Structural basis for relief of phospholamban-mediated inhibition of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase at saturating Ca\(^{2+}\) conditions

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Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is critical for cardiac Ca\(^{2+}\) transport. Reversal of phospholamban (PLB)-mediated SERCA inhibition by saturating Ca\(^{2+}\) conditions operates as a physiological rheostat to reactivate SERCA function in the absence of PLB phosphorylation. Here, we performed extensive atomistic molecular dynamics simulations to probe the structural mechanism of this process. Simulation of the inhibitory complex at superphysiological Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)] = 10 mM) revealed that Ca\(^{2+}\) ions interact primarily with SERCA and the lipid headgroups, but not with PLB’s cytosolic domain or the cytosolic side of the SERCA–PLB interface. At this [Ca\(^{2+}\)], a single Ca\(^{2+}\) ion was translocated from the cytosol to the transmembrane transport sites. We used this Ca\(^{2+}\)-bound complex as an initial structure to simulate the effects of saturating Ca\(^{2+}\) at physiological conditions ([Ca\(^{2+}\)]\(_{\text{total}}\) ≈ 400 μM). At these conditions, ~30% of the Ca\(^{2+}\)-bound complexes exhibited structural features consistent with an inhibited state. However, in ~70% of the Ca\(^{2+}\)-bound complexes, Ca\(^{2+}\) moved to transport site I, recruited Glu\(^{771}\) and Asp\(^{801}\), and disrupted key inhibitory contacts involving the conserved PLB residue Asn\(^{34}\). Structural analysis showed that Ca\(^{2+}\) induces only local changes in intermolecular inhibitory interactions, but does not induce repositioning or changes in PLB structural dynamics. Upon relief of SERCA inhibition, Ca\(^{2+}\) binding produced a site I configuration sufficient for subsequent SERCA activation. We propose that at saturating [Ca\(^{2+}\)] and in the absence of PLB phosphorylation, binding of a single Ca\(^{2+}\) ion in the transport sites rapidly shifts the equilibrium toward a noninhibited SERCA–PLB complex.

The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) uses the energy derived from hydrolysis of one ATP to transport two Ca\(^{2+}\) ions from the cytosol into the lumen of the sarcoplasmic reticulum (SR) (1). In cardiac muscle cells, SERCA function is reversibly regulated by the transmembrane phospholamban (PLB). PLB binds SERCA in a 1:1 heterodimeric regulatory complex and inhibits SERCA activity (2, 3). Phosphorylation of PLB then relieves SERCA inhibition (3) to increase the rate of cardiac muscle relaxation and to restore the SR Ca\(^{2+}\) load necessary for muscle contraction in subsequent beats (4).

PLB inhibits SERCA by binding to a large pocket located in the transmembrane (TM) domain of the pump (5–10). Spectroscopy studies have shown that in the bound complex, SERCA inhibition is tightly coupled to a structural transition between inhibitory and noninhibitory structural states of PLB (11–14). More recently, X-ray crystallography studies showed that in its unphosphorylated form, PLB forms specific intermolecular interactions between conserved residue Asn\(^{34}\) and residue Gly\(^{801}\) of SERCA (15). Extensive studies by our group showed that these intermolecular interactions induce a substantial structural rearrangement of the transmembrane transport sites and stabilize a metal ion-free E1 intermediate of the pump protonated at residue Glu\(^{771}\), E1H\(^{+}\)(771) (16, 17). This SERCA intermediate serves as a kinetic trap that decreases SERCA’s apparent affinity for calcium at low Ca\(^{2+}\) concentrations and depresses the structural transitions necessary for Ca\(^{2+}\)-dependent activation of SERCA (16, 17).

In the absence of PLB phosphorylation, relief of SERCA inhibition occurs at saturating Ca\(^{2+}\) concentrations (18, 19). Two mechanisms have been proposed for the relief of SERCA inhibition at saturating Ca\(^{2+}\) conditions: the dissociation model and the subunit model. The dissociation model proposes that PLB must physically separate from SERCA to relieve inhibition, whereas the subunit model hypothesizes that PLB acts as a functional subunit of SERCA, and inhibition is relieved by local structural rearrangements within the SERCA–PLB complex. Whereas cross-linking experiments have suggested that saturation Ca\(^{2+}\) conditions induce PLB dissociation from SERCA, the findings from these studies are actually consistent with a structural rearrangement, and not dissociation, of the inhibitory complex (7, 19). Furthermore, extensive spectroscopic experiments in live cells, ER membranes, and reconstituted vesicles unequivocally support the subunit model, as they provide direct detection of the SERCA–PLB interaction at high Ca\(^{2+}\) concentrations (11, 20–22).
**Ca**\(^{2+}\)-induced relief of SERCA inhibition by phospholamban

Figure 1. Map of the Ca\(^{2+}\)–protein and Ca\(^{2+}\)–lipid interactions in the MD trajectory of the complex at 10 mM Ca\(^{2+}\). The map of the weighted mass density of Ca\(^{2+}\) (orange surface representation) was calculated using a grid resolution of 1 Å and a cutoff distance of 3.5 Å between Ca\(^{2+}\) and the protein/lipid atoms. The headgroups of the lipid bilayer are shown as gray spheres; SERCA and PLB are shown in blue and red cartoons, respectively. The arrows indicate the location of functionally important sites in SERCA: phosphorylation site (Asp\(^{351}\)) in green; the K\(^{+}\)-binding site (Ala\(^{711}\), Ala\(^{714}\), Lys\(^{712}\), and Glu\(^{732}\)) in purple; the Nβ5–β6 loop (Asp\(^{426}\)–Lys\(^{436}\)) in cyan; and the cytosolic gate (Asp\(^{77}\) and Glu\(^{109}\)) in magenta.

These studies have shown that saturating Ca\(^{2+}\) conditions do not induce dissociation of the SERCA–PLB complex, but the spatial and temporal mechanisms by which Ca\(^{2+}\) reverses SERCA remain unknown. In this study, we designed a series of atomistic molecular simulations to determine the mechanisms for Ca\(^{2+}\)-dependent relief of inhibitory interactions in the SERCA–PLB complex. First, we used a full-length structure of SERCA bound to the inhibitory structure of unphosphorylated PLB as a starting structure to obtain a structure of the SERCA–PLB complex at saturating Ca\(^{2+}\) conditions. We then used the Ca\(^{2+}\)-bound SERCA–PLB structural model generated through this computational approach to perform six independent 1-μs molecular dynamics (MD) simulations of the complex at physiologically relevant conditions. This set of independent simulations was used to systematically identify the effects of saturating Ca\(^{2+}\) conditions on the inhibitory contacts between SERCA and PLB in the absence of PLB phosphorylation.

**Results**

**Interaction of Ca\(^{2+}\) with the SERCA–PLB complex at saturating Ca\(^{2+}\) concentrations**

Physiologically relevant saturating free Ca\(^{2+}\) concentrations are in the low micromolar range (23), but these ion concentrations cannot be effectively modeled with our explicit MD simulations because the finite size of the systems would require less than one Ca\(^{2+}\) ion/system. To overcome this limitation, we first performed a 0.5-μs MD simulation of the SERCA–PLB complex at 10 mM CaCl\(_2\) to mimic saturating Ca\(^{2+}\) concentrations and to match the experimental conditions used previously in X-ray crystallography studies (24, 25). At this Ca\(^{2+}\) concentration, Ca\(^{2+}\) ions interact with several acidic residues of SERCA exposed on both sides of the lipid bilayer, as well as with the lipid headgroups (Fig. 1). However, we found no indication that Ca\(^{2+}\) interacts with the cytosolic domain of PLB or near the cytosolic side of the SERCA–PLB interface (Fig. 1).

Mapping of Ca\(^{2+}\)–SERCA interactions revealed that Ca\(^{2+}\) ions do not form interactions with functionally important regions of the pump, such as the phosphorylation site (Asp\(^{351}\)) or the K\(^{+}\)-binding site involved in SERCA dephosphorylation (Ala\(^{711}\), Ala\(^{714}\), Lys\(^{712}\), and Glu\(^{732}\)) (26) (Fig. 1). In other cases, Ca\(^{2+}\) ions interact, albeit nonspecifically, with other functional sites in the cytosolic headpiece, such as the Nβ5–β6 loop (Asp\(^{426}\)–Lys\(^{436}\)) (27) (Fig. 1). We found that Ca\(^{2+}\) ions bind to SERCA near the cytosolic gate that leads to the transport sites (Fig. 1). This site is located ~20 Å away from the SERCA–PLB interface and is not physically altered by PLB, and SERCA–metal ion interactions in this site are completely independent from PLB binding (15, 16). On average, 2–3 Ca\(^{2+}\) ions occupy this region of the protein, but only a single Ca\(^{2+}\) ion binds to residues Asp\(^{59}\) and Glu\(^{309}\) at the entrance of the cytosolic pathway (Fig. 2A). We found that in the submicrosecond timescale (τ = 0.34 μs), Glu\(^{309}\) translocates a single Ca\(^{2+}\) ion from Asp\(^{59}\) to Asp\(^{800}\) (Fig. 2B). Ca\(^{2+}\) translocation is facilitated by a change in the dihedral angle N-Cα-Cβ-Cγ (χ\(_{1}\)) of Glu\(^{309}\) from values of +165° and −165° to a χ\(_{1}\) = −65°. Upon translocation, Ca\(^{2+}\) is stabilized in the transport sites by electrostatic interactions with residues Asp\(^{800}\) and Glu\(^{309}\) (Fig. 2C). This Ca\(^{2+}\)-bound configuration of the complex is stable for the remainder of the simulation time, so we used it as starting structure for six independent MD simulations, identified as CAL1–CAL6. These MD simulations were used to determine the effects of saturating Ca\(^{2+}\) conditions at physiologically relevant conditions (e.g. ~400 μM total Ca\(^{2+}\) and 100 mM K\(^{+}\)).

This set of MD simulations revealed that Ca\(^{2+}\) remains bound to the transport sites of SERCA and does not dissociate back to the cytosol at physiological conditions. We calculated the coordination number and coordination shell of Ca\(^{2+}\) to characterize the interactions that stabilize a single Ca\(^{2+}\) ion in the transport sites of SERCA. We define the coordination number of Ca\(^{2+}\) as the number of oxygen atoms within 3.5 Å of the calcium ion. This distance is normally considered to be the maximum possible distance between ligand oxygen atoms and the calcium ion (28–30). We found that in all trajectories, Ca\(^{2+}\) interacts with the transport sites predominantly with a coordination number of 7, although Ca\(^{2+}\) also exhibits a coordination number of 8 in a small percentage of the simulation time (<10%). These coordination numbers fall within the typical values estimated from Ca\(^{2+}\)–protein (31, 32) and Ca\(^{2+}\)–SERCA complexes (24, 25).

In two trajectories, CAL1 and CAL3, the Ca\(^{2+}\) ion interacts predominantly with seven coordinating oxygen atoms primarily in a pentagonal bipyramidal coordination geometry, in
agreement with previous crystallographic studies of Ca$^{2+}$-bound SERCA (25) (Fig. 3A). The seven coordinating ligands for Ca$^{2+}$ are the carboxylic oxygen atoms from residues Glu$^{309}$ and Asp$^{800}$, the carbonyl moiety from residue Asn$^{796}$, and between two and four water molecules (Fig. 3A). We found that in trajectories CAL2 and CAL4–CAL6, Ca$^{2+}$ also coordinates to oxygen atoms within the transport site predominantly in a heptavalent pentagonal bipyramidal geometry. However, Glu$^{771}$ rapidly ($t = 25–250$ ns) replaces Glu$^{309}$ in the first coordination shell of Ca$^{2+}$ (Fig. 3B). In this binding mode, Ca$^{2+}$ interacts with the oxygen atoms from transport site residues Glu$^{771}$ and Asp$^{800}$, the carbonyl group from residue Asn$^{796}$, and 2–3 water molecules (Fig. 3B).

**Effect of saturating Ca$^{2+}$ conditions on PLB-induced inhibitory interactions**

Recent studies have shown that PLB residue Asn$^{34}$, which is absolutely required for SERCA inhibition (34), forms specific hydrogen bond interactions with Gly$^{801}$ and Thr$^{805}$ in the TM domain of SERCA (15–17). These interactions induce alterations in the transport site geometry that prevent metal ion occlusion in the transport sites (16, 17). The SERCA-Ca$^{2+}$ interactions shown in Fig. 3 suggest that saturating Ca$^{2+}$ concentrations alter SERCA–PLB inhibitory contacts at physiological conditions. Therefore, we measured intermolecular residue pair distances between Gly$^{801}$ and Asn$^{34}$ and between Thr$^{805}$ and Asn$^{34}$. SERCA residues Glu$^{771}$ and Asp$^{800}$ play a key role in Ca$^{2+}$ occlusion in the transport sites (35), so we also measured the interresidue distance between Glu$^{771}$ and Asp$^{800}$.

In the trajectories where Ca$^{2+}$ primarily binds to SERCA residues Glu$^{309}$ and Asp$^{800}$ (trajectories CAL1 and CAL3), PLB residue Asn$^{34}$ interacts directly with the backbone oxygen of Gly$^{801}$ and the side-chain hydroxyl group of Thr$^{805}$ (Fig. 4). In both cases, the intermolecular interactions Asn$^{34}$–Gly$^{801}$ and Asn$^{34}$–Thr$^{805}$ are present for most of the simulation time. Furthermore, we found that the carboxyl groups of transport site residues Glu$^{771}$ and Asp$^{800}$ in these trajectories are separated by a distance of at least 9 Å (Fig. 4). This spatial separation is characteristic of the inhibited SERCA–PLB complex in the absence of Ca$^{2+}$ (15, 16). Therefore, the stability of the inhibitory interactions and the large spatial separation between Glu$^{771}$ and Asp$^{800}$ indicate that the structures populated in the trajectories CAL1 and CAL3 correspond to those of an inhibited Ca$^{2+}$-bound state of the SERCA–PLB complex (15–17).

In four MD trajectories, CAL2, CAL4, CAL5, and CAL6, we found substantial changes in the distances between intermolecular residue pairs Asn$^{34}$–Gly$^{801}$ and Asn$^{34}$–Thr$^{805}$. Here, the distance Asn$^{34}$–Thr$^{805}$ increases by 1–2 Å (Fig. 4); however, the most significant change is the 3 Å decrease in the distance between the side chain of Asn$^{34}$ and the backbone oxygen of Gly$^{801}$ (Fig. 4). Most importantly, we found that the spatial separation Asn$^{34}$ of PLB and Gly$^{801}$ of SERCA in these trajectories occurs concomitantly with a 3–4 Å decrease in the distance between transport site residues Glu$^{771}$ and Asp$^{800}$ (Fig. 4). These changes in interresidue distances are also accompanied by a shift in the dihedral angle Ca–Cβ–Cγ–Nδ (ϕ) of Asn$^{34}$ from two narrow distributions at $\chi_2 = +180^\circ$ and $\chi_2 = -180^\circ$ to a single broad distribution with a mean around $-20^\circ$ (Fig. 5). This structural change is mostly characterized by a transition from an extended side-chain conformation to a self-contact (36) involving side-chain nitrogen and backbone oxygen atoms of Asn$^{34}$ (Fig. 5). These findings indicate that in trajectories CAL2, CAL4, CAL5, and CAL6, the side chain of PLB residue Asn$^{34}$ becomes more mobile and no longer establishes inhibitory contacts with SERCA.

These observations provide evidence that is consistent with relief of SERCA–PLB inhibition at saturating Ca$^{2+}$ conditions. However, this phenomenon is not consistently observed in all six trajectories, which suggests that this disruption of inhibitory
contacts probably occurs in equilibrium and in a Ca²⁺-independent manner. To test this hypothesis, we performed six 1-μs MD simulations of the SERCA–PLB complex in the absence of Ca²⁺. We found that whereas intermolecular distance Asn³⁴–Thr⁸⁰⁰ is variable among independent MD trajectories, Asn³⁴ of PLB consistently remains spatially close to SERCA residue Gly⁸⁰¹ throughout the entire simulation time in all trajectories (Fig. 6). We also found that carboxylic groups of transport site residues Glu⁷⁷¹ and Asp⁸⁰⁰ are separated by a distance larger than the 6-Å separation required for Ca²⁺ occlusion in this site (24, 25). Therefore, these findings indicate that relief of SERCA–PLB inhibitory contacts does not occur spontaneously in the absence of Ca²⁺ and that Ca²⁺ binding to SERCA at saturating Ca²⁺ conditions disrupts key SERCA–PLB inhibitory contacts at physiological conditions.

**Effects of saturating Ca²⁺ conditions on the structural dynamics of PLB**

Our results demonstrate that Ca²⁺ binding to the transport sites of SERCA at saturating Ca²⁺ conditions generally disrupt key inhibitory interactions between SERCA and PLB. However, it is not clear whether disruption of the inhibitory interactions is linked to (i) changes in the structural dynamics of PLB, (ii) a reorganization of the SERCA–PLB interface, or (iii) local changes involving interresidue interactions along the interface. Therefore, we performed extensive measurements of structural parameters to determine the changes in the structural dynamics of PLB in response to saturating Ca²⁺ conditions.

In the both inhibited and noninhibited Ca²⁺-bound complexes, the cytosolic and TM helices that contain the regulatory phosphorylation and inhibitory domains populate an α-helical structure for >95% of the time. Average interhelical angles between the cytosolic (Val⁴–Thr¹⁷) and TM (residues Arg²⁵–Leu⁵²) helices of PLB fluctuate between 52 and 77° (Table 1), which corresponds to a T-shaped architecture of PLB (Fig. 7A). We found that the calculated interhelical angles of PLB in both inhibited and noninhibited complexes are within the range of those determined experimentally for the unphosphorylated PLB monomer in solution (37, 38). These findings are in agreement with previous spectroscopic studies (11, 39) and demonstrate that saturating Ca²⁺ conditions do not induce order-to-disorder transitions associated with PLB phosphorylation (12, 39–43).

We measured time-dependent root mean square deviation (RMSD) to determine the extent to which the position of the TM domain of PLB changes in the trajectories of the Ca²⁺-bound complexes. RMSD plots revealed that the position of PLB in the binding groove in all six trajectories does not deviate substantially (e.g. RMSD < 2.5 Å) from that determined by X-ray crystallography (Fig. 7B). In addition, root mean square fluctuation (RMSF) calculations using the main-chain Ca atoms show that the cytosolic helix of PLB is highly mobile in solution (Fig. 7C). This behavior corresponds to the inherent diffusion of the helical domain through the viscous bilayer surface, and it is uncorrelated with the presence or absence of inhibitory contacts. The RMSF values of the TM domain residues in all MD trajectories are substantially smaller than those calculated for the cytosolic helix; this indicates that the TM domain of PLB has low mobility in the nanosecond time scale. The RMSF values calculated for the TM domain of PLB, and particularly those around the residue Asn³⁴, are virtually identical both in the presence and absence of intermolecular inhibitory contacts (Fig. 7C).

We also calculated changes in the tilt angle of the TM helix of PLB to complement RMSD and RMSF measurements. We measured the relative tilt angle of the TM helix using the crystal structure of SERCA–PLB as a reference (Table 1). We found that the TM helix exhibits on average a 3.6° increase in the tilt angle relative to the position of PLB in the crystal structure of the complex (Table 1). We found no correlation between the loss of inhibitory contacts and the small change in tilt angle, which indicates that saturating Ca²⁺ conditions do not have an effect on the position of PLB in the complex. Hence, it is likely that the small changes in tilt angle are inherent to the PLB.
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Table 1

Effects of saturating Ca\(^{2+}\) conditions on the structural dynamics of PLB and the inhibitory SERCA–PLB contacts

| Trajectory | PLB interhelical angle\(^{ab}\) | Change in PLB tilt angle\(^{ac}\) | Fraction of native inhibitory contacts, Q\(_{inh}^{ad}\) |
|------------|-------------------------------|--------------------------------|---------------------------------|
| CAL1       | 77.2 ± 8.1                    | 4.5 ± 1.2                      | 0.85 ± 0.06                    |
| CAL2       | 60.0 ± 8.5                    | 3.1 ± 1.2                      | 0.73 ± 0.09                    |
| CAL3       | 51.5 ± 10.5                   | 5.4 ± 2.1                      | 0.81 ± 0.08                    |
| CAL4       | 72.0 ± 7.5                    | 2.5 ± 1.2                      | 0.71 ± 0.09                    |
| CAL5       | 55.1 ± 9.3                    | 3.9 ± 1.5                      | 0.70 ± 0.13                    |
| CAL6       | 56.5 ± 8.7                    | 2.3 ± 1.2                      | 0.73 ± 0.11                    |

\(^{a}\) Values reported as average ± S.D.

\(^{b}\) Interhelical angle is defined as the polar angle between the cytosolic (Val\(^{18}\)-Thr\(^{17}\)) and TM (residues Arg\(^{25}\)-Leu\(^{32}\)) helices of PLB.

\(^{c}\) Axial tilt angle was calculated using backbone alignment, with SERCA–PLB crystal structure (PDB entry 4KYT (15)) as a reference.

\(^{d}\) These PLB residues play important roles in inhibition of SERCA (45).

Figure 7. Effects of saturating Ca\(^{2+}\) conditions on the structural dynamics of PLB at physiologically relevant conditions. A, structures of the Ca\(^{2+}\)-bound SERCA–PLB complex embedded in a lipid bilayer at the end of each 1-µs MD trajectory. The headgroups of the lipid bilayer are shown as gray spheres; SERCA is shown as a blue surface representation. PLB is shown as a cartoon representation and colored according to its functional domains: cytosolic (pink) and TM (red). Based on the structural analysis reported in this study, each structure is labeled as inhibited or not inhibited. B, time-dependent RMSD evolution of the TM helix of PLB in the Ca\(^{2+}\)-bound SERCA–PLB complexes. The RMSD was calculated using backbone alignment for TM helices of the SERCA. C, Ca RMSF values of PLB calculated from each independent MD trajectory. The shaded areas show the location of the cytosolic and TM helices of PLB.

Figure 7. Effects of saturating Ca\(^{2+}\) conditions on the structural dynamics of PLB at physiologically relevant conditions. A, structures of the Ca\(^{2+}\)-bound SERCA–PLB complex embedded in a lipid bilayer at the end of each 1-µs MD trajectory. The headgroups of the lipid bilayer are shown as gray spheres; SERCA is shown as a blue surface representation. PLB is shown as a cartoon representation and colored according to its functional domains: cytosolic (pink) and TM (red). Based on the structural analysis reported in this study, each structure is labeled as inhibited or not inhibited. B, time-dependent RMSD evolution of the TM helix of PLB in the Ca\(^{2+}\)-bound SERCA–PLB complexes. The RMSD was calculated using backbone alignment for TM helices of the SERCA. C, Ca RMSF values of PLB calculated from each independent MD trajectory. The shaded areas show the location of the cytosolic and TM helices of PLB.

dynamics induced by the annular lipid shell surrounding the complex (44). Relief of inhibitory contacts is not accompanied by substantial changes in the structure of PLB in the complex, so disruption of SERCA–PLB inhibitory contacts must occur locally at the interface of the complex. To test this hypothesis, we measured the fraction of native inhibitory contacts, Q\(_{inh}\), between SERCA and PLB residues Leu\(^{31}\), Asn\(^{34}\), Phe\(^{35}\), and Ile\(^{38}\); these residues, located near the cytosolic side of the complex, play important roles in inhibition of SERCA (45). Analysis of the Q\(_{inh}\) values showed that in the complexes with intact inhibitory contacts (CAL1 and CAL3), there is a high retention of native inhibitory contacts (Q\(_{inh}\) > 0.8) between key PLB residues and SERCA (Table 1). As anticipated, there is a substantial decrease in native inhibitory contacts (Q\(_{inh}\) = 0.52–0.62) for PLB residue Asn\(^{34}\) in the trajectories where inhibitory contacts are disrupted (CAL2, CAL4, CAL5, and CAL6; Table 1). However, we found that a loss in native inhibitory contacts also occurs, albeit more moderately, at PLB positions Leu\(^{31}\), Phe\(^{35}\), and Ile\(^{38}\) (Table 1). This indicates that saturating Ca\(^{2+}\) conditions primarily affect local intermolecular interactions involving the side chain of PLB residue Asn\(^{34}\), but also affect intermolecular interactions involving PLB residues within the inhibitory site at the SERCA–PLB interface.

Structure of the transport sites upon Ca\(^{2+}\)-induced disruption of SERCA–PLB inhibitory interactions

We determined whether the structural changes following disruption of inhibitory SERCA–PLB interactions correspond to those associated with the formation of competent transport sites. To this aim, we measured the RMSD values for residues in sites I and II between the MD trajectories and the crystal structure of SERCA bound to two Ca\(^{2+}\) ions (E1\(2\)-Ca\(^{2+}\), PDB entry 1SU4). We also performed distance measurements to determine whether the location of the Ca\(^{2+}\) in the MD trajectories corresponds to that determined by X-ray crystallography (25).

Visualization of the transport sites in the trajectories CAL1 and CAL3, where the inhibitory SERCA–PLB interactions are intact, show that sites I and II are collapsed (Fig. 8). Calculated RMSD values for sites I and II (RMSD >2.7 Å) indicate that there is a poor overlap between these trajectories and the crystal structure of E1\(2\)-Ca\(^{2+}\) (Table 2). In these trajectories, the position of Ca\(^{2+}\) does not overlap with any of the two sites resolved by X-ray crystallography (Fig. 8). Instead, the Ca\(^{2+}\) ion binds to a location that is distant from sites I (r ≈ 5.5 Å) and site II (r ≈ 3.5 Å). These measurements indicate that in the presence of inhibitory SERCA–PLB contacts, Ca\(^{2+}\) does not bind to either site I or II, and the residues in the transport sites adopt a noncompetent structure.
In the MD simulations where the inhibitory SERCA–PLB are disrupted (trajectories CAL2, CAL4, CAL5, and CAL6), the structure of transport site II also deviates substantially from that in the crystal structure of E1:2Ca\textsuperscript{2+} (Fig. 8 and Table 2). However, we found that Ca\textsuperscript{2+} ion binds in a reproducible manner to a location that partially overlaps that of site I (r \approx 2 Å) determined by X-ray crystallography (Fig. 8 and Table 2). When a single Ca\textsuperscript{2+} ion binds to this site, residues Glu\textsuperscript{771}, Thr\textsuperscript{799}, Asp\textsuperscript{800}, and Glu\textsuperscript{808} of site I adopt a structural arrangement that is similar to that found in the crystal structure of the PLB-free E1:2Ca\textsuperscript{2+} state of SERCA (RMSD < 2.2 Å; Fig. 8). The structural rearrangements in the transport sites that follow Ca\textsuperscript{2+} -induced relief of SERCA inhibition are reproducible in trajectories CAL2 and CAL4–CAL6 (Fig. 8) and correspond to the formation of a competent site I.

**Discussion**

We present a mechanistic study of the SERCA–PLB regulatory interactions at saturating Ca\textsuperscript{2+} conditions, thus providing quantitative insight into fundamental processes of activation of Ca\textsuperscript{2+} transport in the heart. We show that in a solution containing 10 mM Ca\textsuperscript{2+}, calcium ions interact primarily with both cytosolic and luminal sides of SERCA and the lipid headgroups. Here, we show for the first time (to our knowledge) that at superphysiological Ca\textsuperscript{2+} conditions, Ca\textsuperscript{2+} ions interact with the luminal C-terminal region of PLB, but not with the cytosolic domain of PLB or the cytosolic side of the SERCA–PLB interface. This indicates that Ca\textsuperscript{2+} does not compete with PLB at the interface of the complex and does not have a direct effect on the structural dynamics and stability of unphosphorylated PLB. Previous FRET spectroscopy experiments support our data and show that saturating Ca\textsuperscript{2+} conditions alter neither the structural dynamics of unphosphorylated PLB nor the stability of the SERCA–PLB heterodimer (11).

At [Ca\textsuperscript{2+}] = 10 mM, Ca\textsuperscript{2+} ions interact with SERCA at the entrance of the pathway that connects the cytosol with the transport sites, in agreement with previous studies by our group showing that PLB does not block metal ion binding to this region of SERCA (16, 17). Recognition of Ca\textsuperscript{2+} by SERCA is facilitated primarily by residues Asp\textsuperscript{800} and Glu\textsuperscript{808}, in agreement with mutagenesis studies of the pump (46). We found that Glu\textsuperscript{808} translocates a single Ca\textsuperscript{2+} ion from Asp\textsuperscript{800} to Asp\textsuperscript{800}, a critical residue in the transport sites that coordinates Ca\textsuperscript{2+} at sites I and II (25). This mechanism for Ca\textsuperscript{2+} translocation is in qualitative agreement with Brownian dynamics studies showing that fast Ca\textsuperscript{2+} binding to the transport sites is primarily guided by Glu\textsuperscript{808} (47).

In the absence of adequate charge neutralization of the transmembrane transport sites, SERCA denaturalization occurs very rapidly even within the native membrane at physiological pH (48, 49). Previous studies have shown that this electric charge can be compensated in the absence of Ca\textsuperscript{2+} by transport site protonation (16, 17) or by binding of metal ion K\textsuperscript{+} (50), Na\textsuperscript{+} (51–53), or Mg\textsuperscript{2+} (54, 55). This suggests that superphysiological concentrations of Ca\textsuperscript{2+} used in this study simply satisfy transport site charge neutralization and that the Ca\textsuperscript{2+}-bound state of the SERCA–PLB complex might not represent a functional state in the cell. However, only a single Ca\textsuperscript{2+} ion occupies the transport sites at a time despite the superphysiological Ca\textsuperscript{2+} concentrations used in this study. This finding is consistent with previous studies showing that the Ca\textsuperscript{2+} binding to SERCA occurs in a sequential manner (56–58) and indicates that the Ca\textsuperscript{2+}-bound SERCA–PLB structure represents a functional state of the complex at saturating Ca\textsuperscript{2+} conditions. In the absence of other Ca\textsuperscript{2+} ions, a single Ca\textsuperscript{2+} bound to the SERCA–PLB complex produces a total [Ca\textsuperscript{2+}] of \sim 400 μM. This value falls in the middle of previous estimates at elevated cytosolic Ca\textsuperscript{2+} in the cardiomyocyte (59–61), so we used the Ca\textsuperscript{2+}-bound SERCA–PLB complex as a starting structure to probe the structural mechanism for relief of SERCA inhibition by PLB.

In 30% of the MD trajectories, Ca\textsuperscript{2+} binds to Glu\textsuperscript{808} and Asp\textsuperscript{800} in an orientation that is similar to that initially found at the end of the 0.5-μs MD trajectory at [Ca\textsuperscript{2+}] = 10 mM. In this configuration, the inhibitory contacts remain intact in the microsecond time scale, and transport sites I and II lack the competent structural organization that is distinctive of the Ca\textsuperscript{2+}-bound state of SERCA (25, 53, 62). This structural arrangement corresponds to a Ca\textsuperscript{2+}-bound, inhibited SERCA–PLB complex. Previous studies have shown that PLB binding decreases SERCA’s apparent affinity for Ca\textsuperscript{2+} only by 2–3-fold.
Ca\textsuperscript{2+}-induced relief of SERCA inhibition by phospholamban

in the micromolar range (63), whereas others have suggested that PLB suppresses Ca\textsuperscript{2+} binding to SERCA (64). Our simulations help reconcile these conflicting studies because they show that PLB-induced changes in the transport sites delay Ca\textsuperscript{2+} binding to either sites I or II, thus altering the apparent Ca\textsuperscript{2+} affinity of SERCA (34).

SERCA–PLB inhibitory interactions are disrupted in 70% of the MD trajectories of the Ca\textsuperscript{2+}-bound complex. In these cases, the initially bound Ca\textsuperscript{2+} moves to site I and recruits the carboxylic groups of transport site residues Glu\textsuperscript{771} and Asp\textsuperscript{800}. These Ca\textsuperscript{2+}-induced structural changes occur concomitantly with a loss in the intermolecular interaction between the side chain of PLB residue Asn\textsuperscript{34} and the backbone oxygen of SERCA residue Gly\textsuperscript{801}. Our data indicates that this Ca\textsuperscript{2+}-dependent relief inhibitory contacts does not result from a large structural rearrangements in the SERCA–PLB interface (22) or changes in the native structural dynamics of PLB in the complex. Instead, PLB remains bound to SERCA, but PLB residue Asn\textsuperscript{34} becomes dynamically more disordered and is unable to establish inhibitory contacts with SERCA. FRET spectroscopy experiments support these findings and show that Ca\textsuperscript{2+} acts upon SERCA–PLB complex exclusively at the TM domain level and that unlike PLB phosphorylation, Ca\textsuperscript{2+} does not induce changes in the structural dynamics of PLB (11). Whereas these structural changes have not been observed directly by spectroscopy, the Ca\textsuperscript{2+}-induced repositioning and mobility of PLB residue Asn\textsuperscript{34} observed in our simulations have been seen in X-ray crystallography studies of the complex at [Ca\textsuperscript{2+}] = 1 mM.\textsuperscript{3}

What are the specific interactions between Ca\textsuperscript{2+} and the transport sites that induce relief of inhibition? The crystal structure of the complex between sarcolipin, a PLB analog, and SERCA revealed a single Mg\textsuperscript{2+} ion bound to transport site residues Glu\textsuperscript{771} and Asp\textsuperscript{800} (55). In this structure, the intermolecular inhibitory interactions are partially altered, which suggests that binding of divalent metal ions in the transport sites is sufficient to reverse SERCA inhibition. However, extensive studies by our group have demonstrated that in the inhibitory complex, Mg\textsuperscript{2+} does not simultaneously interact with Asp\textsuperscript{800} and Glu\textsuperscript{771} (17). Instead, Mg\textsuperscript{2+} adopts a rigid octahedral coordination geometry that has a preference for binding water molecules as opposed to bulky protein side chain dipeols (17). In addition, the ionic radius of Mg\textsuperscript{2+} is smaller than that of Ca\textsuperscript{2+} (65), so adding side chain dipeols to the coordination shell is thermodynamically more favorable for Ca\textsuperscript{2+} than for Mg\textsuperscript{2+}, so Ca\textsuperscript{2+} can produce drier, bulkier coordination complexes (66, 67). This explains why Ca\textsuperscript{2+}, but not Mg\textsuperscript{2+}, recruits both Glu\textsuperscript{771} and Asp\textsuperscript{800} in the transport sites (17, 54). Owing to these distinctive properties of Ca\textsuperscript{2+}, the tug of war between the attraction of Glu\textsuperscript{771} and Asp\textsuperscript{800} for Ca\textsuperscript{2+} drag the Gly\textsuperscript{801} backbone along with them as they move in toward Ca\textsuperscript{2+}. These Ca\textsuperscript{2+}-induced changes destabilize the interaction between Gly\textsuperscript{801} and PLB residue Asn\textsuperscript{34} and induce relief of SERCA inhibition by PLB. It is our postulate that Glu\textsuperscript{771} and Asp\textsuperscript{800} to a large degree define the range of coordination spheres that help preserve or disrupt the inhibitory contacts in the SERCA–PLB complex.

Finally, we asked whether the Ca\textsuperscript{2+}-induced structural changes detected in our simulations produce an intermediate state in the pathway toward SERCA activation. First, we found that upon relief of inhibitory contacts, the side chain of Glu\textsuperscript{309} populates a geometry in which the carboxylic group points toward the cytosol. We propose that this orientation of Glu\textsuperscript{309} is essential for binding and gating of a second Ca\textsuperscript{2+} ion in the transport site II (68). Second, we found that relief of inhibitory SERCA–PLB interactions occurs only when a single Ca\textsuperscript{2+} binds near transport site I and in agreement with mutagenesis studies showing that binding of a single Ca\textsuperscript{2+} in the transport site I is sufficient to reverse SERCA inhibition by PLB (19). The Ca\textsuperscript{2+}-induced structural rearrangements we detected in the simulations correspond to those associated with the formation of a competent transport site I and a vacant site II. This transport site reorganization facilitates binding of a second Ca\textsuperscript{2+} ion and subsequent Ca\textsuperscript{2+}-induced activation of the pump (25, 50, 53, 62).

In summary, we demonstrate that at saturating Ca\textsuperscript{2+} concentrations, binding of a single Ca\textsuperscript{2+} ion shifts the equilibrium toward a noninhibited structure of the SERCA–PLB complex. Our findings indicate that Ca\textsuperscript{2+}-induced reversal of SERCA inhibition depends solely on the ability of Ca\textsuperscript{2+} to diffuse into the transport sites and that the ability of Ca\textsuperscript{2+} to enter the transport sites is not influenced by PLB. Our findings also indicate that reversal of SERCA–PLB inhibition at saturating Ca\textsuperscript{2+} conditions is uncoupled from other regulatory mechanisms, such as the order-to-disorder structural transitions of PLB (12, 39–43). Therefore, the lack of a regulatory mechanism would explain the inability of saturating [Ca\textsuperscript{2+}] to effectively reverse impaired SERCA-mediated Ca\textsuperscript{2+} transport (4, 70, 71) and maladaptations (72) characteristic of chronic heart failure.

**Experimental procedures**

**Setting up SERCA–PLB at superphysiological concentrations of Ca\textsuperscript{2+}**

We used an atomic model of the full-length PLB bound to SERCA generated previously by our group (16) to simulate the inhibited SERCA–PLB complex at superphysiological Ca\textsuperscript{2+} conditions. We modeled transport site residues Glu\textsuperscript{309}, Glu\textsuperscript{771}, and Asp\textsuperscript{800} as unprotonated and residue Glu\textsuperscript{908} as protonated. In addition, we adjusted the pK\textsubscript{a} of other ionizable residues to a pH value of ~7.2 using PROPKA version 3.1 (73, 74). The complex was inserted in a pre-equilibrated 120 × 120-Å bilayer of palmitoyl-2-oleoyl-sn-glycerol-phosphatidylcholine lipids. We used the first-layer phospholipids that surround SERCA in the E1 state (75) as a reference to insert the complex in the lipid bilayer. This initial system was solvated using TIP3P water molecules with a minimum margin of 15 Å between the protein and the edges of the periodic box in the z axis. Ca\textsuperscript{2+} and Cl\textsuperscript{−} ions were added to produce a CaCl\textsubscript{2} concentration of ~10 mM required to match the experimental conditions previously used to obtain crystal structures of Ca\textsuperscript{2+}-bound SERCA (24, 25).

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\textsuperscript{3}Y. Kabashima, H. Ogawa, A. Nakaji, and C. Toyoshima (2017) Crystal structures of calcium pump in complex with sarcolipin/phospholamban. Poster session presented at 15th International Conference on Na,K-ATPase and Related Transport ATPases, September 24–30, 2017, Otsu City, Shiga, Japan.


**Ca²⁺-induced relief of SERCA inhibition by phospholamban**

**Setting up the SERCA–PLB complex at saturating Ca²⁺ conditions**

We used the structure of the complex bound to a single Ca²⁺ ion obtained at superphysiological [Ca²⁺] as a starting structure to simulate the SERCA–PLB complex at saturating Ca²⁺ conditions. We found that a single Ca²⁺ ion bound to the transport sites of SERCA corresponds to a total Ca²⁺ concentration of ∼400 μM. This total Ca²⁺ concentration is much higher than that estimated at rest (76) and is also in good agreement with previous estimates at elevated cytosolic Ca²⁺ (59–61). The SERCA–PLB-Ca²⁺-lipid complex was solvated using TIP3P water molecules. K⁺ and Cl⁻ ions were added to neutralize the system and to produce a KCl concentration of ∼100 mM.

**Setting up the SERCA–PLB complex at free Ca²⁺ conditions**

We used an atomic model of the full-length SERCA–PLB structure (16) to simulate the inhibited complex at free Ca²⁺ conditions. On the basis of our previous studies (16), we modeled transport site residues Glu699 and Asp800 as unprotonated and residues Glu771 and Glu908 as protonated. The lipid–water–protein complex was prepared using the same protocol and KCl concentrations used for the complex at saturating Ca²⁺ conditions.

**Molecular dynamics simulations**

MD simulations of all systems were performed by using the program NAMD version 2.12 (77), with periodic boundary conditions (78), particle mesh Ewald (79, 80), a nonbonded cutoff of 9 Å, and a 2-fs time step. CHARMM36 force field topologies and parameters were used for the proteins (81), lipid (69), water, Ca²⁺, K⁺, and Cl⁻. The NPT ensemble was maintained with a Langevin thermostat (310 K) and an anisotropic Langevin piston barostat (1 atm). Fully solvated systems were first subjected to energy minimization and warmup for 200 ps. This procedure was followed by 10 ns of equilibration with backbone atoms harmonically restrained using a force constant of 10 kcal mol⁻¹ Å⁻². We performed one 0.5-μs MD simulation of SERCA–PLB at 10 mM Ca²⁺ and 12 independent 1-μs MD simulations: six of Ca²⁺-bound SERCA–PLB and six of SERCA–PLB in the absence of Ca²⁺.

**Structural analysis and visualization**

VMD (33) was used for analysis, visualization, and rendering of the structures. To visualize the Ca²⁺–protein and Ca²⁺–lipid interactions, we created a map of the weighted mass density of Ca²⁺ using a grid resolution of 1 Å and a cutoff distance of 3.5 Å between Ca²⁺ and the protein/lipid atoms. This is achieved by replacing each atom in the selection with a normalized Gaussian distribution of width equal to the atomic radius. The distributions are then additively distributed on a grid. The final map is calculated by computing the mass density of Ca²⁺ for each step in the trajectory and averaged over the entire simulation time.

We calculated the fraction of native inhibitory contacts ($Q_{inh}$) between PLB residues Leu31, Asn34, Phe35, and Ile38 and SERCA to measure the effect of calcium binding on the stability of the SERCA–PLB interface. $Q_{inh}$ is defined by a list of native contact pairs $(i,j)$ in the crystal structure of the complex. All pairs of heavy atoms $i$ and $j$ belonging to residues $X_i$ and $X_j$ are in contact if the distance between $i$ and $j$ is $<7$ Å. $Q_{inh}$ is expressed as a number between 1 and 0, and it is calculated as the total number of native contacts for a given time frame divided by the total number of contacts in the crystal structure of the complex (PDB code 4KYT (15)).

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