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Hydrophobic Imbalance in the Cytoplasmic Domain of Phospholamban Is a Determinant for Lethal Dilated Cardiomyopathy*[S]

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Background: Heterozygous mutations in phospholamban cause lethal dilated cardiomyopathy.
Results: Hydrophobic substitutions in the cytoplasmic domain of phospholamban lead to loss of inhibition and a dominant negative effect on SERCA.
Conclusion: Hydrophobic changes in the cytoplasmic domain of phospholamban appear to be one determinant for hereditary dilated cardiomyopathy.
Significance: This may provide a prediction model for genetic testing of patients with heart failure.

The sarco(endo)plasmic reticulum calcium ATPase (SERCA) and its regulatory partner phospholamban (PLN) are essential for myocardial contractility. Arg^{9} → Cys (R9C) and Arg^{14} deletion (R14del) mutations in PLN are associated with lethal dilated cardiomyopathy in humans. To better understand these mutations, we made a series of amino acid substitutions in the cytoplasmic domain of PLN and tested their ability to inhibit SERCA. R9C is a complete loss-of-function mutant of PLN, whereas R14del is a mild loss-of-function mutant. When combined with wild-type PLN to simulate heterozygous conditions, the mutants had a dominant negative effect on SERCA function. A series of targeted mutations in this region of the PLN cytoplasmic domain (^{8}TRSAIRR^{14}) demonstrated the importance of hydrophobic balance in proper PLN regulation of SERCA. We found that Arg^{9} → Leu and Thr^{8} → Cys substitutions mimicked the behavior of the R9C mutant, and an Arg^{14} → Ala substitution mimicked the behavior of the R14del mutant. The results reveal that the change in hydrophobicity resulting from the R9C and R14del mutations is sufficient to explain the loss of function and persistent interaction with SERCA. Hydrophobic imbalance in the cytoplasmic domain of PLN appears to be a predictor for the development and progression of dilated cardiomyopathy.

Approximately one in three cases of congestive heart failure are due to dilated cardiomyopathy (DCM),[4] where the defining features are left ventricular dilation, contractile dysfunction, and weakened pumping force. Familial DCM accounts for one-third to one-half of these cases, and more than 40 genes have been implicated in disease (1). Although the genetic diversity of DCM involves many cellular processes, mutations that depress force generation are prominent. Because force generation and calcium homeostasis are coupled in the myocardium, it is not surprising that disease-associated mutations have been found in calcium-handling proteins of the sarcoplasmic reticulum (SR).

SR calcium stores provide the majority of calcium used in muscle contraction-relaxation. During relaxation, an ATP-dependent calcium pump (SERCA) in the SR is essential for the recovery of calcium. The reuptake of calcium by SERCA determines the rate of relaxation and the size of the calcium store available for subsequent contractions. In cardiac muscle, a second protein called phospholamban (PLN) acts as a reversible inhibitor of SERCA and thereby modulates contractility in response to physiological cues (2, 3). SERCA inhibition by PLN is a dynamic process that depends on the cytosolic calcium concentration as well as the phosphorylation and oligomeric states of PLN. The synergistic effect is a modulation of calcium reuptake and cardiac contractility that can be finely tuned in response to activity, stress, or disease (4).

Dysregulation of SERCA by PLN is linked to heart failure. In humans, abnormal SERCA/PLN ratios and chronic SERCA inhibition are common features of heart failure (5–8), despite a diverse array of root causes. More directly, mutations have been found in the PLN gene itself. A Leu^{39}-stop nonsense mutation has been described as a PLN null (9, 10), where heterozygous individuals develop hypertrophy and homozygous individuals develop DCM. A loss-of-function Arg^{9} → Cys (R9C) missense mutation has been implicated in lethal DCM (11). Although the loss of function associated with this mutant may lead to disease, the main effect is proposed to be an abnormal interaction with protein kinase A (PKA). An Arg^{14} deletion (R14del) mutation has been linked to both mild (12) and severe (13, 14) DCM. This mutant is thought to be a superinhibitor of SERCA in the

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[4] The abbreviations used are: DCM, dilated cardiomyopathy; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum calcium ATPase; PLN, phospholamban.

heterozygous but not the homozygous condition (13). Finally, $Arg^9 \rightarrow$ Leu (R9L) and $Arg^9 \rightarrow$ His (R9H) have recently been identified in heart failure patients (15), yet they remain uncharacterized. Interestingly, there have been no homozygous patients identified with any of these mutations except Leu^{39}-stop. Nonetheless, the available mutants demonstrate that both loss of SERCA inhibition and chronic SERCA inhibition can cause DCM.

The development of therapeutic solutions for DCM requires detailed knowledge of the underlying disease-associated mechanisms (16). Toward this goal, we characterized the functional properties of PLN mutants in isolation with SERCA. The R9C mutation replaces a positive charge with a cysteine, and at first glance, the aberrant chemistry of a sulfhydryl could contribute to disease (17). In a similar line of reasoning, the R14del mutation removes a positive charge, shortens the cytoplasmic domain, and disrupts the PKA recognition motif of PLN. Herein, we made a series of amino acid substitutions in the cytoplasmic domain of PLN. In proteoliposomes with SERCA, we found that $Arg^9 \rightarrow$ Leu and $Thr^8 \rightarrow$ Cys mutations mimicked the functional consequences of R9C, and an $Arg^{14} \rightarrow$ Ala mutation mimicked R14del. The results from many additional mutations indicated that hydrophobic balance in the cytoplasmic domain of PLN is a critical determinant of function. Importantly, when mutant and wild-type PLN were combined with SERCA, the R9C and R14del mutants had a dominant negative effect on SERCA activity. Taken together, hydrophobicity and persistent association with SERCA are contributing factors in PLN mutations known to cause lethal DCM.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of PLN—Human PLN was expressed and purified as described (18). Mutants were confirmed by DNA sequencing (TAGC Sequencing, University of Alberta) and MALDI-TOF mass spectrometry (Institute for Biomolecular Design, University of Alberta). To phosphorylate PLN, purified PLN was solubilized in detergent and treated with the catalytic subunit of PKA (Sigma-Aldrich) (19).

Co-reconstitution of SERCA and PLN—SERCA1a was purified from rabbit skeletal muscle SR (20, 21) and co-reconstituted with PLN as described (19). The purified proteoliposomes yielded a final molar ratio of 1 SERCA, 4.5 PLN, and 120 lipids. The SERCA and PLN concentrations were determined by quantitative SDS-PAGE (22).

Cardiac SR—Cardiac SR membranes were purified from canine and porcine ventricles (23), and the concentrations of both SERCA2a and PLN were quantitated (22, 24, 25). Using affinity-purified SERCA1a and recombinant PLN as protein standards, incremental amounts of each protein were run next to cardiac SR membranes on the same SDS-polyacrylamide gel. Colloidal Coomassie Blue G250-stained gels were digitized, and the SERCA2a bands were quantitated and compared with a SERCA1a standard curve. To determine the amount of PLN in cardiac SR, Western blots were probed with an anti-PLN antibody (2D12, Abcam) followed by an IR800 dye-labeled secondary antibody (LI-COR Biosciences), and the PLN bands were quantitated and compared with a recombinant PLN standard curve.

Activity Assays—Calcium-dependent ATPase activities of the proteoliposomes were measured by a coupled enzyme assay (26). All co-reconstituted PLN mutants were compared with a negative control (SERCA alone) and a positive control (SERCA in the presence of wild-type PLN). The mutants investigated included alanine substitutions from Lys^3 to Thr^{17} of PLN; a series of amino acid substitutions of Thr^8, Arg^9, Ser^{10}, and Arg^{13}; and deletion of Arg^{14} (Table 1). A minimum of three independent reconstitutions and activity assays were performed for each mutant, and the ATPase activity was measured over a range of calcium concentrations (0.1–10 μM). The calcium concentration at half-maximal activity (K_{Ca}) and the maximal activity (V_{max}) were calculated based on non-linear least-squares fitting of the activity data to the Hill equation using Sigma Plot (SPSS Inc., Chicago, IL). Errors were calculated as the S.E. for a minimum of three independent reconstitutions. Comparison of K_{Ca} and V_{max} was carried out using one-way analysis of variance (between subjects), followed by the Holm-Sidak test for pairwise comparisons.

Kinetic Simulations—Reaction rate simulations have been described for the transport cycle of SERCA (27, 28), and we have previously used this approach to understand SERCA inhibition by PLN mutants (29). As before, we performed a global non-linear regression fit of the model to each plot of SERCA ATPase activity *versus* calcium concentration using Dynafit (Biokin Inc., Pullman, WA). The kinetic simulations for SERCA in the absence and presence of wild-type PLN provided starting points for optimization of the fit for SERCA in the presence of phosphorylated and mutant PLN.

RESULTS

Under normal physiological conditions, the cytoplasmic domain of PLN contributes to SERCA inhibition and provides a means of reversing inhibition through its phosphorylation. The R9C and R14del mutations in the cytoplasmic domain alter both the inhibitory capacity of PLN and the regulation of PLN function by phosphorylation. Herein, we wished to gain mechanistic insight into how mutations in the cytoplasmic domain of PLN affect SERCA inhibition and lead to DCM. Our first objective was to compare the disease-causing mutations with phosphorylation of PLN, which is the main physiological mechanism for alleviating SERCA inhibition. We next performed alanine-scanning mutagenesis of the cytoplasmic domain of PLN (residues 3–17 (30)) in an attempt to replicate the behavior of the lethal mutations. Finally, a more focused mutagenesis strategy was used to uncover the physicochemical properties that confer dysfunctional characteristics on PLN and lead to DCM.

Reconstitution versus Cardiac SR—The proteoliposomes used herein contain both SERCA1a and PLN oriented with their cytoplasmic domains on the external surface of the vesicles at lipid/protein ratios similar to those of cardiac SR membranes (19, 29). Although there may be subtle differences in the interaction of PLN with SERCA1a *versus* SERCA2a (31), the skeletal muscle isoform is well established as a surrogate for structural and functional studies of the complex (for examples, see Refs. 32–36 and Refs. 19, 23, 29, and 37, respectively). Herein, we wished to ensure that the PLN-SERCA ratio in our

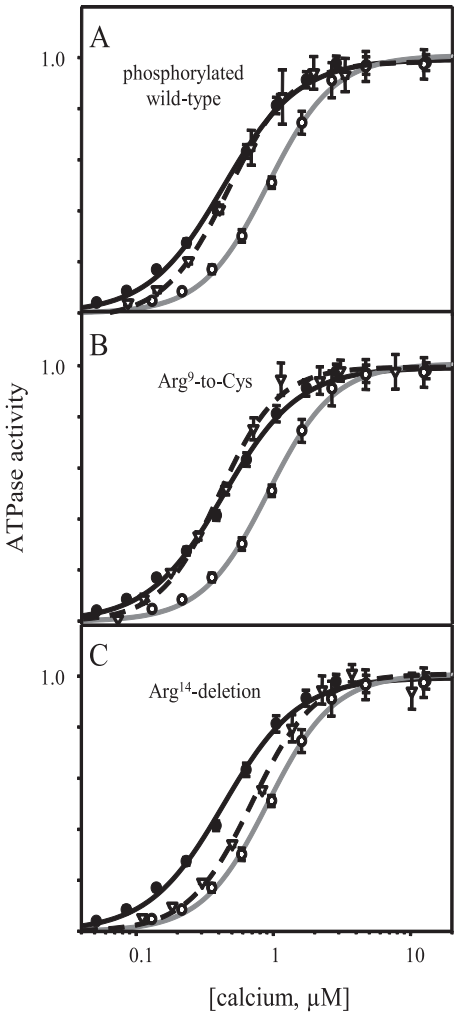

FIGURE 1. **ATPase activity as a function of calcium concentration for SERCA alone (*solid line*) or in the presence of wild-type PLN (*gray line*), phosphorylated PLN (*dashed line*) (*A*), Arg^{9} → Cys mutant of PLN (*dashed line*) (*B*), or Arg^{14} deletion mutant of PLN (*dashed line*) (*C*).** The data are normalized to V_{max}, and each data point is the mean ± S.E. (*error bars*) ($n \geq 4$). The V_{max}, K_{Ca}, and Hill coefficients (n_H) are given in Table 1.

TABLE 1

Kinetic parameters from Hill plots

Relative hydrophobicity of the cytoplasmic domain of PLN using the scale of Liu and Deber (56).

	V_{max}	n_H	K_{Ca}	Hydrophobicity
	$\mu mol/min^{-1}/mg^{-1}$		μM	
SERCA	4.1 ± 0.1	1.7 ± 0.1	0.46 ± 0.02	
WT PLN	6.1 ± 0.1	2.0 ± 0.1	0.88 ± 0.03	−17.0
Phosphorylated PLB	6.3 ± 0.1	2.1 ± 0.1	0.45 ± 0.02	
K3A	5.2 ± 0.1	2.0 ± 0.1	0.67 ± 0.02	−11.8
V4A	4.2 ± 0.1	2.5 ± 0.2	0.87 ± 0.03	−19.9
Q5A	4.8 ± 0.1	2.0 ± 0.2	0.81 ± 0.04	−14.1
Y6A	5.4 ± 0.1	2.1 ± 0.1	0.76 ± 0.02	−18.8
L7A	5.5 ± 0.1	1.9 ± 0.2	0.79 ± 0.05	−21.6
T8A	5.5 ± 0.1	2.3 ± 0.2	0.66 ± 0.03	−15.8
R9A	7.1 ± 0.2	1.6 ± 0.1	0.81 ± 0.04	−14.1
S10A	6.3 ± 0.2	2.0 ± 0.2	0.78 ± 0.04	−14.0
I12A	5.1 ± 0.1	2.2 ± 0.2	0.75 ± 0.03	−21.3
R13A	7.4 ± 0.1	1.7 ± 0.1	0.83 ± 0.03	−14.1
R14A	6.9 ± 0.1	1.9 ± 0.1	0.71 ± 0.02	−14.1
S16A	6.6 ± 0.2	1.8 ± 0.1	0.80 ± 0.04	−14.0
T17A	5.4 ± 0.1	1.8 ± 0.1	1.07 ± 0.04	−15.8
Average	5.8	2.0	0.79	
R9C	3.5 ± 0.1	2.0 ± 0.2	0.39 ± 0.02	−11.8
R9E	5.6 ± 0.1	1.8 ± 0.2	0.64 ± 0.04	−15.7
R9H	5.6 ± 0.1	1.5 ± 0.1	0.9 ± 0.06	−18.9
R9I	4.6 ± 0.1	1.4 ± 0.1	0.44 ± 0.02	−9.8
R9K	5.1 ± 0.1	1.9 ± 0.2	0.82 ± 0.05	−19.2
R9L	4.3 ± 0.1	1.9 ± 0.2	0.40 ± 0.02	−9.5
R9M	4.4 ± 0.1	1.4 ± 0.1	0.58 ± 0.01	−11.0
R9Q	5.7 ± 0.1	1.4 ± 0.1	1.14 ± 0.03	−17.0
R9S	5.8 ± 0.1	1.9 ± 0.1	0.67 ± 0.02	−17.1
R9V	4.5 ± 0.1	1.5 ± 0.1	0.42 ± 0.01	−11.2
T8C	4.5 ± 0.1	2.1 ± 0.2	0.46 ± 0.03	−13.4
S10C	5.9 ± 0.1	2.0 ± 0.1	0.55 ± 0.02	−11.7
R13I	6.7 ± 0.2	1.9 ± 0.2	0.57 ± 0.04	−9.8
R14del	7.0 ± 0.2	1.9 ± 0.2	0.74 ± 0.04	−14.2
R14del + WT	5.3 ± 0.1	1.5 ± 0.1	0.74 ± 0.02	
R9C + WT	5.1 ± 0.2	2.1 ± 0.3	0.50 ± 0.04	

co-reconstituted proteoliposomes was physiologically relevant. The reported PLN/SERCA molar ratios include values of 0.2 (38), ~2 (39–41), ~4 (24, 25). Most studies agree that there is at least a 2-fold excess of PLN, yet there is a lack of consistency between observations. We measured the concentrations of both SERCA2a and PLN in cardiac SR (supplemental Fig. S1). The calculated molar ratio of PLN per SERCA was 4.1 for both canine and porcine cardiac SR. This ratio matches the PLN content of our proteoliposomes and is in excellent agreement with values reported for rabbit, rat, and porcine cardiac SR (24, 25).

R9C, R14del, and Phosphorylated PLN—The PKA-mediated phosphorylation of PLN is the best understood mechanism for alleviating SERCA inhibition, and we wished to compare this mechanistic framework with the disease-associated mutants of PLN. To accomplish this, we measured the calcium-dependent ATPase activity of SERCA alone and in the presence of wild-type PLN, phosphorylated PLN, R9C, and R14del (Fig. 1 and Table 1). As expected, inclusion of wild-type PLN in proteoliposomes with SERCA decreased the K_{Ca} value 1.9-fold and increased the V_{max} value of SERCA 1.5-fold. Phosphorylation of PLN at Ser^{16} alleviated the inhibition and restored the K_{Ca} of SERCA to control levels (Fig. 1*A*). Inclusion of R9C in proteoliposomes had no effect on the K_{Ca} and V_{max} of SERCA, which is in agreement with the initial characterization of this mutant (11). Inclusion of R14del in proteoliposomes decreased the K_{Ca} and increased the V_{max} of SERCA. The effect of R14del on the K_{Ca} of SERCA was somewhat less than that of wild-type PLN, yet this mutant was clearly inhibitory (67% of wild-type inhibition). Interestingly, it was apparent from the ATPase isotherm data (Fig. 1 and Table 1) that the two non-inhibitory forms of PLN (phosphorylated wild type and R9C) had a persistent effect on SERCA function. This is consistent with previous demonstrations that phosphorylated PLN (43–45) and R9C (17) retain the ability to physically interact with SERCA.

To provide a mechanistic framework for the disease-associated mutants, the ATPase activity measurements were fit to the reaction scheme for SERCA calcium binding (Fig. 2) (27). SERCA calcium binding occurs as cooperative steps linked by a conformational transition (ECa ↔ E'Ca). PLN slows this conformational change via an effect on B_{rev}, thereby reducing the K_{Ca} of SERCA (28, 29). For wild-type PLN, phosphorylated PLN, R9C, and R14del, we observed effects on all three calcium-binding steps, particularly A_{for}, B_{rev}, and C_{for} (29). The primary effect was on B_{rev}, yet a decrease in the forward rate constant for binding of the second calcium ion (C_{for}) contributed to SERCA inhibition. Phosphorylation of PLN restored the K_{Ca} of SERCA, yet significant inhibition persisted at low calcium concentrations (Fig. 1*A*). Whereas B_{rev} decreased to near control levels following phosphorylation, the value for C_{for} remained low and

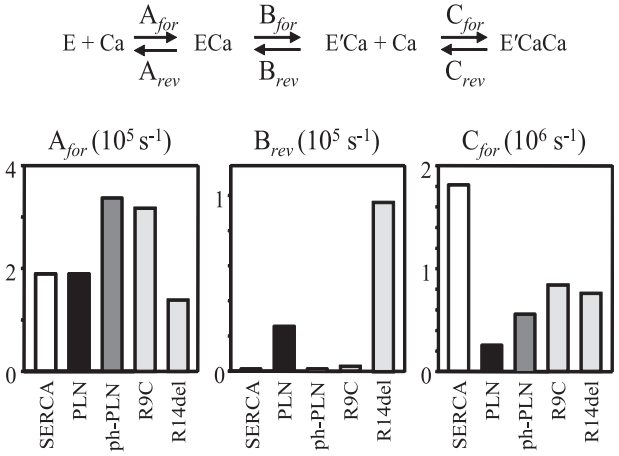

FIGURE 2. **Part of the reaction scheme for calcium transport by SERCA.** Calcium binding involves three distinct steps: binding of the first calcium ion (A_{for} and A_{rev}), a conformational change that establishes cooperativity (B_{for} and B_{rev}), and binding of the second calcium ion (C_{for} and C_{rev}). *Bottom*, summary of the effects observed in kinetic simulations of SERCA in the absence (*SERCA*) and presence of wild-type PLN (*PLN*), phosphorylated PLN (*ph-PLN*), the $Arg^9 \rightarrow$ Cys mutant (*R9C*), and the Arg^{14} deletion mutant (*R14del*).

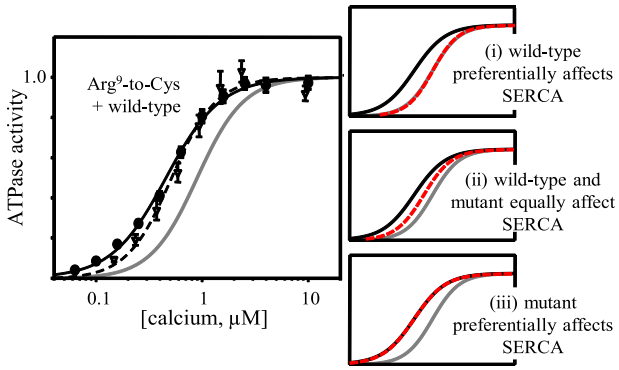

FIGURE 3. **ATPase activity as a function of calcium concentration for SERCA in the presence of an equimolar mixture of R9C and wild-type PLN (*dashed line*).** SERCA in the absence (*solid line*) and presence (*gray line*) of wild-type PLN is shown for comparison. The data are normalized to V_{max}, and each data point is the mean ± S.E. (*error bars*) ($n \geq 4$). The V_{max}, K_{Ca}, and n_H values are given in Table 1. *Right*, the potential outcomes of this experiment are schematically shown.

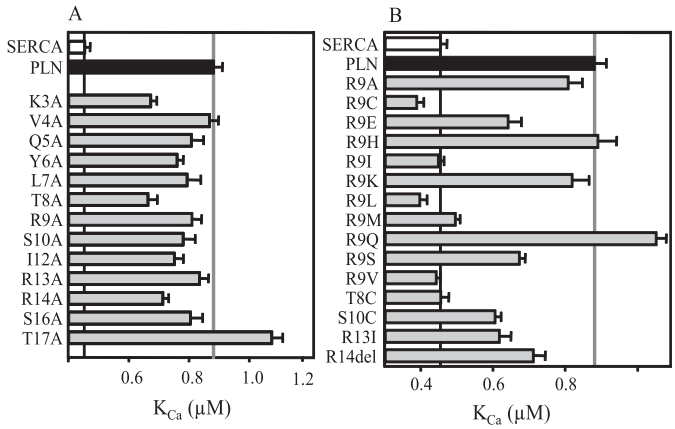

FIGURE 4. **The effects of mutation in the cytoplasmic domain of PLN on the K_{Ca} of SERCA.** The *vertical line* on the *left* is the K_{Ca} for SERCA alone (*white bar*), and the *vertical line* on the *right* is the K_{Ca} for SERCA in the presence of wild-type PLN (*black bar*). The K_{Ca} values are plotted for each mutant of PLN (*gray bars*). *A*, alanine mutations in the cytoplasmic domain of PLN; *B*, disease-associated and disease-mimicking PLN mutations. *Error bars*, S.E.

provided a mechanism for the residual inhibitory activity of phosphorylated PLN (Fig. 2 and supplemental Table S1). In terms of the kinetic simulations, the R9C mutant closely mimicked phosphorylated PLN, whereas the partial inhibition by the R14del mutant was due to an effect on B_{rev} (Fig. 2).

Mixtures of Mutant and Wild-type PLN—Based on heterologous expression in HEK-293 cells (13), the R14del mutant was reported to be a partial inhibitor in the homozygous state and a superinhibitor in the heterozygous state. Consistent with the homozygous state, we found R14del to be a slight loss-of-function mutant. Because the R14del mutant is presumably a superinhibitor of SERCA in the presence of wild-type PLN (heterozygous background), and no homozygous individuals have been found for either R9C or R14del, we examined proteoliposomes containing SERCA and equal amounts of mutant and wild-type PLN. The mixture of R9C and wild-type PLN was similar to SERCA in the presence of only R9C (Fig. 3). Similarly, the mixture of R14del and wild-type PLN was similar to SERCA in the presence of only R14del (Table 1). For comparison, we evaluated a loss-of-function mutant in the transmembrane domain of PLN, $Arg^{34} \rightarrow$ Ala (N34A) (46). The persistent effect on SERCA seen for the R9C and R14del mutants was not observed with N34A (supplemental Fig. S2). From these data, there were two main conclusions. First, in the presence of wild-type PLN, the R9C and R14del mutants were capable of a dominant negative effect on SERCA function. Second, when separated from other cellular components in proteoliposomes, the combination of R14del and wild-type PLN did not result in superinhibition of SERCA. Although we did not observe superinhibition in this simplified experimental system, the complex sequelae associated with disease could result in chronic SERCA suppression. In addition, other environmental or genetic factors may be involved because both mild (12) and severe DCM (13, 14) have been linked to the R14del mutation.

Alanine Substitutions in Cytoplasmic Domain of PLN—In general, mutations in the cytoplasmic domain of PLN tend to have a much smaller effect on SERCA inhibition (30) than mutations in the transmembrane domain (29, 46). These experimental observations contrast sharply with findings in hereditary DCM, where mutations are more common in the cytoplasmic domain of PLN. What physicochemical characteristics define the disease-associated mutations and underlie disease? As a first step in examining this question, we mutated residues 3–17 of PLN to alanine, co-reconstituted each mutant with SERCA, and measured the calcium-dependent ATPase activity of the proteoliposomes (Fig. 4*A* and Table 1). Collectively, the alanine mutants were consistent with the notion that the cytoplasmic domain of PLN makes a small contribution to SERCA inhibition. Averaging the kinetic parameters for the alanine substitutions, we obtained a K_{Ca} of 0.79 μM (78% of wild-type PLN inhibition; *i.e.* 22% average contribution from the cytoplasmic domain).

Despite the limited contribution of the cytoplasmic domain of PLN to SERCA inhibition, a few mutations had a more substantial impact. The largest effects on the K_{Ca} of SERCA were observed for alanine substitution of Lys^3, Thr^8, Arg^{14} (partial loss-of-function mutants), and Thr^{17} (gain-of-function

mutant) (Fig. 4*A*). None of the substitutions recapitulated the R9C mutant. However, the R14A mutant was an effective mimic of the disease-associated R14del. The K_{Ca}, V_{max} and n_H values for SERCA in the presence of R14A were indistinguishable from the values obtained in the presence of R14del (Table 1). This led to the conclusion that the defect associated with the R14del mutation was due to a change in amino acid sequence rather than a change in the length of the cytoplasmic domain.

There were several other interesting observations with this set of mutants (Fig. 4*A* and Table 1). Thr^{17} of PLN is the site of phosphorylation by Ca^{2+}/calmodulin-dependent kinase II, and mutation of this residue to alanine results in gain of function. Several alanine substitutions in PLN also had a surprising effect on the V_{max} of SERCA. The general observation with our reconstitution system is that mutants yield a lower V_{max} for SERCA compared with wild-type PLN and are therefore classified as loss of function (29). In this regard, the most severe loss-of-function mutant was V4A. However, several mutants resulted in gain of function. Notably, the removal of any one of the three arginine residues (Arg^9, Arg^{13}, and Arg^{14}) further enhanced the maximal activity of SERCA. Although we lack a molecular understanding of this effect, this is the first time we have observed gain-of-function mutations.

Kinetic simulations initially focused on the collective effect that cytoplasmic mutations have on the ability of PLN to regulate SERCA (supplemental Table S1). In comparison with wild-type PLN, the cytoplasmic domain mutants on average had a slightly lesser effect on the SERCA conformational change and binding of the second calcium ion (*i.e.* a small decrease in B_{rev} and a small increase in C_{for}). In our prior studies of the transmembrane domain of PLN, we described a correlation between the ability of the mutants to alter K_{Ca} and their effect on C_{for} (29). There was no such unifying relationship for the mutants in the cytoplasmic domain of PLN. Nonetheless, a few mutants stood out and are worthy of further consideration. The comparable R14A and R14del mutants strongly suppressed the conformational change in SERCA that follows binding of the first calcium ion (higher value for B_{rev}). However, the partial loss of function observed for these mutants was attributable to more favorable binding of the second calcium (higher value for C_{for}). As another noteworthy mutant, the gain-of-function noted for T17A was due to unfavorable binding of the first calcium ion (lower value for A_{for}).

As described above, the PLN mutants also altered the V_{max} of SERCA. Although not everyone agrees on the physiological relevance of this phenomenon, it is easily understandable in the kinetic simulations (29). As in our previous studies (29), we observed a clear correlation between the ability of the PLN mutants to alter V_{max} and the forward rate constant for the SERCA conformational change that follows binding of the first calcium ion (B_{for}). Thus, accelerating the formation of the $E'Ca$ state increases the turnover rate and maximal activity of the enzyme.

Mimicking Disease-associated Mutations—Given the link between cytoplasmic mutants of PLN (R9C and R14del) and DCM, we wished to further understand the underlying mechanism of these mutations using the approach described above. Toward this end, a series of targeted mutations in PLN were

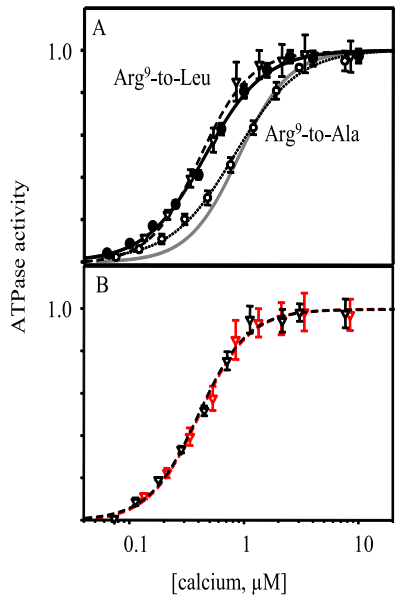

FIGURE 5. *A*, ATPase activity for SERCA alone (*solid line*) and in the presence of R9L (*dashed line*) or R9A (*dotted line*). SERCA in the presence of wild-type PLN is shown for comparison (*gray line*). *B*, superposition of the ATPase activity data for SERCA in the presence of R9C (*black*) and R9L (*red*). The data are normalized to V_{max}, and each data point is the mean ± S.E. (*error bars*) ($n \geq 4$). The V_{max}, K_{Ca}, and n_H values are given in Table 1.

tested, including R9E (charge reversal); R9H, R9K, and R9Q (conservative substitutions); R9I, R9L, R9M, and R9V (hydrophobic substitutions); R9S (isosteric to R9C); T8C and S10C (which moved Cys substitution one residue in each direction); and R13I (which had the same local primary structure as the R14del mutation but retained the full length of PLN).

This set of mutants allowed us to determine why the R9C and R14del mutants were particularly detrimental (Fig. 4*B* and Table 1). The conservative substitutions resulted in either wild-type inhibition (R9H and R9K) or gain of function (R9Q). The R9E and R9S mutants were partially functional with reduced SERCA inhibition (43 and 50% of wild type, respectively). Finally, the hydrophobic mutants mimicked the complete loss of function seen for R9C, with no effect on SERCA function (Fig. 5*A* and Table 1). Remarkably, the ATPase isotherms for R9C and R9L were identical to one another (Fig. 5*B*) and distinct from that for SERCA alone. Thus, the hydrophobic substitutions (in particular R9L) completely mimicked the disease-associated mutation (R9C). This suggested that it is not necessarily the cysteine or sulfhydryl that contributes to the loss of function observed with R9C but rather the hydrophobic character of the residue that interferes with the SERCA-PLN interaction. To test this notion further, we studied T8C and S10C variants of PLN (Fig. 4*B*). Neither mutant duplicated the data for R9C and R9L, yet they were both severe loss-of-function mutations. Therefore, Thr^8, Arg^9, and Ser^{10} were considered potential disease-causing loci. Finally, we studied an R13I mutant as a surrogate for R14del. Recall that R14A, but not R13A, was an effective mimic of R14del. Compared with R14del (67% of wild-type PLN function), the R13I mutant resulted in more severe loss of function (26% of wild-type PLN function). Because the R14A and R13I mutants replaced a basic residue with increasingly hydrophobic residues, this suggested that

hydrophobic balance in the cytoplasmic domain of PLN is a critical determinant for proper function. In support of this notion, all of the most severe loss-of-function mutants studied herein (R9C, R9I, R9L, R9M, R9V, T8C, S10C, and R13I) replaced a polar residue with a hydrophobic residue.

Kinetic simulation of the ATPase isotherms revealed further insight into the severe loss of function observed for the disease-associated and -mimicking mutations (supplemental Table S1). For instance, the complete loss of function associated with the R9C and R9L mutants appeared to be analogous to the phosphorylation of wild-type PLN. All three calcium binding steps were affected, with an increase in A_{for} (binding of the first calcium ion) and C_{for} (binding of the second calcium ion) and a decrease in B_{rev} (SERCA conformational change). The net result was that all three calcium binding steps were more favorable for transport. Additionally, as expected from the ATPase isotherms (Fig. 5), the R9C and R9L mutants had a persistent effect on SERCA rate constants (implying that they remain physically associated) despite being complete loss-of-function mutants. This is consistent with FRET studies demonstrating that R9C retains the ability to interact with SERCA despite the loss of inhibitory function (17).

DISCUSSION

Mutations in human PLN have been linked to hereditary cardiomyopathies. These include Leu^{39}-stop in the transmembrane domain of PLN (9, 10) and R9C (11) and R14del in the cytoplasmic domain (12, 13). Although there are limited data available, the frequency of PLN mutations associated with cardiomyopathies in the human population suggests that it could be included in current genetic test panels (10). Because up to 35% of DCM cases may be hereditary (47), it is necessary that we understand the molecular mechanism of these mutations and establish prediction models for additional mutations that may cause disease. Surprisingly, the disease-associated mutations are more common in the cytoplasmic domain of PLN, whereas the experimentally characterized loss-of-function mutations predominantly occur in the transmembrane domain (29, 46). The experimental observations reflect the fact that SERCA inhibition is primarily encoded by the transmembrane domain of PLN, whereas the known human mutations in the cytoplasmic domain of PLN emphasize the importance of this domain in maintaining normal cardiac contractility.

Cytoplasmic Domain of PLN and SERCA Regulation—The protein-protein interaction between SERCA and PLN slows a conformational change from the calcium-free E2 state of SERCA to the calcium-bound E1 state. Although we lack a molecular structure of the SERCA-PLN complex, there is a potential explanation for this effect in that PLN is thought to bind to SERCA in a groove formed by transmembrane segments M2, M4, M6, and M9 (33). This groove is open in the calcium-free forms of SERCA and closed in the calcium-bound forms. From the perspective of kinetic simulations, PLN alters the apparent calcium affinity of SERCA by slowing a conformational change that follows binding of the first calcium ion to SERCA and increases the cooperativity for binding of a second calcium ion. Note that this conformational change does not necessarily correspond to the E2-E1 structural transition. Nonetheless, the kinetic simulations reveal an effect of PLN on B_{rev} in the reaction scheme for SERCA (28) as well as an effect on C_{for}. Considering the suggestion that PLN dissociates from SERCA following calcium binding (48, 49), the simulations support a physical interaction that persists at least through all three calcium binding steps of the reaction cycle (Fig. 2).

Nonetheless, the above discussion largely refers to the inhibitory interaction of the PLN transmembrane domain. How does the cytoplasmic domain fit into this model, with particular emphasis on phosphorylation and the disease-associated mutations? It is difficult to envision how mutation or phosphorylation of the cytoplasmic domain of PLN negates the inhibitory interaction of the transmembrane domain with SERCA. One model for the phosphorylation of PLN suggests that it restores SERCA function by dissociating the inhibitory complex (50, 51). An alternative model for the phosphorylation of PLN suggests that it restores the apparent calcium affinity of SERCA by altering the structural interaction between the two proteins, yet the two proteins remain associated (43, 44, 52, 53). Our data for SERCA alone *versus* SERCA in the presence of phosphorylated PLN (Fig. 1) demonstrated that the ATPase activity isotherms were not equivalent and that phosphorylated PLN had a persistent effect on SERCA. The inference is that phosphorylated PLN can remain physically associated with SERCA in a non-inhibitory complex.

Disease-associated Mutations and Development of DCM—The PLN mutants R9C and R14del are known to be associated with lethal DCM. In the case of R9C, the underlying mechanism is reported to be a dominant effect on PKA phosphorylation via the β-adrenergic pathway (11). In the case of R14del, the underlying mechanism is reported to be superinhibition of SERCA in the heterozygous state based on co-transfection in HEK-293 cells (13); however, the homozygous transfection of R14del resulted in partial inhibition of SERCA. This latter result is consistent with our data for R14del as a partial inhibitor of SERCA, even in the presence of wild-type PLN. Nonetheless, it is reasonable to expect that these changes in the function of PLN could contribute to myocardial calcium dysregulation and the development or progression of heart disease. The available data suggest that either loss or gain of PLN function can contribute to the development of DCM, even in a heterozygous background where wild-type PLN is present. Herein, one surprising finding was that the R9C and R14del mutants preferentially affect SERCA in the presence of equal amounts of the wild-type protein (designed to approximate heterozygous conditions). This is best exemplified by the data for R9C (Fig. 3). With co-reconstituted proteoliposomes containing SERCA and equal amounts of mutant and wild-type PLN, there were several possible functional outcomes: (i) wild-type PLN may preferentially affect SERCA, resulting in normal SERCA inhibition; (ii) wild-type and mutant PLN may equally affect SERCA, resulting in an intermediate level of SERCA inhibition; and (iii) the mutant may preferentially affect SERCA, resulting in SERCA inhibition that resembles the mutant. For both R14del and R9C, the effect of the mutant on SERCA function dominated in the presence of the wild-type protein.

Focusing on R9C, what is it about this mutation that causes loss of function and a persistent effect on SERCA? We con-

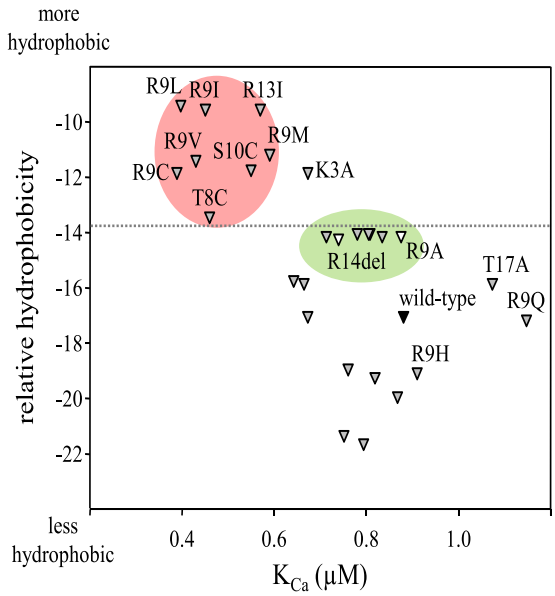

FIGURE 6. **The relative hydrophobicity of the cytoplasmic domain mutants (residues 1–17) is plotted *versus* K_{Ca} for SERCA in the presence of the various mutants.** The most severe loss-of-function mutations (R9C, R9I, R9L, R9M, R9V, T8C, S10C, and R13I) cluster (*red circle*) *above* a hydrophobicity threshold (*dotted line*) for the cytoplasmic domain of PLN, and a cluster of partial loss-of-function mutants (including R14del; *green circle*) fall close to the predicted threshold.

cluded that the dominant negative effect of these mutants is a result of a persistent physical interaction with SERCA because R9C and other loss-of-function mutants have been shown to interact with SERCA (17, 54, 55). In terms of SERCA inhibition, the most severe loss-of-function mutants in the present study included R9C, R9I, R9L, R9M, R9V, T8C, S10C, and R13I. The common denominator appeared to be the replacement of a polar residue with a hydrophobic residue. Using the hydrophobicity scale of Liu and Deber (56), we calculated the relative hydrophobicity of the cytoplasmic domain of PLN for all of the studied amino acid substitutions. The hydrophobicity was then plotted against the K_{Ca} of SERCA in the presence of the mutant (Fig. 6). The severe loss-of-function mutations clustered above a critical hydrophobicity threshold for the cytoplasmic domain of PLN, whereas a cluster of partial loss-of-function mutants (including R14A, R14del, and R9A) fell near the threshold. This suggests that hydrophobic balance in this region of PLN (Thr^8–Arg^{14}) is essential for proper inhibitory function. From a prediction standpoint, spontaneous or hereditary missense mutations that replace any of these residues of PLN with a hydrophobic residue (such as R9L) should also give rise to disease, and these mutations may eventually be found in the human population. In a heterozygous background, these loss-of-function mutants would be expected to have a dominant negative effect over wild-type PLN, thereby conferring loss of calcium transport properties on SERCA. Combined with abnormal regulation by phosphorylation for the R9C and R14del mutants, the effect of the mutants would be constitutive. Thus, it is not surprising that the cumulative effect on myocardial calcium regulation leads to early onset, lethal DCM.

In Support of Hydrophobic Imbalance Hypothesis—Given the similarity to R9C, we anticipated that the R9L mutation might eventually be found in the human population. This was confirmed during the final stages of our work when two individuals in a cohort of 1,014 Brazilian patients with heart failure were found to harbor the R9L mutation (15). One of these R9L patients presented with idiopathic DCM followed by death at 30 years of age, reminiscent of what we know about R9C patients (11). An R9H mutation was also found, although the disease relevance of this mutant remains unclear.

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