Biochemical and Biophysical Research Communications 420 (2012) 236–240

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Phospholamban mutants compete with wild type for SERCA binding in living cells

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ARTICLE INFO

*Article history:*
Received 17 February 2012
Available online 1 March 2012

*Keywords:*
SERCA
PLB
HEK
FRET
Competition

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 complete 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.



## 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 [$Ca^{2+}$] in resting myocytes and potentiating contraction. In cardiac muscle, SERCA is inhibited at submicromolar [$Ca^{2+}$] 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 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 $Ca^{2+}$ ("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

### 2.1. Generation of stable cell lines expressing fluorescent fusion proteins

ECFP and EYFP mammalian vectors (Clonetech), containing the monomeric A206K mutation, were fused to the N-terminus of

*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.

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doi:10.1016/j.bbrc.2012.02.125

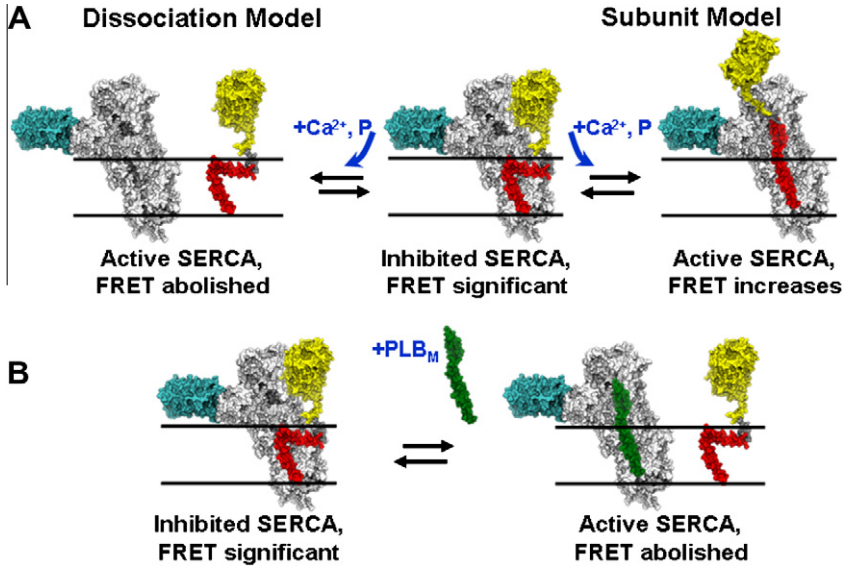


**Fig. 1.** SERCA inhibition relief scheme. (A) Alternative mechanisms for relief of SERCA inhibition can be distinguished by FRET from CFP-SERCA to YFP-PLB. (B) Proposed gene therapy approach, based on subunit model, is testable by FRET.

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% $CO_2$. 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.

### 2.2. 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 48 h after transfection.

### 2.3. Western blots to quantify PLB and SERCA content

Cells were pelleted and homogenized 48 h 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 h in 1x 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 pmol), 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).

### 2.4. 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 430 nm/24, emission 470 nm/24) and YFP (excitation 500 nm/20, emission 535 nm/30) filters in automated filter wheels (Ludl) driven by MetaMorph software (Molecular Devices). Images were acquired using a 40x 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-s intervals before and after the start of 20-s 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