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Functional and physical competition between phospholamban and its mutants provides insight into the molecular mechanism of gene therapy for heart failure

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ABSTRACT

We have used functional co-reconstitution of purified sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) with phospholamban (PLB), its inhibitor in the heart, to test the hypothesis that loss-of-function (LOF) PLB mutants (PLB_M) can compete with wild-type PLB (PLB_W) to relieve SERCA inhibition. Co-reconstitution at varying PLB-to-SERCA ratios was conducted using synthetic PLB_W, gain-of-function mutant I40A, or LOF mutants S16E (phosphorylation mimic) or L31A. Inhibitory potency was defined as the fractional increase in K_{Ca}, measured from the Ca^{2+}-dependence of ATPase activity. At saturating PLB, the inhibitory potency of I40A was about three times that of PLB_W, while the potency of each of the LOF PLB_M was about one third that of PLB_W. However, there was no significant variation in the apparent SERCA affinity for these four PLB variants. When SERCA was co-reconstituted with mixtures of PLB_W and LOF PLB_M, inhibitory potency was reduced relative to that of PLB_W alone. Furthermore, FRET between donor-labeled SERCA and acceptor-labeled PLB_W was decreased by both (unlabeled) LOF PLB_M. These results show that LOF PLB_M can compete both physically and functionally with PLB_W, provide a rational explanation for the partial success of S16E-based gene therapy in animal models of heart failure, and establish a powerful platform for designing and testing more effective PLB_M targeted for gene therapy of heart failure in humans.

1. Introduction

Muscle relaxation occurs when the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) hydrolyzes ATP and pumps Ca^{2+} from the sarcoplasm back into the SR against its concentration gradient. In the heart, SERCA activity is regulated by phospholamban (PLB), a single-span membrane protein that functions to inhibit SERCA by decreasing its apparent Ca^{2+}-affinity (increasing K_{Ca}) [1]. PLB inhibition of SERCA is relieved physiologically either by micromolar Ca^{2+} or by phosphorylation of PLB at Ser 16 by PKA [2–4], and can also be relieved by a number of point mutations [5], including S16E (a cytoplasmic domain mutation that partially mimics phosphorylation [6]) and L31A (a transmembrane domain mutation [5]).

Insufficient SERCA activity is a hallmark of heart failure (HF), which is a leading cause of hospitalization and death in most parts of the world [7], and overexpression of SERCA, using recombinant AAV vectors, has been shown to relieve heart failure in clinical trials [8]. HF is associated with an increased ratio of PLB to SERCA [9], so the inhibitory interaction between SERCA and PLB has become an attractive therapeutic target [2]. Indeed, interfering with the SERCA–PLB interaction in HF animal models can result in improved cardiac function [10–14]. However, complete ablation of PLB can lead to premature death in humans [15], suggesting that a more subtle approach is needed.

Relief of PLB-dependent SERCA inhibition, whether by micromolar Ca^{2+}, PLB phosphorylation, or functional mutation in PLB, has long been thought to require dissociation of the SERCA–PLB complex (Fig. 1A), a hypothesis supported primarily by cross-linking studies [16,17]. However, this hypothesis is inconsistent with measurements of fluorescence resonance energy transfer (FRET) from SERCA to PLB that demonstrated no Ca^{2+}-dependence of SERCA–PLB affinity [18] and with intra-PLB FRET and electron paramagnetic resonance (EPR) studies that showed no SERCA–PLB dissociation by either Ca^{2+} or phosphorylation of PLB at S16 [19,20], suggesting that PLB remains bound upon SERCA activation

Abbreviations: Ca^{2+}, divalent calcium ion; Dabcyl-SE, 4-((4-(dimethylamino) phenyl)azo)-benzoic acid succinimidyl ester; Fmoc, 9-fluorenylmethyloxycarbonyl; FRET, fluorescence resonance energy transfer; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid; K_d, dissociation constant; LOF, loss of function; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; NaOH, Sodium hydroxide, pCa, $-\log[Ca^{2+}]$; PKA, protein kinase A; PEG–PS, polyethylene glycol–polystyrene (graft support); pK_{Ca}, $-\log(K_{Ca})$, calcium concentration at half-maximal ATPase activity; PLB, phospholamban; SDS, sodium dodecyl-sulfate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; WT, wild-type.

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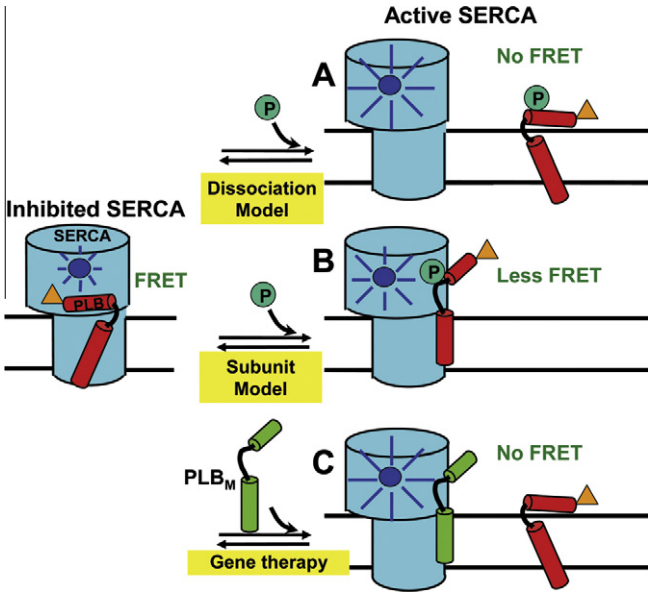

Fig. 1. Models for relief of SERCA inhibition. Left: PLB_W binding to SERCA is detected when the fluorescence of donor (blue) on SERCA is quenched by the acceptor (orange) on PLB via FRET. (A) In the Dissociation Model, loss of function (e.g., due to phosphorylation) requires dissociation of the SERCA–PLB complex, which would completely eliminate FRET. (B) In the Subunit Model, inhibition can be relieved by a structural rearrangement, without dissociation of the SERCA–PLB complex. (C) For HF gene therapy applications, if a LOF mutant PLB_M has affinity for SERCA comparable to that of PLB_W, it can compete with PLB_W to increase SERCA function. These hypotheses can be tested by FRET.

(Fig. 1B). In addition, several loss-of-function (LOF, with less inhibitory potency than WT) PLB mutants have been shown to retain at least some SERCA binding affinity [6,21,22]. These results suggest that it might be feasible to identify a LOF PLB mutant (denoted PLB_M below) with sufficient SERCA affinity to compete with WT (denoted PLB_W below) for SERCA binding, and that such a mutant would be a useful therapeutic reagent. Intriguingly, *in vivo* cardiac rAAV delivery of a gene corresponding to S16E, a pseudophosphorylated PLB mutant, prevents HF occurrence or progression in small and large animal models [12,14,23]. To understand the molecular basis of the therapeutic effectiveness of S16E, we previously studied its structural dynamics, showing that the S16E mutation does not abolish SERCA binding, but it partially unfolds the cytoplasmic domain of PLB (detected by EPR and NMR) [6,22], almost as much as is caused by phosphorylation at S16 [20,22,24]. We suggest that S16E can relieve SERCA inhibition by competing with PLB_W for SERCA binding.

In the present study, we test this hypothesis directly and quantitatively by performing both FRET and functional assays on reconstituted membranes containing donor-labeled SERCA, acceptor-labeled PLB_W, and unlabeled S16E. We ask whether this LOF mutant of PLB can compete with the native WT for SERCA binding, which should reduce both FRET and inhibition (Fig. 1C). We use a similar approach to evaluate the LOF PLB mutant L31A [5] as a phosphorylatable alternative to S16E. The results have important implications for future efforts in gene therapy.

2. Materials and methods

2.1. SERCA purification and labeling

SERCA was purified [25] and labeled with IAEDANS [18] as described previously. To determine the dye concentration in labeled samples, the absorbance (ε_{334nm} = 6100 M^{-1} cm^{-1}) [26], was measured after treatment with 0.1 M NaOH and 1% SDS. Samples of AEDANS-SERCA were flash-frozen and stored in the dark at −80 °C until further usage.

2.2. Synthesis, purification, and labeling of PLB mutants

PLB was assembled on Fmoc-Leu-PEG-PS resin by Fmoc chemistry using a PE Biosystems Pioneer™ peptide synthesis system, as previously reported [18]. The N-terminal amino group of unlabeled PLBs was acetylated using acetic anhydride. For FRET, PLB_W was labeled at the N-terminus with the non-fluorescent acceptor DabcylSE (denoted Dab-PLB_W). Peptide composition and concentration were confirmed by MALDI-TOF and amino acid analysis, and samples were stored in methanol at −20 °C.

2.3. Co-reconstitution of SERCA and PLB

SERCA and PLB were co-reconstituted, as previously described [27–29], at 700 lipids/SERCA. Each sample contained 40 μg of SERCA and varying amounts of PLB to obtain molar ratios of 0–20 PLB to SERCA. Ca^{2+}-ATPase activity and FRET measurements were performed immediately after co-reconstitution.

2.4. Ca^{2+}-ATPase functional measurements

Functional and FRET measurements were carried out at 25 °C. ATPase activity was measured using an NADH-detecting enzyme-linked assay, as a function of [Ca^{2+}], in 96-well microtiter plates [28,30]. The time-dependence of absorbance at 340 nm was measured in a SpectraMaxPlus™ microplate reader (Molecular Devices, Sunnyvale, CA). Data were fitted using the Hill function:

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

where V is the initial ATPase rate and n is the Hill coefficient. The inhibitory effect of each PLB variant was indicated by the observed increase in the apparent Ca^{2+} dissociation constant K_{Ca}, measured relative to SERCA reconstituted in the absence of PLB.

Based on K_{Ca} measured as above, we define inhibitory potency, P, as the % increase in K_{Ca} (decrease in apparent Ca^{2+} affinity) caused by PLB:

$$P(n) = [K_{Ca}(n)/K_{Ca}(0) - 1] * 100 \quad (2)$$

where n = PLB/SERCA.

To determine the apparent affinity of each PLB variant for SERCA, $P(n)$ data in Fig. 2B were fitted using the specific binding function:

$$P(n) = P_{max} * n/(n + K_d) \quad (3)$$

where K_d is the apparent dissociation constant.

We assume that the effects of a mixture of PLB_W with a LOF PLB_M (Fig. 3) on the SERCA K_{Ca} depends on the relative potencies and affinities of the PLB variants competing for a single inhibitory binding site on SERCA:

$$P(w + m) = \{[w * P(w) + A * m * P(m)]/(w + A * m)\} \quad (4)$$

where w = PLB_W/SERCA; m = PLB_M/SERCA; A = $K_d(PLB_W)/K_d(PLB_M)$.

2.5. Fluorescence resonance energy transfer (FRET) measurements

Fluorescence emission spectra were acquired using a Gemini EM microplate fluorimeter (Molecular Devices, Sunnyvale, CA) with excitation at 350 nm from a Xenon flash lamp (1 J/flash). Samples were plated in triplicate (75 μL per well) on 384-well, black wall, optical bottom well plates (Nalge Nunc International, Rochester, NY). Emission spectra were recorded in triplicate, from