Published in final edited form as:

Biochem Biophys Res Commun. 2014 June 27; 449(2): 196–201. doi:10.1016/j.bbrc.2014.04.166.

Time-resolved FRET Reveals the Structural Mechanism of SERCA-PLB Regulation

Xiaoqiong Dong¹ and David D. Thomas^{2,*}

¹Department of Biomedical Engineering, University of Minnesota, Minneapolis, Minnesota 55455, USA

²Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, USA

Abstract

We have used time-resolved fluorescence resonance energy transfer (TR-FRET) to characterize the interaction between phospholamban (PLB) and the sarcoplasmic reticulum (SR) Ca-ATPase (SERCA) under conditions that relieve SERCA inhibition. Unphosphorylated PLB inhibits SERCA in cardiac SR, but inhibition is relieved by either micromolar Ca^{2+} or PLB phosphorylation. In both cases, it has been proposed that inhibition is relieved by dissociation of the complex. To test this hypothesis, we attached fluorophores to the cytoplasmic domains of SERCA and PLB, and reconstituted them functionally in lipid bilayers. TR-FRET, which permitted simultaneous measurement of SERCA-PLB binding and structure, was measured as a function of PLB phosphorylation and $[\text{Ca}^{2+}]$. In all cases, two structural states of the SERCA-PLB complex were resolved, probably corresponding to the previously described *T* and *R* structural states of the PLB cytoplasmic domain. Phosphorylation of PLB at S16 completely relieved inhibition, partially dissociated the SERCA-PLB complex, and shifted the *T/R* equilibrium within the bound complex toward the *R* state. Since the PLB concentration in cardiac SR is at least 10 times that in our FRET measurements, we calculate that most of SERCA contains bound phosphorylated PLB in cardiac SR, even after complete phosphorylation. 4 μM Ca^{2+} completely relieved inhibition but did not induce a detectable change in SERCA-PLB binding or cytoplasmic domain structure, suggesting a mechanism involving structural changes in SERCA's transmembrane domain. We conclude that Ca^{2+} and PLB phosphorylation relieve SERCA-PLB inhibition by distinct mechanisms, but both are achieved primarily by structural changes within the SERCA-PLB complex, not by dissociation of that complex.

Keywords

phospholamban; SERCA; phosphorylation; FRET

© 2014 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: ddt@umn.edu. Phone (612)-625-0957. Fax: (612)-624-0632..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

A hallmark of heart failure is dysregulation of intracellular Ca^{2+} handling [1], primarily caused by inadequate removal of Ca^{2+} from the cytosol by the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) [2], which actively sequesters Ca^{2+} back into the sarcoplasmic reticulum (SR) at a ratio of 2 Ca^{2+} per ATP hydrolyzed [3]. In the heart, SERCA is regulated by phospholamban (PLB), a single-pass integral membrane protein. Unphosphorylated PLB inhibits SERCA by decreasing its apparent Ca^{2+} affinity [4]. Under physiological conditions, this inhibition is relieved by either micromolar Ca^{2+} or by phosphorylation of PLB, primarily by protein kinase A (PKA) at S16 [5]. Recent gene therapies targeting increased SERCA activity show promise for alleviation of heart failure [6]. Ablation of PLB or introduction of phosphomimetic mutant S16E-PLB suppress progression of heart failure in animal models [7,8,9]. Overexpression of SERCA2a, the cardiac isoform, has successfully completed Phase IIa clinical trials [10]. These studies validate the SERCA-PLB complex as a potent therapeutic target for heart failure. However, rational design of improved therapies is hampered by uncertainty regarding the mechanism by which PLB regulates SERCA.

Two structural mechanisms for relief of SERCA inhibition have been proposed. The dissociation model hypothesizes that PLB dissociates from SERCA to relieve inhibition. This model is supported by crosslinking and co-immunoprecipitation studies that found decreased physical interaction between SERCA and PLB at either micromolar Ca^{2+} or after phosphorylation at S16 by PKA [11,12,13,14,15,16]. However, recent spectroscopic studies support the subunit model, in which inhibition of SERCA is relieved by structural rearrangements within the SERCA-PLB complex, not by dissociation. Electron paramagnetic resonance (EPR) and fluorescence resonance energy transfer (FRET) studies showed that PLB remains bound to SERCA when the pump is activated [17,18,19,20]. EPR and NMR studies showed that the cytoplasmic domain of PLB exists in equilibrium between a *T* state that is ordered and an *R* state that is dynamically disordered [21,22,23]. Phosphorylation shifts the equilibrium toward the *R* state and relieves inhibition [24]. FRET studies showed that variation of lipid headgroup charge shows a strong correlation between the population of the *R* state and SERCA-PLB activation, without dissociation, further validating the subunit model [25]. That study showed the power of time-resolved (TR) FRET to distinguish between changes in structure and association. In the present study, we have used TR-FRET, using fluorophore-labeled SERCA and PLB reconstituted in lipid bilayers, to resolve the effects of both micromolar Ca^{2+} and PLB phosphorylation on the structure and stability of the SERCA-PLB complex. These results provide definitive insights into the molecular mechanisms underlying relief of inhibition in cardiac SR.

2. Materials and methods

2.1 SERCA purification and labeling

Crude SR vesicles were prepared from the fast-twitch skeletal muscle of New Zealand white rabbits [26]. SERCA was further purified from crude SR vesicles using reactive-red chromatography [27]. For FRET studies, purified SERCA was labeled with 5-iodoacetamidofluorescein (IAF) (Invitrogen, CA) specifically and completely at C674 [28].

2.2 Expression, purification, phosphorylation and labeling of PLB

Native PLB equilibrates between monomers and homopentamers [29]. To simplify the analysis and focus on the SERCA-PLB interaction, a monomeric mutant of PLB was used, with the three cysteine residues (C36, C41 and C46) in the transmembrane domain mutated to alanine, phenylalanine, and alanine, respectively [30]. Site-directed mutagenesis was performed to mutate Y6 to C for thiol-reactive fluorophore attachment. This site was chosen because Y6 is not involved in the interaction with SERCA [31]. Recombinant PLB was expressed in *E. coli* and purified as previously published [32]. For site-directed fluorophore labeling, lyophilized PLB powder was dissolved at a concentration of 0.2 mM in 20 mM MOPS, 1% octyl β -D-glucopyranoside (OG), pH 7.0. Alexa Fluor® 350 C₅ maleimide (Invitrogen, CA) freshly dissolved in DMSO was then added at 10-fold molar excess. The reaction was allowed to proceed at room temperature for 1 hour, and the labeled PLB was purified by reversed-phase HPLC. For phosphorylation studies, labeled PLB was phosphorylated as described previously [29] and purified by reversed-phase HPLC. Complete labeling and phosphorylation of PLB was confirmed by ESI-MS. The concentration of PLB was measured by the BCA assay.

2.3 Co-reconstitution of SERCA and PLB

SERCA and PLB were co-reconstituted into lipid vesicles using 4:1 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (mol/mol) to yield unilamellar vesicles [33]. Ca²⁺-ATPase activity and FRET measurements were performed immediately after co-reconstitution. The final buffer composition of FRET samples was 50 mM MOPS, 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, pH 7.0, with varying concentrations of CaCl₂ to yield the desired free Ca²⁺ concentration (pCa 8.0, 6.4, or 5.4).

2.4 Ca²⁺-ATPase functional measurements

To ensure that labeled SERCA and PLB remain functional, we measured the ATPase activity of co-reconstituted samples at 25 °C, using an NADH-linked enzyme-coupled assay [33,34]. The Ca²⁺-dependent rate of ATP consumption was fitted to the Hill equation,

$$V = V_{max} / \left[1 + 10^{-n(pK_{Ca} - pCa)} \right], \quad \text{Eq 1}$$

where $pCa = -\log_{10} [Ca^{2+}]$, V_{max} is the limiting activity at saturating calcium, pK_{Ca} is the pCa value where $V = 0.5V_{max}$, and n is the Hill coefficient. The inhibition of SERCA by PLB is shown as pK_{Ca} , the shift of pK_{Ca} upon addition of PLB.

2.5 Time-resolved fluorescence resonance energy transfer (TR-FRET) measurements

SERCA and PLB were labeled with fluorophores at the sites shown in Fig. 1A. PLB was labeled with Alexa Fluor 350 maleimide (donor) at Y6C, and SERCA was labeled with IAF (acceptor) at C674. The quantum yield of bound Alexa Fluor 350 maleimide was measured in 20 mM MOPS, 1% OG, pH 7.0, using quinine sulfate dehydrate (AnaSpec, CA) as the standard, yielding a quantum yield of 0.48 for PLB, 0.80 for phosphorylated PLB (pPLB). The corresponding R_0 values [35] are calculated to be 4.6 nm and 5.0 nm, respectively. The

time-resolved fluorescence decay of co-reconstituted samples was measured by time-correlated single-photon counting (Becker-Hickl, Berlin, Germany), following excitation at 385 nm using a subnanosecond pulsed diode laser (PicoQuant, Berlin, Germany), filtering the emitted light using a 440/40 filter (Semrock, NY), and detection with a PMH-100 photomultiplier (Becker-Hickl). The instrument response function (IRF, Fig. 1B) was recorded from water. TR-FRET data was analyzed as previously published [25]. The observed donor-only waveform (Fig. 1B) was fitted by a simulation $F_{Dsim}(t)$, consisting of a multi-exponential decay $F_D(t)$ convoluted with the IRF

$$F_D(t) = \sum_{i=1}^n A_i \exp(t/\tau_{Di})$$

$$F_{Dsim}(t) = \int_{-\infty}^{+\infty} IRF(t-t') F_D(t') dt' \quad \text{Eq 2}$$

where τ_{Di} is the fluorescence lifetime of the i th component. In this study, two exponentials were sufficient to fit the donor-only waveform (Fig. S1). The waveform of the samples containing donor and acceptor (Fig. 1B), was fitted by $F_{D+A, sim}(t)$:

$$F_{D+A}(t) = (1 - x_{DA}) F_D(t) + x_{DA} \sum_{j=1}^m F_{DA,j}(t)$$

$$F_{D+A, sim}(t) = \int_{-\infty}^{+\infty} IRF(t-t') F_{D+A}(t') dt', \quad \text{Eq 3}$$

where x_{DA} is the mole fraction of donor-labeled PLB that is bound to acceptor-labeled SERCA, and $F_{DA,j}(t)$ is the fluorescence waveform of the j th donor-acceptor complex. We found that $m=2$ was sufficient (Fig. S2).

$$F_{DA}(t) = \int_{-\infty}^{+\infty} \rho(R) \sum_{i=1}^n A_i \exp\left\{(-t/\tau_{Di}) \left[1 + (R_{oi}/R)^6\right]\right\} dR \quad \text{Eq 4}$$

where R_{oi} is the Förster distance of Alexa Fluor 350 and IAF, and $\rho(R)$ is the distance distribution function, assumed to be sum of Gaussians, each with a mole fraction x_j , a center r_j and a full width at half maximum (FWHM _{j}) (Fig. 1C):

$$\rho(R) = \sum_{j=1}^2 x_j \left(\frac{1}{\sigma_j \sqrt{2\pi}} \right) \exp\left[-\frac{(R-r_j)^2}{2\sigma_j^2}\right]$$

where $\sigma_j = \frac{FWHM_j}{2\sqrt{2\ln 2}}$ Eq 5

We assumed that PLB and pPLB populate the same two states, because the distance parameters resolved from independent fittings are similar for PLB and pPLB (Fig. S2) and this assumption does not change the goodness of the fit (Fig. S3). Our previous FRET studies showed that the shorter distance observed between probes on the SERCA and PLB cytoplasmic domains is the R state (correlating with decreased inhibition), while the longer distance is due to the T state, in which the PLB cytoplasmic domain interacts with membrane surface and hence increases interprobe distance [25]. Therefore, we refer the parameters of the shorter distance with a subscript R , and those of the longer distance with a subscript T (Fig. 1).

2.6 Statistical Analysis

Data is presented as the mean $\pm$ SE. For comparison between two groups, a student's *t*-test was performed. A *p*-value less than 0.05 was taken as significant.

3. Results

3.1. Function of labeled SERCA and PLB

To confirm that the labeled proteins preserve their functions, we co-reconstituted SERCA and PLB into lipid vesicles and measured the Ca^{2+} -dependent ATPase activity. The function of unlabeled PLB is characterized by inhibition of SERCA (decreasing pK_{Ca}) and reversal after phosphorylation at S16 by PKA (Fig. 2). For unlabeled SERCA, $\text{pK}_{\text{Ca}} = 6.43 \pm 0.02$, unlabeled PLB shifted pK_{Ca} to 6.32 ± 0.003 , and unlabeled pPLB restored pK_{Ca} to 6.39 ± 0.02 . Labeled proteins demonstrated similar results (Fig. 2). The pK_{Ca} of acceptor-labeled SERCA is 6.45 ± 0.02 , donor-labeled PLB shifted it to 6.32 ± 0.003 , and phosphorylated donor-labeled PLB shifted it back to 6.45 ± 0.01 . Therefore, the fluorophore-labeled SERCA and PLB retained their functions, and these samples should provide reliable information of the conformational rearrangement induced by phosphorylation of S16 by PKA.

3.2 TR-FRET within the SERCA-PLB complex

Time-resolved fluorescence of donor-labeled PLB was measured in the presence of either unlabeled or acceptor-labeled SERCA with a PLB to SERCA molar ratio of 0.2. The fluorescence waveforms were then fitted by the equations described in Materials and Methods. TR-FRET data was fitted to two Gaussian distance distributions [25], assuming that PLB and pPLB populate the same two structural states (Fig. S2 and S3). The shorter distance, r_R , is 3.52 ± 0.42 nm and the longer distance, r_T , is 8.27 ± 0.27 nm (Fig. 3A&B). The distance between the labeling sites predicted by the SERCA-PLB model is 2.69 nm (Fig. 1A right) [36]. Crosslinking showed previously that K3 of PLB was within 1.5 nm of SERCA K400 [11]. The distance between C674 and K400 predicted by a crystal structure in the absence of Ca is 3.48 nm (3B9R) [37], consistent with our measurement for r_R . The fraction of pPLB that binds to SERCA (x_{DA}) decreased significantly from that of PLB, from 0.99 ± 0.01 to 0.50 ± 0.06 (Fig. 3C), indicating that the SERCA-PLB complex partially dissociates after PLB phosphorylation. Within the bound population, the fraction of the *R* state, x_R , is substantially increased after PLB phosphorylation, from 0.33 ± 0.04 to 0.68 ± 0.04 (Fig. 3A&C), indicating a substantial shift of the *T/R* equilibrium toward the *R* state.

Interprobe distances were measured at three Ca^{2+} concentrations, and no significant effects of Ca^{2+} were observed in binding or structure (Fig. 3, Table S1). These results show clearly that PLB remains bound to SERCA after the E2 (Ca-free) to E1 (Ca-bound) transition. This result is consistent with previous FRET findings that the SERCA-PLB complex does not dissociate at physiological Ca^{2+} concentrations [17,19].

4. Discussion

We have performed TR-FRET on fluorophore-labeled SERCA and PLB co-reconstituted into lipid vesicles, to probe the structural mechanism for relief of inhibition by PLB phosphorylation and by micromolar Ca^{2+} . The functions of the proteins were preserved after covalent modification with fluorophores (Fig. 2). TR-FRET analysis showed that phosphorylation of PLB partially dissociates the SERCA-PLB complex and shifts the SERCA-bound PLB toward the less inhibitory *R* state. Ca^{2+} does not dissociate the complex or perturb the *T-R* equilibrium (Fig. 3).

The mechanism of relief of inhibition due to PLB phosphorylation has been controversial. Phosphorylation of PLB has been found to perturb the physical interaction between SERCA and PLB, as detected by decreases in crosslinking [14] and co-immunoprecipitation [15]. One study in living cells, using fluorescent fusion proteins, reported that phosphomimetic PLB mutations decreased FRET between SERCA and PLB [38], while another study reported increased FRET upon PLB phosphorylation [20]. Due to the steady-state nature of these FRET measurements, it is not clear whether changes in FRET were due to changes in SERCA-PLB association or to changes in the donor-acceptor distance within the complex. In the present study, this ambiguity was removed by the detection of time-resolved fluorescence, which simultaneously and independently resolves changes in binding and structure [25]. TR-FRET showed that 40-50% of PLB remains bound to SERCA after phosphorylation (Fig. 3C), even though phosphorylation completely relieves SERCA inhibition (Fig. 2). Therefore, the activity of the SERCA-pPLB complex is comparable to that of SERCA alone, and the relief of inhibition in the bound complex must be due to a structural change within the SERCA-pPLB complex.

To assess the physiological significance of the two observed effects – dissociation and structural change, it is important to compare the composition of our reconstituted samples with those in cardiac SR. In the present study with a fluorescent donor on PLB, acceptor-labeled SERCA was in 5-fold excess over PLB, to maximize the precision of FRET measurements. However, in cardiac SR, as in our functional measurements (Fig. 2), PLB is in large excess (3-5 fold) over SERCA [39]. Based on the affinity of SERCA for pPLB indicated from Fig. 3, and on the known concentrations of SERCA and PLB in cardiac SR [39,40], $76 \pm 2\%$ of SERCA in cardiac SR contains bound pPLB, even after complete phosphorylation of PLB (Table S2). This is consistent with EPR and NMR findings that pPLB remains bound to SERCA [18,23], with allosteric relief of inhibition achieved by shifting the *T/R* equilibrium toward the *R* state [23,24]. The present study clearly supports this “subunit” model for SERCA regulation (Fig. 4, left) over the dissociation model.

Although both dissociation and subunit models have been proposed to explain the mechanism of relief of inhibition by micromolar Ca^{2+} [12,13,16,17,19], our TR-FRET data showed no Ca -dependent changes in either binding or structure (Fig. 3B, C, D), clearly ruling out the subunit model, but also showing clearly that Ca^{2+} relieves SERCA-PLB inhibition by a different mechanism from that of phosphorylation. This result is consistent with previous steady-state FRET studies showing little or no dissociation of PLB from SERCA due to Ca^{2+} [17,19]. Ca^{2+} is known to induce structural changes in its activation of

SERCA [41,42], but these changes apparently do not perturb the distance between our labeling sites in the cytoplasmic domains of SERCA and PLB. This conclusion is consistent with structural models, since the distance between SERCA C674 (our acceptor labeling site) and K400 (proposed to lie in the binding groove of the PLB cytoplasmic domain, where our donor is located), changes only from 3.48 nm in the Ca-free crystal structure to 3.41 nm in the Ca-bound structure [37,43]. Since SERCA inhibition by PLB is caused by interaction of the transmembrane domains of the two proteins [44], we propose that Ca-induced structural changes in the SERCA transmembrane domain disrupt this inhibitory interaction, without changing interactions of the cytoplasmic domains, and without producing a substantial decrease in SERCA-PLB affinity (Fig. 4, right). This rearrangement of transmembrane helices would explain the Ca-induced decrease in crosslinking between the transmembrane domains of SERCA and PLB [12,13,16].

In summary, we conclude that phosphorylation of PLB and Ca^{2+} relieve SERCA inhibition through different mechanisms (Fig. 4), neither of which relies primarily on dissociation of the SERCA-PLB complex. PLB phosphorylation acts by promoting the dynamic disorder of the PLB cytoplasmic domain, while Ca^{2+} acts by changing the structure of the transmembrane domain of SERCA. Both mechanisms must disrupt the inhibitory interactions between the two transmembrane domains, by distinct mechanisms that remain to be elucidated (Fig. 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Razvan Cornea and Elizabeth Lockamy for helpful discussions, Zachary James for help with mutagenesis and PLB expression, Ji Li and Kurt C. Peterson for technical support. Work was supported by grants NIH GM27906 and NIH AR0507220 to DDT..

Abbreviations

IAF	5-iodoacetamidofluorescein
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
EGTA	ethylene glycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ESI-MS	electrospray ionization mass spectroscopy
FRET	fluorescence resonance energy transfer
TR-FRET	time-resolved FRET
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
pCa	$-\log_{10} [\text{Ca}^{2+}]$
pK_{Ca}, $-\log(K_{\text{Ca}})$	calcium concentration at half-maximal ATPase activity

PLB	phospholamban
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} -ATPase
SR	sarcoplasmic reticulum
OG	octyl β -D-glucopyranoside
PKA	protein kinase A

References

- [1]. Gwathmey JK, Copelas L, MacKinnon R, Schoen FJ, Feldman MD, Grossman W, Morgan JP. Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. *Circ Res*. 1987; 61:70–76. [PubMed: 3608112]
- [2]. Bers DM, Despa S. Cardiac myocytes Ca^{2+} and Na^{+} regulation in normal and failing hearts. *J Pharmacol Sci*. 2006; 100:315–322. [PubMed: 16552170]
- [3]. Inesi G, Tadini-Buoninsegni F. $\text{Ca}^{2+}/\text{H}^{+}$ exchange, lumenal Ca^{2+} release and $\text{Ca}^{2+}/\text{ATP}$ coupling ratios in the sarcoplasmic reticulum ATPase. *J Cell Commun Signal*. 2013
- [4]. Cantilina T, Sagara Y, Inesi G, Jones LR. Comparative studies of cardiac and skeletal sarcoplasmic reticulum ATPases. Effect of a phospholamban antibody on enzyme activation by Ca^{2+} . *J Biol Chem*. 1993; 268:17018–17025. [PubMed: 8349590]
- [5]. Mattiazzi A, Mundina-Weilenmann C, Guoxiang C, Vittone L, Kranias E. Role of phospholamban phosphorylation on Thr17 in cardiac physiological and pathological conditions. *Cardiovasc Res*. 2005; 68:366–375. [PubMed: 16226237]
- [6]. Gwathmey JK, Yerevanian A, Hajjar RJ. Targeting sarcoplasmic reticulum calcium ATPase by gene therapy. *Hum Gene Ther*. 2013; 24:937–947. [PubMed: 24164241]
- [7]. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J Jr, Kranias EG, Giles WR, Chien KR. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell*. 1999; 99:313–322. [PubMed: 10555147]
- [8]. Hoshijima M, Ikeda Y, Iwanaga Y, Minamisawa S, Date MO, Gu Y, Iwatate M, Li M, Wang L, Wilson JM, Wang Y, Ross J Jr, Chien KR. Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med*. 2002; 8:864–871. [PubMed: 12134142]
- [9]. Iwanaga Y, Hoshijima M, Gu Y, Iwatate M, Dieterle T, Ikeda Y, Date MO, Chrast J, Matsuzaki M, Peterson KL, Chien KR, Ross J Jr. Chronic phospholamban inhibition prevents progressive cardiac dysfunction and pathological remodeling after infarction in rats. *J Clin Invest*. 2004; 113:727–736. [PubMed: 14991071]
- [10]. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Ditttrich H, Hajjar RJ. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): A Phase 2 Trial of Intracoronary Gene Therapy of Sarcoplasmic Reticulum Ca^{2+} -ATPase in Patients With Advanced Heart Failure. *Circulation*. 2011
- [11]. James P, Inui M, Tada M, Chiesi M, Carafoli E. Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. *Nature*. 1989; 342:90–92. [PubMed: 2530454]
- [12]. Chen Z, Akin BL, Jones LR. Ca^{2+} binding to site I of the cardiac Ca^{2+} pump is sufficient to dissociate phospholamban. *J Biol Chem*. 2010; 285:3253–3260. [PubMed: 19948724]
- [13]. Chen Z, Stokes DL, Rice WJ, Jones LR. Spatial and dynamic interactions between phospholamban and the canine cardiac Ca^{2+} pump revealed with use of heterobifunctional cross-linking agents. *J Biol Chem*. 2003; 278:48348–48356. [PubMed: 12972413]
- [14]. Morita T, Hussain D, Asahi M, Tsuda T, Kurzydowski K, Toyoshima C, MacLennan DH. Interaction sites among phospholamban, sarcolipin, and the sarco(endo)plasmic reticulum Ca^{2+} -ATPase. *Biochem Biophys Res Commun*. 2008; 369:188–194. [PubMed: 18053795]

- [15]. Asahi M, McKenna E, Kurzydowski K, Tada M, MacLennan DH. Physical interactions between phospholamban and sarco(endo)plasmic reticulum Ca^{2+} -ATPases are dissociated by elevated Ca^{2+} , but not by phospholamban phosphorylation, vanadate, or thapsigargin, and are enhanced by ATP. *J Biol Chem*. 2000; 275:15034–15038. [PubMed: 10809745]
- [16]. Bal NC, Maurya SK, Sopariwala DH, Sahoo SK, Gupta SC, Shaikh SA, Pant M, Rowland LA, Bombardier E, Goonasekera SA, Tupling AR, Molkentin JD, Periasamy M. Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat Med*. 2012; 18:1575–1579. [PubMed: 22961106]
- [17]. Mueller B, Karim CB, Negrashov IV, Kutchai H, Thomas DD. Direct detection of phospholamban and sarcoplasmic reticulum Ca -ATPase interaction in membranes using fluorescence resonance energy transfer. *Biochemistry*. 2004; 43:8754–8765. [PubMed: 15236584]
- [18]. James ZM, McCaffrey JE, Torgersen KD, Karim CB, Thomas DD. Protein-protein interactions in calcium transport regulation probed by saturation transfer electron paramagnetic resonance. *Biophys J*. 2012; 103:1370–1378. [PubMed: 22995510]
- [19]. Bidwell P, Blackwell DJ, Hou Z, Zima AV, Robia SL. Phospholamban binds with differential affinity to calcium pump conformers. *J Biol Chem*. 2011; 286:35044–35050. [PubMed: 21832088]
- [20]. Gruber SJ, Haydon S, Thomas DD. Phospholamban mutants compete with wild type for SERCA binding in living cells. *Biochem Biophys Res Commun*. 2012 epublished.
- [21]. Karim CB, Kirby TL, Zhang Z, Nesmelov Y, Thomas DD. Phospholamban structural dynamics in lipid bilayers probed by a spin label rigidly coupled to the peptide backbone. *Proc Natl Acad Sci U S A*. 2004; 101:14437–14442. [PubMed: 15448204]
- [22]. Nesmelov YE, Karim CB, Song L, Fajer PG, Thomas DD. Rotational dynamics of phospholamban determined by multifrequency electron paramagnetic resonance. *Biophys J*. 2007; 93:2805–2812. [PubMed: 17573437]
- [23]. Gustavsson M, Verardi R, Mullen DG, Mote KR, Traaseth NJ, Gopinath T, Veglia G. Allosteric regulation of SERCA by phosphorylation-mediated conformational shift of phospholamban. *Proc Natl Acad Sci U S A*. 2013; 110:17338–17343. [PubMed: 24101520]
- [24]. Karim CB, Zhang Z, Howard EC, Torgersen KD, Thomas DD. Phosphorylation-dependent conformational switch in spin-labeled phospholamban bound to SERCA. *J Mol Biol*. 2006; 358:1032–1040. [PubMed: 16574147]
- [25]. Li J, James ZM, Dong X, Karim CB, Thomas DD. Structural and Functional Dynamics of an Integral Membrane Protein Complex Modulated by Lipid Headgroup Charge. *J Mol Biol*. 2012
- [26]. Fernandez JL, Roseblatt M, Hidalgo C. Highly purified sarcoplasmic reticulum vesicles are devoid of Ca^{2+} -independent ('basal') ATPase activity. *Biochim Biophys Acta*. 1980; 599:552–568. [PubMed: 6105877]
- [27]. Stokes DL, Green NM. Three-dimensional crystals of CaATPase from sarcoplasmic reticulum. Symmetry and molecular packing. *Biophys J*. 1990; 57:1–14.
- [28]. Bishop JE, Squier TC, Bigelow DJ, Inesi G. (Iodoacetamido)fluorescein labels a pair of proximal cysteines on the Ca^{2+} -ATPase of sarcoplasmic reticulum. *Biochemistry*. 1988; 27:5233–5240. [PubMed: 2971396]
- [29]. Cornea RL, Jones LR, Autry JM, Thomas DD. Mutation and phosphorylation change the oligomeric structure of phospholamban in lipid bilayers. *Biochemistry*. 1997; 36:2960–2967. [PubMed: 9062126]
- [30]. Mascioni A, Karim C, Zamoan J, Thomas DD, Veglia G. Solid-state NMR and rigid body molecular dynamics to determine domain orientations of monomeric phospholamban. *J Am Chem Soc*. 2002; 124:9392–9393. [PubMed: 12167032]
- [31]. Zamoan J, Nitu F, Karim C, Thomas DD, Veglia G. Mapping the interaction surface of a membrane protein: Unveiling the conformational switch of phospholamban in calcium pump regulation. *Proc Natl Acad Sci U S A*. 2005; 102:4747–4752. [PubMed: 15781867]
- [32]. Buck B, Zamoan J, Kirby TL, DeSilva TM, Karim C, Thomas D, Veglia G. Overexpression, purification, and characterization of recombinant Ca -ATPase regulators for high-resolution

- solution and solid-state NMR studies. *Protein Expr Purif*. 2003; 30:253–261. [PubMed: 12880775]
- [33]. Reddy LG, Cornea RL, Winters DL, McKenna E, Thomas DD. Defining the molecular components of calcium transport regulation in a reconstituted membrane system. *Biochemistry*. 2003; 42:4585–4592. [PubMed: 12693956]
- [34]. Lockamy EL, Cornea RL, Karim CB, Thomas DD. Functional and physical competition between phospholamban and its mutants provides insight into the molecular mechanism of gene therapy for heart failure. *Biochem Biophys Res Commun*. 2011; 408:388–392. [PubMed: 21510919]
- [35]. Lakowicz, JR. *Principles of Fluorescence Spectroscopy*. 3rd ed.. Springer Science + Business Media, LLC; New York: 2006.
- [36]. Toyoshima C, Asahi M, Sugita Y, Khanna R, Tsuda T, MacLennan DH. Modeling of the inhibitory interaction of phospholamban with the Ca²⁺ ATPase. *Proc Natl Acad Sci U S A*. 2003; 100:467–472. [PubMed: 12525698]
- [37]. Olesen C, Picard M, Winther AM, Gyrupe C, Morth JP, Oxvig C, Moller JV, Nissen P. The structural basis of calcium transport by the calcium pump. *Nature*. 2007; 450:1036–1042. [PubMed: 18075584]
- [38]. Hou Z, Kelly EM, Robia SL. Phosphomimetic mutations increase phospholamban oligomerization and alter the structure of its regulatory complex. *J Biol Chem*. 2008; 283:28996–29003. [PubMed: 18708665]
- [39]. Ferrington DA, Yao Q, Squier TC, Bigelow DJ. Comparable levels of Ca-ATPase inhibition by phospholamban in slow-twitch skeletal and cardiac sarcoplasmic reticulum. *Biochemistry*. 2002; 41:13289–13296. [PubMed: 12403631]
- [40]. Gross RW. Identification of plasmalogen as the major phospholipid constituent of cardiac sarcoplasmic reticulum. *Biochemistry*. 1985; 24:1662–1668. [PubMed: 3159423]
- [41]. Espinoza-Fonseca LM, Thomas DD. Atomic-level characterization of the activation mechanism of SERCA by calcium. *PLoS ONE*. 2011; 6:e26936. [PubMed: 22046418]
- [42]. Hou Z, Hu S, Blackwell DJ, Miller TD, Thomas DD, Robia SL. Two-color calcium pump reveals closure of the cytoplasmic headpiece with calcium binding. *PLoS ONE*. 2012 accepted.
- [43]. Jensen AM, Sorensen TL, Olesen C, Moller JV, Nissen P. Modulatory and catalytic modes of ATP binding by the calcium pump. *Embo J*. 2006; 25:2305–2314. [PubMed: 16710301]
- [44]. Karim CB, Marquardt CG, Stamm JD, Barany G, Thomas DD. Synthetic null-cysteine phospholamban analogue and the corresponding transmembrane domain inhibit the Ca-ATPase. *Biochemistry*. 2000; 39:10892–10897. [PubMed: 10978176]
- [45]. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996; 14:33–38. 27–38. [PubMed: 8744570]

HIGHLIGHTS

- What is the mechanism by which PLB inhibition of SERCA is relieved in the heart?
- Time-resolved FRET was measured between PLB and SERCA cytoplasmic domains.
- Binding and structure were simultaneously resolved.
- Both PLB phosphorylation and Ca^{2+} relieve SERCA inhibition without dissociation.
- PLB and Ca^{2+} induce distinct structural changes in the SERCA-PLB complex.

Fig. 1.

TR-FRET experimental design. **A** Models of SERCA-PLB complex. SERCA (silver)-bound PLB (blue) is proposed to exist in an equilibrium between *T* and *R* states, as shown previously by EPR [24]. There is no high-resolution structure of the SERCA-PLB complex, but a model for the *R* state (right) is from [36], and the *T* state model (left) is based on the NMR structure of PLB. The labeling sites on SERCA (C674) and PLB (Y6C) are shown as green and magenta spheres, respectively. The interprobe distances in *T* and *R* states are denoted as r_T and r_R , respectively. Image created in VMD [45]. **B** Representative fluorescence waveforms. D, donor only. D+A, donor plus acceptor. Grey indicates instrument response function. **C** Interprobe distance distributions resolved from fluorescence waveforms in **B**. Two Gaussian distance distributions were resolved. The shorter and longer distances correspond to *R* and *T* states, respectively [25].

Fig. 2. Functional effect of PLB phosphorylation (as measured by ΔpK_{Ca}) is not affected by FRET labeling of SERCA and PLB. The molar ratio of Lipid:PLB:SERCA is 700:5:1. $n = 3$.

Fig. 3.

TR-FRET shows that phosphorylation of PLB partially dissociates the SERCA-PLB complex and shifts the population to the shorter distance distribution. **A:** Two Gaussian distance distributions (two structural states of SERCA-PLB, *R* and *T*) were needed to fit the TR-FRET data. Red: PLB. Blue: pPLB. **B:** Centers (*r*) and widths (FWHM) of distance distributions. **C:** Compared to PLB, phosphorylated PLB decreased the mol fraction of SERCA-bound PLB (x_{DA}), but did not abolish it. **D:** The mol fraction of the short distance component (x_R) increased after phosphorylation. SERCA: PLB = 5. Lipid : SERCA = 200. n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4.

Two mechanisms to relieve SERCA inhibition by PLB in cardiac SR. Phosphorylation shifts the PLB cytoplasmic domain toward the dynamically disordered *R* state. Micromolar Ca^{2+} induces a structural change in the SERCA transmembrane domain.