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Modulation of Cardiac Contractility by the Phopholamban/SERCA2a Regulatome

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Abstract

Heart disease remains the leading cause of death and disability in the Western world. Current therapies aim at treating the symptoms rather than the subcellular mechanisms, underlying the etiology and pathological remodeling in heart failure. A universal characteristic, contributing to the decreased contractile performance in human and experimental failing hearts, is impaired calcium sequestration into the sarcoplasmic reticulum (SR). SR calcium uptake is mediated by a Ca^{2+}-ATPase (SERCA2), whose activity is reversibly regulated by phospholamban (PLN). Dephosphorylated PLN is an inhibitor of SERCA and phosphorylation of PLN relieves this inhibition. However, the initial simple view of a PLN/SERCA regulatory complex has been modified by our recent identification of SUMO, S100 and the histidine rich Ca-binding protein as regulators of SERCA activity. In addition, PLN activity is regulated by two phosphoproteins, the inhibitor-1 of protein phosphatase 1 and the small heat shock protein 20, which impact the overall SERCA-mediated Ca-transport. This review will highlight the regulatory mechanisms of cardiac contractility by the multimeric SERCA/PLN-ensemble and the potential for new therapeutic avenues targeting this complex by using small molecules and gene transfer methods.

Keywords

heart failure; contractility; sarcoplasmic reticulum; calcium

An important role of Ca^{2+} in muscle contraction was first indicated a century ago by Ringer (1883), who demonstrated that the frog's heart would not contract in the absence of extracellular Ca^{2+}. Since then, it has been shown that Ca^{2+} is a physiological regulator of contraction, energetics, cell survival and other processes in muscle. Aberrant Ca-homeostasis is a universal characteristic of human and experimental heart failure and studies over the years by many laboratories which have the understanding that the subcellular mechanisms underlying regulation of Ca-handling in the normal and diseased heart. The precipitating events that lead to single cardiac myocyte dysfunction usually begin with an injury to the ventricle (myocardial infarction, ischemia, infection, valvular disease, familial, idiopathic), which activates the renin angiotensin and sympathetic nervous system along with cytokines. These in turn can cause direct damage to the individual cardiac myocytes resulting in contractile dysfunction and abnormal calcium cycling and eventual apoptosis and death (Figure 1). This review will focus on the regulation of Ca-cycling and contractility

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by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase/Phospholamban (SERCA/PLN) complex and their interacting partners under physiological and pathophysiological conditions in the heart. The role of each Ca-cycling protein in this multimeric complex, which contributes to the pathophysiology of heart failure, and novel therapeutic strategies aimed at improving calcium homeostasis, will be discussed.

SR Ca^{2+}-ATPase and Ca^{2+} Homeostasis

There are three isoforms of the SR or endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA2). SERCA2 is the predominant variant of all SERCA isoforms and phylogenetically the oldest. Three different splice transcripts have been reported so far, SERCA2a, SERCA2b, and SERCA2c, which only differ at the *C*-terminus. SERCA2a is expressed in cardiac muscle and slow twitch skeletal muscle; SERCA2b is present in adult smooth muscle and nonmuscle tissues; and SERCA2c is found in cardiac muscle as well as non-muscle tissue including epithelial, mesenchymal, and hematopoietic cells. The proposed general model of the enzyme has three cytoplasmic domains joined to a set of 10 transmembrane helices by a narrow extramembrane pentahelical stalk [1]. In heart, SERCA2a activity controls both the rate of cytosolic Ca^{2+} removal and the degree of SR Ca^{2+} load, representing a fundamental determinant of both cardiac relaxation and contraction.

Studies in genetically altered models have defined the functional role of the SERCA pump in Ca^{2+} homeostasis and cardiac physiology. Transgenic mice overexpressing SERCA2a by 1.2-or 1.5-fold exhibited increased SR Ca^{2+} transport and enhanced rates of cardiac contractility and relaxation [2,3,4]. No cardiac pathology was observed in these animals, suggesting that SERCA2a overexpression can be tolerated by the heart. On the other hand, absence of the SERCA2 gene is lethal, with homozygous null (SERCA2 −/−) mice dying early in development [5]. Heterozygous (SERCA2 +/−) mice are viable, showing 35% decrease in SERCA2 protein levels as a result of the loss of one copy of the SERCA2 allele and exhibited decreased myocyte contractility and SR Ca^{2+} load. Although no cardiac pathology was exhibited under basal conditions, reduction in SERCA2 levels in combination with an increased hemodynamic load resulted in an accelerated pathway to heart failure [6]. These mice show impaired intracellular Ca^{2+} homeostasis and decreased rates of cardiac contractile function, indicating the requirement for two functional copies of the SERCA2 gene for effective SR Ca^{2+} cycling and cardiac function [5,7]. More recently, an inducible cardiomyocyte-specific excision of SERCA2 resulted in severe reduction in both systolic and diastolic function and high mortality 8 weeks following gene excision.

SERCA2a Binding Partners

While experimental evidence suggests that proteins involved in SR Ca^{2+} release, such as the ryanodine receptor, function as part of a macromolecular complex, the existence of such a protein complex in the regulation of SR Ca^{2+} uptake has only recently begun to emerge (Figure 2). In particular, SERCA2a has been found to interact with proteins of the SR lumen, such as histidine rich calcium binding protein (HRC) [8] and calreticulin [9], while its cytosolic region has been shown to bind to S100A [10]. Furthermore, PLN and sarcolipin (SLN) have been found to bind to the cytosolic and/or transmembrane domains of SERCA2a, with accumulating evidence suggesting that these interactions lead to inhibition of the pump's affinity for Ca^{2+} [11,12]. PLN has proven to be a major regulator of SERCA2a activity and so far, it is the only SERCA2a-associated protein directly involved in cardiac disease development, including heart failure.

The Histidine-Rich Calcium Binding Protein

The histidine-rich calcium binding protein (HRC), similarly to calsequestrin, is a low-affinity, high-capacity Ca-binding protein located in the cardiac SR lumen [8]. Interestingly, recent studies have revealed that HRC may have a complex role in the cardiomyocyte and may mediate a cross-talk between SR Ca-uptake and release, as reviewed by Arvanitis et. al. [13]. Indeed, HRC is a regulator of both SERCA2a activity, through its direct binding interaction, and RyR function through its binding to triadin. These interactions may be regulated in a Ca-dependent manner during the cardiac cycle [8]. The role of this protein in heart function has been elucidated through adenoviral expression studies and transgenic models. Acute HRC overexpression in cardiomyocytes was associated with decreases in Ca^{2+}-induced Ca^{2+}-release (CICR) and the Ca^{2+}-decay rate, resulting in depressed contractility [14]. Importantly, a modest increase of HRC expression had a more pronounced effect on Ca^{2+} transients and cardiomyocyte contractility than a 20-fold overexpression of calsequestrin [15]. Accordingly, transgenic overexpression of HRC in the heart [16] depressed SR Ca^{2+} uptake rates, providing further support for the inhibitory role of HRC on SERCA2a and intracellular Ca^{2+} cycling. In addition, chronic overexpression of HRC in the heart, compromised the heart's response to stress, and eventually progressed to hypertrophy and heart failure upon aging [16]. Thus, increases in the apparent stoichiometry of HRC/SERCA2, as observed in human failing hearts, may contribute to the depressed SR Ca^{2+} uptake and impaired cardiomyocytes Ca^{2+} cycling [14,16]. Paradoxically, overexpression of HRC conferred a cardioprotective effect against an ischemic insult, which was at least partially attributed to reduced mitochondrial Ca load and attenuated apoptotic and necrotic injuries. Furthermore, HRC null mice exhibited normal basal cardiac function but they were more susceptible to isoproterenol-induced hypertrophy. Importantly, ablation of HRC impaired weight gain, possibly due to alterations in cell metabolism [17].

Recently, a human HRC variant (S96A) was identified, associated with life-threatening ventricular arrhythmias in dilated cardiomyopathy patients [18]. The substitution of Ala in position 96 of the HRC alters its regulatory effects resulting in overall disturbed SR Ca-homeostasis, as evidenced by reduced Ca transient amplitude and prolongation of Ca decay time [19]. Importantly, acute expression of the mutant HRC in failing cardiomyocytes was associated with aberrant Ca transients and arrhythmogenesis [19], consistent with the phenotype of human carriers.

Collectively, these studies have established a critical role of HRC in maintaining Ca homeostasis in the SR by regulating Ca storage, release and uptake.

PLN Regulation of SERCA2

In the early 1970s, it was suggested that the effects of various catecholamines on cardiac function may be partly attributed to phosphorylation of the SR by cAMP-dependent protein kinase(s). In 1972, two independent groups reported the ability of cyclic AMP-dependent protein kinases to phosphorylate the cardiac SR [20,21]. It soon became clear that the substrate for protein kinase A (PKA) was not the SR Ca^{2+}-ATPase but a low molecular weight protein. At the annual picnic of Arnie's Katz's lab in the summer of 1973, his wife Phyllis (a classicist) suggested that the new phosphoprotein be named phospholamban from the Greek root words phosphate and "lambano", which mean "to receive phosphate." [22]. PLN is a small protein, comprising 52 amino acid residues. Indeed, subsequent studies confirmed that PLN is a critical mediator of the β-adrenergic stimulatory effects and changes in its phosphorylation were associated with functional alterations of the cardiac SR and contractility.

Ca^{2+}-CaM-dependent PKs have also been shown to phosphorylate PLN and this occurs independently of PKA phosphorylation [23,24]. Phosphorylation by cAMP-dependent PK occurs on Ser 16, whereas Ca^{2+}-CaM-dependent PK catalyzes exclusively the phosphorylation of Thr 17 [23,24]. Dephosphorylated PLN exerts an inhibitory effect on SERCA2a and phosphorylation by either kinase was shown to result in stimulation of the SR Ca^{2+}-ATPase activity and the initial rates of SR Ca^{2+} transport by relieving inhibition on the SR Ca-uptake pump (Figure 3). Stimulation was associated with an increase in the apparent affinity of the SR Ca^{2+}-ATPase for Ca^{2+} (K_{Ca}).

In vitro, PLN is also phosphorylated by two additional PKs: PK-C and a cGMP-dependent PK. Protein kinase C (Ca^{2+}/phospholipids–dependent PK) phosphorylates the protein at a site distinct (Ser 10) from those phosphorylated by either cAMP-dependent PK or Ca^{2+}-CaM–dependent PK, while cGMP-kinase phosphorylates PLN at Ser 16. Importantly, cardiac SR appears to contain an intrinsic protein phosphatase activity, which can dephosphorylate both cAMP-dependent and Ca^{2+}/CAM-dependent sites on PLN [25].

On the basis of these observations, it was initially proposed that phosphorylated PLN acts as a stimulator of the cardiac SR Ca^{2+}-ATPase (SERCA2) activity. However, in the late 1980s, there was a significant breakthrough demonstrating that dephosphorylated PLN is actually an inhibitor of cardiac SERCA2 and that phosphorylation relieves this inhibition, giving the appearance of phosphorylation-induced stimulation [26,27]. This finding, together with the identification of a cardiac SR–associated protein phosphatase that can dephosphorylate PLN [25], has led to our current understanding of PLN as a reversible inhibitor of the cardiac SR Ca^{2+} ATPase activity.

Mechanisms of PLN Regulation

PLN is in dynamic equilibrium between monomeric and oligomeric states. In the dephosphorylated form, a substantial fraction of PLN monomers exists and this has been proposed to be the active species of PLN that binds SERCA2 and inhibits it. Upon phosphorylation, PLN appears to form mainly pentamers, which is due to changes in the isoelectric point (from 10 to 6.7) of the protein [28] and this oligomer has been suggested to be inactive or at least less active than the monomeric unit (Figure 3).

The complete amino acid sequence of PLN has been determined for various tissues and species from fish to humans, where it has been shown to exhibit a high degree of homology further supporting the notion that PLN is a critical regulator of cardiac function. There is currently no evidence for the existence of any isoforms for this protein and the PLN gene has been mapped to human chromosome 6 [29]. The calculated molecular weight of PLN is 6080 Da, and the protein has been proposed to contain two major domains: a hydrophilic domain (domain I) with three unique phosphorylatable sites (Ser 10, Ser 16 and Thr 17), and a hydrophobic C-terminal domain, anchored into the SR membrane. The hydrophilic domain (amino acids 1–30) has been further divided into two sub domains: domain Ia (amino acids 1–20) and Ib (amino acids 21–30). Domain Ia has a net positive charge in the dephosphorylated form and consists of an α-helix followed by a Pro residue at position 21 (stalk region). Domain Ib has been suggested to be relatively unstructured [1,30]. The hydrophobic domain (II; amino acids 31–52) forms an α-helix in the SR membrane (Figure 3).

PLN migrates as a 24- to 28-kDa pentamer on SDS gels and dissociates into dimers and monomers upon boiling in SDS prior to electrophoresis. Site-specific mutagenesis experiments identified Cys (Cys 36, Cys 41, and Cys 46), Leu (Leu 37, Leu 44, and Leu 51) and Ile (Ile 40 and Ile 47) residues in the hydrophobic transmembrane domain as essential amino acids for PLN pentamer formation [30]. The leucine and isoleucine amino acids are

suggested to form five zippers in the membrane that stabilize the pentameric form of the protein with a central pore, defined by the surface of the hydrophobic amino acids [28].

Cyclic AMP-dependent phosphorylation of PLN reverses its inhibitory effect on the Ca^{2+} pump. The inhibitory role of PLN on SR and cardiac function has been directly confirmed using transgenic animal models. Overexpression of the protein (PLN-overexpressing mice) was associated with inhibition of SR Ca^{2+} transport, Ca^{2+} transient, and depression of basal left ventricular function [31]. On the other hand, partial (PLN-heterozygous mice) or complete ablation of the protein (PLN-deficient mice) in mouse models was associated with increases in SR Ca^{2+} transport and cardiac function [32,32, 33,34]. Actually, a close linear correlation between the levels of PLN and contractile parameters in PLN overexpressors, WTs, PLN-heterozygous and PLN-homozygous knockout hearts was observed [35], indicating that PLN is a prominent regulator of myocardial contractility. These findings suggest that changes in the level of this protein may result in parallel changes in SR function and cardiac contractility.

The region of PLN interacting with the Ca^{2+}-ATPase may involve amino acids 2–18. This association is disrupted by phosphorylation of Ser 10, Ser 16 or Thr 17 (phosphorylated by protein kinase C, cAMP-dependent, and Ca^{2+}-calmodulin dependent protein kinase, respectively) in PLN, because the positive charges of the PLN cytosolic domain are partially neutralized by the phosphate moiety in this vicinity. Phosphorylation of PLN by the cAMP-dependent protein kinase at Ser 16 is associated with local unwinding of the α-helix at position 12–16 resulting in conformational changes in the recognition unit of the protein [36].

Interestingly, PLN peptides, corresponding to the hydrophobic membrane-spanning domain, also affect Ca^{2+}-ATPase activity by lowering its affinity for Ca^{2+} [30]. The importance of the membrane-spanning region of PLN in inhibiting SR Ca^{2+}-ATPase activity was demonstrated through mutagenesis studies [30]. It was shown that substitution of the pentamer-stabilizing residues (Leu 37, Leu 44, Leu 51, Ile 40, and Ile 47) in the membrane-spanning region (domain II) by Ala resulted in monomeric mutants, which were more effective inhibitors of the SR Ca^{2+}-ATPase activity than wild-type PLN. These PLN monomeric mutants were called “supershifters” because they decreased the apparent affinity of SR Ca^{2+}-ATPase to a greater extent than wild-type PLN. Thus, it was proposed that monomeric PLN is the active form, which is involved in the interaction with SR Ca^{2+}-ATPase. Furthermore, scanning alanine-mutagenesis studies have identified the amino acid residues in the transmembrane domain of PLN (Leu 31, Asn 34, Phe 35, Ile 38, Leu 42, Ile 48, Val 49, and Leu 52), which are associated with loss of function [30]. These amino acids are located on the exterior face of each helix in the pentameric assembly of PLN (opposite from the pentamer-stabilizing face). The importance of these transmembrane domain residues of PLN to SR Ca^{2+} -ATPase function has been demonstrated in vivo using several transgenic models. N27A PLN can act as a superinhibitor and N27A hearts have depressed Ca^{2+} -ATPase activity and cardiac function, which is not recovered completely by β-adrenergic stimulation, and the hearts progress to failure [37,38]. Mice expressing the V49G variant in the PLN transmembrane domain also exhibited diminished cardiac function and hypertrophy which progressed to dilated cardiomyopathy [39]. Moreover, L37A and I40A PLN transgenic mice showed similar decreased function and pathology [40].

As indicated above, the PLN monomer has been proposed to be the active species for interaction with the SR Ca^{2+}-ATPase and the pentamers are regarded as functionally inactive forms of PLN [30]. Phosphorylation of PLN monomers promotes association into inactive pentamers. Thus, two important steps for SR Ca^{2+}-ATPase inhibition have been suggested: (1) dissociation of monomeric PLN from dephosphorylated pentamers; and (2) binding of PLN monomers to the SR Ca^{2+}-ATPase. There are at least two interaction sites

between PLN and the SR Ca^{2+}-ATPase: one in the cytoplasmic domains of the two proteins and another one within the transmembrane sequences.

In Vivo Phosphorylation of PLN and Regulation of Contractility

The stimulatory effects of β-adrenergic agonists in the heart led scientists to initially explore cAMP-induced phosphorylation of contractile proteins, identifying troponin I as a cAMP-PK substrate in 1975 [41]. It was not until 1982 that the first in vivo evidence of PLN phosphorylation was presented in vivo [42]. In these experiments, the ATP pool was labeled with [^{32}P] orthophosphate. Microsomal fractions enriched in SR were prepared from hearts freeze-clamped during stimulation with different agonists (catecholamines, forskolin, phosphodiesterase inhibitors, phorbol esters) and analyzed by gel electrophoresis and autoradiography for ^{32}P incorporation. β-adrenergic agonist (isoproterenol) stimulation of the perfused hearts produced an increase in ^{32}P incorporation into PLN [42,43]. The stimulation of ^{32}P incorporation into PLN was associated with an increased rate of Ca^{2+} uptake into SR membrane vesicles and an increased SR Ca^{2+}-ATPase activity [43,44].

These biochemical changes were associated with increases in left ventricular functional parameters (contractility and relaxation). The in vivo phosphorylation of PLN was specific only for inotropic agents that increased the cAMP content of the myocardium (β-adrenergic agonists, forskolin, and phosphodiesterase inhibitors). On the other hand, positive inotropic interventions which increased the intracellular Ca^{2+} level by cAMP-independent mechanisms (α-adrenergic agonists, ouabain, and elevated [Ca^{2+}]) did not stimulate PLN phosphorylation or relaxation. In addition although protein kinase C (PKC) and cGMP-dependent PK (PKG) were shown to phosphorylate PLN in vitro, stimuli that activate PK-C or elevate the cGMP levels did not increase PLN phosphorylation in beating guinea pig hearts [45,46]. Thus, the physiological relevance of PK-C and PK-G dependent PLN-phosphorylation is not clear at present. Importantly, phosphorylation of Ser 16 correlated most closely with changes in cardiac function in beating hearts compared to PKA-phosphorylation of troponin I [47]. Based on these results and findings in transgenic animals [48], it has been proposed that: 1) prevention of Ser 16 phosphorylation (Ser 16 → Ala mutation) results in attenuation of the β-adrenergic response in mammalian hearts; and 2) that phosphorylation of Ser 16 is a prerequisite for Thr 17 phosphorylation. Indeed in mice containing an alanine substitution for Ser 16, there were diminished responses to the β-adrenergic stimulation. Also, there was no phosphorylation at Thr 17 in these animals [48]. Conversely, substituting alanine for Thr 17 did not interfere with phosphorylation of Ser 16, and hearts were responsive to β-adrenergic stimulation [49]. Moreover, overexpression of a non-phosphorylatable form of PLN (both Ser 16 and Thr 17 sites mutated to Ala) resulted in maximum inhibition of the SR Ca^{2+} ATPase calcium affinity [50]. It should be noted that Thr 17 has been shown to be phosphorylated independently of Ser 16 under conditions which activate CaMKII and suppress phosphatase activity such as increased stimulation frequency of the heart, elevated intracellular Ca^{2+}, ischemia-reperfusion injury, and acidosis [2351].

The functional alterations in the SR Ca^{2+}-ATPase activity may explain, at least partly, the activating and relaxing effects of β-adrenergic agents in cardiac muscle. The cAMP-dependent phosphorylation of PLN under either in vitro or in vivo conditions increases the rate of SR Ca^{2+} transport and SR Ca^{2+}-ATPase activity. Such an increase in Ca^{2+} transport is expected to contribute primarily to the relaxing effects of catecholamines. The increased phosphorylation of PLN and the increased Ca^{2+} levels accumulated by the SR would lead to the availability of higher levels of Ca^{2+} to be subsequently released for binding to the contractile proteins. The critical and prominent role of PLN in the mediation of β-adrenergic functional responses was also confirmed in transgenic animal studies. Cardiac myocytes or work-performing heart preparations from PLN -deficient mice exhibited largely attenuated

responses to β-adrenergic agonist stimulation [33,52], indicating that PLN is a key phosphoprotein in the heart's responses to β-adrenergic agonists.

Besides PLN, increases in the phosphorylation of other myocardial phosphoproteins, including the L-type Ca-channel and phospholemman in the outer cell membrane, troponin I and C-protein in the myofilaments, and the SR Ca^{2+}-release channel (ryanodine receptor) have been also shown to contribute to the stimulatory effects of β-adrenergic agonists in the heart.

PLN Human Mutations

In recent years, several human mutations in the PLN gene have been identified, and additional insights into PLN regulation of SERCA2 have been obtained. Two of these mutations (R9C and R14del) are associated with increases in PLN inhibition of the Ca-affinity of SERCA2 [53,54]. Their mechanisms appear to be partially due to decreases in cAMP-dependent protein kinase (PKA) phosphorylation of wild type PLN, preventing relief of the PLN inhibitory effects. The chronic inhibition of SERCA by mutant PLN led to dilated cardiomyopathy and premature death in families of carriers of R9C or R14del-PLN [53,54]. These findings were also observed in mouse models carrying the R9C or R14del-PLN mutations [53,54]. Interestingly, in the absence of endogenous PLN, the R14del mutant fails to co-localize with SERCA2a, resulting in lack of inhibition of SR Ca-uptake and enhanced contractility in the mouse [54]. The mutant PLN is misrouted to the sarcolemma where it interacts with Na/K-ATPase, leading to cardiac remodeling despite the enhanced contractility [54]. In addition, two more substitutions at Arg9 were identified in a heart failure cohort, namely R9L and R9H[55]. A loss of function human PLN mutation (Leu 39 stop) was also found to result in dilated cardiomyopathy and premature death in the homozygous state [56]. The Leu 39 stop mutation produces a truncated PLN protein that when introduced in HEK 293 cells resulted in an unstable protein. The low levels of L39stop-PLN detected were misrouted to other membranes, leaving SERCA2a activity in the unhibited state. Finally, mutations have also been identified in the promoter region of the PLN gene, which are associated with increased [57,58] or decreased promoter activity [59], presumably leading to alterations in PLN levels. The cardiac phenotypes associated with these naturally occurring mutations in PLN have revealed an important difference between human and mice. Mutations that are deleterious for humans are not necessarily so for mice. This may be due to the differences in cardiac reserve and regulation of Ca-balance in the cardiomyocytes of the two species. The mouse heart beats at nearly its maximal rate while the human heart has a large cardiac reserve, allowing it to increase its rate by 2–3 fold. PLN is a major regulator of SR Ca-content during increases in heart rate and defective PLN regulation would compromise the cardiac reserve. In addition, several differences underlie Ca-cycling regulation in the two species, such as differences in myosin heavy chain isoforms and the role of Na-Ca-exchanger on a beat-to-beat basis. Thus, a fine balance by PLN regulation is of greater importance in humans than in mice.

HAX-1 Regulation of PLN/SERCA Activity

The HS-1 associated protein X-1 (HAX-1), which has a MW of ~35 kDa is ubiquitously expressed in mitochondria and was originally identified as an intracellular protein with anti-apoptotic function. Indeed, overexpression of HAX-1 protected cardiomyocytes against hypoxia/reoxygenation-induced apoptosis [60]. Interestingly, more recent studies have revealed that HAX-1 also localizes to the SR and is an important regulator of Ca cycling. These effects are primarily mediated through its interaction with PLN and to a lesser extent through direct SERCA2a modulation (Figure 2). Indeed, HAX-1 has been shown to interact directly with PLN, with the minimal binding region of PLN to Hax-1 being amino acids 16–22, which includes both the Ser16 and Thr17 phosphorylation sites. This suggested that

binding of HAX-1 to PLN may control the activity of PLN [61]. Similarly to the SERCA2/PLN interaction, binding of HAX-1 to PLN was found to be diminished upon phosphorylation of PLN by cAMP-dependent protein kinase and increasing Ca^{2+} concentrations, indicating that HAX-1 may regulate the functional properties of PLN in the heart. Indeed, the PLN/HAX-1 interaction was shown to play a role in modulating Ca^{2+} cycling and cardiac contractility in vivo. Cardiac overexpression of HAX-1 decreased the affinity of SERCA2 for Ca and depressed myocyte calcium kinetics and mechanics. Accordingly, down-regulation of HAX-1 enhanced calcium cycling and contractility. The inhibitory effects of HAX-1 were abolished upon phosphorylation of PLN, consistent with the relief of the HAX-1/PLN interaction. The mechanism underlying the inhibitory effects of HAX-1 appeared to involve increased formation of PLN monomers, the active/inhibitory units of the calcium pump. Indeed ablation of PLN rescued the HAX-1 inhibitory effects on Ca-cycling and contractility in vivo [62] (Figure 4). Interestingly, HAX-1 fails to translocate to the SR in the absence of PLN, suggesting that the interaction of HAX-1 with PLN is imperative for its physiological function [63]. It is important to note that HAX-1 also interacts directly with SERCA2a [64] but evidence from genetic models and adenovirally-infected cardiomyocytes suggest that the major effect of HAX-1 on SR calcium transport involves the Ca affinity of the pump and not the maximal velocity, as would be expected from direct SERCA modulation. Importantly, the HAX-1/PLN complex may regulate SR/ER and mitochondrial Ca^{2+} homeostasis, influencing the initiation of the apoptotic cell death signaling cascades. Indeed, expression studies in HEK cells showed that the anti-apoptotic effects of HAX-1, following hypoxia/reoxygenation-induced cell death, were enhanced in the presence of PLN [61]. Similarly to Bcl-2, the beneficial effects of HAX-1 are associated with decreased SR calcium content leading to reduced mitochondria Ca load. Collectively, these findings indicate that HAX-1 represents a new regulator of PLN/SERCA activity, which may link Ca^{2+} handling and cell survival.

Inhibitor-1/Protein Phosphatase 1

Early studies indicated that the major phosphatase dephosphorylating PLN is PP1 [65,66] and the activity of this enzyme is significantly increased in human failing hearts [67]. These increases in PP1 may be one of the underlying factors in cardiac dysfunction and remodeling, as suggested by the generation of a transgenic model, expressing similar levels of PP1 as the human failing hearts [68]. The transgenic hearts exhibited depressed basal contractile function and a blunted β-adrenergic response, which resulted in hypertrophy, heart failure and early death. PP1 is a holoenzyme comprised of its catalytic domain, which possesses its phosphatase activity, complexed with as many as 100 established or putative regulatory subunits [69]. At the level of the cardiac SR, PP1 is regulated by its endogenous inhibitors, I-1 and I-2. Indeed, further insights into the role of this phosphatase were provided by studies of its endogenous inhibitors, I-1 and I-2. Cardiac-specific expression of a truncated and constitutively active form of I-2 (I-2*) depressed PP1 activity, enhanced contractile parameters and increased Ca^{2+} transient kinetics [70]. These effects were associated with increased PLN phosphorylation at Ser16 but not at Thr17, suggesting a site-specific preference for PLN. Collectively, these results indicated that PP1 is a critical negative modulator of cardiac function.

Another endogenous regulator of PP1 is I-1, which gets activated upon phosphorylation at Thr35 by the cAMP-dependent kinase, PKA, resulting in attenuated PP1 activity [71,72,73]. Indeed, I-1 knock-out (KO) mice exhibited depressed basal cardiac function in vivo. Furthermore β-adrenergic responses were blunted in isolated perfused hearts, associated with enhanced PP1 activity and decreased PLN phosphorylation [68]. Accordingly, adenovirally-mediated overexpression of I-1 *ex vivo* enhanced contractility and increased PLN phosphorylation, upon β-adrenergic stimulation [74]. Further studies in vivo showed that

overexpression of a truncated (AA: 1–65) and constitutively active (T35D) form of I-1 (I-1c) resulted in decreases in the SR-associated PP1 activity, enhanced PLN-phosphorylation at both Ser16 and Thr17 and enhanced contractility both basally and after β-adrenergic stimulation [75]. Phosphorylation of the RyR at Ser2808 and TnI phosphorylation at Ser22/Ser23 were unaffected. These results were confirmed by studies in an inducible transgenic model, which allowed for expression of I-1c in the adult heart [76]. Collectively, these experiments suggested that I-1 may be acting as a molecular inotrope by suppressing PP1 activity and allowing for unopposed increases in the phosphorylation of PLN, which amplifies the β-agonist response (Figure 5). However, other studies have indicated opposite findings on the beneficial[72] effects of increased I-1 function in the heart [77]. Inducible I-1c expression on an I-1 deficient background in the adult heart was associated with enhanced function, but these hearts underwent remodeling through the aging process and they exhibited increased susceptibility to arrhythmias upon prolonged β-agonist stimulation [77]. These findings may be due to increased pSer2815-RyR and potential diastolic Ca-leak from the SR [78,79]. The reason for this apparent discrepancy between labs on the regulatory role of I-1 in vivo may be due to differences in overexpression levels, genetic background and/or environmental conditions.

Interestingly, there are additional phosphorylation sites on I-1: Ser67 and Thr75 that are phosphorylated by PKC. Studies in genetically-altered mouse models indicated that phosphorylation of I-1 at Ser67 by PKC-α was associated with increased PP1 activity and depressed contractility *in vivo*[80]. Further studies in isolated cardiomyocytes showed that phosphorylation at these sites was associated with enhanced PP1 activity, decreased PLN phosphorylation and depressed contractile function [81,82]. Interestingly, these studies also showed that activation of the cAMP pathway was not able to fully reverse the depressed contractility, which was attributed to the inability of Thr35 to be phosphorylated efficiently in the PKC phosphorylation site mutants. Overall, these data suggest that I-1 may be an important mediator of the crosstalk between the PKA and PKC pathways in the heart. In addition, the cAMP and Ca^{2+} signaling pathways are also integrated at the level of I-1, as Thr35 is dephosphorylated by PP2A and PP2B, relieving PP1 inhibition and restoring cardiac function [83]. Thus, I-1 appears to be a more complex regulator than previously thought and may modulate PP1's activity according to differential cellular conditions, which activate these signaling pathways.

More recently, a human polymorphism has been identified in the human I-1 gene (G147D), which was associated with blunted β-adrenergic responses and depressed PLN phosphorylation in isolated cardiomyocytes [84], suggesting that this transversion may contribute to decreased SR Ca^{2+} cycling and functional deterioration in the failing heart. Thus, restoration of proper PP1 activity by I-1 may be a novel therapeutic strategy to rectify the disturbed Ca^{2+} homeostasis and Ca^{2+} cycling in the failing heart.

Heat Shock Protein 20

It is now widely acknowledged that the induction of heat shock proteins is cytoprotective, which is attributed at least partly, to chaperone activities of these "stress proteins". Of particular interest in this family is a protein of ~20 kDa, namely Hsp20 or HspB6. This is the only small heat shock protein that has the consensus motif (RRAS) for protein kinase A/protein kinase G (PKA/PKG)-dependent phosphorylation at its Ser16 site [85]. This suggested that Hsp20 may be subject to neurohormonal control via the β-adrenergic cascade in the heart. Indeed, it has been shown that Hsp20 expression and phosphorylation at Ser 16 is increased upon sustained β-adrenergic signaling in cardiomyocytes [86]. In a congestive heart failure (CHF) model, both the expression of phosphorylated and unphosphorylated forms of Hsp20 were found to be significantly increased compared with the normal group [86]. Several

studies have focused on elucidating the role of Hsp20 and its phosphorylation on cardiac contractility and cardioprotection. Interestingly, it has been suggested that Hsp20 regulates myocardial contraction via regulation of the PP1/PLN axis [87]. In vitro studies in cardiomyocytes, using phospho-peptide analogs [88] or adenoviruses, indicated that Hsp20 was associated with significant increases in mechanical and Ca-kinetic parameters [85,88] Importantly, cardiac overexpression of Hsp20 significantly enhanced contractile parameters and Ca-kinetics [87]. Conversely, the knockdown of Hsp20 by anti-sense RNA or microRNA-320 was associated with depressed contractility [89]. The underlying mechanisms appear to involve direct binding of Hsp20 to type 1 phosphatase (PP1) and inhibition of PP1 activity, associated with enhanced phosphorylation of PLN and SR Ca-cycling (Figure 5). This hypothesis was supported by the observation that PP1 activity was reduced in Hsp20 transgenic mice. Furthermore, the inotropic effects of Hsp20 were abrogated in adenovirally-infected cardiomyocytes expressing a non-phosphorylatable form of PLN (S16A/T17A), further supporting the notion that the effects of Hsp20 on contractility are mediated through modulation of the PP1/PLN axis.

Furthermore, increases in Hsp20 levels are associated with cardioprotection. Indeed, overexpression of Hsp20 in cardiomyocytes is protective against Iso-induced apoptosis [90] and simulated I/R injury [91]. In vivo studies in Hsp20 transgenic mice showed that these hearts are resistant to chronic beta-agonist-induced cardiac remodeling, interstitial fibrosis and apoptosis. Contractility was also preserved in hearts with increased Hsp20 levels. Furthermore, Hsp20-transgenic hearts, subjected to global no-flow ischemia/reperfusion, exhibited improved recovery of contractile performance with reduced necrosis and apoptosis [92]. Moreover, the infarct region-to-risk region ratio was significantly reduced in Hsp20-hearts, compared to wild-type hearts. Interestingly, the levels of Hsp20 phosphorylation were significantly increased in post- ischemic/reperfused transgenic hearts, suggesting that Hsp20 phosphorylation may play an important role in cardioprotection against ischemic injury. Indeed overexpression of a S16A-Hsp20 mutant abrogated the protective effects of Hsp20 in the heart and increased infarct size as well as apoptosis [86]. The beneficial effects of Hsp20 in cardioprotection were further demonstrated in response to Doxorubicin-induced cardiotoxicity. The mechanism underlying these cardioprotective effects is associated with reduced apoptosis through the Bax/BCl-2 and Akt pathways. Furthermore, a human mutant with P20L substitution was identified, which was associated with diminished phosphorylation at Ser16 and complete abrogation of the Hsp20 cardioprotective effects against ischemia/reperfusion [91]. Since PP1 and PLN have also been implicated in regulation of the apoptotic pathways, it is intriguing to speculate that the anti-apoptotic, cardioprotective effects of Hsp20 may also been mediated, at least partly, through PP1 and PLN. Together, these results support the beneficial effects of Hsp20 and its phosphorylation in at multiple levels by modulating cardiac contractility and survival.

S100A1

The S100A1 contains two EF-hand calcium-binding motifs [93] and is highly prevalent in cardiac cells, localizing at the SR, the myofilaments, and the mitochondria [94]. S100A1 exerts its biological function by its interaction with various target proteins to regulate cellular processes. Previous reports suggested that S100A1 interacts with SERCA2a and PLN [10], as well as with RyR [95]. Therefore, it seems that in response to intra-SR Ca^{2+} signals, S100A1 affects SR Ca^{2+} uptake and/or release. Indeed, it has been shown that diminished cardiac S100A1 protein levels are characteristic of end-stage failing human hearts [96]. S100A1 may be affecting SERCA and RyR due to its potent molecular chaperone character [97], functioning predominantly as a Ca^{2+}-sensor rather than a Ca^{2+}-storage protein [98]. In addition, S100A1 has been localized within the mitochondria and the myofilaments but their roles within these cellular structures have not been well defined yet.

S100A1 has been shown to be a promising target for cardiac gene therapy [99]. S100A1 gene transfer in isolated cardiomyoctes resulted in enhanced contraction and calcium transients. In rodent models of heart failure, AAV6-S100A1 gene therapy was able to improve cardiac function and to reverse left ventricular remodeling in the long term (8 weeks after gene therapy) [99] in a rat model of heart failure. In the same way, cardiac hypertrophy was reduced and cardiomyocyte Ca^{2+} cycling maintained. Furthermore, to compare current pharmacological interventions, S100A1 gene therapy was tested against chronic β-AR blockade by metoprolol and as a combination therapy. S100A1 therapy alone resulted in superior cardiac performance in comparison to metoprolol and combination therapy revealed synergistic effects of β-AR-blockage and restoration of S100A1 protein levels [99]. In the latest study by Pleger et al., MI was induced by balloon occlusion of the left circumflex coronary artery for two hours [100]. AAV9-S100A1 cardiac-restricted gene therapy was performed two weeks later via the anterior vein into the left ventricular remote myocardium [100]. After 14 weeks, gene transfer resulted in cardiac restricted gene expression without any signs for extra-cardiac expression analyzed by AAV-luciferase expression. Cardiac function was severely impaired after MI and hearts showed signs of left ventricular remodeling. In comparison, AAV9-S100A1 treatment prevented cardiac deterioration and reversed ventricular remodeling by re-establishing S100A1 expression level. Furthermore, S100A1 treatment was able to reconstitute cytosolic and SR Ca^{2+} cycling as well as energy homeostasis in isolated cardiomyocytes [100].

SUMO1

The levels and activity of SERCA2a in cardiomyocytes can also be modulated in parallel with the levels of a cytoplasmic protein, small ubiquitin-like modifier type 1 (SUMO1) [101]. SUMOs are a family of peptides that alter the function of other proteins in cells through a post-translational modification described as sumoylation. Sumoylation is involved in the modulation of various cellular processes such as transport from the nucleus to the cytosol, transcription, and protein stabilization and degradation through the process of reversible covalent linking of SUMO to the target protein [101]. The activities of many important intracellular proteins are modified in this way, including steroid receptors, proto-oncogenes, tumor suppressors, and cardiac transcription factors.

Kho et al. found that sumoylation appeared to prolong the lifetime of SERCA2a in the cell as well as increase the intrinsic activity of SERCA2a ATPase [102]. The authors also found that SERCA2a and SUMO1 levels were both reduced in mouse and pig models of heart failure and in cardiomyocytes isolated from failing human ventricles [102]. To determine whether reduced SUMO1 levels are responsible for reduced SERCA2a protein levels and reduced cardiac function, SUMO1 was increased by gene transfer in a mouse model of heart failure (induced by thoracic aortic constriction). Increasing SUMO1 levels led to a restoration of SERCA2a levels, improved hemodynamic performance, and reduced mortality among the animals with heart failure. Reduction of SUMO1 expression using a short hairpin RNA approach reduced SERCA2a levels and adversely affected the pump function of the mouse hearts. The link to the sodium–calcium exchanger was recapitulated in these experiments, since sodium–calcium exchanger levels were higher after SUMO1 down-regulation, again suggesting that the expression of these two key calcium-handling proteins is related. A key additional finding was that reduced cardiac function due to SERCA2a down-regulation could not be improved by up-regulation of SUMO1 without its stimulatory effect on SERCA2a levels. This is an important observation, because sumoylation leads to translational modification of a number of intracellular proteins that might have changed cardiac function independently of SERCA2a.

Kho and colleagues [102] report a SUMO-induced modification of a cardiac protein and have shown a new mechanism for modulation of SERCA2a activity and cardiac-pump function. As our understanding of SUMOylation increases, targeting it for therapeutic benefits to the heart may be possible.

The SERCA/PLN Regulatory Complex in Heart Failure

In human and experimental heart failure both the level and the activity of the SR Ca^{2+} pump are decreased contributing to the deteriorated cardiac function [103,104]. Furthermore, the protein level of PLN remains unaltered and this results in an increased fraction of SERCA in the inhibited form by PLN [13]. In addition, the phosphorylated PLN levels are decreased further compounding reduced SR Ca^{2+} ATPase activity [104]. This diminished phosphorylation of PLN can be attributed to an attenuation of the beta-adrenergic cascade due to receptor desensitization, receptor downregulation and uncoupling, which occurs during disease progression [105,106]. Importantly, it has been recognized that activation of phosphatases may also contribute to the dephosphorylation of PLN and depressed cardiac function [68,107,108]

Furthermore, decreases in the protein levels of HAX-1 and inhibitor-1 as well as PKA-phosphorylation of I-1 have been observed in failing hearts, which are expected to further contribute to the depressed SR Ca-cycling homeostasis. On the other hand, the levels of HRC [16] and Hsp20 as well as PKA-phosphorylation of Hsp20 [86] are significantly increased in heart failure, suggesting important compensatory responses for the deteriorated cardiac function. Thus, complex regulatory mechanisms underlie the function of the SERCA/PLN modulators under physiological and pathophysiological conditions.

The SR Ca-Transport Interactome (“Assemble”) as Target in Heart Failure

There is a need for innovative and targeted therapy to improve the impaired contractile function and halt remodeling in heart failure. Reversing the contractile failure of cardiac myocytes with the use of standard pharmacological inotropic agents has been controversial, most likely due to the pleiotropic intracellular effects of the targeted molecules [109]. As such, it has been suggested that a targeted approach may be more effective in alleviating the depressed function and progression of remodeling.

Given the central importance of the SR Ca^{2+} ATPase to proper SR Ca^{2+} cycling, excitation-contraction coupling, and thus cardiac function, SERCA2 has been a focus of potential gene-targeted therapy for heart failure, especially since pump activity is depressed in failing hearts. SERCA2a gene-transfer in animal models and human failing cardiomyocytes has shown beneficial effects, including improved contractility and energetics as well as prevention of arrhythmias and hypertrophy. For example, adenoviral delivery of SERCA2a to human failing cardiomyocytes rescued the depressed contractility and Ca^{2+} transients [110]. Adenovirus-mediated gene-transfer also improved function and survival in rat failing hearts. Additionally SERCA2a delivery suppressed arrhythmias as well as infarct size in a rat model of ischemia/reperfusion injury [110]. These beneficial effects in heart failure have also been demonstrated in multiple models of heart failure in large animals [110]. Along with the demonstration of improved myocardial mechanical function, the overexpression of SERCA2a had multiple effects including improved myocardial energetics, endothelial function and anti-arrhythmic effects. There has been a well documented decrease in creatine kinase activity in failing hearts resulting in a decrease in the supply of ATP to essential areas in cardiac cells. The decrease in SERCA2a-mediated Ca^{2+} regulation in the failing heart increases the energy cost for Ca^{2+} regulation, together resulting in inefficient energy use [111]. Sakata et al investigated the effects of SERCA2a overexpression on mechanoenergetics in rats. In these studies they measured the oxygen cost of left ventricular (LV) contractility per

beat for Ca^{2+} handling in excitation–contraction coupling per unit change in LV contractility. In diseased hearts from diabetic and aortic-banded rats, the oxygen cost of LV contractility was increased; SERCA2a overexpression restored the increased oxygen cost of LV contractility to the normal level, indicating an improvement in energy utilization (Figure 6). Of note, chronic stimulation of the myocardium by [beta]-adrenergic agents increases mortality, probably due to enhanced mechanical function without the improvement of energy utilization, leading to increase total energy consumption. This improvement in energy use could, therefore, hold great significance for future clinical trials of SERCA2a gene therapy for congestive HF. This clear difference mechanoenergetic efficacy could directly influence patient survival. In fact, Del Monte and coworkers showed improved survival in rats after SERCA2a overexpression, coupled with the improvement in energy consumption. The effects of SERCA2a gene transfer seem to reverse the adverse remodeling in the cardiac cells with resulting improvements in energetics. The effects of SERCA2a on energetics cannot be viewed as an acute effect, such as the infusion of dobutamine but has to be considered in terms of a chronic effect on overall cardiac remodeling and reduction in ventricular size. Recently Lyon et al have shown that SERCA2a gene transfer does in fact reverse abnormalities of sarcolemmal structure in heart failure with reappearance of z-grooves and T-tubules in reverse remodeled hearts [112].

Furthermore, overexpression of SERCA2a increases coronary blood flow and in doing so improves cardiac function [113]. One of the reasons for this improvement has been shown to be the increased production of NO caused by SERCA2a overexpression through an increase in eNOS levels and phosphorylation. In a recent study, Hadri et al demonstrated that eNOS expression was increased in SERCA2a-overexpressing human coronary endothelial cells as a result of SERCA2a-mediated activation of the eNOS promoter. SERCA2a appears to play a role in the induction of eNOS transcription *via* an element in the promoter region from bp −5,039 to −4,861. This region was shown to contain a 269-bp activator element acting as an enhancer of transcription. The enhancer contains myeloid zinc finger–like, AP-2, Sp1, and Ets binding sites and is important for the endothelial specificity of eNOS promoter [113]. This additional effect of overexpression of SERCA2a might have a synergetic effect in the prevention of cardiovascular disease.

The antiarrhythmic effects of SERCA2a overexpression were demonstrated in acute ischemia-reperfusion [114] and in heart failure [114,115,116]. In chronic heart failure after myocardial infarction in rats, the overexpression of SERCA2a was associated with a reduction of spontaneous and provoked ventricular arrhythmia along with a reduction in calcium leak from the SR [116]. The latter findings support the clinical safety of SERCA2a gene therapy in the arrhythmia-prone heart failure population.

In other recent studies, SERCA2a gene transfer has been shown to decrease alternans, a substrate of ventricular arrhythmias both in vitro and in vivo[117]. The improved contractile performance, along with improved myocardial energetics, endothelial function and coronary flow has resulted in improved survival of HF animals following gene transfer of SERCA2a. In porcine and ovine models of HF, gene transfer of SERCA2a with AAV vectors carrying SERCA2a resulted in a decrease in ventricular volumes, enhanced ejection fraction and de-activation of HF biomarkers [118,119]. Most importantly, these studies showed that there was a gene-dose-effect whereby an increase in the concentration of vectors delivered resulted In higher gene expression of SERCA2a and improved performance in the HF animals.

Based on the extensive evidence that SERCA2a induces beneficial effects in multiple models of heart failure, the first clinical trial of gene therapy in patients with HF was launched in the United States in 2007 -CUPID (Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease) [120,121]. Patients treated with

AAV1.SERCA2a demonstrated improvement or stabilization in New York Heart Association (NYHA) class, MLWHFQ (Minnesota Living With Heart Failure Questionnaire), six-minute walk test (6MWT), maximal Oxygen consumption during exercise (VO2 max), N-Terminal pro-hormone of Brain Natriuretic Peptide levels (NT-proBNP), and LV end-systolic volumes [122]. Significant increases in time to adjudicated CV events, and a decreased frequency of CV events per patient were observed in all patients receiving AAV1.SERCA2a. No increases in adverse events, disease-related events, laboratory abnormalities or arrhythmias were observed in AAV1.SERCA2a treated patients compared to placebo. Additionally, after 12 months of receiving a single infusion of AAV1.SERCA2a, patients treated with the highest dose versus placebo had an 88 percent risk reduction of major cardiovascular events such as death, need for left ventricular assist device (LVAD) or cardiac transplant, episodes of worsening of heart failure, and number of heart failure-related hospitalizations [122]. These positive results have persisted at 24 months. Furthermore, there were no changes in arrhythmias as recorded by the implantable cardioverter defibrillators (ICDs) that were required in all patients.

Targeting PP1/PLN

Given the aberrant regulation of type 1 phosphatase activity in cardiac pathology, several groups have investigated the potential of phosphatase inhibition as a therapeutic approach to enhance cardiac function in heart failure. Specifically, studies have examined the inhibition of PP1 through its regulators, inhibitor-2 and inhibitor-1. Gene delivery of I-2 in a cardiomyopathic heart failure hamster model [123], during the transition from moderate to severe dysfunction, resulted in prevention of heart failure progression and prolonged survival. These beneficial effects were associated with decreased PP1 activity and increased PLN phosphorylation at Ser16 without any changes in the phosphorylation of RyR. In contrast to the acute beneficial effects, cardiac-specific expression of the constitutively active I-2 (I-2*) was associated with heart failure and depressed PLN phosphorylation at Ser16 after pressure overload induced by aortic constriction. The apparent discrepancies may be related to confounding, compensatory effects of ‘chronic’ inhibition of PP1 in the transgenic mice.

I-1-targeted interventions also exhibited therapeutic promise in heart failure. I-1 ablation reduced the incidence of isoprenaline-induced arrhythmias, hypertrophy and death, possibly due to negation of the adverse effects associated with the PKC sites [124]. Adenoviral-mediated expression of the constitutively active (T35D) and truncated form of I-1 (I-1c), which lacks the detrimental PKC-phosphorylation sites, enhanced contractile parameters and Ca kinetics of human failing cardiomyocytes [68]. In vivo, overexpression of I-1c in the heart also enhanced Ca-cycling and contractile parameters. Notably, upon trans-aortic constriction, I-1c hearts maintained the enhanced cardiac performance and these mice exhibited an attenuated progression to heart failure, characterized by a diminished extent of cardiac hypertrophy and fibrosis, with no decompensation. Furthermore, these mice maintained their enhanced cardiac function upon chronic isoproterenol-stimulation, associated with cardiac remodeling [75].

More recently, it has been reported that inducible expression of I-1c in the adult heart protects against ischemia/reperfusion (I/R) injury [76]. In particular, expression of I-1c ameliorated contractile dysfunction and attenuated cellular damage post-I/R. However, other studies [124] indicated detrimental effects of prolonged I-1c expression in the heart, associated with enhanced RyR phosphorylation at the CaMKII-site, S2815. Nevertheless, gene delivery of I-1c in pre-existing heart failure in a rat model of pressure overload restored contractility to non-failing levels. Of special interest is the fact that these beneficial effects were mediated by enhanced phosphorylation of PLN, while the PKA-phosphorylation levels of the RyR were unchanged. Interestingly, a recent study by Kawashima and colleagues in

2009 [125] showed that specific inhibition of PP1 by I-1 stimulated SR Ca-uptake but had no effect on Ca-release, suggesting that I-1 may be preferentially regulating PLN and not the RyR. The beneficial effects of I-1c in rodent models have been confirmed in large animal models of heart failure whereby acute and chronic gene transfer of I-1c by viral gene transfer induced both short-term and long-term beneficial hemodynamic effects. In porcine ischemic models of heart failure, AAV9.I1c induced long term hemodynamic benefits and improved cardiac remodeling (Figure 6). Future studies may further address the beneficial effects of PP1 inhibition through I-1c in functional recovery and remodeling in heart failure.

CONCLUSION AND PERSPECTIVE

In summary, several lines of evidence indicate that the SERCA/PLN regulatory complex is of paramount importance in proper cardiac Ca-cycling and function. In addition, it has recently become apparent that this fine-tuned regulation by SERCA/PLN represents a nodal point in the interaction of several protein partners that mediate a fine cross-talk between Ca and several signaling pathways, underlying cardiac performance and cell death. Indeed, disturbances in the regulatory function of SERCA/PN have been implicated as important contributors to the depressed cardiac function and remodeling in failing hearts. As such, targeting SERCA, PLN or the PLN/PP1 through I-1 or Hsp20 appears beneficial in alleviating the detrimental effects of heart failure, through specific increases in SR Ca-transport. Notably, the benefits of targeting SERCA have been shown in a recent clinical trial and the benefits of the other regulatory partners are at various stages of development. These new paradigms have led to experimental trials in enhancing SERCA2a uptake. There is now intensive scrutiny of the various proteins that lead to calcium cycling abnormalities and this focus will ultimately lead to novel treatment modalities in heart failure.

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Non standard abbreviations and acronyms

Ad	Adenovirus
AAV	Adeno-Associated Virus
HAX-1	HS-1-associated protein X-1
HRC	Histidine Rich Calcium Binding Protein
Hsp20	Heat shock protein 20
I-1	Inhibitor-1
I/R	Ischemia/reperfusion
KO	Knockout
MLWHFQ	Minnesota Living with Heart Failure Questionnaire
NYHA	New York Heart Association
OE	Over-expression
PLN	Phospholamban

PP1c	Protein Phosphatase 1 catalytic subunit
RyR	Ryanodine Receptor
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SUMO1	Small ubiquitin-like modifier type 1
VO_2max	Maximal oxygen consumption
WT	Wild-type

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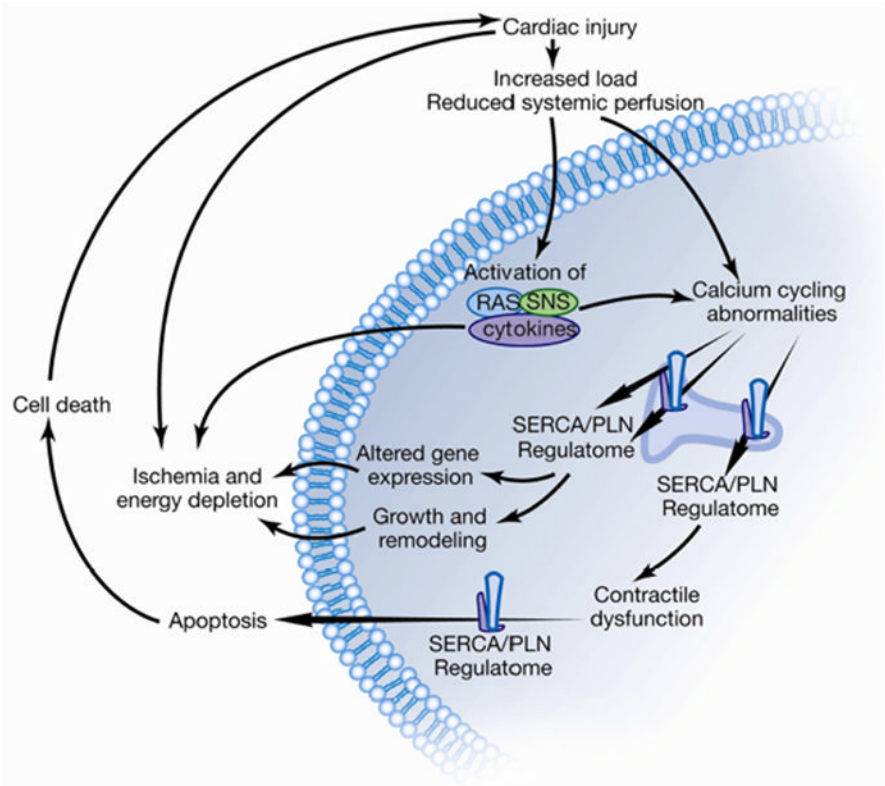

Figure 1. The SERCA2a/PLN regulatory complex in cardiac calcium cycling and survival

Injury to the ventricle such as myocardial infarction, ischemia, infection, valvular disease, familial, idiopathic activates the renin angiotensin and sympathetic nervous system along with cytokines. These in turn can cause direct damage to the individual cardiac myocytes resulting in contractile dysfunction and abnormal calcium cycling and eventual apoptosis and death. Targeting the SERCA2a/PLN proteome may have beneficial effects in abrogating the damage done to the individual cardiomyocytes.

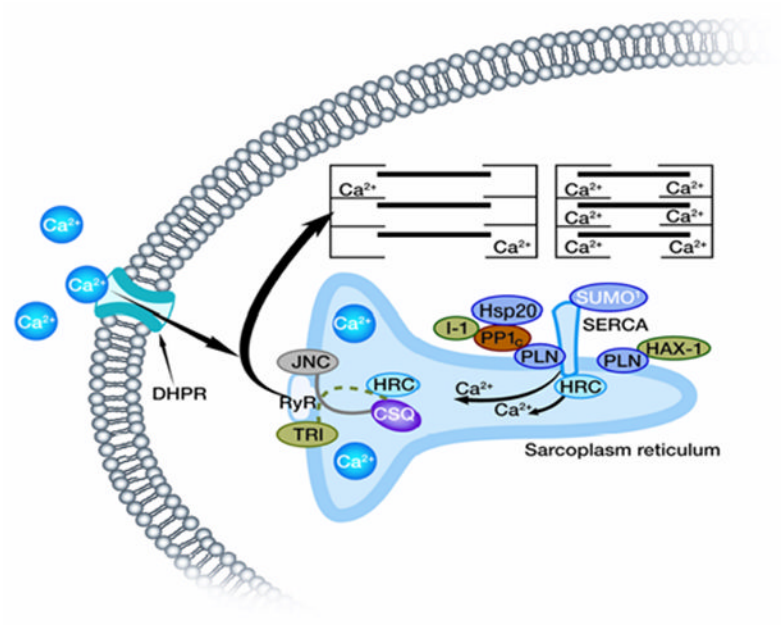

Figure 2. Regulation of SR Ca-transport by a multimeric protein complex

SERCA2a activity is regulated by its reversible inhibitor PLN, SUMO and the histidine rich Ca-binding protein (HRC). Phosphorylation of PLN is mediated by cAMP-dependent or Ca-CAM-dependent PKs and dephosphorylation occurs by protein phosphatase 1 (PP1). The activity of PP1 is regulated by inhibitor-1 (I-1).

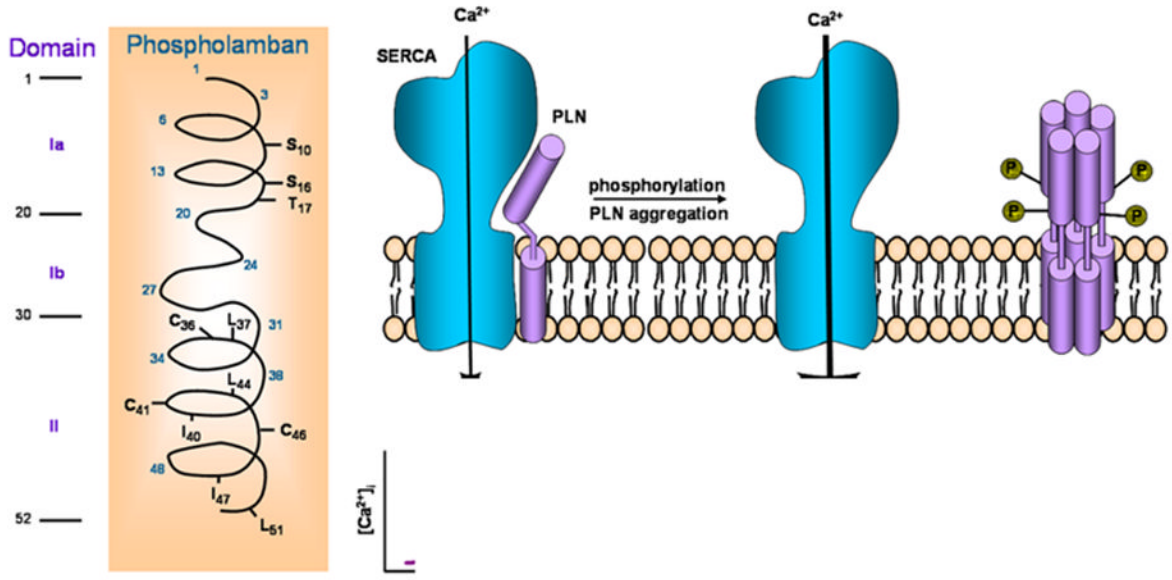

Figure 3. Modeling of PLN

There are three domains: cytosolic domain Ia, containing the phosphorylated Ser16 and Thr17 sites, cytosolic domain Ib and transmembrane domain II, containing AA that are important in pentamer stabilization and functional regulation of SERCA2a. The PLN monomer interacts with SERCA2a and inhibits its activity, while PLN phosphorylation leads to pentameric assembly and relief of the PLN inhibitory effects.

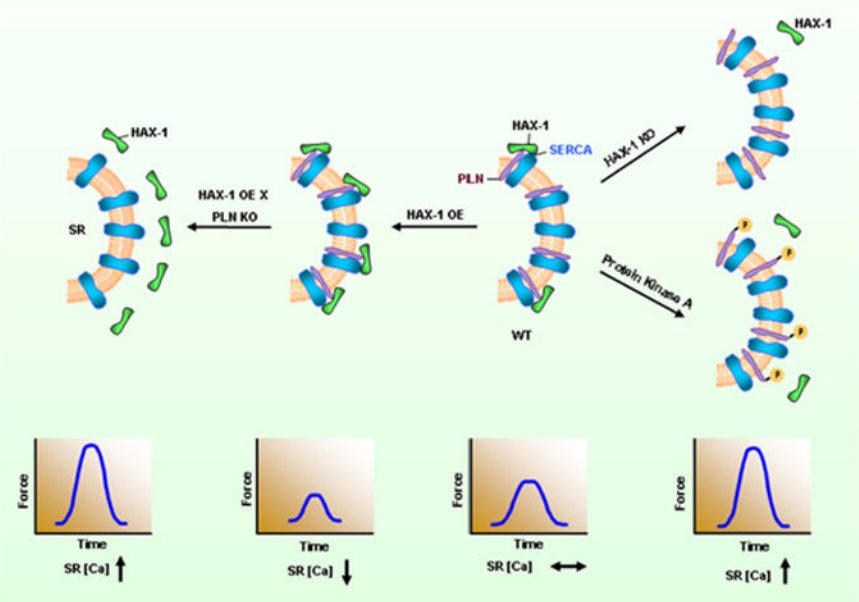

Figure 4. HAX-1 regulates PLN/SERCA activity

The anti-apoptotic protein HAX-1 interacts with PLN and enhances its inhibitory effects on SERCA2a and contractility. PKA phosphorylation of PLN or ablation of HAX-1 abolish the inhibition of HAX-1 on PLN/SERCA and Ca-cycling. Accordingly, overexpression of HAX-1 increases PLN inhibition and depresses contractility.

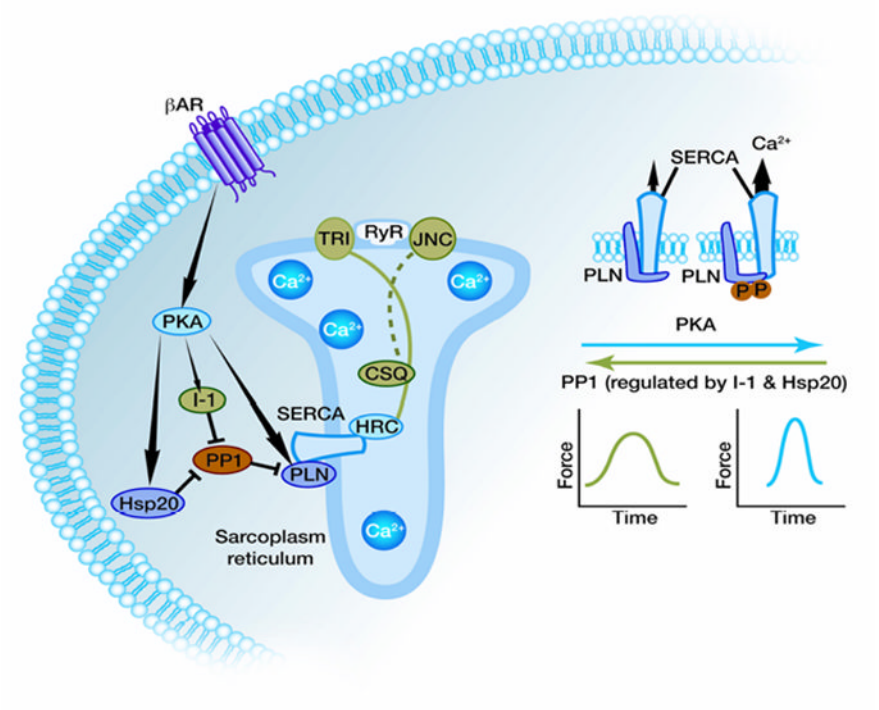

Figure 5. Beta adrenergic agonist stimulation and protein phosphatase 1 in regulation of the PLN/SERCA activity

PKA activation results in increased phosphorylation of PLN, Inhibitor-1 and Hsp20 amplifying the stimulatory effects b-AR stimulation on SR Ca-transport and contractility.

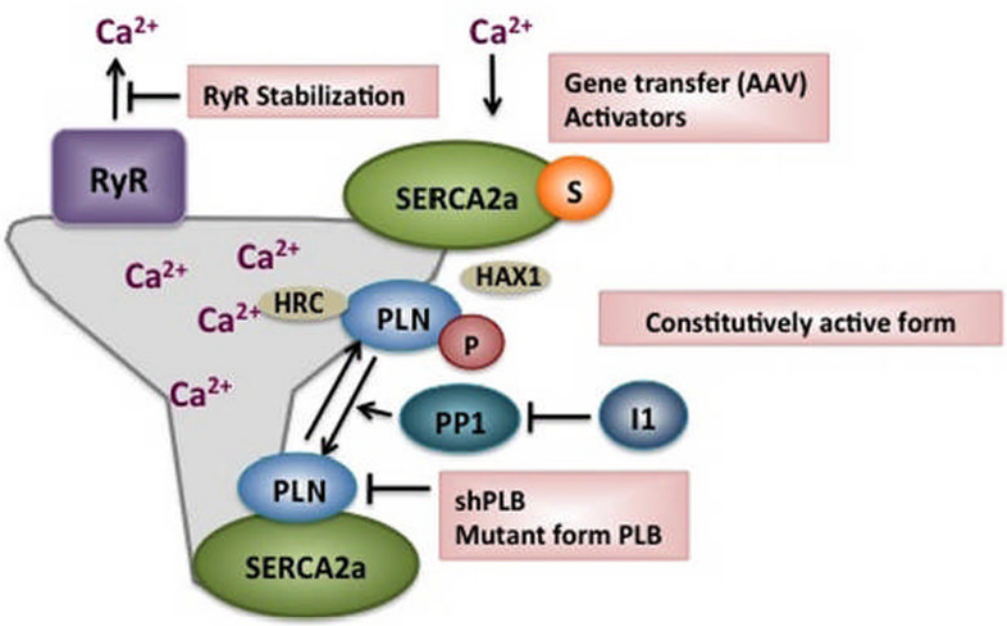

Figure 6. Strategies of improving calcium handling and SR Ca-content in heart failure

These strategies have focused pharmacologically on inhibiting the Na/K ATPase which results in increased intracellular Ca^{2+} and more recently on stabilizing the Ryanodine Receptor in resulting in decreased SR Ca^{2+} leak. Gene editing techniques have focused on enhancing SERCA2a's activity by either increasing the level of SERCA2a or altering the expression of its partners.