



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 Ca²⁺-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 Ca²⁺ 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 Ca²⁺ 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⁺₇₇₁. X-ray crystallography studies at 40–80 mM Mg²⁺ have proposed that SLN-bound SERCA populates E1·Mg²⁺, an intermediate with Mg²⁺ bound near transport site I. To test this proposed mode of SLN regulation, we performed a 0.5-μs MD simulation of E1·Mg²⁺-SLN in a solution containing 100 mM K⁺ and 3 mM Mg²⁺, 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·Mg²⁺ incapable of producing a competent Ca²⁺ transport site I. Following removal of Mg²⁺, a 2-μs MD simulation of Mg²⁺-free SERCA-SLN showed that Mg²⁺ does not re-bind to the transport sites, indicating that SERCA-SLN does not populate E1·Mg²⁺ at physiological conditions. Instead, protein pK_a calculations indicate that SLN stabilizes a metal ion-free SERCA state (E1·H⁺₇₇₁) 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 Ca²⁺ 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 Ca²⁺-ATPase, SERCA) is an integral membrane protein responsible for the transport of Ca²⁺ 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 Ca²⁺ affinity in a phosphorylation-dependent manner but has little or no effect on maximal velocity (*V*_{max}) [7], thus effectively regulating cardiac function during rest and exercise [8]. SLN also controls Ca²⁺ affinity of SERCA yet increases slippage of Ca²⁺ 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

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the apparent Ca^{2+} affinity of SERCA [11]; (ii) they have high sequence identity, including homologous loss-of-function mutations: SLN mutants L8A and N11A 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 force field 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 α 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^{SLN}–G801^{SERCA} to determine

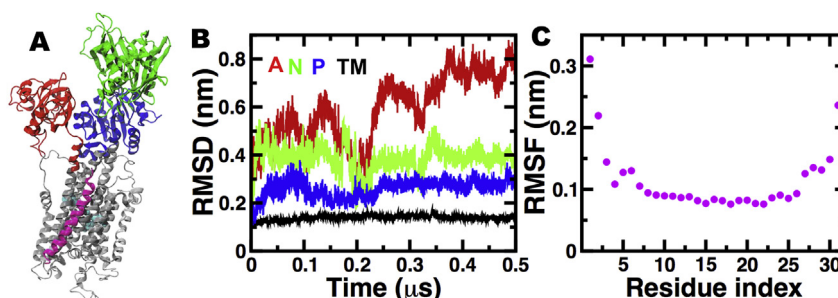


Fig. 1. Structural dynamics of E1· Mg^{2+} -SLN. (A) Crystal structure of the E1· Mg^{2+} -SLN. SERCA is colored according to its four functional domains: N (green), P (blue), A (red), and TM (gray); SLN is shown in magenta. (B) Time-dependent changes in RMSD of SERCA domains. RMSD of the TM domain was 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_α 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.)

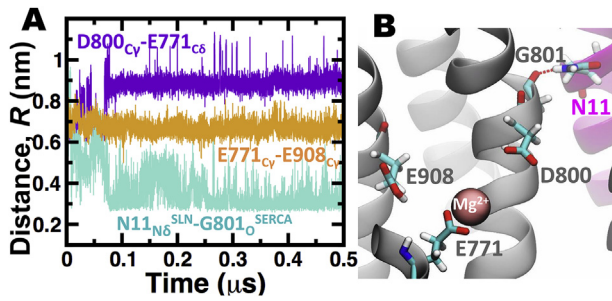


Fig. 2. Structure of transport site I in $E1 \cdot Mg^{2+}$ -SLN. (A) Distance evolution between residues D800-E771 and E771-E908 of SERCA, and G801 of SERCA and N11 of SLN. C_{γ} - C_{δ} , and C_{δ} - C_{δ} distances were calculated for D800-E771 and E771-E908, respectively. The distance $N11^{SLN}$ - $G801^{SERCA}$ was calculated between the backbone oxygen of G801 and the N_{δ} of N11. (B) Structure of transport site I at the end of the 0.5- μ s MD simulation. SERCA and SLN are shown as gray and magenta ribbons, residues in the transport sites as sticks, and Mg^{2+} as a sphere. (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 $E1 \cdot Mg^{2+}$. Analysis of time evolution of interresidue distances showed a 0.5 nm decrease in the distance $N11^{SLN}$ - $G801^{SERCA}$ (Fig. 2A), thus facilitating the formation of a hydrogen bond between the backbone oxygen of $G801^{SERCA}$ and the N_{δ} atom of $N11^{SLN}$ (Fig. 2B). The formation of this hydrogen bond occurs simultaneously with a 0.3-nm increase in the distance between E771 and D800, indicating that SLN pulls D800 away from site I even in the presence of bound Mg^{2+} (Fig. 2A and B). The distances D800-E771 and D800-E908 are ~ 0.3 nm longer than those expected for competent transport site geometries [16]. These results indicate that SLN disfavors the formation of a competent transport site I of SERCA.

3.3. Protein pK_a calculations of SERCA-SLN $\pm Mg^{2+}$

We recently found that the distorted transport site geometry shown in Fig. 2B promotes protonation of residue E771 for PLB-bound SERCA [17]. Therefore, we used PROPKA to perform empirical pK_a calculations of acidic residues in the transport sites of $E1 \cdot Mg^{2+}$ -SLN in the presence and absence of bound Mg^{2+} . The acidic residues analyzed here are E309, E771, D800, and E908. Predicted pK_a values of the crystal structure of Mg^{2+} -bound SERCA-SLN indicate that at resting pH of skeletal muscle (~ 7.0 [28]), only residue E908 is protonated (Table 1). Identical protonation patterns were observed in pK_a calculations performed on 6 independent 20-ns MD trajectories of Mg^{2+} -bound SERCA-SLN (Table 1); these trajectories were generated using the structure of $E1 \cdot Mg^{2+}$ -SLN at $t = 0.5 \mu$ s as a starting point.

We performed six 20-ns MD trajectories of SERCA-SLN in the absence of bound Mg^{2+} , starting from the structure of $E1 \cdot Mg^{2+}$ -SLN at $t = 0.5 \mu$ s. We found that upon Mg^{2+} removal, acidic residues

E908 and E771 become protonated at physiological pH (Table 1). This protonation pattern is identical to that of SERCA-PLB reported recently by our group [17], indicating that PLB binding induces E771 protonation in the absence of bound Mg^{2+} . Therefore, we performed an additional MD simulation of SERCA-SLN protonated on E771 and E908 in the absence of bound Mg^{2+} . This simulation was performed to evaluate the structural properties of the protonated complex, and to determine whether $E1 \cdot Mg^{2+}$ -SLN is populated at physiologically relevant Mg^{2+} concentrations.

3.4. Structural dynamics of $E1 \cdot H^+$ - $_{771}$ -SLN

We performed a 2- μ s MD simulation of the SERCA-SLN complex starting from the structure of $E1 \cdot Mg^{2+}$ -SLN at $t = 0.5 \mu$ s, but in the absence of bound Mg^{2+} . Time evolution of RMSD values for each functional domain of SERCA showed that the TM domain undergoes minor structural changes (RMSD < 0.2 nm), but it is structurally stable in the trajectory (Supplementary Fig. S1). As in $E1 \cdot Mg^{2+}$ -SLN, the cytosolic domain is mobile; however, all three N, A and P domains become less mobile after $t = 1.3 \mu$ s (Supplementary Fig. S1). Calculation of RMSF values for SLN showed that, except for residues M1, E2 and Y31, SLN has very low mobility in the complex (RMSF < 0.15 nm) and that it populates a stable α -helical structure in the trajectory. The RMSF pattern is nearly identical to that of $E1 \cdot Mg^{2+}$ -SLN, indicating that Mg^{2+} binding does not play a role on the structural dynamics of SLN.

Analysis of the Mg^{2+} -SERCA interactions in or near the transport sites showed that Mg^{2+} binds, albeit transiently ($t < 4$ ns), near residues E58, D59, and E109 (Supplementary Fig. S2). These residues are located near the entrance to the transport sites, but Mg^{2+} binding to these sites occur only three times in the trajectory, and Mg^{2+} does not enter the transport sites in the trajectory. This data and our pK_a calculations indicate that at physiological conditions, SLN binding to SERCA does not stabilize a $E1 \cdot Mg^{2+}$ state, but instead an $E1$ state protonated on E771 and E908. We designate this state $E1 \cdot H^+$ - $_{771}$ -SLN.

3.5. Structural comparison of transport site I in $E1 \cdot H^+$ - $_{771}$ -SLN and $E1 \cdot H^+$ - $_{771}$ -PLB

We measured the distances D800-E771 and E771-E908 to evaluate the structural arrangement of transport site I in $E1 \cdot H^+$ - $_{771}$ -SLN (Fig. 3A). Analysis of the time-dependent distance plots show that interresidue distances in transport site I do not fluctuate substantially in the trajectory. Comparison between the interresidue distances in $E1 \cdot H^+$ - $_{771}$ -SLN with distance distributions calculated from three independent MD trajectories of $E1 \cdot H^+$ - $_{771}$ -PLB (Fig. 3B) indicate that both SLN and PLB produce identical structural arrangements of transport site I of the $E1$ state (Fig. 3C).

We recently showed that the transport sites of PLB-bound SERCA bind K^+ ions transiently and non-specifically [17]. Therefore, we investigated the K^+ interactions with the transport sites of $E1 \cdot H^+$ - $_{771}$ -SLN and compared them with those of $E1 \cdot H^+$ - $_{771}$ -PLB. We found that transport sites of $E1 \cdot H^+$ - $_{771}$ -SLN are occupied by K^+ at least 80% of the time, but the residence time of K^+ is ≤ 5 ns. K^+ binds only transiently to the transport sites of $E1 \cdot H^+$ - $_{771}$ -SLN, an identical behavior observed in the inhibited transport sites of $E1 \cdot H^+$ - $_{771}$ -PLB [17]. We found that K^+ ions bind non-specifically to nine identical positions we have previously identified in PLB-bound $E1 \cdot H^+$ - $_{771}$ (Fig. 4A). The binding preference for each site is different for SLN- and PLB-bound SERCA (Fig. 4B). Despite this variability, we found that K^+ sites 1 and 2 are occupied for $\sim 40\%$ of the time in both SLN- and PLB-bound $E1 \cdot H^+$ - $_{771}$. These sites are located near residue D800, which is ionized in $E1 \cdot H^+$ - $_{771}$. Therefore, neutralization of D800 by K^+ is a conserved feature in both $E1 \cdot H^+$ - $_{771}$ -SLN and

Table 1
Predicted pK_a values of acidic residues in the transport sites.

State	E309	E771	D800	E908
SERCA-SLN, $+Mg^{2+}$ (x-ray) ^{a,c}	5.6	6.6	3.0	8.8
SERCA-SLN, $+Mg^{2+}$ (MD) ^{b,c}	6.1 ± 0.1	4.8 ± 0.2	4.8 ± 0.1	7.5 ± 0.1
SERCA-SLN, $-Mg^{2+}$ (MD) ^{b,c}	5.3 ± 0.1	8.9 ± 0.1	5.7 ± 0.3	7.7 ± 0.1
SERCA-PLB (MD) ^{b,d}	5.4 ± 0.3	9.2 ± 0.2	6.1 ± 0.1	7.9 ± 0.2

^a Reported values are mean \pm SE.

^b pK_a values calculated using a single crystal structure (PDB code: 3w5a).

^c $+Mg^{2+}$ and $-Mg^{2+}$ indicate the presence and absence of a single Mg^{2+} ion bound in the transport sites.

^d pK_a values from Ref. [17].

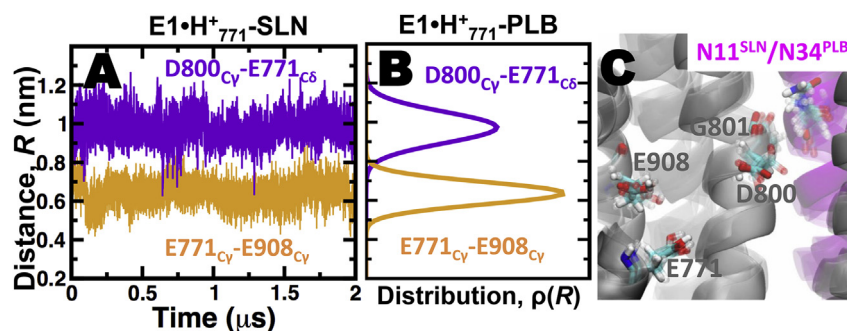


Fig. 3. Geometric comparison of transport site I of $E1 \cdot H^+$ -SLN and $E1 \cdot H^+$ -PLB. (A) Distances were calculated between C_δ - C_δ and C_δ - C_γ atoms for E771-E908 and D800-E771, respectively. (B) For comparison, we plotted distance distributions for residue pairs D800-E771 and E771-908 of $E1 \cdot H^+$ -PLB. Distributions were obtained by combining three 1- μ s trajectories of $E1 \cdot H^+$ -PLB reported in Ref. [17]. (C) Superposition of $E1 \cdot H^+$ -SLN at $t = 2 \mu$ s onto three structures of $E1 \cdot H^+$ -PLB at the end three independent 1- μ s MD simulations. We show that SLN and PLB produce an identical structural arrangement of transport site I.

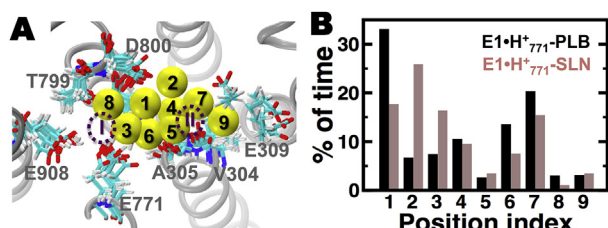


Fig. 4. Transient K^+ ion interactions in the transport sites of $E1 \cdot H^+$ -SLN and $E1 \cdot H^+$ -PLB. (A) Location of the nine different positions occupied by K^+ (yellow spheres) in the transport sites of $E1 \cdot H^+$. Each position is labeled from 1 to 9. The dashed circles show the approximate 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^+ spends at each position. The values for $E1 \cdot H^+$ -PLB represent the average from three independent 1- μ s simulations reported in Ref. [17]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$E1 \cdot H^+$ -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 $E1 \cdot H^+$.

4. Discussion

Protein pK_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^{2+} , indicating that the effect of SLN on SERCA is independent of the type of metal ion bound in the transport sites.

Protein pK_a calculations and MD simulations showed that $E1 \cdot Mg^{2+}$ 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, $E1 \cdot H^+$. 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, $E1 \cdot H^+$. We recently showed that PLB-bound $E1 \cdot H^+$ 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^{2+} affinity of SERCA [11,12].

It is possible that other newly-discovered SERCA regulators sarcolamban (SCL) [29] and myoregulin (MLN) [30] control Ca^{2+} affinity by populating $E1 \cdot H^+$ because they share structural and

functional similarity with PLB and SLN. Therefore, we propose that this mechanism for inhibition of Ca^{2+} transport applies to both cardiac and skeletal muscle. These findings have profound therapeutic implications because Ca^{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^{2+} cycling in diseased cardiomyocytes, thus improving cardiac function in heart failure [31–34]. In skeletal muscle, over-expression of SERCA enhances SR Ca^{2+} -uptake, excitation-contraction coupling, and Ca^{2+} clearance from sarcoplasm, thus mitigating Duchenne muscular dystrophy [35]. Identification of $E1 \cdot H^+$ as the inhibitory mechanism opens new doors for structure-based strategies to stimulate SERCA and Ca^{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_a calculations and microsecond MD simulations to demonstrate that SLN-bound SERCA is protonated at residue E771 and does not bind Mg^{2+} . These findings indicate that SLN binding to SERCA does not populate $E1 \cdot Mg^{2+}$ but an $E1$ intermediate protonated on E771, $E1 \cdot H^+$. 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 $E1 \cdot H^+$, and (iii) transient binding of K^+ but not Mg^{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^{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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