Published in final edited form as:

J Muscle Res Cell Motil. 2012 December ; 33(6): 485–492. doi:10.1007/s10974-012-9319-4.

Tuning the Structural Coupling between the Transmembrane and Cytoplasmic Domains of Phospholamban to Control Sarcoplasmic Reticulum Ca^{2+} -ATPase (SERCA) Function

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Abstract

Phospholamban (PLN) is the endogenous inhibitor of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA), the integral membrane enzyme responsible for 70% of the Ca^{2+} shuttling into the SR, inducing cardiac muscle relaxation in humans. Dysfunctions in SERCA:PLN interactions have been implicated as having a critical role in cardiac disease, and targeting Ca^{2+} transport has been demonstrated to be a promising avenue in treating conditions of heart failure. Here, we designed a series of new mutants able to tune SERCA function, targeting the loop sequence that connects the transmembrane and cytoplasmic helices of PLN. We found that a variable degree of loss of inhibition mutants is attainable by engineering glycine mutations along PLN's loop domain. Remarkably, a double glycine mutation results in a complete loss-of-function mutant, fully mimicking the phosphorylated state of PLN. Using nuclear magnetic resonance (NMR) spectroscopy, we rationalized the effects of these mutations in terms of entropic control on PLN function, whose inhibitory function can be modulated by increasing its conformational dynamics. However, if PLN mutations go past a threshold set by the phosphorylated state, they break the structural coupling between the transmembrane and cytoplasmic domains, resulting in a species that behaves as the inhibitory transmembrane domain alone. These studies provide new potential candidates for adenovirus gene therapy to reverse the effects of heart failure.

Keywords

phospholamban; membrane protein; loss-of-function mutations; SERCA

Introduction

Ca^{2+} ions signal muscle contraction and relaxation, coupling the membrane action potential and the mechanical contraction of muscle (Bers 2002; Kelly et al. 2008). The interplay between the sarcoplasmic reticulum ATPase (SERCA) and phospholamban (PLN) is crucial to intracellular Ca^{2+} cycling and proper cardiac muscle contraction and relaxation, and dysfunction in their protein-protein interactions have been implicated in cardiac disease (Haghighi et al. 2004). SERCA is a 110 kDa integral membrane P-type ATPase which utilizes ATP hydrolysis to transport Ca^{2+} into the SR (MacLennan and Kranias 2003). SERCA is responsible for 70% of the calcium reuptake during diastole in human

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This article is dedicated to the late Michael and Kate Bárány, in tribute to their outstanding contributions to understanding the biochemistry and biophysics of muscle proteins. I had the privilege to share my ideas on phospholamban with Michael and I continue to enjoy my friendship with his son George, my colleague at the University of Minnesota.

cardiomyocytes (MacLennan and Kranias 2003). SERCA comprises a large cytoplasmic domain that includes the actuator domain, the phosphorylation domain, and a nucleotide binding domain, along with 10 transmembrane helices constituting the Ca^{2+} transport pathway (Toyoshima et al. 2000). The cardiac isoform of SERCA (SERCA2a) is regulated by PLN, a single-pass membrane protein that binds the enzyme *via* intramembrane interactions, reducing its apparent Ca^{2+} affinity (Simmerman and Jones 1998; Traaseth et al. 2008; Veglia et al. 2010). β -Adrenergic stimulation unleashes protein kinase A (PKA) that phosphorylates PLN at Ser16, reversing its inhibition of SERCA and augmenting the diastolic phase (Bers 2008).

PLN is a 52-amino acid integral membrane protein which is comprised of three structural domains (Traaseth et al. 2009; Verardi et al. 2011; Zamoon et al. 2003) that are further subdivided into four dynamic domains (cytoplasm: domain Ia (residues 1-16), loop (residues 17-22), domain Ib (residues 23-30); transmembrane: domain II (residues 31-52)) (Metcalf et al. 2004; Traaseth et al. 2009; Verardi et al. 2011) (Fig 1). The transmembrane helix of PLN is the principle structural domain which inhibits SERCA (Karim et al. 2000; Karim et al. 2004), while the regulatory phosphorylation Ser 16 site, which determines the inhibitory or non-inhibitory state, lies in the cytoplasmic helix (Chu et al. 2000). Despite its small size, PLN can populate several conformational states, and domain Ia and Ib participate in folding/unfolding equilibria, which are important for SERCA inhibition as well as recognition by PKA (Gustavsson et al. 2011; Masterson et al. 2011; Traaseth and Veglia 2010). The loop connecting these two domains also has functional relevance, in particular the Pro21 residue that lies in the center of the loop. Squier and co-workers reported that mutation of Pro21 to Ala in a monomeric cysteine-null background led to partial inhibition of SERCA, increase in overall helicity, decreased dynamics at the C-terminus, and local structural changes at the loop leading to diminished solvent accessibility (Li et al. 2005). Additionally, our group found that the SERCA inhibition and binding could be tuned modulating the conformational dynamics of the loop through mutation in the Pro21 site, so-called *dynamics rheostat* (Ha et al. 2007). Our studies identified the mutant species AFA-PLN^{P21G} to be a possible candidate for treatment of heart failure by delivery via rAAV gene therapy. AFA-PLN^{P21G} was found to have the same structural dynamics and functional characteristics as PLN^{S16E}, a PLN pseudo-phosphorylated mutant already proven to be successful in reversing heart failure in large and small animal models by Chien and coworkers (Hoshijima et al. 2002; Kaye et al. 2007).

Naturally occurring mutations in PLN have also been linked to familial forms of cardiomyopathy: R9C (Schmitt et al. 2003), R9L and R9H (Medeiros et al. 2011b), R14del (Haghighi et al. 2006), and L39stop (Haghighi et al. 2003). PLN has also become a target for gene therapy (Hoshijima et al. 2002; Hoshijima et al. 2006; Kaye et al. 2007), namely by delivering PLN mutants which decrease SERCA inhibition. The challenge remains in how to rationally design PLN mutants with tunable effects on SERCA to account for the diversity of patient population and the various manifestations of heart failure (Hajjar et al. 2012).

Numerous mutagenesis studies of PLN have been carried out by the MacLennan, Jones, and Young groups, offering invaluable insight by identifying residues crucial to PLN's inhibitory ability (MacLennan et al. 1998; Simmerman et al. 1996; Trieber et al. 2009). Although these studies served as a crucial starting point to understanding the effect of point mutations in PLN have on SERCA activity, they often did not identify the reasons for observed functional effects, *i.e.* whether the mutation induced a change to structural dynamics, altered the binding surface, decreased SERCA binding, *etc.* The data clearly demonstrate that the transmembrane domain is *Janus*-faced, with one side of the helix primarily involved in oligomerization and the other side binding to SERCA. Mutagenesis studies show that domain Ia and the loop are the optimum targets for designing loss-of-

function (LOF) mutants. In contrast, mutations in domain Ib and domain II yield varying results, but all known gain-of-function (GOF) and super-inhibitors are concentrated in those two domains.

The rationale of this study is to build on our previous success tuning the structural dynamics of PLN to control SERCA function by introducing a series of glycine residues to the loop domain. Our original hypothesis was that an increase in conformational dynamics in the loop would interrupt the structural coupling between the transmembrane helix, which is primarily responsible for SERCA inhibition, and the cytoplasmic helix, which contains the phosphorylation sites that dictate the inhibition state. To test this hypothesis, we sought to introduce single Gly mutations at the M20 site and Q22 site to investigate whether the loss-of-function (LOF) rendered by the P21G mutation was site-specific (Fig 2). Secondly, we determined the SERCA inhibition of the phosphorylated species to see if post-translational control of these single Gly-mutants of PLN still remains intact, a property which has been suggested to be crucial for therapeutic success (Ha et al. 2007). Thirdly, we expanded the Gly mutations into double and triple mutations: AFA-PLN^{M20G P21G} and AFA-PLN^{M20G P21G Q22G}. To characterize the conformational dynamics of the various mutants and correlate with their functional state, we used nuclear magnetic resonance (NMR) spectroscopy that enables the quantification of molecular motions of proteins at the atomic level (Palmer and Massi 2006; Palmer et al. 2001).

Our results suggest that the structural dynamics of the glycine mutants can be tuned, resulting in varying degrees of fast timescale dynamics and degrees of SERCA inhibition. However, species where the mutation increased the conformational dynamics past the threshold set by the phosphorylated species behave as the transmembrane helix alone. The mutants represent new templates for rational design of protein therapies using gene transfer approaches, modifying the effects of an inhibitor to control enzyme function.

Materials and Methods

Cloning of PLN mutants

Primary sequences of the mutants cloned in this study are described in Fig 2. Cloning was performed using the Stratagene QuikChange protocol as previously described (Ha et al. 2007). For the AFA-PLN^{M20G}, AFA-PLN^{P21G}, and AFA-PLN^{Q22G} constructs, the pMal-c2E-TEV-AFA-PLN plasmid was used as a parental template, which expresses a maltose binding protein (MBP) PLN fusion protein with a tobacco etch virus (TEV) protease cleavage site encoded in-between. The forward primer for the AFA-PLN^{M20G} is as follows: 5' – ACC ATT GAA GGC CCG CAG CAG GCG CGC CAG AAC – 3". The forward primer for the AFA-PLN^{Q22G} is as follows: 5' – ACC ATT GAA ATG CCG GGC CAG GCG CGC CAG AAC – 3". The forward primer for the AFA-PLN^{M20G P21G} is as follows: 5' – GGC AGC ACC ATT GAA GGC GGC CAG CAG GCG CGC CAG AAC CTG – 3". The cloning of GGG-PLN required two PCR reactions on the P21G PLN AFA background, first converting M20 to G and then Q22 to G. Pentameric mutants were cloned using the pMal-c2E-TEV-wtPLN plasmid as the parental template. PLN^{M20G P21G} was cloned stepwise, using PLN^{P21G} as the initial template. Stepwise mutagenesis was necessitated due to the high T_m values of primers containing codons encoding for glycine, because of their high GC content. After mutagenesis was confirmed from EtBr stained agarose gels, the PCR reaction was transformed into DH5 α cells and grown on ampicillin-agarose plates. Subsequent colonies were grown overnight in 5 mL Luria-Bertani (LB) media with 0.1% ampicillin, and then spun down on a tabletop centrifuge at 13,000 RPM. The remaining pellet was resuspended and the plasmid was extracted from the pellet using the Qiagen Quick-Spin Miniprep kit. Constructs were confirmed through sequencing at the University

of Minnesota Biomedical Genomics Center. Correct target constructs were then transformed into BL21(DE3) *Escherichia coli* cells.

Protein Preparations and ATPase Activity Assays

Protein expression and purification were performed on all constructs as previously described (Buck et al. 2003; Veglia et al. 2010). Phosphorylation of PLN samples at Ser16 was performed as previously described (Gustavsson et al. 2011; Ha et al. 2007; Metcalfe et al. 2005) using recombinantly expressed protein kinase A purified by an established protocol (Masterson et al. 2008). Phosphorylation was confirmed by a band shift in the SDS-PAGE, and by MALDI-TOF mass spectrometry. The degree of SERCA inhibition by PLN analogs was determined using a coupled enzyme assay correlating the depletion in NADH absorbance to ATPase activity as previously described (Fabiato et al. 1978; Ha et al. 2007). SERCA inhibition was measured in reconstituted lipid samples according to Reddy et al. (Reddy et al. 2003). PLN variants were co-reconstituted with purified SERCA in lipid bilayer membranes (DOPC:DOPE, 4:1) at molar ratios of 10:1 PLN:SERCA and 700:1 lipids:SERCA. The calcium dependence of the ATPase activity was measured at 37 °C using a coupled enzyme assay, monitoring the consumption of NADH by the decrease in absorbance at 340 nm using a Spectromax microplate reader (Molecular Devices). Initial rate of SERCA (V) was measured as a function of calcium concentration (pCa), and data was fit to the Hill equation (Reddy et al. 2003).

NMR Studies

Unless otherwise noted, NMR samples were prepared with a buffer containing 20 mM Na₂HPO₄ (J.T. Baker), 120 mM NaCl (Malinkrodt), 0.1% NaN₃, and 300 mM dodecylphosphocholine (DPC) (Avertec). Lyophilized PLN was weighed and added in increments to 300 µL buffer until the sample reached an approximate concentration of 1 to 1.5 mM protein. After addition of HPLC purified protein, all samples were adjusted to pH 6.0 with dilute NaOH to neutralize residual trifluoroacetic acid remaining in the protein powder. All heteronuclear single quantum coherence (HSQC) spectra were collected on a Varian spectrometer operating at a ¹H Larmor frequency of 600 MHz. Sample quality was assessed by signal-to-noise and resolution in the 2D spectrum with between 32 to 64 increments in the indirect dimension. Heteronuclear steady-state NOE spectra were collected as previously described (Ha et al. 2007; Metcalfe et al. 2004). Briefly, two spectra were collected using the established pulse sequence based on Farrow, *et al.* (Farrow et al. 1994) with a spectral width of 6000 Hz in the indirect proton dimension and 1500 Hz in the indirect nitrogen dimension. 128 scans were done with 64 points in the indirect dimension. The saturation spectrum was collected with a 3 s presaturation period on the proton frequency. Peak intensities were analyzed using NMRView5 software (Johnson 2004).

Results

Single Gly mutations were successfully cloned on a monomeric AFA-PLN background, yielding AFA-PLN^{M20G}, AFA-PLN^{P21G}, and AFA-PLN^{Q22G}. The species were expressed in *E. coli*, and subsequently isolated by affinity and HPLC chromatography. SERCA activity measurements served as the initial screen to assess the effects of the mutations. Functional assays were performed with SERCA reconstituted in lipids alone as the negative control, and also in the presence of AFA-PLN or the super-inhibitor mutant AFA-PLN^{N27A} as a positive control, giving a consistent ΔpK_{Ca} shift of 0.3 and 0.5 pCa units, respectively. Fig 3A displays a representative normalized ATPase activity curve which yields the pK_{Ca} shifts. Fig 3B shows the average ΔpK_{Ca} values for the three mutants as compared to the control AFA-PLN (filled bars). Compared to the AFA-PLN species, the AFA-PLN^{M20G}, AFA-PLN^{P21G}, and AFA-PLN^{Q22G} analogs reduced the PLN inhibition by approximately 0.1 pCa

units. Therefore, irrespective of their positioning in the dynamic loop, the single site mutations manifested an LOF character. The extent of this effect is variable, with a gradient of SERCA inhibition ranging from ΔpK_{Ca} of 0.23 ± 0.01 for M20G to ΔpK_{Ca} of 0.16 ± 0.02 for Q22G. Remarkably, previous studies showed that Ala mutations of M20 or Q22 do not render LOF characteristics (MacLennan et al. 1998; MacLennan and Kranias 2003).

To test their propensity to be phosphorylated by protein kinase A (PKA-C) and thereby reverse SERCA inhibition, the Gly mutants of PLN were incubated with catalytic amounts of the kinase and analyzed by gel shift assays. We found that all of the single Gly PLN mutants were quantitatively phosphorylated *in vitro* by recombinant protein kinase A. Importantly, SERCA functional assays revealed that phosphorylation at Ser 16 for each single Gly mutant was sufficient to completely reverse inhibition (Fig 3B, open bars).

To investigate whether the combination of two or more Gly mutations would increase the LOF effect, we cloned a double mutant, AFA-PLN^{M20G P21G}, and a triple Gly mutant, AFA-PLN^{M20G P21G Q22G}. We found that (1) the AFA-PLN^{M20G P21G} species was a complete LOF mutant, and (2) AFA-PLN^{M20GP21GQ22G} completely recovers the inhibitory function (Fig 3B). Based on these results, we speculate that combining the Gly at position M20 and P21 conferred even stronger LOF behavior, creating an additive effect in the double mutant. In contrast, a triple mutation eliminated the structural coupling between the cytoplasmic and transmembrane helix, resulting in a species that is similar to the transmembrane helix alone (Karim et al. 2000). Electrophoretic gel shift assays show that incubation of PKA with these double and triple mutants does result in a gel shift typically observed for the phosphorylated species, consistent with quantitative phosphorylation. These results were confirmed by mass spectrometry. The cytoplasmic helix contains the phosphorylation site, while the transmembrane helix is the principal domain responsible for inhibition. We sought to test the hypothesis that phosphorylation of the Gly mutants would not reverse the effects of inhibition, due to the possible breaking of the conformational coupling between these domains. Our results indicate that phosphorylation does reverse the effects of inhibition for PLN^{M20G P21G} and also PLN^{M20G P21G Q22G}. Fig 3B shows the averaged SERCA activity measurements of several different samples of unphosphorylated (closed bars) and phosphorylated (open bars) of the double and triple glycine AFA-PLN loop mutations. Values of ΔpK_{Ca} are reported in Table 1.

A critical issue in the design of possible therapeutic mutations for delivery through gene therapy is the effect the species will have *in vitro* versus *in vivo*, particularly when dealing with possible functional effects that are the result of unknown protein-protein interactions and also interactions with the endogenous species. Hence, we studied the function of mutant with the most LOF character, AFA-PLN^{M20G P21G} by comparison to the endogenous wild-type background sequence in order to determine whether the functional results may be due to altered oligomeric states. Functional results with SERCA in the presence of the PLN^{M20G P21G} mutant show that this is a strong LOF mutant, with a ΔpK_{Ca} of 0.095 ± 0.021 (Fig 3A, right), confirming that the M20G P21G mutation results in a LOF species regardless of its monomeric or pentameric state.

To characterize the structural features of the mutations, AFA-PLN^{M20G}, AFA-PLN^{M20G P21G}, and AFA-PLN^{M20G P21G Q22G} were expressed in ¹⁵N isotopically labeled media, purified, and reconstituted in DPC micelles. The ¹H-¹⁵N HSQC spectra of all three mutants were well resolved (Supplementary Fig 1A) and assigned using ¹⁵N edited NOESY-HSQC experiments at short (70 ms) mixing times to identify short-range NOEs. Amide resonance assignments were correlated to the ¹H-¹⁵N HSQC assignment for AFA-PLN (Supplementary Fig 1B). Most changes are localized near the mutation site. However, some distal changes, especially in domain Ib, are detected, specifically residues Arg25 and Asn27.

The structural dynamics of these mutations were characterized using nuclear spin relaxation measurements (Palmer and Massi 2006). R_1 , R_2 , and NOE values were measured for AFA-PLN^{M20G}, AFA-PLN^{M20G P21G}, and AFA-PLN^{M20G P21G Q22G} (Supplementary Fig 2) in DPC micelles. While the structure is largely intact, as evidenced by only minor perturbations in the ^1H - ^{15}N HSQC spectrum compared to AFA-PLN, we do observe an increase in the fast (ps-ns) conformational dynamics of the backbone for all of the mutants examined. Increased motions are detected in the intervening loop and domains Ia and Ib. While domain Ib and the loop of the Gly mutants have conformational dynamics similar to that of pSer16 AFA-PLN, the phosphorylated species is significantly more mobile in domain Ia. Thus, introduction of the single or multiple Gly mutants change only the local motion and does not mimic the conformational dynamics found in the phosphorylated species (pSer16-AFA-PLN). A possible explanation is that the negatively charged phosphoserine interacts with the positively charged side chains of Arg13 and 14, forming transient salt bridges that affect the folding and dynamics of the entire cytoplasmic regions including domain Ia. In fact, the presence of the phosphoryl group shifts the conformational equilibrium of domain Ia of PLN from a folded (T state) to an unfolded (R state) (Gustavsson et al. 2011; Masterson et al. 2011). The ^{15}N relaxation data do not support a significant T to R state transition upon Gly mutations. The latter was confirmed by ^1H - ^{13}C HSQC experiments that image the side chains bearing methyl groups. Indeed, we found that the Gly mutations in the loop do not affect the T to R state equilibrium substantially, i.e. we observed only minor chemical shift changes for the domain Ia methyl groups (Supplementary Fig 3). These findings indicate that the LOF character of the Gly mutants is not directly related to the T/R equilibrium of domain Ia, and that the Gly mutations in the intervening loop cause a local increase in backbone flexibility.

Discussion

We sought to expand on previously published results on tuning the function of SERCA by altering the conformational dynamics of the loop domain in PLN (Ha et al. 2007). Our previous results showed that AFA-PLN^{P21G} displayed similar structural dynamics to the pseudo-phosphorylated species PLN^{S16E}, which has shown promise in reversing heart failure in animal models upon delivery via rAAV-mediated gene therapy (Hoshijima et al. 2006). In addition to mimicking the structural dynamics of the pseudo-phosphorylated form, AFA-PLN^{P21G} also has the additional characteristic of being able to be post-translationally-modified by phosphorylation at Ser16 by PKA, a regulatory feature which is crucial for maintaining Ca^{2+} homeostasis (Ha et al. 2011).

The functional results of AFA-PLN^{M20G}, AFA-PLN^{P21G}, and AFA-PLN^{Q22G} (Fig 3, Table 1) show that introduction of the Gly residue did not need to be specifically positioned at Pro 21 in order to have a LOF effect. Additionally, the *in vitro* phosphorylation results of the three species suggest that these mutants may still also be post-translationally modified *in vivo*. Remarkably, similar alanine mutations at M20 and Q22 did not show appreciable differences in SERCA inhibition (Kimura et al. 1998; MacLennan et al. 1998), demonstrating the sensitivity of SERCA activity on the PLN sequence. While all of the mutants resulted in LOF characteristics, they inhibited SERCA to different degrees, resulting in a gradient of function. This is an important factor when designing mutations to alter contractility. While introducing PLN mutants which are completely LOF would increase SERCA activity and activate diastole in the short term, constitutive activation of SERCA is known to promote hypertrophy (Shanmugam et al. 2011) and a PLN null genotype is found in humans to result in heart failure (Haghighi et al. 2003; Medeiros et al. 2011a). Hence, development of mutations of PLN for use in gene therapy would necessitate mutants which sampled a wide array of levels in SERCA inhibition.

The results of AFA-PLN^{M20G P21G} show that the LOF effects for the M20 and P21 site were additive, resulting in the least inhibitory mutant of this study. Phosphorylation of this species AFA-PLN^{M20G P21G} mutant resulted in a ΔpK_{Ca} similar to that of pSer16 AFA-PLN. The NMR results show minimal differences in the structures of the various mutants. In fact, comparison of the protein fingerprints (¹H, ¹⁵N] HSQC spectra) of the AFA-PLN^{M20G P21G} mutant and AFA-PLN show some slight differences, especially in residues near the mutation site in the primary sequence. A few differences are also evident for residues located in the juxtamembrane domain Ib (Fig 1). A map of the chemical shift changes upon increasing glycines in the loop did not follow the same trajectory that was seen for pseudo-phosphorylated mutants and the Ser16 phosphorylated species (Gustavsson et al. 2011). Also, the NMR dynamic parameters (R_1 , R_2 and heteronuclear NOE values) in domain Ia indicate that AFA-PLN^{M20G P21G} is significantly less dynamic than pSer16 AFA-PLN in this domain (Supplementary Fig 2). Still, AFA-PLN^{M20G P21G} mimics pSer16-PLN functionally, which suggests that mutations in the loop leads to the reversal of SERCA inhibition by a different mechanism than an order-to-disorder transition of domain Ia, a mechanism that has been proposed for phosphorylated and pseudo-phosphorylated mutants of PLN (Gustavsson et al. 2011). In contrast to domain Ia, the conformational dynamics of the loop and domain Ib of the Gly mutants are similar to that of pSer16 PLN and thus more mobile than AFA-PLN. Taken together with the chemical shift changes in domain Ib, this alteration of domain Ib conformational dynamics may be responsible for the functional effects associated with the Gly mutations.

Based on the data collected on these single, double, and triple Gly mutants, we hypothesize that the functional effects of the Gly mutations are related to a gradual structural uncoupling of the two helical domains of PLN: the inhibitory transmembrane domain and the regulatory cytoplasmic regions. A single Gly mutation increases the local dynamics and induces LOF character. Also, a double mutation augments the LOF character with concomitant increase of local dynamics. However, three sequential mutations cause a complete *loss of the structural coupling between the regulatory cytoplasmic domain and the inhibitory transmembrane domain*, rendering the mutant similar to the isolated transmembrane domain, which has an inhibitory potency similar to that of PLN (Karim et al. 2000). To support this hypothesis, we showed that single and double Gly mutants have increasing LOF character, while the inhibitory potency of the triple Gly mutant is virtually identical to that of AFA-PLN.

Interestingly, AFA-PLN^{M20GP21GQ22G} can be still phosphorylated, reversing SERCA inhibition. The substitution of the three Gly residues in the loop does not prevent the signal of phosphorylation to be relayed from PLN to SERCA to reverse its inhibition. This suggests that phosphorylation is a more complex event that involve both structural and electrostatic changes in both binding partners, with a possible allosteric mechanism where interactions of the phosphorylated domain Ia lead to changes within SERCA that are relayed to domain II. The latter will be resolved when atomic resolution structural information on the SERCA/PLN will be available.

Although the original Ala-scanning experiments performed by MacLennan and coworkers (MacLennan et al. 1998) show Ala mutations in the loop region to be non-effectual on SERCA function (with the exception of P21A), Gly mutations in the same region reveal that fairly conservative alterations in the loop region can have a significant impact on SERCA function. Fig 4 shows a structural dynamics and function correlation between different species of PLN, and the blue triangle highlights the optimum area to target when designing LOF mutants to improve cardiac contractility by improving SERCA function. The results of this study are promising in revealing the loop region as an area to target for development of therapeutic PLN mutants, since the phosphorylation site remains intact. The results of

studying PLN mutations have established that for a mutant to maintain proper function: (a) it must be able to be phosphorylated in order to relieve inhibition, and (b) the dynamic changes throughout domain Ia, the loop, and domain Ib can mimic the order-to-disorder transition induced by phosphorylation, but they should not exceed the dynamics of pSer16 PLN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants to G.V. from the National Institutes of Health (GM64742) and predoctoral fellowships to K.N.H. from the National Heart Lung and Blood Institute (5F31HL095361) and M.G. from the American Heart Association (10PRE3860050).

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Abbreviations

PLN	phospholamban
SERCA	sarcoplasmic reticulum Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
LOF	loss-of-function
DPC	dodecylphosphocholine
NMR	nuclear magnetic resonance
PKA	protein kinase A

Fig 1.

A. Hybrid solution and solid state NMR structures for pentameric wt-PLN (PDB 2KYV, left) and monomeric AFA-PLN (PDB 2KB7, center) and structure of the R state of PLN determined from solution NMR and molecular dynamics simulations (de Simone et al. submitted). The four dynamic domains of PLN are color-coded: domain Ia, green; loop, orange; domain Ib, red; domain II, blue. Sites of loop mutations are rendered in the space-filling motif.

Fig 2.

Primary structures of glycine mutants in this study. The secondary structure motifs of PLN (helix, loop, helix) and dynamics domains are displayed above. Mutations are highlighted in the loop region.

Fig 3. Functional measurements of SERCA in reconstituted lipids in the presence of PLN Gly loop mutants. **A.** Coupled enzyme activity assay measurement of SERCA in absence (black circles) and presence of PLN species. Left panel: unphosphorylated (closed green triangles) and phosphorylated (open green triangles) monomeric M20G P21G AFA-PLN. Specific activity of reconstituted SERCA averages 2.9 ± 1.4 IU as reported in Gustavsson, et. al. 2011. Right panel: wt-PLN (blue circles) and M20G P21G mutant on pentamer background (PLN^{M20GP21G}, green triangles). As in the monomeric conditions, the M20G P21G mutation in pentameric conditions is still severe LOF. **C.** Histogram of ΔpK_{Ca} measurements of all PLN species. Error bars are indicative of standard deviation from averaged values of ΔpK_{Ca} .

Fig 4. Structural dynamics-function correlation plot of PLN Gly mutants. Left axis is degree of SERCA inhibition, with a larger ΔpK_{Ca} indicating more SERCA inhibition. NOE is difference in average NOE values for each PLN species in the loop and domain Ib (residues 17-30) from AFA-PLN. Higher values in NOE indicates more fast dynamics for that mutant. Blue triangle highlights optimum region for designing PLN mutants to improve cardiac contractility.

Table 1

ΔpK_{Ca} values for all PLN species averaged over 6 measurements. ΔpK_{Ca} values are calculated from as the difference in pK_{Ca} values from the fits of the SERCA activity curves in the absence and presence of PLN inhibitor.

	ΔpK_{Ca}	$\Delta K_{Ca} \mu M$
AFA-PLN	0.31 ± 0.02	0.464 ± 0.022
pSer16 -AFA-PLN	0.025 ± 0.012	0.064 ± 0.037
AFA-PLN ^{M20G}	0.23 ± 0.01	0.108 ± 0.086
pSer16-AFA-PLN ^{M20G}	0.031 ± 0.018	0.012 ± 0.037
AFA-PLN ^{P21G}	0.18 ± 0.01	0.184 ± 0.026
pSer16-AFA-PLN ^{P21G}	0.042 ± 0.013	0.036 ± 0.038
AFA-PLN ^{Q22G}	0.16 ± 0.02	0.053 ± 0.078
pSer16-AFA-PLN ^{Q22G}	0.068 ± 0.014	0.020 ± 0.055
AFA-PLN ^{M20G P21G}	0.047 ± 0.009	0.048 ± 0.024
pSer16- AFA-PLN ^{M20G P21G}	0.015 ± 0.010	0.019 ± 0.022
AFA-PLN ^{M20G P21G Q22G}	0.33 ± 0.01	0.288 ± 0.021
pSer16- AFA-PLN ^{M20G P21G Q22G}	0.090 ± 0.017	0.047 ± 0.019