ELSEVIER

Cardiovascular Research 68 (2005) 366 – 375

Cardiovascular Research

www.elsevier.com/locate/cardiores

Review

Role of phospholamban phosphorylation on Thr^{17} in cardiac physiological and pathological conditions

Alicia Mattiazzi [a,*], Cecilia Mundiña-Weilenmann [a], Chu Guoxiang [b], Leticia Vittone [a], Evangelia Kranias [b]

[a] *Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas Medicina, 60 y 120, (1900), La Plata, Argentina*
[b] *Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, USA*

Received 1 April 2005; received in revised form 9 August 2005; accepted 15 August 2005
Available online 13 October 2005
Time for primary review 23 days

Abstract

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].

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

0008-6363/$ - see front matter © 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2005.08.010

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 β-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. β-Adrenergic stimulation

2.1.1. PLN vs. other regulatory proteins

It is well established that β-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 sarcolemma, 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 β-adrenergic agents [17,18,20–27]. It was further demonstrated that PLN phosphorylation is predominant in determining the mechanical effects of β-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 β-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 β-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 β-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 β-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 β-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 β-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 knock out 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,

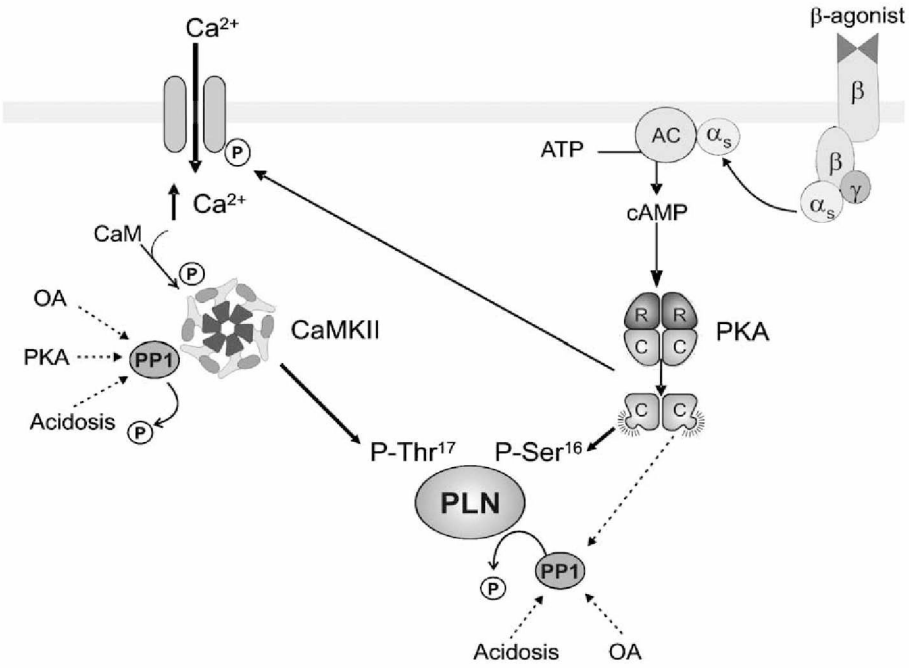

Fig. 1. Cascade of events leading to phosphorylation of PLN during β-adernergic stimulation. Binding of a β-agonist to its receptor activates the heterotrimeric G protein, which through the dissociation of its α_s subunit enhances adenylate cyclase (AC) activity. AC catalyzes cAMP formation, which binds 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, Ser^{16} 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 Thr^{17} residue.

demonstrated that the phosphorylation of Ser^{16} of PLN is a prerequisite for the phosphorylation of Thr^{17} [33], in line with earlier findings which indicated the dependence of the phosphorylation of Thr^{17} on intracellular cAMP levels [18,22,32]. Experiments in PLN-T17A hearts further showed that Ser^{16} can be phosphorylated independently of Thr^{17} in vivo and that phosphorylation of Ser^{16} was sufficient for mediating the maximal cardiac responses to β-adrenergic stimulation [34]. These experiments suggested a predominant role of the phosphorylation of Ser^{16} over that of Thr^{17}, in the mechanical effect produced by β-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 Ser^{16} and Thr^{17} 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 β-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 Thr^{17} 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 β-agonists [27]. In line with these findings, Kuschel et al. [25] and Bartel et al. [26], demonstrated that the dose–response curve of Thr^{17} phosphorylation to isoproterenol was shifted to the right, compared to that of Ser^{16} phosphorylation, clearly indicating that Ser^{16} was the only phosphorylated site at the lowest isoproterenol concentrations. The lack of contribution of Thr^{17} to the total PLN phosphorylation, at the lowest isoproterenol concentrations, may be attributed to the modest increase in PKA activity produced by the low β-adrenergic stimulation, which would produce only a small increase in intracellular Ca^{2+}, not enough to activate CaMKII and phosphorylate Thr^{17} 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 Thr^{17} residue (Fig. 1).

A similar modest or null contribution of Thr^{17} phosphorylation to the total PLN phosphorylation evoked by β-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, Ser^{16} was the only PLN site showing an

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 phosphorylate 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 ($\leq$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^{17} 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 Thr17 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 $PP1_C$. $PP1G_M$, the regulatory subunit, is responsible for the localization of the catalytic subunit, $PP1_C$, to the SR. The binding of $PP1_C$ 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 $PP1_C$ from $PP1G_M$, thereby inactivating its phosphatase activity [43]. In contrast, the phosphorylation of Ser^{48} increases the activity of $PP1_C$, but this activation is overridden by the phosphorylation of Ser^{67} and the consequent dissociation of $PP1_C$ 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 Ser^{16} phosphorylation) and unmasks the CaMKII-dependent phosphorylation of Thr^{17} (Fig. 1). The increase in Ca^{2+}, on the other hand, is a potential mechanism by which these signals may be damped. It was shown that the increase in extracellular Ca^{2+} in the presence of isoproterenol produced a further increase in the phosphorylation of Thr^{17} of PLN, which is associated with a significant decrease in the phosphorylation of Ser^{16} residue [47]. One possible explanation for this finding might lie in the subtle interaction between the different players intervening in the phosphorylation of PLN (Figs. 1 and 2): the increase in Ca^{2+} by activating PP2B may dephosphorylate the INH-1 and PP1. Both dephosphorylations would enhance PP1 activity, overriding the inhibitory effect of PKA on this phosphatase. 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^{67} (a site different from that phosphorylated by PKA), by the Ca^{2+}- and phospholipid-dependent PKCα, 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 phosphatase 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+}.

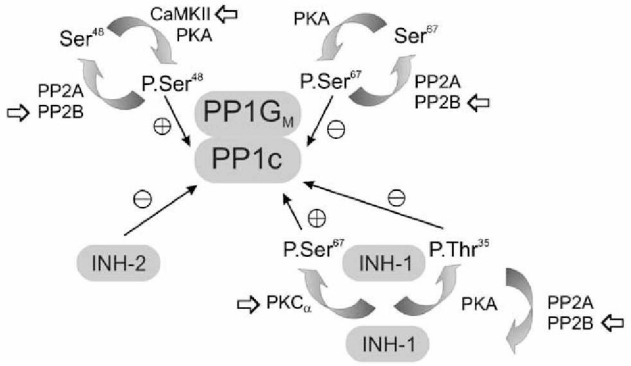

Fig. 2. Regulation of the phosphatase that dephosphorylates PLN. PP1 consists of a catalytic subunit ($PP1_C$) and a regulatory protein that targets the enzyme to the SR ($PP1G_M$). $PP1G_M$ can be phosphorylated at Ser^{48} and Ser^{67}. Phosphorylation of Ser^{67} disrupts the interaction $PP1_C$–$PP1G_M$, and the catalytic subunit released is less active towards PLN. In contrast, Ser^{48} phosphorylation increases PP1 activity. PKA phosphorylates both sites being the net effect, inhibition of PP1 activity. SR-bound CaMKII phosphorylates only the Ser^{48} site. Dephosphorylation of both sites on $PP1G_M$ is acomplished by PP2A and PP2B. PP1 activity is also regulated by accessory proteins, the inhibitor 1 and 2 (INH-1 and INH-2). PKA phosphorylation of Thr^{35} of INH-1 turns on its inhibitory activity towards PP1. Phosphorylation of Ser^{67} of INH-1 by the Ca^{2+}- and phospholipid-dependent PKCα increases the activity of PP1. Dephosphorylation of INH-1 is carried out by PP2A and PP2B. Open arrows indicate the points of Ca^{2+} regulation.

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 acidotic 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 TnI [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+} reuptake by the SR. This would tend to compensate the depression of SERCA2a and would ameliorate Ca^{2+} overload, 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

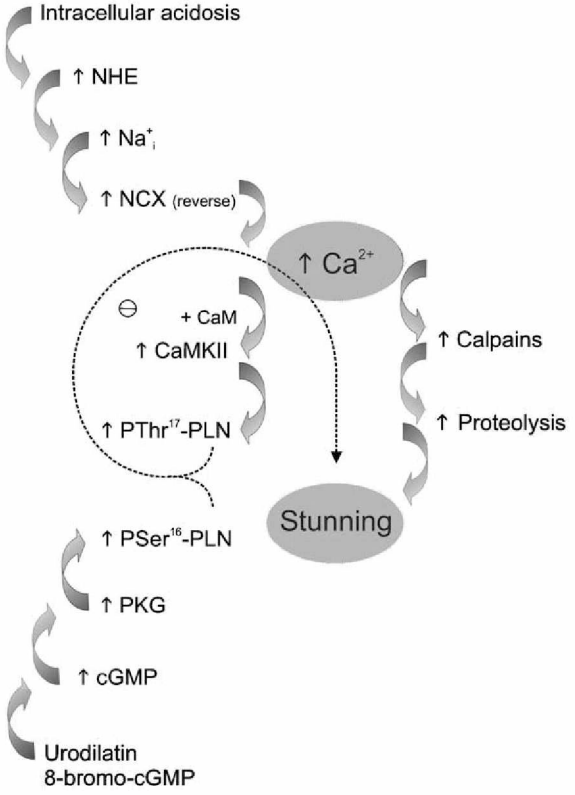

Fig. 3. Cascade of events leading to the generation of stunning and the putative protective role of PLN. The accumulation of protons during ischemia activates the Na^+/H^+exchanger (NHE) to extrude H^+, with the consequent increase in intracellular Na^+. The increase in intracellular Na^+ favors the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) that as a counterpart of Na^+ extrusion promotes Ca^{2+} influx, leading to Ca^{2+} overload. The increase in intracellular Ca^{2+} activates Ca^{2+}-dependent proteases, such as calpains that has been proposed to be responsible for the proteolysis of different proteins, like myofilament proteins, the suggested endpoint of myocardial stunning in rodents. The phosphorylation of PLN at Thr^{17} through CaMKII, and at Ser^{16} by PKG, by increasing SR Ca^{2+} uptake, could limit the Ca^{2+} overload.

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 β-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 β-adrenergic pathway [84]. According to these results, an increase in PLN expression or function would lead to an increase in β-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 β-adrenoceptor stimulation and contributes to the relaxant effect of the high concentrations of the β-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 acidotic period. These results, together with the fact that phosphorylation of Ser^{16} of PLN by cGMP has been also shown to be protective in ischemia/reperfusion injury, 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.

Acknowledgements

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

References

[1] Fabiato A, Fabiato F. Calcium release from the sarcoplasmic reticulum. Circ Res 1977;40:119–29.
[2] Bers DM. Excitation–contraction coupling and cardiac contractile force. 2nd ed. Dordrecht, Netherlands: Kluwer Academic Publishers; 2001.
[3] Tada M, Kirchberger MA, Katz AM. Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3′,5′-monophosphate-dependet protein kinase. J Biol Chem 1975;250:2640–7.
[4] James P, Inui M, Tada M, Chiesi M, Carafoli E. Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. Nature 1989;342:90–2.
[5] Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, et al. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of β-agonist simulation. Circ Res 1994;75:401–9.
[6] Hoit BD, Khoury SF, Kranias EG, Ball N, Walsh RA. In vivo echocardiographic detection of enhanced left ventricular function in genetargeted mice with phospholamban deficiency. Circ Res 1995;77:632–7.
[7] Chu G, Luo W, Slack JP, Tilgmann C, Sweet WE, Spindler M, et al. Compensatory mechanisms associated with the hyperdynamic function of phospholamban-deficient mouse hearts. Circ Res 1996;79:1064–76.
[8] Kadambi VJ, Ponniah S, Harrer J, Hoit B, Dorn GW, Walsh RA, et al. Cardiac-specific overexpression of phospholamban alters calcium kinetics and resultant cardiomyocyte mechanics in transgenic mice. J Clin Invest 1996;97:533–9.
[9] Luo W, Wolska BM, Grupp IL, Harrer JM, Haghighi K, Ferguson DG, et al. Phospholamban gene dosage effects in the mammalian heart. Circ Res 1996;78:839–47.
[10] Simmerman HK, Collins JH, Theibert JL, Wegener AD, Jones LR. Sequence analysis of phospholamban. Identification of phosphorylation sites and two major structural domains. J Biol Chem 1986;261:13333–41.
[11] Drago GA, Colyer J. Discrimination between two sites of phosphory lation on adjacent amino acids by phosphorylation site-specific antibodies to phospholamban. J Biol Chem 1994;269:25073–7.
[12] Huggins JP, Cook EA, Piggott JR, Mattinsley TJ, England PJ. Phospholamban is a good substrate for cyclic GMP-dependent protein kinase in vitro, but not in intact cardiac or smooth muscle. Biochem J 1989;260:829–35.
[13] Movsesian MA, Nishikawa M, Adelstein RS. Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. Stimulation of cardiac sarcoplasmic reticulum calcium uptake. J Biol Chem 1984;259:8029–32.
[14] Edes I, Kranias EG. Phospholamban and troponin I are substrates for protein kinase C in vitro but not in intact beating guinea pig hearts. Circ Res 1990;67:394–400.
[15] Mundiña-Weilenmann C, Vittone L, Rinaldi G, Said M, Chiappe de Cingolani GE, Mattiazzi AR. Endoplasmic reticulum contribution to the relaxant effect of cGMP and cAMP elevating agents in aorta. Am J Physiol Heart Circ Physiol 2000;278:H1856–65.
[16] Pierkes M, Gambaryan S, Boknik P, Lohmann SM, Schmitz W, Potthast R, et al. Increased effects of C-type natriuretic peptide on cardiac ventricular contractility and relaxation in guanylyl cyclase A-deficient mice. Cardiovasc Res 2002;53:852–61.
[17] Lindemann JP, Jones LR, Hathaway DR, Henry BG, Watanabe AM. β-adrenergic simulation of phospholamban phosphorylation and Ca^{2+}-ATPase activity in guinea pig ventricles. J Biol Chem 1983;258:464–71.
[18] Lindemann JP, Watanabe AM. Phosphorylation of phospholamban in intact myocardium. Role of Ca^{2+}-calmodulin-dependent mechanisms. J Biol Chem 1985;260:4516–25.
[19] Wegener AD, Zimmermann HKB, Lindenmann JP, Jones LR. Phospholamban phosphorylation in intact ventricles: phosphorylation of serine 16 and threonine 17 in response to β-adrenergic stimulation. J Biol Chem 1986;261:5154–9.
[20] Mundiña de Weilenmann C, Vittone L, de Cingolani G, Mattiazzi A. Dissociation between contraction and relaxation: the possible role of phospholamban phosphorylation. Basic Res Cardiol 1987;82:507–16.
[21] Karczewski P, Bartel S, Haase H, Krause EG. Isoproterenol induces both cAMP- and calcium-dependent phosphorylation of phospholamban in canine heart in vivo. Biomed Biochim Acta 1987;46:S433–9.
[22] Vittone L, Mundiña C, Chiappe de Cingolani G, Mattiazzi A. cAMP and calcium dependent mechanisms of phospholamban phosphorylation in intact hearts. Am J Physiol Heart Circ Pysiol 1990;258:H318–25.
[23] Mundiña-Weilenmann C, Vittone L, Ortale M, Chiappe de Cingolani G, Mattiazzi A. Immunodetection of phosphorylation sites gives new insights into the mechanisms underlying phospholamban phosphorylation in the intact heart. J Biol Chem 1996;271:33561–7.
[24] Calaghan SC, White E, Colyer J. Co-ordinated changes in cAMP, phosphorylated phospholamban, Ca^{2+} and contraction following beta-adrenergic stimulation of rat heart. Pflugers Arch 1998;436:948–56.
[25] Kuschel M, Karczewski P, Hempel P, Schlegel WP, Krause EG, Bartel S. Ser^{16} prevails over Thr^{17} phospholamban phosphorylation in the beta-adrenergic regulation of cardiac relaxation. Am J Physiol Heart Circ Physiol 1999;276:H1625–33.
[26] Bartel S, Vetter D, Schlegel WP, Wallukat G, Krause EG, Karczewski P. Phosphorylation of phospholamban at threonine-17 in the absence and presence of beta-adrenergic stimulation in neonatal rat cardiomyocytes. J Mol Cell Cardiol 2000;32:2173–85.
[27] Said M, Mundiña-Weilenmann C, Vittone L, Mattiazzi A. The relative relevance of phosphorylation of the Thr^{17} residue of phospholamban is different at different levels of β-adrenergic stimulation. Pflugers Arch 2002;444:801–9.

[28] MacDougall LK, Jones LR, Cohen P. Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. Eur J Biochem 1991;196:725–34.
[29] Sham JS, Jones LR, Morad M. Phospholamban mediates the beta-adrenergic-enhanced Ca^{2+} uptake in mammalian ventricular myocytes. Am J Physiol Heart Circ Physiol 1991;261:H1344–9.
[30] Peña JR, Wolska BM. Troponin I phosphorylation plays an important role in the relaxant effect of beta-adrenergic stimulation in mouse hearts. Cardiovasc Res 2004;61:756–63.
[31] Bilezikjian LM, Kranias EG, Potter JD, Schwartz A. Studies on phosphorylation of canine cardiac sarcoplasmic reticulum by calmodulin-dependent protein kinase. Circ Res 1981;49:1356–62.
[32] Napolitano R, Vittone L, Mundiña-Weilenmann C, Chiappe de Cingolani G, Mattiazzi A. Phosphorylation of phospholamban in the intact heart. A study on the physiological role of the Ca^{2+}–calmodulin-dependent protein kinase system. J Mol Cell Cardiol 1992;24:387–96.
[33] Luo W, Chu G, Sato Y, Zhou Z, Kadambi VJ, Kranias EG. Transgenic approaches to define the functional role of dual site phospholamban phosphorylation. J Biol Chem 1998;73:4734–9.
[34] Chu G, Lester JW, Young KB, Luo W, Zhai J, Kranias EG. A single site (Ser^{16}) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to β-agonists. J Biol Chem 2000;275:38938–43.
[35] Capogrossi MC, Stern MD, Spurgeon HA, Lakatta EG. Spontaneous Ca^{2+} release from the sarcoplasmic reticulum limits Ca^{2+}-dependent twitch potentiation in individual cardiac myocytes. A mechanism for maximum inotropy in the myocardium. J Gen Physiol 1988;91:133–55.
[36] Bradshow JM, Kubota Y, Meyer T, Schulman H. An ultrasensitive Ca^{2+}/calmodulin-dependent protein kinase II-protein phosphatase 1 switch facilitates specificity in postsynaptic calcium signaling. Proc Natl Acad Sci U S A 2003;100:10512–7.
[37] Walker KS, Watt PW, Cohen P. Phosphorylation of the skeletal muscle glycogen-targeting subunit of protein phosphatase 1 in response to adrenaline in vivo. FEBS Lett 2000;466:121–4.
[38] De Koninck P, Schulman H. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science 1998;79:227–30.
[39] Hagemann D, Kuschel M, Kuramochi T, Zhu W, Cheng H, Xiao RP. Frequency-encoding Thr^{17} phospholamban phosphorylation is independent of Ser^{16} phosphorylation in cardiac myocytes. J Biol Chem 2000;275:22532–6.
[40] Zhao W, Uehara Y, Chu G, Song Q, Qian J, Young K, et al. Threonine-17 phosphorylation of phospholamban: a key determinant of frequency-dependent increase of cardiac contractility. J Mol Cell Cardiol 2004;7:607–12.
[41] DeSantiago J, Maier LS, Bers DM. Frequency-dependent acceleration of relaxation in the heart depends on CaMKII, but not phospholamban. J Mol Cell Cardiol 2002;34:975–84.
[42] Valverde CA, Mundiña-Weilenmann C, Said M, Ferrero P, Vittone L, Salas M, et al. Frequency-dependent acceleration of relaxation in mammalian heart: a property not relying on phospholamban and SERCA2a phosphorylation. J Physiol 2005;562:801–13.
[43] Hubbard MJ, Cohen P. Regulation of protein phosphatase-1G from rabbit skeletal muscle. 1. Phosphorylation by cAMP-dependent protein kinase at site 2 releases catalytic subunit from the glycogen-bound holoenzyme. Eur J Biochem 1989;186:701–9.
[44] Dent P, Lavoinne A, Nakielny S, Caudwell FB, Watt P, Cohen P. The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature 1990;348:302–7.
[45] Cohen P. The structure and regulation of protein phosphatases. Annu Rev Biochem 1989;58:453–508.
[46] Neumann J. Altered phosphatase activity in heart failure, influence on Ca^{2+} movement. Basic Res Cardiol 2002;97(Suppl 1):I91–5.
[47] Vittone L, Mundiña-Weilenmann C, Said M, Mattiazzi A. Mechanisms involved in the acidosis enhancement of the isoproterenol-induced phosphorylation of phospholamban in the intact heart. J Biol Chem 1998;273:9804–11.
[48] Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, et al. PKC-α regulates cardiac contractility and propensity toward heart failure. Nat Med 2004;10:248–54.
[49] Sacchetto R, Bovo E, Donella-Deana A, Damiani E. Glycogen- and PP1c-targeting subunit GM is phosphorylated at Ser48 by sarcoplasmic reticulum-bound Ca^{2+}–calmodulin protein kinase in rabbit fast twitch skeletal muscle. J Biol Chem 2005;280:7147–55.
[50] Gaskell WH. On the tonicity of the heart and blood vessels. J Physiol 1880;3:48–75.
[51] Fabiato A, Fabiato F. Myofilament-generated tension oscillations during partial calcium activation and activation dependence of the sarcomere length–tension relation of skinned cardiac cells. J Gen Physiol 1978;72:667–99.
[52] Hulme JT, Orchard CH. Effect of acidosis on Ca^{2+} uptake and release by sarcoplasmic reticulum of intact rat ventricular myocytes.. Am J Physiol Heart Circ Physiol 1998;275:H977–87.
[53] DeSantiago J, Maier LS, Bers DM. Phospholamban is required for CaMKII-dependent recovery of Ca transients and SR Ca reuptake during acidosis in cardiac myocytes. J Mol Cell Cardiol 2004;36:67–74.
[54] Mundiña-Weilenmann C, Ferrero P, Said M, Vittone L, Kranias EG, Mattiazzi A. Role of phosphorylation of Thr^{17} residue of phospholamban in mechanical recovery during hypercapnic acidosis. Cardiovasc Res 2005;66:114–22.
[55] Brofman JD, Leff AR, Munoz NM, Kirchhoff C, White SR. Sympathetic secretory response to hypercapnic acidosis in swine. J Appl Physiol 1990;69:710–7.
[56] Braunwald E, Kloner RA. The stunned myocardium: prolonged postischemic ventricular dysfunction. Circulation 1982;66:1146–9.
[57] Bolli R, Marbán E. Molecular and cellular mechanism of myocardial stunning. Physiol Rev 1999;79:609–34.
[58] Gao WD, Atar D, Backx P, Marbán E. Relationship between intracellular calcium and contractile force in stunned myocardium. Direct evidence for decreased myofilament Ca^{2+} responsiveness and altered diastolic function in intact ventricular muscle. Circ Res 1995;76:1036–48.
[59] Rapundalo ST, Briggs FN, Feher JJ. Effects of ischemia on the isolation and function of canine cardiac sarcoplasmic reticulum. J Mol Cell Cardiol 1986;18:837–51.
[60] Krause SM, Jacobus WE, Becker LC. Alterations in cardiac sarcoplasmic reticulum calcium transport in the postischemic "stunned" myocardium. Circ Res 1989;65:526–30.
[61] Limbruno U, Zucchi R, Ronca-Testoni S, Galbani P, Ronca G, Mariani M. Sarcoplasmic reticulum function in the "stunned" myocardium. J Mol Cell Cardiol 1989;21:1063–72.
[62] Zucchi R, Ronca-Testoni S, Di Napoli P, Yu G, Gallina S, Bosco G, et al. Sarcoplasmic reticulum calcium uptake in human myocardium subjected to ischemia and reperfusion during cardiac surgery. J Mol Cell Cardiol 1996;28:1693–701.
[63] Osada M, Netticadan P, Tamura K, Dallha NG. Modification of ischemia–reperfusion-induced changes in cardiac sarcoplasmic reticulum by preconditioning. Am J Physiol Heart Circ Physiol 1998;274:H2025–34.
[64] Vittone L, Mundiña-Weilenmann C, Said M, Ferrero P, Mattiazzi A. Time course and mechanisms of phosphorylation of phospholamban residues in ischemia–reperfused rat hearts. Dissociation of phospholamban phosphorylation pathways. J Mol Cell Cardiol 2002;34:39–50.
[65] Said M, Vittone L, Mundiña-Weilenmann C, Ferrero P, Kranias EG, Mattiazzi A. Role of dual-site phospholamban phosphorylation in the stunned heart: insights from phospholamban site-specific mutants. Am J Physiol Heart Circ Physiol 2003;285:H1198–205.
[66] Abdallah Y, Gkatzoflia A, Pieper H, Zoga E, Walther S, Kasseckert S, et al. Mechanism of cGMP-mediated protection in a cellular model of myocardial reperfusion injury. Cardiovasc Res 2005;66:123–31.

[67] Xie Y, Zhu Y, Zhong WZ, Chen L, Zhou Z-N, Yuan W-J, et al. Role of dual-site phospholamban phosphorylation in intermittent hypoxia-induced cardioprotection against ischemia–reperfusion injury. Am J Physiol Heart Circ Physiol 2005;288:H2594–602.
[68] Kim SJ, Kudej RK, Yatani A, Kim YK, Takagi G, Honda R, et al. A novel mechanism for myocardial stunning involving impaired Ca^{2+} handling. Circ Res 2001;89:831–7.
[69] Lindner M, Erdmann E, Beuckelmann DJ. Calcium content of the sarcoplasmic reticulum in isolated ventricular myocytes from patients with terminal heart failure. J Mol Cell Cardiol 1998;30:743–9.
[70] O'Rourke B. Mechanisms of altered excitation-contraction coupling in canine tachicardia-induced heart failure. I. Experimental studies. Circ Res 1999;84:562–70.
[71] Hasenfuss G. Alterations in calcium regulatory proteins in heart failure. Cardiovasc Res 1998;37:279–89.
[72] Marx SO. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing heart. Cell 2000;101:365–76.
[73] Schwinger RHG, Munch G, Bolk B, Karczewski P, Krause EG, Erdmann E. Reduced Ca^{2+}-sensitivity of SERCA2a in failing human myocardium due to reduced Serin-16 phospholamban phosphorylation. J Mol Cell Cardiol 1999;31:479–91.
[74] Sande JB, Sjaastad I, Hoen IB, Bokenes J, Tonnessen T, Holt E, et al. Reduced level of serine (16) phosphorylated phospholamban in the failing rat myocardium: a major contributor to reduced SERCA2a activity. Cardiovasc Res 2002;53:383–91.
[75] Netticadan T, Temsah R, Kawabata K, Dallha NS. Sarcoplasmic reticulum Ca^{2+}/calmodulin-dependent kinase is altered in heart failure. Circ Res 2000;86:596–605.
[76] Mishra S, Sabbah HN, Jain JC, Gupta RC. Reduced Ca^{2+}-calmodulin-dependent protein kinase activity and expression of LV myocardium of dogs with heart failure. Am J Physiol Heart Circ Physiol 2003;284: H876–83.
[77] Huang B, Wang S, Qin D, Boutjdir M, El-Sherif N. Dimished basal phosphorylation level of phospholamban in the postinfarctium remodeled rat ventricle. Role of β-adrenergic pathway, Gi protein, phosphodiesterase and phosphatases. Circ Res 1999;85:848–55.
[78] Bristow MR, Ginsburg R, Umans V. β1- and β2-adrenergic -receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective β1-receptor down regulation in heart failure. Circ Res 1986;59:248–54.
[79] Gupta RC, Mishra S, Rastogi S, Imai M, Habib O, Sabbah HN. Cardiac SR-coupled PP1 and expression are increased and inhibitor-1 protein expression is decreased in failing hearts. Am J Physiol Heart Circ Physiol 2003;285:H2373–81.
[80] Boateng S, Seymour AM, Dunn M, Yacoub M, Boheler K. Inhibition of endogenous cardiac phosphatase activity and measurement of sarcoplasmic reticulum calcium uptake: a possible role of phospholamban phosphorylation in the hypertrophied myocardium. Biochem Biophys Res Commun 1997;239:701–5.
[81] Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93:215–28.
[82] Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of δ-isoforms of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase in failing and non failing human myocardium. Circ Res 1999;84:713–21.
[83] Kirchhefer U, Schmitz W, Scholtz H, Newmann J. Activity of cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase in failing and non failing human hearts. Cardiovasc Res 1999;42:254–61.
[84] Dash R, Kadambi V, Schmidt AG, Tepe NM, Biniakiewicz D, Gerst MJ, et al. Interactions between phospholamban and beta-adrenergic drive may lead to cardiomyopathy and early mortality. Circulation 2001;103:889–96.