

PHOSPHOLAMBAN: A TINY PROTEIN WITH A PROMINENT ROLE IN THE REGULATION OF CARDIAC RELAXATION AND CONTRACTILITY

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SUMMARY

Phospholamban (PLN) is a small sarcoplasmic reticulum (SR) protein that in the dephosphorylated state tonically inhibits the SR Ca^{2+} -ATPase (SERCA2a). Different type of evidence to be described, points to PLN as a crucial regulator of basal cardiac Ca^{2+} cycling, contractility and relaxation and as a main determinant of β -adrenergic stimulatory responses *in vivo*. The involvement of PLN and PLN phosphorylation under pathological conditions will be also discussed.

INTRODUCTION

The central players of excitation-contraction-coupling (ECC)

Excitation-contraction-coupling can be defined as the series of events beginning at the level of the sarcolemma by membrane depolarization, and finishing at the level of the sarcomeres with the interaction and sliding of myofilaments to produce contraction. Ca^{2+} ions are responsible for the coupling between both events. Figure 1 is a schematic

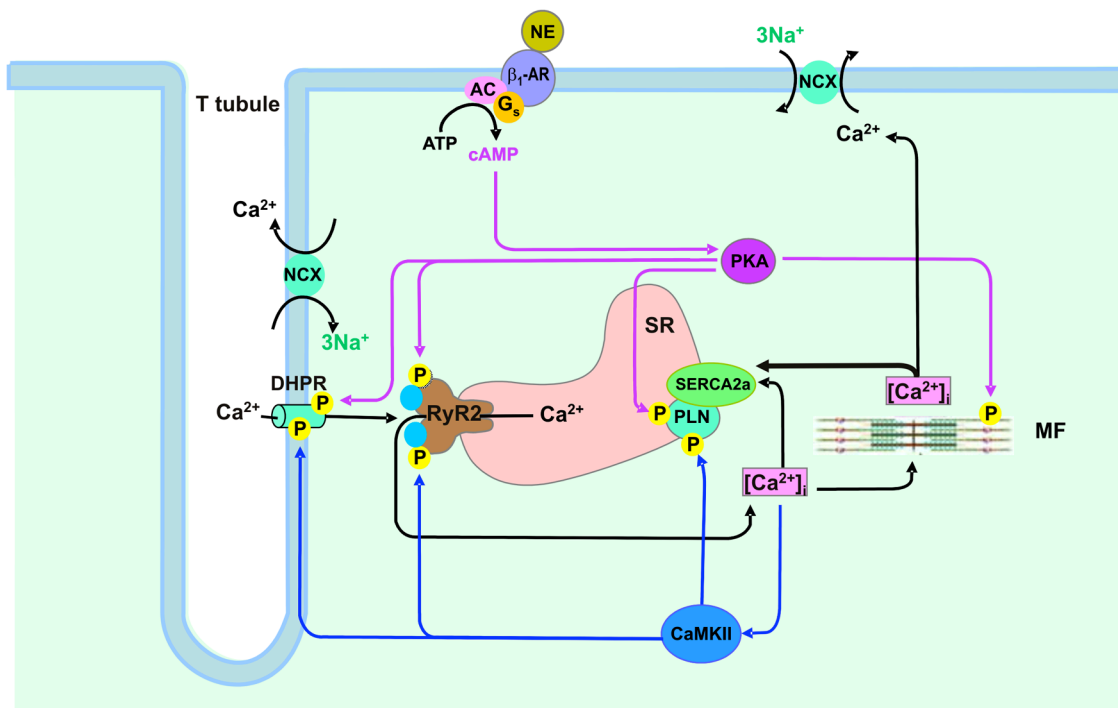


Figure 1: The different steps of ECC in the heart are depicted in black. In violet and blue: cAMP/PKA/CaMKII cascades triggered by β -adrenoceptor stimulation.

illustration of this mechanism. Upon depolarization, Ca^{2+} enters the cell through the voltage-dependent L-type Ca^{2+} channels (Dihydropyridine receptors, DHPRs) and triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through the activation of the SR Ca^{2+} channels or ryanodine receptors (RyR2). This process, known as Ca^{2+} -induced- Ca^{2+} -release¹, amplifies and coordinates the Ca^{2+} signal. Ca^{2+} then binds to the myofilaments allowing their interaction. The decrease in cytosolic Ca^{2+} triggers relaxation. This decrease is mainly induced by the SR Ca^{2+} -ATPase (SERCA2a), which mediates Ca^{2+} uptake into the SR, and, to a lesser extent, by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which transfers Ca^{2+} to the extracellular space². By mediating SR Ca^{2+} uptake, the activity of SERCA2a also influences cardiac contractility since it determines the size of the luminal Ca^{2+} store that is available for release in the next beat. The activity of SERCA2a, which in humans determines the rate of removal of >70% of cytosolic Ca^{2+} , is under the control of a closely associated SR protein named phospholamban (PLN), the endogenous inhibitor of SERCA2a³. In this review we will focus on PLN and its role in the regulation of cardiac function under physiological and pathological conditions.

PLN structure and interaction with the SR Ca^{2+} -ATPase

PLN is a small protein spanning the SR membranes, expressed almost exclusively in cardiac, smooth and slow-twitch skeletal muscles. The cloning of PLN revealed that it is a 52-amino acid protein of 6.1 kDa that forms a homopentamer, which accounts for the original observation that phosphorylated PLN has an apparent mass of 22 kDa⁴. From analysis of the amino-acid sequence, it was suggested that the protein is organized in three domains: cytosolic domain Ia [amino acids (AA) 1–20] containing serine (Ser) 16, the PKA phosphorylation site, and threonine (Thr) 17, the CaMKII phosphorylation site; cytosolic domain Ib (AA 21–30) and domain II (AA 31–52), which traverses the membrane⁵.

It has been proposed that a physical protein-protein interaction between the cytoplasmic domain of PLN (AA 2-18 of each subunit) and SR Ca^{2+} -ATPase (AA 336-412 and 467-762) is critically involved in the inhibitory effect of PLN on the Ca^{2+} -pump. However, the interaction of this PLN domain with the SERCA2a appears to be insufficient *per se* to alter the apparent Ca^{2+} affinity of SERCA2a. Moreover, coexpression of the hydrophobic membrane spanning PLN domain with SERCA2a has revealed that this PLN domain was able to modify the Ca^{2+} affinity of the pump suggesting that both domains are involved in the inhibitory effect of PLN on SR Ca^{2+} -ATPase⁵.

In *in vitro* expression systems, PLN monomer appears to be the more effective Ca^{2+} -pump inhibitor. Moreover, gain of function experiments demonstrated that overexpression of two PLN monomeric mutants (L37A and I40A) in transgenic mouse models are superinhibitors of SERCA2a. This type of evidence indicates that PLN exists in the SR as an inactive pentamer (storage form), which depolymerizes into functional monomers prior to interaction with SERCA2a⁵. Of interest, overexpression of HAX-1, a ubiquitously expressed anti-apoptotic protein, reduced SERCA2a activity in isolated cardiomyocytes and *in vivo*, leading to depressed myocyte Ca^{2+} kinetics and mechanics. Mechanistically, HAX-1 promotes the formation of PLN monomers⁶.

PLN Function

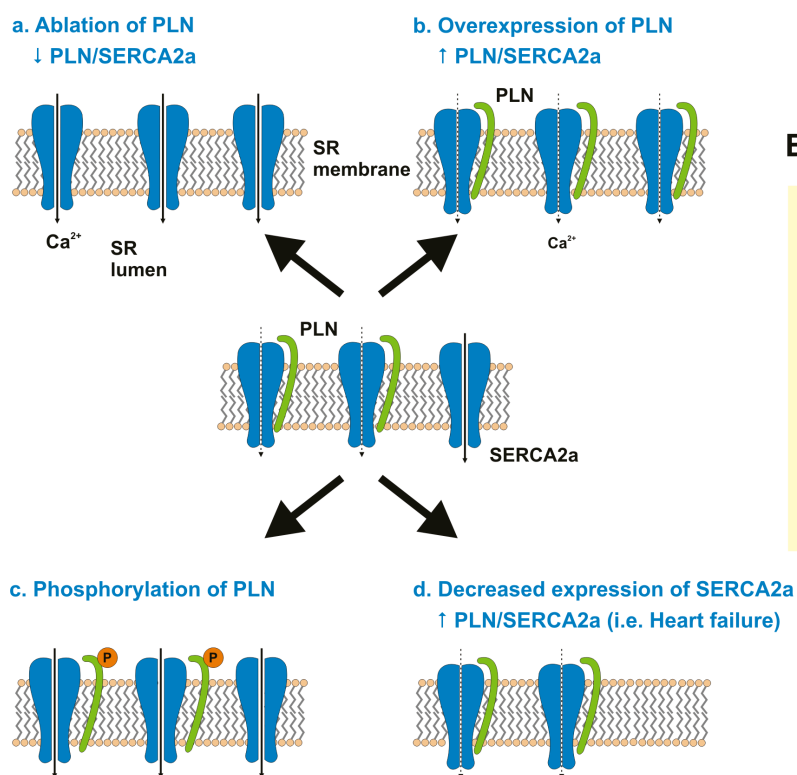
Being PLN an endogenous inhibitor of the SR Ca^{2+} -pump, there are two main mechanisms by which it may regulate cardiac relaxation and contractility (Figure 2):

1. Alterations in the relationship PLN/SERCA2a, which would either increase or decrease the number of SERCA2as inhibited by PLN.
2. Modifications of the phosphorylation status of PLN, owing to the fact that PLN phosphorylation relieves PLN inhibition on the pump.

1. Relationship PLN/SERCA2a

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 PLN as an endogenous inhibitor of SERCA2a^{7,8}. Ablation of PLN enhanced contractility and relaxation. This hypercontractile function of PLN-deficient hearts (PLN^{-/-}) was associated with increases in the apparent affinity of SERCA2a for Ca²⁺ and in the intraluminal SR Ca²⁺ content⁷. Opposite effects were observed with the overexpression of PLN⁸ (Figure 2Aa-b).

A



B

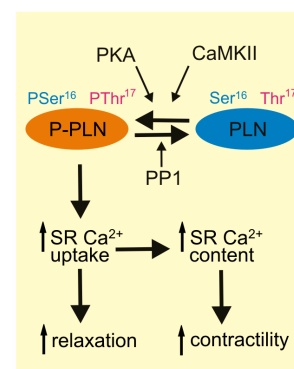


Figure 2: A. Different ways of modifying the inhibitory effect of PLN on SERCA2a B. Regulation of the phosphorylation state of PLN and its functional consequences.

2. PLN phosphorylation pathways

In vitro experiments demonstrated that phosphorylation of PLN at Ser¹⁶ site by PKA and at Thr¹⁷ site, by CaMKII reverses PLN inhibitory function, probably through conformational changes that disrupt PLN/ SERCA2a interactions⁵ with the consequent increase in SR Ca²⁺ uptake (Figure 2Ac and 2B). *In vitro* studies also indicate that PKA and CaMKII phosphorylations are independent of each other and when acting together they are additive. A crucial determinant of the status of phosphorylation of PLN, which is frequently overlooked, is the activity of the type 1 phosphatase (PP1), the major SR phosphatase that specifically dephosphorylates PLN⁹, in turn under the control of different kinases and phosphatases (See for review Mattiazzi et al., 2005)¹⁰.

PLN and β -adrenergic stimulation

Cardiac function is regulated on a beat-to-beat basis through the sympathetic nervous system. Sympathetic stimulation of cardiac β -adrenergic receptors (β -AR) induces positive chronotropic (heart beats more frequently), inotropic (heart beats stronger), and lusitropic (heart relaxes faster) effects, -the so-called “fight or flight response”-, the most effective mechanism to acutely increase output of the heart. Activation of β -AR by β 1- agonists at the cell membrane, initiates a signal-transduction pathway (Figure 1) that proceeds through Gs protein to stimulate cyclic AMP formation by adenylate cyclase and PKA activation, which then phosphorylates and alters the function of several cardiac proteins among which PLN is predominant in determining the mechanical effects of β -agonists. Two different experimental approaches eloquently support this contention: 1. Dialysis of ventricular myocytes with a monoclonal antibody against PLN, virtually suppressed the mechanical effects of the β -agonists¹¹; 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 preparations⁷.

Wegener et al.¹² first demonstrated that there were only two sites of PLN phosphorylated during β -AR stimulation: Ser¹⁶ (PKA site) and Thr¹⁷ (CaMKII site). Moreover, phosphorylation at Ser¹⁶ precedes that at Thr¹⁷, although at steady state, both sites were phosphorylated in approximately equimolar amounts. Phosphorylation of PLN by increasing SR Ca²⁺ uptake, increases SR Ca²⁺ refilling in each beat and therefore enhanced relaxation and contractility (Figure 2B). Recent data demonstrate that it is the β 1AR subtype that mediates CaMKII activation and PLN Thr¹⁷ phosphorylation in response to isoproterenol infusion¹³.

Isoproterenol-induced phosphorylation of PLN by PKA and CaMKII activation was also observed by using different techniques which corroborate that dual phosphorylation of PLN does occur *ex vivo* as it was the case of the *in vitro* experiments¹⁴⁻¹⁷.

Interdependence of PLN phosphorylation pathways during β -adrenergic stimulation.

Earlier attempts to phosphorylate PLN by CaMKII in the intact heart had systematically failed unless cAMP levels within the cell increase^{14,15,18}. These findings suggested an interaction between PLN phosphorylation pathways. Experiments in transgenic mice, expressing either PLN-wild type (PLN-WT), or the Ser16→Ala mutant PLN, demonstrated that the phosphorylation of Ser¹⁶ of PLN is a prerequisite for the phosphorylation of Thr¹⁷¹⁹, in line with earlier findings which indicated the dependence of the phosphorylation of Thr¹⁷ on intracellular cAMP levels¹⁵. Experiments in Thr17→Ala mutant PLN 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 β -adrenergic stimulation²⁰. These experiments suggested a predominant role of the phosphorylation of Ser¹⁶ over that of Thr¹⁷, in the mechanical effect produced by β -adrenergic stimulation. The combination of phosphorylation site-specific antibodies with quantification of ³²P incorporation into PLN further helped to clarify the relative role of Ser¹⁶ and Thr¹⁷ phosphorylations^{16,17}. Lowering extracellular Ca²⁺ significantly decreased total PLN phosphorylation and the shortening of half relaxation time induced by the β -agonist isoproterenol (Figure 3A). Figure 3B shows that the decrease in total PLN phosphorylation was exclusively produced by the decrease in CaMKII-dependent Thr¹⁷ site phosphorylation. These experiments indicated that phosphorylation of Thr¹⁷ accounted for approximately 50% of the total PLN phosphorylation and rate of relaxation, at the highest isoproterenol concentrations (≥ 10 nM). However similar type of experiments demonstrated that there

was no contribution of the CaMKII pathways to the total PLN phosphorylation at the lowest isoproterenol concentrations¹⁷. This finding 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 Thr¹⁷ site (See Figure 1). Since it has been shown that PKA inhibits PP1 (See for references¹⁰), the modest increase in PKA activity would also fail to significantly inhibit the phosphatase that dephosphorylates PLN, further favoring the dephosphorylated state of Thr¹⁷ residue. These results may explain the failure to find PLN phosphorylation in transgenic mice in which Ser¹⁶ site was mutated to Ala, i.e., the lack of phosphorylation of the Ser¹⁶ site precludes the increase in intracellular Ca^{2+} necessary to phosphorylate Thr^{17,19}.

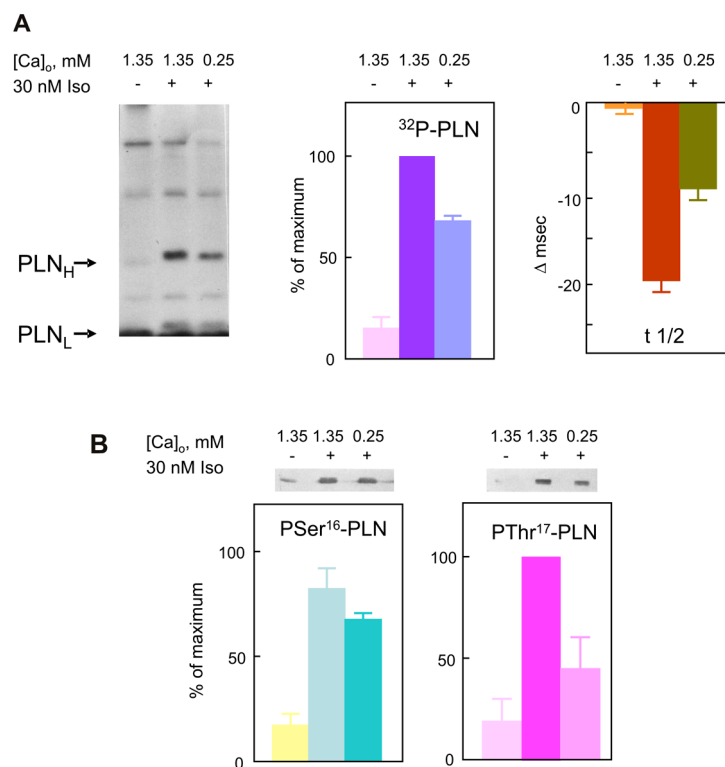


Figure 3. Phosphorylation of Ser¹⁶ and Thr¹⁷ of PLN similarly contribute to the relaxant effect of maximal β -AR stimulation.

Taken together, these findings demonstrated the additive nature of PKA and CaMKII pathways of PLN phosphorylation, in agreement with the *in vitro* results⁵. In addition, they indicated that both PLN phosphorylation sites equally contribute to the total PLN phosphorylation at the highest levels of β -adrenergic stimulation¹⁶. Finally, that CaMKII is a β -AR mediator being PKA its upstream activator, through the PKA-dependent increases in Ca^{2+} .

Interestingly, β -AR may activate Epac, a guanine nucleotide exchange protein directly activated by cAMP, independently of PKA. Activation of Epac has been recently associated with an increase in CaMKII activity and phosphorylation of Thr¹⁷ of PLN. The mechanism of this activation remains unclear (See for references²¹).

Finally, it is important to mention that cGMP-dependent protein kinase and Akt, phosphorylate PLN at Ser¹⁶ and Thr¹⁷ residues, respectively, with the consequent increase in SR Ca^{2+} handling and contractility^{10,22}.

PLN under pathological conditions

There are several pathological situations in which PLN and PLN phosphorylation are involved. Among them ischemia/reperfusion and heart failure are particularly relevant. Interestingly, phosphorylation of PLN and the consequent increase in SR Ca^{2+} uptake, results beneficial for heart performance in some cases, whereas it is detrimental in others. Obviously, in all situations the effects of PLN phosphorylation is the same, i.e. to increase SR Ca^{2+} refilling. As will be discussed below, dissimilar outcomes of the same effect (increase in SR Ca^{2+} uptake) may arise from differences in the extent or type of Ca^{2+} handling alterations, which may be summarized in at least two mutually non exclusive reasons: 1. The different level of SR Ca^{2+} uptake and load produced by PLN phosphorylation; 2. The status/activity of other proteins, which share with PLN the task of intracellular Ca^{2+} handling, like the RyR2 and the NCX.

Ischemia/reperfusion injury (I/R)

In the last few years, our laboratory has described a dual effect of CaMKII-dependent phosphorylations in I/R, which may be attributed to a CaMKII-dependent phosphorylation of PLN at the Thr¹⁷ site: 1. A beneficial effect in the reversible I/R also known as stunned heart. This effect consists in an enhancement of the contractile and intracellular Ca^{2+} transient recovery. Direct measurements of intracellular Ca^{2+} and transgenic mice in which Thr¹⁷ site of PLN and/or Ser¹⁶ site of PLN were mutated to Ala, demonstrated that the phosphorylation of Thr¹⁷ residue of PLN, that occurs at the onset of reperfusion was essential for the mechanical and intracellular Ca^{2+} handling recovery observed^{23,24} 2. A detrimental effect (apoptosis and necrosis), involving CaMKII-activation, Thr¹⁷ phosphorylation of PLN and mitochondrial alterations, in the irreversible I/R. These effects appear to be associated with a degradation of RyR2 and a increase in SR Ca^{2+} release^{25,26}. Similar opposite results of PLN phosphorylation and increase in SR Ca^{2+} uptake on myocardial performance, have been obtained by others. For instance, in PLN^{-/-} mice, the absence of PLN exacerbates I/R injury²⁷. Similarly, the protective effect (decreased number of apoptotic cells) of chronic CaMKII inhibition in transgenic mice expressing a CaMKII inhibitory peptide (AC3-I mice) and submitted to myocardial infarction, was absent in interbred AC3-I mice with PLN^{-/-} mice²⁸. In these animals, the absence of the inhibitory effect of PLN greatly enhances SR Ca^{2+} uptake and load. In contrast, other results associate the increase in PLN phosphorylation observed after the inducible expression of inhibitor-1, -a PLN phosphatase inhibitor-, with the beneficial effect of this inhibitor in I/R injury²⁹. Figure 4 offers an interpretation to these apparently contradictory results: Phosphorylation of PLN would be beneficial, favoring SR Ca^{2+} handling, provided that SR Ca^{2+} uptake and load are moderate (or even high), and no other alterations occurred at the level of SR. This would be the case of the beneficial effect observed in the reversible I/R²⁴ (Figure 4A). In a similar way, the increase in SR Ca^{2+} uptake due to the absence of PLN that occurs in PLN^{-/-} mice, produces a very significant improvement of basal contractility (Figure 4B), which is tolerated throughout the life of the animal without deleterious consequences if left “unstressed”. In contrast, high or even moderate increases in SR Ca^{2+} content in hearts submitted to stress with alterations of RyR2s (degradation, phosphorylation, redox changes), may evolve to a detrimental effect due to an increase in Ca^{2+} release/leak that favors mitochondrial damage. This would be the case of our experiments of irreversible I/R^{25,26} (Figure 4C) and might be also the case of PLN^{-/-} mice submitted to I/R²⁷.

Heart Failure

Most findings now indicate that the levels of PLN protein remain unchanged, whereas the levels of SERCA2a protein decrease in human heart failure, altering the functional PLN/SERCA2a ratio and resulting in higher inhibition of the Ca^{2+} pump (Figure 2Ad). PLN is also less phosphorylated in heart failure, becoming more inhibitory overall. As a consequence, SERCA2a decreases the amount of Ca^{2+} reuptake by the SR, lowering SR Ca^{2+} refilling, relaxation and contractility³⁰. In most heart failure models, there is also an increase in NCX expression, which would compensate for the decrease in SERCA2a and the consequent impaired relaxation, although at the enormous cost of a loss of intracellular Ca^{2+} , which is extruded from the cell by the exchanger (See Figure 1)³⁰. Together, these alterations are main determinants of the decrease in contractility of failing hearts. The observed reduction in the Ca^{2+} available for myofilament activation and reduced SR Ca^{2+} reuptake are obvious targets to improve myocyte contraction and relaxation and, thus, cardiac pump function. Potential therapeutic strategies are to increase SERCA2a levels or to attenuate the PLN inhibitory effect on SERCA2a.

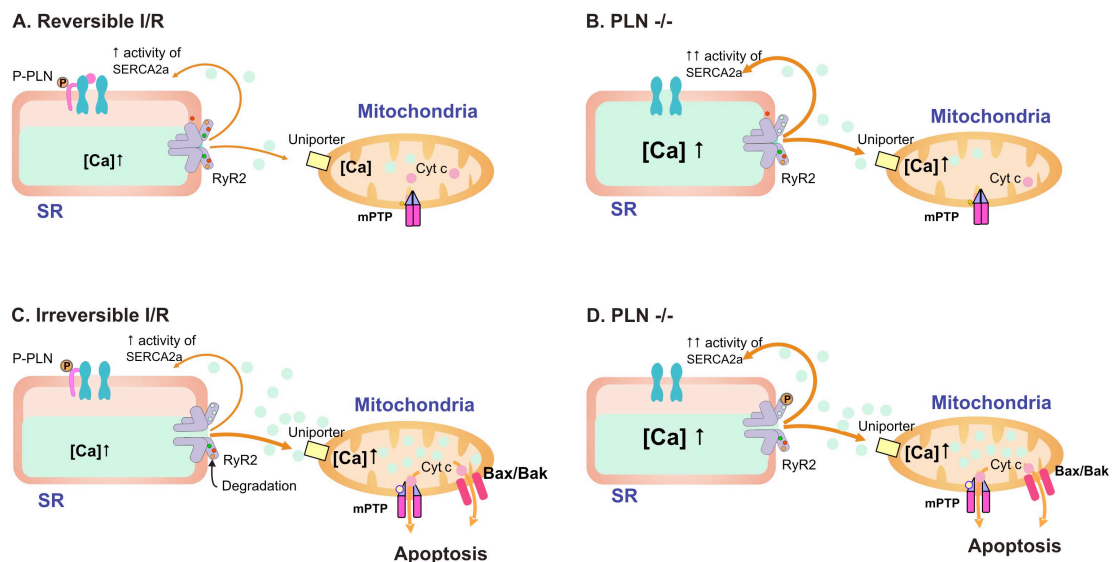


Figure 4: Dual role of PLN phosphorylation and increased SR Ca^{2+} uptake on cardiac function.

However, whereas some studies have clearly shown the beneficial effects of improved SR Ca^{2+} cycling through PLN ablation on cardiac function and remodelling, others have shown that normalization of myocyte Ca^{2+} handling might not translate into improved cardiac function *in vivo* or into reversal of remodelling. Just to mention a few results, overexpression of SERCA2a, using recombinant adenovirus-mediated gene transfer, led to enhanced contractility in isolated myocytes³¹ and improved cardiac function, metabolism and survival in a rat aortic constriction model of heart failure³². These studies support the idea that an enhanced expression of SERCA2a restores disturbed intracellular Ca^{2+} handling by decreasing the relative ratio of PLN/SERCA2a. Interestingly, these results have led to the ongoing clinical trials in patients with established heart failure which recently reported promising results³³. In contrast, in mice that overexpress both a G protein, $\text{G}\alpha_q$, involved in α -adrenergic and angiotensin II signalling, and a mutant myosin binding protein C that causes familial hypertrophic cardiomyopathy in humans, PLN ablation was less successful: cardiac myocyte contractility was rescued, but no improvement was observed in whole-heart contractile

function or in cardiac dilation³⁴. A recent study by Zhang et. al³⁵ confirms the dichotomy between the rescue of global Ca²⁺ handling by PLN-/- mice and the impairment of the heart failure phenotype. Overexpression of CaMKII δ C in mice produced dilated cardiomyopathy which was not rescued by the crossbreed with PLN-/-, but rather exacerbated. In exploring the possible mechanisms of this therapeutic failure, the authors show that SR Ca²⁺ release and uptake were normalized. However, the SR Ca²⁺ content was supranormal and RyR2s were phosphorylated. The authors postulated that these changes may be the cause of the observed increase in SR Ca²⁺ leak through the phosphorylated RyR2s which in turn produces mitochondrial Ca²⁺ overload and apoptosis and thus exacerbates heart failure (This possibility is schematized in Figure 4D).

In summary, PLN plays a major role in the regulation of cardiac relaxation and contractility, being the main effector of the β -adrenergic cascade at the cellular level. PLN has also a significant role in different heart diseases and several studies point to this protein as a potential target to increase SR Ca²⁺ uptake. However, it is important to define how and in which conditions this increase may be useful.

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