. As stated above, PKA, through the phosphorylation of PP1 and of the INH-1 and the consequent inhibition of PP1, amplifies its own signal (PKA-dependent activation of PP1 through this cascade might be responsible for the decrease in the phosphorylation of Ser$^{16}$ residue of PLN, observed in the presence of isoproterenol and high Ca$^{2+}$. In line with this idea, recent experiments demonstrated that the phosphorylation of the INH-1 at Ser$^{57}$ (a site different from that phosphorylated by PKA), by the Ca$^{2+}$-and phospholipid-dependent PKC$_{\alpha}$, increased PP1 activity and decreased Ser$^{16}$ phosphorylation of PLN [48]. Finally, Ser$^{48}$ of PP1G~M~ is phosphorylated by CaMKII in the striated skeletal muscle [49]. If this mechanism is also active in cardiac muscle, it would directly contribute to PP1 activation. Obviously, PP1 activation would not only affect the phosphorylation of Ser$^{16}$ of PLN but also limit the CaMKII-dependent increase in Thr$^{17}$ phosphorylation. Overall, different types of evidence indicate that this phosphorylation regulatory cascade may constitute a fine mechanism by which the increase in Ca$^{2+}$ attenuates the signals that act via cAMP. This cascade would also damp the phosphorylations produced by sustained increases in Ca$^{2+}$.

3. Phosphorylation of Thr$^{17}$ of PLN in pathological conditions

3.1. 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 [50]. Acidosis produces a rapid decrease in the contraction, which is largely due to a decrease in myofilament Ca$^{2+}$ responsiveness [51]. The decrease in contractility is associated with 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 [52]. This initial impairment of contractility and relaxation is followed by a spontaneous mechanical recovery which requires an intact SR and has been shown to be dependent on the activity of the CaMKII. Experiments by DeSantiago et al. [53] showed that this recovery did not occur in hearts of PLN-KO mice. These experiments first suggested an important role of the phosphorylation of PLN, particularly the Thr$^{17}$ residue, in
the recovery from acidosis. This suggestion was based on earlier experiments showing that the simultaneous increase in intracellular Ca\(^{2+}\) and the acidosis-induced inhibition of phosphatases produces an increase in the phosphorylation of Thr\(^{17}\) [47]. Recent experiments showed that phosphorylation of Thr\(^{17}\) site of PLN transiently increased at the onset of acidosis and is associated with a great part to the contractile and relaxation recoveries that follow the acidic insult, most of which occurred within the first 3 min of acidosis [54]. This phosphorylation would provide a mechanism to overcome the direct depressant effect of acidosis on SERCA2a [52]. A more prominent role of the phosphorylation of PLN residues might be expected in vivo. Systemic acidosis is known to increase sympathetic nerve activity [55]. This increased sympathetic tone may produce a more persistent phosphorylation of both, Thr\(^{17}\) and Ser\(^{16}\), residues of PLN, with a further contribution to the mechanical recovery. Finally, the experiments by DeSantiago et al. [53], showing the absence of mechanical recovery of myocytes lacking PLN, would indicate that the presence of PLN, as opposite to the chronic de-repression of SERCA2a in PLN-KO mice, may be required throughout the entire acidosis period for both the relaxation and contractile recoveries.

### 3.2. Stunning

Myocardial stunning describes the reversible decrease in myocardial contractility that follows a brief ischemic insult, clinically manifested as sluggish recovery of the pump function after revascularization [56]. The relevant characteristic of this phenomenon is the reversible nature of the process, in contrast with the irreversible myocardial dysfunction that occurs with the presence of necrosis or apoptosis. The mechanisms responsible for the delayed recovery of contractile function are not completely clear [57]. Different types of evidence in rodents and human indicate that the ultimate cause of the alteration of myocardial contractility was a decrease in myofilament Ca\(^{2+}\) responsiveness, since the Ca\(^{2+}\) transient was not altered, although contractility was decreased. Although the results are not unanimous, this decrease in myofilament Ca\(^{2+}\) responsiveness was attributed, at least in rodents, to the degradation of Tnl [58]. On the other hand, experimental evidence indicates that the function of the SR is altered, both in the reversible as well as in the irreversible ischemia–reperfusion injury [59–63]. 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, including rats, mice, dogs and humans, submitted to moderate and reversible injury during cardiac surgery [60–62]. An intriguing question is, therefore, why does the intracellular Ca\(^{2+}\) transient remain unaltered in species in which the SR function is depressed? A possible explanation to this puzzle is that compensatory mechanisms can overcome the depressed SERCA2a activity. Experiments in perfused rat hearts demonstrated an increase in the phosphorylation of Thr\(^{17}\) residue of PLN at the beginning of reperfusion (1–3 min), which was decreased to basal levels by the inhibitor of the reverse mode of the NCX, KB-R7943. The decrease of this phosphorylation by CaMKII inhibition prolonged half relaxation time, which indicates that the phosphorylation of Thr\(^{17}\), when present, attenuates the impaired relaxation that occurs at the beginning of reperfusion [64]. Furthermore, in transgenic mice in which Thr\(^{17}\) of PLN was mutated to Ala, the contractile recovery after an ischemic period was significantly diminished and relaxation was prolonged when compared to the recovery of mice with intact PLN [65]. These results indicate that phosphorylation of 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 the depression of SERCA2a and would ameliorate Ca\(^{2+}\) over-load, typical of the beginning of reflow [57]. Moreover, these experiments indicate that activation of SERCA2a by any other means, at this crucial moment of reperfusion, may greatly contribute to Ca\(^{2+}\) handling. In accordance to 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 [66]. Fig. 3 is a scheme showing the possible cascade of events, leading to the phosphorylation of Thr\(^{17}\) at the beginning of reperfusion, and the hypothetical protective effect of PLN phosphorylation on stunning.

Longer periods of ischemia may lead, to a different PLN phosphorylation pattern [63,67]. In hearts submitted to 30 min of ischemia followed by reperfusion, Xie et al. [67] showed that phosphorylation of Thr\(^{17}\) significantly decreased at the end of ischemia and at the beginning of reperfusion, when phosphorylation of Ser\(^{16}\) was enhanced. Interestingly, these authors described that intermittent hypoxia protects against the ischemia/reperfusion injury by increasing both Ser\(^{16}\) and Thr\(^{17}\) phosphorylation, supporting the concept that phosphorylation of both residues is important for myocardial recovery after the ischemic period.

Finally, Kim et al. [68] described that in large animals whose contractility is more dependent on extracellular Ca\(^{2+}\), an alteration of Ca\(^{2+}\) handling is the main cause of stunning, instead of the decrease in Ca\(^{2+}\) responsiveness of the myofilaments, observed in rodents. In association with the altered Ca\(^{2+}\) handling, the authors described a dephosphorylation of Ser\(^{16}\) site of PLN at the end of reperfusion and speculated that these alterations might constitute the cellular basis of stunning in large mammals. In these large animals, it would be interesting to examine the status of Thr\(^{17}\) phosphorylation all along the reperfusion period and particularly in the crucial first minutes of reflow.

### 3.3. Heart failure

Heart failure (HF) develops when the heart is unable to provide an adequate cardiac output to meet the metabolic needs of the organism. Substantial progress has been made...
over the past few years in elucidating the pathophysiology of contractile dysfunction and its various subcellular molecular counterparts. 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\[69,70\]. These abnormalities in intracellular Ca\(^{2+}\) have been associated in most of 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\[71\]. 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 \[72\]. Consistently with the decrease in SERCA2a expression, several studies indicated that SERCA2a activity and/or SR Ca\(^{2+}\) uptake are impaired in the failing heart \[71\]. The decrease in SERCA2a expression is associated with either a smaller decrease or no change in PLN expression, which results in a decrease in the ratio SERCA2a/PLN and a further decrease in SR Ca\(^{2+}\) uptake. In this scenario, the level of phosphorylation of PLN becomes a crucial factor which, unfortunately, has not been well defined either in hypertrophy or HF. Phosphorylation of PLN has been found to be decreased by some authors, either at Ser\(^{16}\) \[73,74\], Thr\(^{17}\) \[75,76\] or both \[77\]. This should be consistent with the \(\beta\)-adrenergic down-regulation and the increase in PP1 activity, described in some HF models \[78,79\]. The decrease in the phosphorylation of PLN would be detrimental, since it would add to the decrease in SERCA2a/PLN ratio, to further inhibit SERCA2a activity. Other studies have observed, however, either no changes at the Ser\(^{16}\) site \[75\], or even an increase in total PLN phosphorylation \[80\]. The cause for these contradictory results may lay in the different HF models and the different times at which the phosphorylation of PLN was studied in the development of HF. Referent in particular to the phosphorylation of Thr\(^{17}\) site, numerous studies indicate that elevation of intracellular Ca\(^{2+}\) is implicated in cardiac hypertrophic signaling \[81\]. This elevated Ca\(^{2+}\) may not only activate the pathway that produces the hypertrophic signals, but may also modify phosphorylation pathways of proteins involved in the excitation–contraction-coupling, through the activation of Ca\(^{2+}\)-dependent kinases, among which Thr\(^{17}\) of PLN is a possible candidate. However, and as stated above, the increase in Ca\(^{2+}\) might also trigger the activation of phosphatases and lead to a depressed CaMKII activity and diminished Thr\(^{17}\) phosphorylation \[36,45\]. Indeed, although in some HF models, CaMKII activity and expression have been reported to be decreased \[75,76\], these were found to be increased in others, like human HF with dilated cardiomyopathy \[82,83\]. It would be important to know the time course of the phosphorylation of Thr\(^{17}\) of PLN (as well as of the other CaMKII-dependent phosphorylations), and the consequences of this putative phosphorylation in the transition and progression from hypertrophy to HF. Of particular interest is the fact that experiments in transgenic mice in which PLN was overexpressed, a feedback loop was described between the proximal and distal ends of the \(\beta\)-adrenergic pathway \[84\]. According to these results, an increase in PLN expression or function would lead to an increase in \(\beta\)-adrenergic activity, to phosphorylate PLN and therefore maintain cardiac function. Moreover, if PLN phosphorylation decreased, as in some HF models, adrenergic activity would increase to maintain cardiac function. As sustained increases in cardiac adrenergic activity are detrimental to the heart \[84\], pharmacological tools that selectively increase PLN phosphorylation might turn to be beneficial in the evolution to HF.

4. Conclusions and perspectives

Taken together, these findings suggest that the Thr\(^{17}\) site in PLN is phosphorylated under conditions of \(\beta\)-adrenergic receptor 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 Thr\(^{17}\) 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 acute period. These results, together with the fact that phosphorylation of Ser\(^{16}\) of PLN by cGMP has been also shown to be protective in ischaemia/reperfusion injury, shed new lights for the search of novel strategies for cardioprotection in the clinical setting. Moreover, it is important to examine whether the increase in Ca\(^{2+}\), involved in the genesis of myocardial hypertrophy, may increase CaMKII activity and therefore the phosphorylation of Thr\(^{17}\) of PLN, and whether this increased PLN-phosphorylation may influence the evolution from hypertrophy to HF.

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