Published in final edited form as:

Biochem Biophys Res Commun. 2012 April 6; 420(2): 236–240. doi:10.1016/j.bbrc.2012.02.125.

Phospholamban Mutants Compete with Wild Type for SERCA Binding in Living Cells

Simon J. Gruber, Suzanne Haydon, and David D. Thomas*

Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Abstract

We have used fluorescent fusion proteins stably expressed in HEK cells to detect directly the interaction between the sarcoplasmic reticulum Ca-ATPase (SERCA) and phospholamban (PLB) in living cells, in order to design PLB mutants for gene therapy. Ca^{2+} cycling in muscle cells depends strongly on SERCA. Heart failure (HF), which contributes to 12% of US deaths, typically exhibits decreased SERCA activity, and several potential therapies for HF aim to increase SERCA activity. We are investigating the use of LOF-PLB mutants (PLB_M) as gene therapy vectors to increase SERCA activity. Active SERCA1a and WT-PLB, tagged at their N termini with fluorescent proteins (CFP and YFP), were coexpressed in stable HEK cell lines, and fluorescence resonance energy transfer (FRET) was used to detect their interaction directly. Phosphorylation of PLB, induced by forskolin, caused an increase in FRET from CFP-SERCA to YFP-PLB, indicating that SERCA inhibition can be relieved without dissociation of the complex. This suggests that a LOF mutant might bind to SERCA with sufficient affinity to compete effectively with WT-PLB, thus relieving SERCA inhibition. Therefore, we transiently expressed a series of PLB_M in the CFP-SERCA/YFP-PLB cell line, and found decreased FRET, implying competition between PLB_M and WT-PLB for binding to SERCA. These results establish this FRET assay as a rapid and quantitative means of screening PLB_M for optimization of gene therapy to activate SERCA, as needed for gene therapy in HF.

Keywords

SERCA; PLB; HEK; FRET; Competition

1. Introduction

The sarcoplasmic reticulum (SR) Ca-ATPase (SERCA) is an integral membrane protein that pumps Ca^{2+} from the cytosol into the SR lumen in muscle cells, thus maintaining low cytosolic $[\text{Ca}^{2+}]$ in resting myocytes and potentiating contraction. In cardiac muscle, SERCA is inhibited at submicromolar $[\text{Ca}^{2+}]$ by the single-pass transmembrane protein phospholamban (PLB), which can be phosphorylated to relieve SERCA inhibition [1]. Because SERCA activity or expression is reduced in many instances of heart failure (HF) [2, 3], SERCA activation is a widely pursued goal for development of new therapies [3]. A gene

© 2012 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: ddt@umn.edu, 312 Church St. SE, 5-290 NHH, Minneapolis, MN 55455, Phone: 612-625-0957; Fax: 612-624-5121.

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.

therapy approach using rAAV to overexpress SERCA2a in heart tissue recently concluded phase II clinical trials with promising results [4], and small-molecule SERCA activators are also being sought [5]. PLB-based approaches involve overexpression of a pseudophosphorylated PLB (S16E) [6] or a protein phosphatase I inhibitor to increase the phosphorylation of PLB [7]. Here we explore expression of loss-of-function PLB mutants (PLB_M) to displace WT-PLB and activate SERCA.

Clear understanding of the mechanism by which PLB inhibits SERCA (Fig. 1A) is critical to designing an effective therapy. In the conventional model, supported by crosslinking and immunoprecipitation [8, 9], SERCA inhibition can only be relieved by dissociation of PLB (“Dissociation Model,” Fig. 1A left), but recent spectroscopic studies suggest that PLB remains bound to SERCA even after activation by PLB phosphorylation or Ca²⁺ (“Subunit Model”, Fig. 1A right) [10–13]. If this model is valid, an alternative therapeutic approach is suggested – a loss-of-function PLB mutant (PLB_M), introduced by gene therapy, could relieve SERCA inhibition if it binds tightly to SERCA, thus competing with endogenous PLB (Fig. 1B). Evidence favoring this hypothesis in a reconstituted membrane system was recently published [14]. In the present study we have used fluorescent fusion proteins to detect directly the SERCA-PLB interaction in living cells. We used fluorescence microscopy to determine whether phosphorylation of YFP-PLB dissociates it from CFP-SERCA (Fig. 1A), and to measure the ability of several PLB_M to compete with YFP-PLB for CFP-SERCA binding (Fig. 1B).

2. Materials and Methods

Generation of Stable Cell Lines Expressing Fluorescent Fusion Proteins

ECFP and EYFP mammalian vectors (Clontech), containing the monomeric A206K mutation, were fused to the N-terminus of rabbit SERCA1a and canine PLB respectively. HEK293 cells (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) without phenol red (Gibco/Invitrogen), supplemented with 10% fetal bovine serum (Atlanta Biologicals), at 37° C and 10% CO₂. Cells were transiently transfected using Lipofectamine (Invitrogen), and stable cell lines were generated by G418 (Sigma) selection. Surviving clones expressing CFP-SERCA and YFP-PLB were further selected by fluorescence expression seen via fluorescence microscopy. The goal was to obtain measurable CFP fluorescence with a substantial excess of YFP over CFP.

Transient Expression of non-fluorescent PLB_M for Competition Measurements

Mutations in WT-PLB cDNA (resulting in PLB_M in plasmid pRH132) were made using the QuickChange XLII mutagenesis kit (Agilent). PLB_M were expressed in stable CFP-SERCA/YFP-PLB cell lines using 293fectin (Invitrogen). The amount of PLB_M DNA used, volume of 293fectin, and time after transfection to maximize competition were all optimized so that PLB_M expression was within 20% of that observed for the stable expression of YFP-PLB. Final conditions in a six-well plate required 1.25 pmol DNA with 9 μL 293fectin, and photobleaching measurements were acquired 48h after transfection.

Western Blots to Quantify PLB and SERCA Content

Cells were pelleted and homogenized 48h after transient transfection with PLB_M DNA or after 5 min. incubation in 40 μM forskolin [15]. Cell homogenates were run on 4–20% Tris-HCl gels (Criterion, Biorad) at 5 μg total homogenate protein along with standard curves of PLB (synthetic, WT or phosphorylated) and rabbit light SR ([16]), transferred to Immobilon-FL membranes (Millipore), and blocked for 1 hour in 1× TBS/casein (Bio-rad). Primary antibodies for unphosphorylated PLB (Ab2D12, Abcam), PLB phosphorylated at S16 (Ab285, Merck), SERCA1 (IIH11, Abcam), or GFP variants (1GFP63, Abcam) were

visualized using IR secondary antibodies (goat-anti-mouse or goat-anti-rabbit) from LI-COR Biosciences. Blots were scanned on the Odyssey (LI-COR Biosciences). Concentrations of PLB (phosphorylated and unphosphorylated forms) and PLB_M were determined with high accuracy using synthetic standards run on the same blots as the cell homogenates [17, 18]. The slopes of standard curves were calculated from summed monomer and pentamer band intensities of three load concentrations for each standard (0.2, 0.4, and 0.8 pmoles), using LI-COR Odyssey software and median, one-pixel background subtraction. Band intensities for both monomer and pentamer of YFP-PLB in cell homogenates were summed, and concentrations of both YFP-uPLB (C_U) and YFP-pPLB (C_P) were calculated by solving simultaneous equations, using the standard slopes [17, 18]. X_P (the fraction of phosphorylated YFP-PLB) was then calculated by $X_P = C_P / (C_U + C_P)$ [17, 18]. A GFP antibody was used to label duplicate blots of the cell homogenates and indicated that total [YFP-PLB] was unaffected by incubation with forskolin. Blots with the SERCA antibody showed that all samples used in phosphorylation experiments had essentially the same CFP-SERCA expression levels (36 ± 5 nmol SERCA/g total protein). The molar ratio of YFP-PLB to SERCA was found to be 7.8 ± 0.9 , and the transient expression of PLB_M was consistently observed to be similar (7.1 ± 1.2).

Fluorescence Resonance Energy Transfer (FRET) Measurements in Live HEK Cells

Acceptor-selective photobleaching of cells co-expressing CFP-SERCA and YFP-PLB was done essentially as described previously [19] on an Eclipse TE200 microscope (Nikon Instruments), using CFP (excitation 430nm/24, emission 470nm/24) and YFP (excitation 500nm/20, emission 535nm/30) filters in automated filter wheels (Ludl) driven by MetaMorph software (Molecular Devices). Images were acquired using a 40× dry objective (0.55 numerical aperture), an X-Cite metal-halide lamp (EXFO), and a Cascade II CCD camera (Photometrics). A 10-ms exposure time was used in both channels with no neutral density filters, or a 100-ms exposure time was used with two neutral density filters, to reduce CFP photobleaching over the course of the experiment. Images at both emission wavelengths were then acquired at 20-second intervals before and after the start of 20-second exposures to high intensity light at YFP-specific excitation wavelengths. Photobleaching intervals were continued until YFP intensity was reduced to less than 5% of its starting value. The fractional decrease of fluorescence emission intensity of the donor (CFP-SERCA) caused by the presence of an acceptor (YFP-WT-PLB) is defined as the FRET efficiency $E = 1 - (F_{DA}/F_D)$, where F_{DA} and F_D are the fluorescence intensities in the presence and absence of acceptor, respectively. F_{DA} was measured as the CFP intensity prior to the start of the YFP photobleach. F_D was determined by the y-axis intercept of the linear fit to a data plot of YFP intensity (on x-axis) to CFP intensity (y-axis) over the course of the photobleach for each cell, to account for any CFP photobleaching and incomplete YFP photobleaching [19]. FRET values were calculated for all cells in each experimental condition and averaged together to determine FRET_{ave}. To eliminate any changes in FRET_{ave} due to differences in YFP-PLB expression levels, only cells with YFP intensities common to all experimental conditions were included in the FRET_{ave} calculation.

3. Results

3.1. PLB Phosphorylation

We expressed CFP-SERCA and YFP-PLB stably in HEK cells (at a molar ratio of 7.8 PLB/SERCA, to ensure saturation), then measured FRET using acceptor-selective photobleaching (Fig. 2). The control FRET value of the parent cell line was $15.4 \pm 1.2\%$, measured repeatedly and on different days over several weeks. This stable cell line was critical to evaluating perturbations due to PLB phosphorylation or competition with PLB_M. FRET was calculated for every cell and averaged together based on initial YFP intensity.

FRET between CFP-SERCA and YFP-PLB increased significantly when PLB phosphorylation was induced by incubating the cells in forskolin (fsk), which activates the PKA pathway at adenylyl cyclase (Fig. 3A). Immunoblots from the same cells, using antibodies specific for phosphorylated and unphosphorylated PLB, verify that phosphorylation of YFP-PLB increased substantially (from 51% to 87%) after forskolin treatment (Fig. 3B). Increased FRET indicates increased SERCA-PLB association or a shorter donor-acceptor distance within the SERCA-PLB complex. If the Dissociation Model of SERCA regulation were valid, we would expect a decrease in FRET (Fig. 1A, left). Thus this data clearly supports the Subunit Model (Fig. 1A, right), in which phosphorylation changes the structure of the SERCA-PLB complex but does not dissociate it.

FRET measurements were used to calculate the distance between CFP and YFP before and after induction of PLB phosphorylation. Since FRET is a linear combination of the values for unphosphorylated (U) and phosphorylated (P) PLB, $\text{FRET} = X_U \cdot \text{FRET}_U + X_P \cdot \text{FRET}_P$, where $X_P (= 1 - X_U)$ is the fraction of PLB that is phosphorylated. Solving two simultaneous equations (before and after fsk), we find that the FRET values for the bound SERCA-PLB complex in the absence and presence of phosphorylation are $\text{FRET}_U = 0.07 \pm .02$ and $\text{FRET}_P = 0.23 \pm 0.04$. Since YFP-PLB is in large excess over CFP-SERCA, and since phosphorylation actually increases FRET, we assume that all CFP donors are participating in FRET. Assuming a single interprobe distance R in each case, $R = (1/\text{FRET} - 1)^{-1/6} \cdot 4.92 \text{ nm}$, giving $R = 7.7 \pm 0.4 \text{ nm}$ (unphosphorylated) and $6.0 \pm 0.2 \text{ nm}$ (phosphorylated) [20]. Thus phosphorylation of PLB causes a change in the structure of the SERCA-PLB complex that decreases the CFP-YFP distance by $\Delta R = 1.7 \pm 0.6 \text{ nm}$ ($p < 0.01$, $n = 8$), consistent with the Subunit Model in Fig. 1A (right).

3.2. PLB_M Competition

In contrast to the increased FRET induced by PLB phosphorylation, FRET between CFP-SERCA and YFP-PLB decreased significantly when unlabeled PLB_M was added (Fig. 2, Fig. 4). FRET measurements, following transient transfection of unlabeled PLB_M into the stable CFP-SERCA/YFP-PLB HEK cell line, showed that all PLB_M studied here compete effectively with YFP-PLB for SERCA binding, and several reduced FRET by approximately half (Fig. 4). Expression levels of YFP-PLB and PLB_M were similar, so a 50% decrease in FRET indicates that PLB_M has a SERCA affinity similar to that of YFP-PLB. Further addition of YFP-PLB or a cytosolic protein (activation-induced deaminase, AID, used as a control) did not affect FRET (Fig. 4), indicating that CFP-SERCA is saturated with YFP-PLB at the start of each competition experiment. The data in Fig. 4 were obtained from PLB variants based on the human sequence. Essentially the same results were obtained when these same mutations (WT, P21G, and S16E) were studied on the background of the monomeric AFA-PLB mutant (not shown).

Several PLB_M compete effectively with YFP-PLB, with a potency (affinity) equal to or greater than that of unlabeled WT-PLB (Fig. 4). The pseudo-phosphorylated mutant S16E, which rescues HF phenotypes in animal models when introduced via rAAV [6], is a potent competitor, reducing CFP-YFP FRET by 45%. Two other LOF PLB_M, P21G and L31A, also showed competition; this is important, since these mutants, unlike S16E, can still be phosphorylated by PKA at S16 and are thus preferable as gene therapy candidates. I40A, a superinhibitory (GOF) mutant of PLB [21], showed similar competitive potency as WT and several LOF mutants (Fig. 4).

In the competition experiments, we assume that the effect of unlabeled PLB_M is to decrease the fraction of CFP-SERCA that is bound to YFP-PLB:

$$\text{FRET}' = X_B * \text{FRET},$$

where FRET and FRET' are values observed before and after transient expression of unlabeled PLB_M, and X_B is the mole fraction of CFP-SERCA that remains bound to YFP-PLB after PLB_M expression. Since the levels of YFP-PLB and PLB_M are very similar, this means that, to a good approximation,

$$K_A(\text{PLB}_M)/K_A(\text{PLB}) = \text{FRET}/\text{FRET}' - 1,$$

where K_A is the affinity (1/K_D) of the PLB variant for CFP-SERCA. Since most of the PLB_M tested, as well as WT-PLB, decreased FRET by about half (FRET/FRET' = 2) (Fig. 4), each has about the same SERCA affinity as YFP-PLB. The exception is L31A, which gave FRET/FRET' = 0.7, so its SERCA affinity is about half that of the others.

4. Discussion

We showed previously that the SERCA-PLB interaction can be measured by FRET in living cells transiently expressing fluorescent fusion proteins CFP-SERCA and YFP-PLB [22]. The present study establishes a stable cell line to make precise FRET measurements over time (Fig. 2), making it possible to compare the effects of numerous perturbations, such as PLB phosphorylation (Fig. 3) or the introduction of unlabeled PLB_M to compete for SERCA binding (Fig. 2, Fig. 4). We used this assay to resolve several controversies regarding SERCA/PLB regulation. We found that FRET between SERCA and PLB actually increases upon phosphorylation (Fig. 3), clearly contradicting the Dissociation Model in favor of the Subunit Model (Fig. 1A). Since PLB inhibition can be relieved by phosphorylation without decreasing SERCA affinity, a loss-of-function mutation might do the same (Fig. 1B). This hypothesis was confirmed by the competition experiments, in which all mutants tested showed competition with WT-PLB for SERCA binding (Fig. 2, Fig. 4).

This system offers the potential for probing the fundamental regulatory mechanism of the SERCA-PLB system in living cells [22, 23], but our main motivation is therapeutic development. This direct and rapid competition assay in HEK cells provides a platform for evaluating further PLB_M for gene therapy treatment of HF to activate SERCA (Fig. 1). The effectiveness of S16E, the pseudophosphorylated PLB_M, to compete significantly for SERCA binding in HEK cells (Fig. 4) helps explain its success as a gene therapy tool in animal models of HF [6]. Introduction of S16E-PLB into the human heart could cause chronic inotropic stimulation and is otherwise unregulated, so it is not optimal for gene therapy [24]. Better PLB_M must be developed, with intact phosphorylation sites.

L31A-PLB is a well-known LOF mutant [14], but it still competes with WT-PLB, albeit less effectively than S16E-PLB (Fig. 4), in agreement with recent FRET experiments on purified proteins [14]. The P21G mutant, designed from magnetic resonance-based structural analysis [25], provides encouraging evidence that a LOF PLB_M with an intact phosphorylation site can be just as effective as S16E (Fig. 4). Most of the PLB_M tested show similar SERCA affinity as WT-PLB, but none shows significantly tighter binding (Fig. 4). We are currently developing double mutants, combining LOF mutations with other mutations that increase SERCA affinity [25]. We envision an assortment of mutants to fine-tune SERCA activity to treat distinct HF syndromes. Our FRET competition assay in live cells provides a rapid and inexpensive method for screening potential therapeutic PLB_M mutants before they are tested in animals or humans. This FRET assay also has the potential

for high-throughput screening of small molecules as potential HF drugs to activate SERCA [14, 26].

Highlights

- PLB phosphorylation in HEK cells increased FRET between YFP-PLB and CFP-SERCA.
- Competition: Expressing loss-of-function PLB mutants in the system decreased FRET.
- The FRET assay could screen potential therapeutic PLB mutants to activate SERCA.

Abbreviations

CFP	cyan fluorescent protein
FRET	fluorescence resonance energy transfer
K_d	dissociation constant
LOF	loss of function
PKA	protein kinase A
PLB	phospholamban
PLB_M	PLB mutant
SDS	Sodium dodecyl-sulfate
SERCA	Sarco-endoplasmic reticulum Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
WT	wild-type
YFP	yellow fluorescent protein

Acknowledgments

We thank Razvan Cornea, Seth Robia, J. Michael Autry, John Rubin, and Gianluigi Veglia for helpful discussions, and Octavian Cornea for helping to prepare the manuscript for publication. Spectroscopy experiments were performed at the Biophysical Spectroscopy Center, University of Minnesota.

This work was supported by NIH grants to DDT (GM27906, AR057220). SH and SJG were supported by the Minnesota Muscle Training Grant (NIH AR007612), and SJG is currently supported by a predoctoral fellowship from the American Heart Association (Midwest Affiliate 11PRE5710019).

References

1. Simmerman HK, Collins JH, Theibert JL, Wegener AD, Jones LR. Sequence analysis of phospholamban. Identification of phosphorylation sites and two major structural domains. *J Biol Chem.* 1986; 261:13333–13341. [PubMed: 3759968]
2. MacLennan DH, Kranias EG. Phospholamban: a crucial regulator of cardiac contractility. *Nat Rev Mol Cell Biol.* 2003; 4:566–577. [PubMed: 12838339]
3. Frank KF, Bolck B, Brixius K, Kranias EG, Schwinger RH. Modulation of SERCA: implications for the failing human heart. *Basic Res Cardiol.* 2002; 97 Suppl 1:I72–I78. [PubMed: 12479238]
4. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich 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; 124:304–313. [PubMed: 21709064]

5. Hasenfuss G, Teerlink JR. Cardiac inotropes: current agents and future directions. *Eur Heart J*. 2011; 32:1838–1845. [PubMed: 21388993]
6. 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]
7. Nicolaou P, Hajjar RJ, Kranias EG. Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology. *J Mol Cell Cardiol*. 2009; 47:365–371. [PubMed: 19481088]
8. 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]
9. 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]
10. Negash S, Yao Q, Sun H, Li J, Bigelow DJ, Squier TC. Phospholamban remains associated with the Ca^{2+} - and Mg^{2+} -dependent ATPase following phosphorylation by cAMP-dependent protein kinase. *Biochem J*. 2000; 351:195–205. [PubMed: 10998362]
11. 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]
12. 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]
13. 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]
14. 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]
15. Maruyama K, MacLennan DH. Mutation of aspartic acid-351, lysine-352, and lysine-515 alters the Ca^{2+} transport activity of the Ca^{2+} -ATPase expressed in COS-1 cells. *Proc Natl Acad Sci U S A*. 1988; 85:3314–3318. [PubMed: 2966962]
16. Birmachu W, Nisswandt FL, Thomas DD. Conformational transitions in the calcium adenosinetriphosphatase studied by time-resolved fluorescence resonance energy transfer. *Biochemistry*. 1989; 28:3940–3947. [PubMed: 2526653]
17. Ablorh NA, Nitu F, Engebretsen K, Thomas DD, Holger JS. Insulin-dependent rescue from cardiogenic shock is not mediated by phospholamban phosphorylation. *Clin Toxicol (Phila)*. 2009; 1–7.
18. Ablorh N-A, Miller T, Nitu FR, Gruber SJ, Karim CB, Thomas DD. Accurate quantitation of phospholamban phosphorylation by immunoblot. *Anal Biochem*. 2012 accepted.
19. Autry JM, Rubin JE, Pietrini SD, Winters DL, Robia SL, Thomas DD. Oligomeric interactions of sarcolipin and the Ca-ATPase. *J Biol Chem*. 2011; 286:31697–31706. [PubMed: 21737843]
20. Kelly EM, Hou Z, Bossuyt J, Bers DM, Robia SL. Phospholamban oligomerization, quaternary structure, and sarco(endo)plasmic reticulum calcium ATPase binding measured by fluorescence resonance energy transfer in living cells. *J Biol Chem*. 2008; 283:12202–12211. [PubMed: 18287099]
21. Kimura Y, Asahi M, Kurzydowski K, Tada M, MacLennan DH. Phospholamban domain Ib mutations influence functional interactions with the Ca^{2+} -ATPase isoform of cardiac sarcoplasmic reticulum. *J Biol Chem*. 1998; 273:14238–14241. [PubMed: 9603928]
22. Robia SL, Campbell KS, Kelly EM, Hou Z, Winters DL, Thomas DD. Forster transfer recovery reveals that phospholamban exchanges slowly from pentamers but rapidly from the SERCA regulatory complex. *Circ Res*. 2007; 101:1123–1129. [PubMed: 17975108]

23. Winters DL, Autry JM, Svensson B, Thomas DD. Interdomain fluorescence resonance energy transfer in SERCA probed by cyan-fluorescent protein fused to the actuator domain. *Biochemistry*. 2008; 47:4246–4256. [PubMed: 18338856]
24. Crystal RG. Cardiac gene therapy: Pumping the heart. *Gene Therapy*. 2003; 10:2–3.
25. Ha KN, Traaseth NJ, Verardi R, Zamoon J, Cembran A, Karim CB, Thomas DD, Veglia G. Controlling the inhibition of the sarcoplasmic Ca^{2+} -ATPase by tuning phospholamban structural dynamics. *J Biol Chem*. 2007; 282:37205–37214. [PubMed: 17908690]
26. Muretta JM, Negrashov IV, Kyrychenko A, Ladokhin AS, Kast D, Gillispie GE, Thomas DD. High -performance time-resolved fluorescence by direct waveform recording. *Rev Sci Instrum*. 2010 in press.

Figure 1. SERCA inhibition relief scheme

A: Alternative mechanisms for relief of SERCA inhibition can be distinguished by FRET from CFP-SERCA to YFP-PLB. B: Proposed gene therapy approach, based on Subunit Model, is testable by FRET.

Figure 2. FRET from CFP-SERCA to YFP-PLB in live HEK cells

Normalized fluorescence was measured for YFP (yellow) or CFP (black or red). YFP acceptors were selectively photobleached, and FRET was calculated as shown. Black: control. Red: FRET is reduced after transient expression of PLB_M.

Figure 3. Effect of PLB phosphorylation on SERCA-PLB FRET in live cells

(A) FRET in live cells, before and after 5 min incubation in 40 μ M forskolin (fsk), increases from 0.15 ± 0.01 to 0.21 ± 0.02 (SEM, $n = 8$, $p < 0.01$). (B) Immunoblot of cell homogenates from (A) shows increased YFP-PLB phosphorylation, by increased Ab285 (red, specific for pPLB) and decreased Ab2D12 (green, specific for uPLB). Quantitative densitometry, using purified uPLB and pPLB standards [17], shows that phosphorylation increased from $51 \pm 4\%$ to $87 \pm 5\%$ in PLB monomer (shown) and pentamer bands.

Figure 4. FRET competition in live cells

Decreased FRET indicates that unlabeled PLB_M constructs compete effectively with YFP-PLB for binding to CFP-SERCA. $n \geq 3$ for each PLB_M, significant difference from control is indicated by ** ($p < 0.001$) and * ($p < 0.01$).