Sarcolipin and phospholamban inhibit the calcium pump by populating a similar metal ion-free intermediate state

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ABSTRACT

We have performed microsecond molecular dynamics (MD) simulations and protein pK\(_A\) calculations of the muscle calcium pump (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, SERCA) in complex with sarcolipin (SLN) to determine the mechanism by which SLN inhibits SERCA. SLN and its close analog phospholamban (PLN) are membrane proteins that regulate SERCA by inhibiting Ca\(^{2+}\) transport in skeletal and cardiac muscle. Although SLN and PLB binding to SERCA have different functional outcomes on the coupling efficiency of SERCA, both proteins decrease the apparent Ca\(^{2+}\) affinity of the pump, suggesting that SLN and PLB inhibit SERCA by using a similar mechanism. Recently, MD simulations showed that PLB inhibits SERCA by populating a metal ion-free, partially-protonated E1 state of the pump, E1-H\(^{+}\)-SLN, an intermediate with Mg\(^{2+}\) bound near transport site I. To test this proposed mode of SLN regulation, we performed a 0.5-\(\mu\)s MD simulation of E1-Mg\(^{2+}\)-SLN in a solution containing 100 mM K\(^+\) and 3 mM Mg\(^{2+}\), with calculation of domain dynamics in the cytosolic headpiece and side-chain ionization and occupancy in the transport sites. We found that SLN increases the distance between residues E771 and D800, thereby rendering E1-Mg\(^{2+}\) incapable of producing a competent Ca\(^{2+}\) transport site I. Following removal of Mg\(^{2+}\), a 2-\(\mu\)s MD simulation of Mg\(^{2+}\)-free SERCA-SLN showed that Mg\(^{2+}\) does not re-bind to the transport sites, indicating that SERCA-SLN does not populate E1-Mg\(^{2+}\) at physiological conditions. Instead, protein pK\(_A\) calculations indicate that SLN stabilizes a metal ion-free SERCA state (E1-H\(^{+}\)-SLN) protonated at residue E771, but ionized at E309 and D800. We conclude that both SLN and PLB inhibit SERCA by populating a similar metal ion-free intermediate state. We propose that (i) this partially-protonated intermediate serves as the consensus mechanism for SERCA inhibition by other members of the SERCA regulatory subunit family including myoregulin and sarcolamban, and (ii) this consensus mechanism is utilized to regulate Ca\(^{2+}\) transport in skeletal and cardiac muscle, with important implications for therapeutic approaches to muscle dystrophy and heart failure.

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1. Introduction

The muscle calcium pump (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, SERCA) is an integral membrane protein responsible for the transport of Ca\(^{2+}\) from the cytosol into the sarcoplasmic reticulum (SR) lumen of cardiac and skeletal muscle cells [1]. SERCA uses the energy derived from hydrolysis of one ATP molecule and the counter-transported exchange of two protons [2,3]. Two membrane proteins are known to regulate SERCA activity: the 52-residue phospholamban (PLB) and the 31-residue sarcolipin (SLN). PLB is expressed in cardiac muscle and to a lesser extent in slow-twitch skeletal muscles, whereas SLN is predominantly expressed in skeletal muscles, but it is also expressed in atria [4,5]. Transient expression of SLN has also been found in the left ventricle in human Takotsubo cardiomyopathy [6].

SLN and PLB binding to SERCA have different functional outcomes. PLB controls Ca\(^{2+}\) affinity in a phosphorylation-dependent manner but has little or no effect on maximal velocity (V\(_{\text{max}}\)) [7], thus effectively regulating cardiac function during rest and exercise [8]; SLN also controls Ca\(^{2+}\) affinity of SERCA yet increases slippage of Ca\(^{2+}\) into the cytosol instead of the SR lumen [9], thus contributing to non-shivering thermogenesis in skeletal muscle [10]. Despite their distinct functional outcomes, SLN and PLB share important functional and structural features: (i) SLN and PLB both decrease
the apparent Ca\(^{2+}\) affinity of SERCA [11]; (ii) they have high sequence identity, including homologous loss-of-function mutations: SLN mutants L31A and N34A and the analogous PLB mutants L31A and N34A, which all disrupt SERCA inhibition [12]; (iii) SLN and PLB bind to the same groove on the transmembrane domain of SERCA [13–15]; and (iv) the crystal structures of SERCA with bound PLB or SLN are virtually identical (RMSD = 0.15–0.25 nm).

Super-physiological concentrations of Mg\(^{2+}\) have been used recently to obtain crystal structures of SLN-bound SERCA in an E1 conformation [13,14]. One crystal structure, obtained in the presence of 40 mM Mg\(^{2+}\), shows a SLN-bound E1 structure with a single Mg\(^{2+}\) ion in the transport sites; this state was designated E1 · Mg\(^{2+}\)-SLN [14]. It was proposed that SLN inhibits SERCA by stabilizing the E1 · Mg\(^{2+}\) state [14]. Molecular dynamics (MD) simulation studies showed that E1 · Mg\(^{2+}\) is not a functional intermediate of SERCA without bound subunit [16], and that Mg\(^{2+}\) ions do not bind to E1 under physiological conditions [16,17]. Based on this information, we hypothesize that SLN inhibits SERCA activity by populating a metal ion-free, protonated E1 state. To test this hypothesis, we performed protein pK\(_a\) calculations and microsecond-long MD simulations of SERCA-SLN starting from the crystal structure of E1 · Mg\(^{2+}\)-SLN. By comparing our simulations of SERCA-SLN with our recent simulations of SERCA-PLB and subunit-free SERCA, we have identified a common intermediate for SERCA regulation in cardiac and skeletal muscle. We provide the first report that identifies a consensus mechanism for SERCA inhibition by SLN and PLB in atomic-level detail, with important implications for structure-based therapeutic approaches to heart failure and muscle dystrophy.

2. Materials and methods

2.1. Protein pK\(_a\) calculations

We used PROPKA 3.1 [18,19] to calculate the pK\(_a\) values of acidic residues in the transport sites of SERCA. PROPKA estimates empirical pK\(_a\) values of ionizable groups in proteins and protein-ligand complexes based on the 3D structure, and benefits from explicitly incorporating the Coulombic interactions that arise from mutually titrating residues via the Tanford-Roxby procedure [20].

2.2. Setting up SERCA-SLN for simulation

We used the crystal structure of E1 · Mg\(^{2+}\)-SLN (PDB code: 3w5a [14]) as a starting structure for our simulations. We adjusted the charges on ionizable residues to correspond to their calculated values at pH ~7.0, as follows. For E1 · Mg\(^{2+}\)-SLN, residues E309, E771 and D800 were calculated to be ionized, and E908 was protonated. For Mg\(^{2+}\)-free SERCA-SLN, pK\(_a\) calculations predict residues E309 and D800 were ionized, and E771 and E908 were protonated. The complexes were inserted in a 12 × 12 nm bilayer of POPC and solvated using TIP3P water molecules. K\(^+\), Mg\(^{2+}\), and Cl\(^-\) ions were added to produce concentrations of 100 mM, 3 mM, and 110 mM, respectively. CHARMM36 field force topologies and parameters were used for the protein [21], lipid [22], water, K\(^+\), and Cl\(^-\). For Mg\(^{2+}\), we used a set of new CHARMM parameters [23].

2.3. Molecular dynamics simulations

MD simulations of SERCA-SLN were performed with NAMD 2.9 [24], periodic boundary conditions [25], particle mesh Ewald [26,27], a non-bonded cutoff of 0.9 nm, and a 2 fs time step. The NPT ensemble was maintained with a Langevin thermostat (310 K) and an anisotropic Langevin piston barostat (1 atm). We performed two simulations: a 0.5-μs trajectory of E1 · Mg\(^{2+}\)-SLN and a 2-μs trajectory of Mg\(^{2+}\)-unbound SERCA-SLN.

3. Results

3.1. Structural dynamics of E1 · Mg\(^{2+}\)-SLN

We performed a 0.5-μs MD simulation of E1 · Mg\(^{2+}\)-SLN to determine the structural dynamics of the crystallographic complex (Fig. 1A) in solution. We measured the backbone root-mean-square deviations (RMSD) for each functional domain of SERCA to determine the changes in SERCA (Fig. 1B). We found that the 10-helix transmembrane (TM) domain of SERCA rapidly settles to a plateau at 0.13 nm, indicating that its structure is nearly identical to that in the crystal structure of E1 · Mg\(^{2+}\)-SLN. Each domain in the cytosolic headpiece of E1 · Mg\(^{2+}\)-SLN undergoes relatively large-amplitude structural dynamics (RMSD = 0.3–0.8 nm) in this time scale (Fig. 1B). The dynamic nature of the cytosolic headpiece, and in particular of the A domain, is in line with previous studies showing that the headpiece is inherently flexible in the E1 state in the absence and presence of PLB [16,17]. We also evaluated the structure and mobility of SLN in the complex. We found that residues R3-Q30 of SLN exclusively populate an α-helical structure in the trajectory. Root-mean square fluctuations (RMSF) of the main chain C\(_\alpha\) atoms of SLN showed that, except for residues M1, E2, and Y31, SLN has very low mobility in the complex (RMSF < 0.15 nm, Fig. 1C).

3.2. Structure of transport site I in E1 Mg\(^{2+}\)-SLN

We measured intramolecular distances D800-E771 and E771-E908, and intermolecular distance N11\(_\text{SLN}\)·G801\(_\text{SERCA}\) to determine the crystal structure of the E1 · Mg\(^{2+}\)-SLN. SERCA is colored according to its four functional domains: N (green), P (blue), A (red), and TM (gray); SLN is shown in magenta. (B) Time-dependent changes in RMSD of SERCA domains. RMSD of the TM domain was calculated using backbone alignment for TM helices of the pump; RMSD of A, N, and P domains was calculated by aligning the backbone of the cytosolic headpiece. (C) C\(_\beta\) RMSF of SLN calculated from the MD trajectory. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the effects of SLN binding on the geometry of transport site I of E1-Mg\(^{2+}\). Analysis of time evolution of interresidue distances showed a 0.5 nm decrease in the distance N1\(^{11}\)-GLN-G801 of SERCA (Fig. 2A), thus facilitating the formation of a hydrogen bond betw...
Fig. 3. Geometric comparison of transport site I of E1-H$^+$ \textsuperscript{771}-SLN and E1-H$^+$ \textsuperscript{771}-PLB. (A) Distances were calculated between C\textsubscript{6}–C\textsubscript{6} and C\textsubscript{6}–C\textsubscript{6} atoms for E771-E908 and D800-E771, respectively. (B) For comparison, we plotted distance distributions for residue pairs D800-E771 and E771-908 of E1 to examine the structural arrangement of transport site I. We show that SLN and PLB produce an identical structural arrangement of transport site I.

Fig. 4. Transient K\textsuperscript+ ion interactions in the transport sites of E1-H$^+$ \textsuperscript{771}-SLN and E1-H$^+$ \textsuperscript{771}-PLB. (A) Location of the nine different positions occupied by K\textsuperscript+ (yellow spheres) in the transport sites of E1-H$^+$ \textsuperscript{771}. Each position is labeled from 1 to 9. The dashed circles show the approximate location of transport sites I and II. TM helices are represented by gray ribbons and cation-binding residues are shown as sticks. (B) Percent of time K\textsuperscript+ spends at each position. The values for E1-H$^+$ \textsuperscript{771}-PLB represent the average from three independent 1-\mu s simulations reported in Ref. [17]. (C) Superposition of E1-H$^+$ \textsuperscript{771}-SLN at t = 2 \mu s onto three structures of E1-H$^+$ \textsuperscript{771}-PLB at the end three independent 1-\mu s MD simulations. We show that SLN and PLB produce an identical structural arrangement of transport site I.

E1-H$^+$ \textsuperscript{771}-PLB. These results indicate that PLB and SLN binding to SERCA populate identical structurally stable but incompetent transport site I. Thus, SLN and PLB inhibit SERCA similarly by populating E1-H$^+$ \textsuperscript{771}.

4. Discussion

Protein pK\textsubscript{a} calculations and MD simulations showed that SLN induces structural changes in the transport sites that correspond to those previously identified as inhibitory. This local structural change occurs in the presence and absence of bound Mg\textsuperscript{2+}, indicating that the effect of SLN on SERCA is independent of the type of metal ion bound in the transport sites.

Protein pK\textsubscript{a} calculations and MD simulations showed that E1-Mg\textsuperscript{2+} is not the main intermediate stabilized by SLN. Instead, SLN binding to SERCA alters the geometry of transport site I and populates a protonated E1 intermediate, E1-H$^+$ \textsuperscript{771}. Comparison between our data and previous MD simulations of SERCA-PLB revealed that both SLN and PLB inhibit the SERCA by populating an identical intermediate, E1-H$^+$ \textsuperscript{771}. We recently showed that PLB-bound E1-H$^+$ \textsuperscript{771} serves as a kinetic trap that depresses but does not abolish SERCA activity at normal physiological conditions [17]. These findings are in line with experimental data showing that both SLN and PLB decrease the apparent Ca\textsuperscript{2+} affinity of SERCA [11,12].

It is possible that other newly-discovered SERCA regulators, sarcolamban (SCL) [29] and myoregulin (MLN) [30] control Ca\textsuperscript{2+} affinity by populating E1-H$^+$ \textsuperscript{771} because they share structural and functional similarity with PLB and SLN. Therefore, we propose that this mechanism for inhibition of Ca\textsuperscript{2+} transport applies to both cardiac and skeletal muscle. These findings have profound therapeutic implications because Ca\textsuperscript{2+} dysregulation is a hallmark of muscle and cardiovascular diseases. For example, disruption of the SERCA-PLB complex in cardiac muscle can be used to normalize Ca\textsuperscript{2+} cycling in diseased cardiomyocytes, thus mitigating Duchenne muscular dystrophy [35]. Identification of E1-H$^+$ \textsuperscript{771} as the inhibitory mechanism opens new doors for structure-based strategies to stimulate SERCA and Ca\textsuperscript{2+} transport in muscle and heart disease. This includes the discovery of small molecule activators of SERCA and gene therapy strategies to neutralize subunit inhibition.

Further studies will be needed to answer questions regarding the functional differences among these regulatory proteins. For example, if PLB and SLN induce the same structural changes in the transport sites (Fig. 3) and populate the same inhibitory intermediate, why is only SLN able to uncouple SERCA [36]? What are the mechanisms by which the luminal tail in SLN regulates SERCA [11]? What about other post-translational protein modiﬁcations such as SLN acylation [37]? What is the role of SLN self-oligomerization in SERCA regulation [38]? Complementary experiments and simulation studies on SERCA regulation will be needed to test these questions directly.

5. Conclusion

We have used protein pK\textsubscript{a} calculations and microsecond MD simulations to demonstrate that SLN-bound SERCA is protonated at residue E771 and does not bind Mg\textsuperscript{2+}. These findings indicate that SLN binding to SERCA does not populate E1-Mg\textsuperscript{2+} but an E1 intermediate protonated on E771, E1-H$^+$ \textsuperscript{771}. Comparison with our previous MD simulations of SERCA-PLB showed remarkable similarities in the effects of SLN and PLB binding to SERCA, including (i) control of transport site geometry, (ii) formation of E1-H$^+$ \textsuperscript{771}, and (iii) transient binding of K\textsuperscript+ but not Mg\textsuperscript{2+} in the transport sites. We propose that both SLN and PLB inhibit SERCA activity by populating an identical inhibited E1 state of the pump. This partially-protonated intermediate serves as the consensus mechanism for SERCA inhibition by other members of the SERCA regulatory subunit family. We conclude that this consensus mechanism is utilized to regulate Ca\textsuperscript{2+} transport in skeletal and cardiac muscle, with important implications for therapeutic approaches to muscular dystrophy and heart failure.
Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.012.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.05.012.

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