Membrane Perturbation of ADP-insensitive Phosphoenzyme of Ca\(^{2+}\)-ATPase Modifies Gathering of Transmembrane Helix M2 with Cytoplasmic Domains and Luminal Gating

Stefania Danko, Kazuo Yamasaki, Takashi Daiho & Hiroshi Suzuki

Ca\(^{2+}\) transport by sarcoplasmic reticulum Ca\(^{2+}\)-ATPase involves ATP-dependent phosphorylation of a catalytic aspartic acid residue. The key process, luminal Ca\(^{2+}\) release occurs upon phosphoenzyme isomerization, abbreviated as \(E_{1PCa^2}\) (reactive to ADP regenerating ATP and with two occluded Ca\(^{2+}\) at transport sites) \(\rightarrow\) \(E_{2P}\) (insensitive to ADP and after Ca\(^{2+}\) release). The isomerization involves gathering of cytoplasmic actuator and phosphorylation domains with second transmembrane helix (M2), and is epitomized by protection of a Leu\(^{119}\)-proteinase K (prtK) cleavage site on M2. Ca\(^{2+}\) binding to the luminal transport sites of \(E_{2P}\), producing \(E_{2PCa^2}\) before Ca\(^{2+}\)-release exposes the prtK-site.

Here we explore \(E_{2P}\) structure to further elucidate luminal gating mechanism and effect of membrane perturbation. We find that ground state \(E_{2P}\) becomes cleavable at Leu\(^{119}\) in a non-solubilizing concentration of detergent \(C_{12}E_8\) at pH 7.4, indicating a shift towards a more \(E_{2PCa^2}\)-like state. Cleavage is accelerated by Mg\(^{2+}\) binding to luminal transport sites and blocked by their protonation at pH 6.0. Results indicate that possible disruption of phospholipid-protein interactions strongly favors an \(E_{2P}\) species with looser head domain interactions at M2 and responsive to specific ligand binding at the transport sites, likely an early flexible intermediate in the development towards ground state \(E_{2P}\).

Sarco(endo)plasmic reticulum (SR) Ca\(^{2+}\)-ATPase (expressed in adult fast-twitch skeletal muscle, SERCA1a), a representative member of P-type ion transporting ATPases, catalyzes Ca\(^{2+}\) transport coupled with ATP hydrolysis (Fig. 1) (for recent reviews, see Refs 1–3). The enzyme consists of three large cytoplasmic domains, Nucleotide binding (N), Phosphorylation (P), and Actuator (A), and ten transmembrane helices (M1~M10) (Figs 1 and 2). Ca\(^{2+}\) transport requires communication between the catalytic site on the cytoplasmic domains and the transport sites in the transmembrane helices via coupled structural changes, i.e. cytoplasmic domain motions and rearrangements of transmembrane helices. The enzyme is activated by the binding of two cytoplasmic Ca\(^{2+}\) ions at the high affinity transport sites composed of residues located on M4, M5, M6, and M8 (\(E_{2}\) to \(E_{1Ca^2}\) in Fig. 1). Then it is auto-phosphorylated at the catalytic residue Asp\(^{351}\) with ATP to form an ADP-sensitive phosphoenzyme (E1P), which is capable of reacting with ADP to regenerate ATP in the reverse reaction. Upon E1P formation, the two bound Ca\(^{2+}\) are occluded in the transport sites (E1PCa\(_2\)). The subsequent isomeric transition to the ADP-insensitive E2P form involves a large rotation of the A domain to associate with the P domain, thereby rearranging the Ca\(^{2+}\) binding sites to deocclude Ca\(^{2+}\), open the release path (luminal gate), and reduce the affinity, thus allowing Ca\(^{2+}\) release into the lumen. As a consequence, the catalytic site in E2P is prepared for subsequent aspartyl phosphate hydrolysis by tightening of associated A and P domains. In the first step towards hydrolysis, progressing from the ground state to the transition state, namely E2P + H\(_2\)O \(\rightarrow\) E2-\(P\), the transport sites are protonated and the luminal gate closes tightly, preventing luminal Ca\(^{2+}\) access and driving the process forward\(^{4,5}\).
The cytoplasmic part of the second transmembrane helix, M2, plays a crucial role in coupling A-domain motion and tilting of the P domain during the rearrangements of transport sites\(^4\)-\(^9\).

The \(E_2P\) ground state, transition state (\(E_2P^\ddagger\)), and product complex (\(E_2\cdot P_i\)) in the \(E_2P\) hydrolysis process are mimicked by the stable structural analogs \(E_2\cdot BeF_3^-\), \(E_2\cdot AlF_4^-\), and \(E_2\cdot MgF_4^{2-}\), respectively, as produced with the respective phosphate analogs for different configurational states\(^4\). Their crystal structures, without or with the potent inhibitor thapsigargin (TG), have been solved at atomic level\(^7\),\(^8\),\(^10\),\(^11\) following purification of the protein using a non-ionic detergent octaethylene glycol monododecyl ether (C\(_{12}\)E\(_8\)). Commensurate with the structural changes mentioned, the crystal structures are subtly different although the overall molecular structure of the compactly organized cytoplasmic A, P, and N domains with tightly bound BeF\(_3^-\) and occluded Mg\(_2^+\) at the catalytic site and the arrangement of transmembrane helices are similar. Namely, the \(E_2\cdot BeF_3^-\) crystal produced at pH 7.0 in 50 mM Mg\(_2^+\) has wide open transport sites (luminal gate open) with one bound Mg\(_2^+\)\(^11\) and that at pH 5.7, where the transport sites are protonated and Mg\(_2^+\) is absent, the luminal access pathway is less open\(^8\) (Fig. 2). The structures with bound TG at a cavity surrounded by M3, M5, and M7, namely \(E_2\cdot BeF_3^-\) (TG), and those of \(E_2\cdot AlF_4^-\) (TG) and \(E_2\cdot MgF_4^{2-}\) (TG), are different again, and the luminal gate is tightly closed. The closure is associated with formation of hydrophobic interaction network, the Tyr122-hydrophobic cluster (Y122-HC) by Leu119/Tyr122 on the cytoplasmic part of M2 and five residues of the gathered A and P domains (Ile179/Leu180 (A), Val705/Val726 (P)) and A/M3-linker (Ile232 on the loop connecting the A domain with M3). Significantly, in the \(E_2\cdot BeF_3^-\) crystals without TG, where the gate is open, the side chains of Leu119/Tyr122 are close but pointing away from the other gathered five residues, indicative of weaker domain interactions here (Fig. 2).

Extensive mutation and kinetic studies have demonstrated\(^12\)-\(^15\) that all seven residues involved in Y122-HC including Leu\(^{119}\)/Tyr\(^{122}\) are crucial for opening the gate, reducing Ca\(^{2+}\) affinity, and allowing rapid Ca\(^{2+}\)-release (\(E_2\cdot P_{Ca^2+}\) \(\rightarrow\) \(E_2\cdot P\) + 2Ca\(^{2+}\)), and for subsequent gate-closure and the formation of a catalytic site with hydrolytic ability. Investigation of the structural changes during these events has been aided by proteolytic digestion patterns, including a prtK site at Leu\(^{119}\)\(^2\)-\(^16\),\(^17\),\(^19\) The site is exposed in the unphosphorylated \(E_2\) form but protected in \(E_2\cdot BeF_3^-\), \(E_2\cdot AlF_4^-\), and \(E_2\cdot MgF_4^{2-}\) as well as in the TG-bound forms of these analogs. Thus susceptibility to prtK attack or otherwise seems a good indicator of the state of the gathering of the head domains on M2. Significantly, \(E_2\cdot P_{Ca^2+}\), an early \(E_2\) species, is uniquely susceptible to attack, an indication of a loose arrangement of head domains on M2 prior to progression to ground state \(E_2P\)\(^4\),\(^18\),\(^19\).

---

**Figure 1. Reaction sequence of Ca\(^{2+}\)-ATPase.** The sequence is shown with intermediates and transition states (\(E_1\cdot P_{Ca^2+}\).ADP\(^\ddagger\) and \(E_2\cdot P^\ddagger\)). Stable structural analog for each state developed with phosphate analogs BeF\(_3^-\), AlF\(_4^-\), and MgF\(_4^{2-}\)\(^4\),\(^6\),\(^7\),\(^17\),\(^19\) is shown with gray-highlight. In the crystal structures \(E_1Ca_2\cdot AlF_4^-\cdot ADP\) and \(E_2\cdot BeF_3^-\) (PDB code: 2ZBD\(^8\) and 2ZBE\(^8\), respectively), the cytoplasmic domains A (yellow), P (cyan), and N (pink), M1–M10, occluded two Ca\(^{2+}\), and membrane position are indicated. Arrows on the domains in \(E_1Ca_2\cdot AlF_4^-\cdot ADP\) indicate their approximate motions to the \(E_2\cdot BeF_3^-\) structure to show changes in \(E_1PCa^2+\) \(\rightarrow\) \(E_2P\) + 2Ca\(^{2+}\) as an available model.
Unexpectedly, we now find that low, non-solubilizing concentrations of C12E8 render the prtK site at Leu119 in E2·BeF3− susceptible to attack. It is as though the detergent has released constraints at the transmembrane helices to favor a state closer to that on Ca2+ binding to the luminal sites, namely E2PCa2. The phenomenon uncovers a hitherto undescribed intermediate just prior to ground state E2P, stabilized by detergent that is uniquely susceptible to diverse ligand binding and cross-protein conformational changes. It shows that phospholipid-protein interactions directly participate the conformational changes associated with luminal gating events and expedite Ca2+ release.

**Results**

**PrtK-cleavage of Leu119-site in E2·BeF3− with C12E8 at pH 7.4.** In Fig. 3a, prtK-proteolysis of E2·BeF3− (E2P) is completely resistant to prtK both without and with A23187 as found previously4. In the presence of C12E8, a 95-kDa fragment (p95) is produced by specific prtK-cleavage at the Leu119-site without any other cleavages. Cleavage is accelerated by 30 mM Mg2+, but no cleavage occurs in the absence of C12E8 even at 30 mM Mg2+. In Fig. 3d, the Mg2+ concentration dependence of the specific prtK-cleavage rate at the Leu119-site is determined in C12E8 and different monovalent cations (K+, Na+, and Li+) at 0.1 M. The rate increases with increasing Mg2+ concentration – binding to a low affinity site favors exposure. The cleavage is faster in Na+ and K+ as compared with that in Li+ or in the absence of monovalent cation, thus K+ or Na+ binding at the K+ site on the P domain20,21 increases prtK attack at Leu119. The BeF3− coordinated in the catalytic site behind the residues involved in Y122-HC is shown by a space-filling model (cyan for beryllium and purple for fluoride) and Asp351 (the auto-phosphorylation site) is shown in a ball-stick model in the panels (note that they are obscured by Y122-HC in E2·BeF3− (TG)). The Mg2+ bound to the catalytic site is not depicted as it is also hidden by Y122-HC. The TGES184 loop and Val200 loop (Lys189-Lys205) are colored by a red loop and a blue loop, respectively in all panels. The prtK-cleavage sites at Leu119 and Thr242 and the trypsin-cleavage sites at Arg198 and Arg505 are indicated (backbone carbon).

![Figure 2. Crystal structures E2·BeF3− and E2·BeF3− (TG). Structures E2·BeF3− with bound Mg2+ at the transport sites (formed at pH 7.0 and 50 mM Mg2+), E2·BeF3− with most probably protonated transport sites (formed at pH 5.7), and E2·BeF3− (TG) (PDB code: 3B9B, 2ZBE, 2ZBF, respectively) are shown as a cartoon model. The cytoplasmic region indicated by the red broken line on the whole molecule of E2·BeF3− with bound Mg2+ is enlarged in the three top panels. In the three bottom panels, the view of transport sites from the luminal side as indicated by a large green arrow is shown. The A, P, and N domains and cytoplasmic part of M2 are yellow, cyan, pink, and purple, respectively. The Mg2+ and water molecules at the Ca2+ binding sites (transport sites) and Na+ bound at the K+ (Na+) site on the P domain are green, and red, blue spheres, respectively. The seven residues involved in the formation of Tyr122-hydrophobic cluster, Y122-HC (Leu119/Tyr122 on M2, Ile179/Leu180 on the A domain, Val705/Val726 on the P domain, and Ile232 on the A/M3-linker) are shown with van der Waals spheres, and colored green (Leu119/Tyr122), brown (Ile179/Leu180), and orange (Val705/Val726/Ile232). The BeF3− coordinated in the catalytic site behind the residues involved in Y122-HC is shown by a space-filling model (cyan for beryllium and purple for fluoride) and Asp351 (the auto-phosphorylation site) is shown in a ball-stick model in the panels (note that they are obscured by Y122-HC in E2·BeF3− (TG)). The Mg2+ bound to the catalytic site is not depicted as it is also hidden by Y122-HC. The TGES184 loop and Val200 loop (Lys189-Lys205) are colored by a red loop and a blue loop, respectively in all panels. The prtK-cleavage sites at Leu119 and Thr242 and the trypsin-cleavage sites at Arg198 and Arg505 are indicated (backbone carbon).
Figure 3. Effects of C$_{12}$E$_8$ and various factors on proteolysis of E$_2$BeF$_3^-$, E$_2$AlF$_4^-$, and E$_2$MgF$_4^{2-}$. The proteolysis was performed for various times with prtK and trypsin as indicated with E$_2$BeF$_3^-$ (a,e), E$_2$AlF$_4^-$ (b,f), and E$_2$MgF$_4^{2-}$ (c,f) of SR vesicles in the presence or absence of 0.15 mg/ml C$_{12}$E$_8$ or 15 μM A23187 in 50 mM MOPS/Tris pH 7.4 (a–c) or MES/Tris pH 6.0 (e,f), 0.1 M KCl, 1 mM EGTA, and 0 or 30 mM MgCl$_2$ without or with 4 μM TG (“TG”), as indicated. The “E$_2$-TG” state of SR vesicles un-treated with the metal fluoride was subjected to the proteolysis as a control. In (d), the rate of prtK digestion of 110 kDa-ATPase chain in C$_{12}$E$_8$ at pH 7.4 was determined at various concentrations of MgCl$_2$ in 0.1 M KCl, NaCl, or LiCl or in the absence of these salts, otherwise as in (a) and as described under “METHODS”. The fragments indicated on the right of a panel are p95 produced by the prtK-cleavage at the Leu$^{119}$-site on M2, p81/p83 produced by the prtK-cleavage at the Thr$^{242}$-site on A/M3-linker (p83) and Ala$^{746}$ on M5 (p81)$^{16,39}$, and the tryptic A1 fragment produced by cleavage at the Arg$^{198}$-site on the A fragment (N-terminal half), which is formed very rapidly together with the B fragment (C-terminal half) by cleavage at Arg$^{505}$-site$^{40}$. 

www.nature.com/scientificreports/
well as without Mg\(^{2+}\), the 110-kDa ATPase chain is very rapidly cleaved producing p95 and p81/p83 fragments by cleavages at Leu\(^{19}\) and at Thr\(^{246}\) (p83) and Ala\(^{146}\) (p81), respectively, in agreement with previous findings\(^{41}\).

**Tryptic T2** (Arg\(^{198}\)-site) in E2-BeF\(_3^-\) is completely resistant in C\(_{12}E_8\). The association of the Val\(^{200}\) loop (Lys\(^{189}\)_Lys\(^{205}\)) on the A domain with the P domain by ionic interactions is crucial for E2P structure formation and occurs as a consequence of the A domain’s large rotation during the E1P\(_{Ca}\)→ E2P isomeric transition\(^{6,7,24}\). With the changes, the Arg\(^{198}\)-tryptic T2 site in this loop becomes completely resistant to tryptic attack\(^4,6\).

In Fig. 3a, the trypsin proteolysis was performed as described above with prtK. In the BeF\(_3^-\)-free state with bound TG as a control (“E2-TG”) in which the A and P domains are not fixed, the Arg\(^{198}\)-site is cleaved producing the A1 and A2 fragments (the A2 fragment is not seen because it is at the gel front) as found previously\(^6\). In E2-BeF\(_3^-\), the A1 and A2 fragments are not produced regardless of the presence of C\(_{12}E_8\) and 30 mM Mg\(^{2+}\), thus the Arg\(^{198}\)-site is completely resistant, consistent with association of the A and P domains by an ionic network as seen in the E2-BeF\(_3^-\) crystal structures\(^8,11\).

E2-BeF\(_3^-\) in C\(_{12}E_8\) is completely resistant to prtK at pH 6.0. In Fig. 3e, prtK-proteolysis was performed at pH 6.0 otherwise as in Fig. 3a. At this pH the luminal transport sites are expected to be protonated. No cleavage of the 110 kDa-ATPase chain occurred even in C\(_{12}E_8\) and 30 mM Mg\(^{2+}\). The tryptic Arg\(^{198}\)-site was also completely resistant at pH 6.0 as at pH 7.4 without and with C\(_{12}E_8\) and 30 mM Mg\(^{2+}\).

E2-AlF\(_4^-\) and E2-MgF\(_2^-\) are completely resistant to prtK even in C\(_{12}E_8\) at pH 7.4 and 6.0. E2-AlF\(_4^-\), the analog for the product complex (E2-P) is completely resistant to prtK at pH 7.4 and 6.0 even in the presence of C\(_{12}E_8\) both without and with 30 mM Mg\(^{2+}\) (Fig. 3b,f). The Arg\(^{198}\)-site is also protected from trypsin in all these conditions. E2-MgF\(_2^-\), the analog for the product complex (E2-P) is completely resistant to prtK and to trypsin in all these conditions as E2-AlF\(_4^-\) (Fig. 3c,f).

Hydrophobic nature of the nucleotide/catalytic site revealed by TNP-AMP superfluorescence. TNP-AMP binds to the ATP binding site with a very high affinity and develops an extremely high “superfluorescence” in the E2P ground state and its analog E2-BeF\(_3^-\)\(^{2,25}\). The TNP moiety binds at the adenine position in the N domain and the superfluorescence can be ascribed to a favorable TNP moiety Phe 487 interaction and site-occlusion that excludes non-specific water and increases hydrophobicity by the contribution of Arg\(^{174}\) on the A domain in the N-interface on the TNP binding pocket\(^{26}\). The superfluorescence is completely lost during E2P + H\(_2\)O → E2-P\(^\dagger\), as demonstrated with the change E2-BeF\(_3^-\) → E2-AlF\(_4^-\)\(^4\), probably through TNP-Phe\(^{487}\) mal-alignment and water influx here. In Fig. 4, the superfluorescence development in E2-BeF\(_3^-\) upon the TNP-AMP binding at saturating 4 \(\mu\)M was examined without and with C\(_{12}E_8\) at pH 7.4 and 6.0 and various concentrations of Mg\(^{2+}\) in 0.1 M K\(^+\) and Li\(^+\) at sub-mM to ~mM concentration is able to bind and cause reverse isomerization \(\rightarrow 2P\) ground state and its analog \(\rightarrow 2P\), as demonstrated with the change E2-BeF\(_3^-\) → E2-AlF\(_4^-\)\(^4\).

In the absence of both C\(_{12}E_8\) and A23187, and increasing Mg\(^{2+}\) concentrations of Mg\(^{2+}\) in C\(_{12}E_8\), and Mg\(^{2+}\) lost during converting the remaining E1Ca2·BeF\(_3^-\) and E1Ca2·BeF\(_3^-\) in C\(_{12}E_8\) to below ~100 \(\mu\)M \(\rightarrow 2P\), with those in Li\(^+\). Increasing Mg\(^{2+}\) concentration up to 60 mM caused only slight decrease. The results show that the catalytic/nucleotide site, starting from the E2P ground state, is not affected by C\(_{12}E_8\), Mg\(^{2+}\), K\(^+\), and protonation of transport sites.

E2-BeF\(_3^-\) in C\(_{12}E_8\) and Mg\(^{2+}\) is resistant to luminal Ca\(^{2+}\)-induced reverse conversion to E1Ca2·BeF\(_3^-\). The E2P ground state possesses luminally partially open low affinity transport sites and luminal Ca\(^{2+}\) at sub-mM to ~mM concentration is able to bind and cause reverse isomerization E2P + 2Ca\(^{2+}\) → E2PCA2 → E1P\(_{Ca}\), which contributes to the proper setting of luminal Ca\(^{2+}\) concentration through “back-door inhibition”. This reverse process as well as the forward E2P isomeration mimics the structural analogs E2-BeF\(_3^-\) (E2P), E2-BeF\(_3^-\)-Ca\(_2\) (E2PCA2, the transient intermediate state before the Ca\(^{2+}\)-release), and E1Ca2·BeF\(_3^-\) → (E1P\(_{Ca}\))\(^{17-19}\). In Figs 5 and 6, the effect of luminal Ca\(^{2+}\) on E2-BeF\(_3^-\) was examined at pH 7.4 in C\(_{12}E_8\) or A23187, various concentrations of Mg\(^{2+}\), and 0.1 M K\(^+\) or Li\(^+\). Here it should be noted that the E1Ca2·BeF\(_3^-\) complex is not stable and rapidly decomposes to E1Ca2 in the presence of a high concentration of Ca\(^{2+}\) (due to Ca\(^{2+}\)-substitution at the unoccluded catalytic Mg\(^{2+}\) site in E1Ca2·BeF\(_3^-\)), on the other hand, it is very rapidly isomerized to E2BeF\(_3^-\) releasing Ca\(^{2+}\) upon the removal or reduction of luminal free Ca\(^{2+}\) concentration to below ~100 \(\mu\)M as the process mimics the isomeric transition E1PCA2 → E2P + 2Ca\(^{2+}\). Also, the E1Ca2·BeF\(_3^-\) complex decomposes to E1Ca2 upon ADP binding, mimicking the ADP-induced reverse dephosphorylation of E1PCA2, and upon TNP-AMP binding probably analogous to the ADP-induced process, in contrast to a stable E2BeF\(_3^-\) with bound ADP or TNP-AMP binding.

In Fig. 5, taking these known properties into account, we first determined the overall time course of the Ca\(^{2+}\)-induced E2BeF\(_3^-\) reverse conversion and decomposition to E1Ca2 (E2BeF\(_3^-\) + 2Ca\(^{2+}\) → E2BeF\(_3^-\)-Ca\(_2\) → E1Ca2·BeF\(_3^-\) → E1Ca2) by adding an excess EGTA after various times of incubation with 0.5 mM Ca\(^{2+}\) thereby converting the remaining E1Ca2·BeF\(_3^-\)-Ca\(_2\) to the stable E2BeF\(_3^-\) species, and in addition adding TNP-AMP to determine superfluorescence development to estimate the total amount of E2BeF\(_3^-\) and E1Ca2·BeF\(_3^-\) species remaining at the time of EGTA addition. In Fig. 6, prtK proteolysis was performed for a short period during the 0.5 mM Ca\(^{2+}\) incubation and without the EGTA addition to identify the structural states of EP species under representative conditions in Fig. 5 (although the Ca\(^{2+}\)-induced process proceeds).

First in Fig. 5 where TNP-AMP superfluorescence is examined, we found both with K\(^+\) and without K\(^+\) (with LiCl) that the Ca\(^{2+}\)-induced reverse conversion/decomposition of E2-BeF\(_3^-\) is considerably slower in C\(_{12}E_8\) than in A23187, and increasing Mg\(^{2+}\) to ~20 mM in C\(_{12}E_8\) causes a marked retardation or almost complete inhibition. The retardation by Mg\(^{2+}\) in C\(_{12}E_8\) is much stronger and occurs at much lower Mg\(^{2+}\) concentration than in A23187. In the absence of both C\(_{12}E_8\) and A23187, i.e. with an impermeable SR membrane, no conversion nor decomposition of E2-BeF\(_3^-\) occurs with Ca\(^{2+}\), therefore the Ca\(^{2+}\)-induced decomposition is due to the Ca\(^{2+}\) access from...
the luminal side as found previously. Regarding the $K^+$ effect, the luminal $Ca^{2+}$-induced conversion/decomposition of $E_2\cdot BeF_3^-$ is considerably faster in $K^+$ than in its absence, therefore specific $K^+$ binding accelerates the process. Then in Fig. 6a, prtK-proteolysis was performed to identify the structural state stabilized in C12E8 with, most typically, 30 mM $Mg^{2+}$ in the absence of $K^+$ during luminal $Ca^{2+}$-induced $E_2\cdot BeF_3^-$ reverse conversion and decomposition. Here, the sample was incubated first with 0.5 mM $Ca^{2+}$ for 10 s, and then with a high concentration of prtK for various times without removal of $Ca^{2+}$. The proteolytic pattern was compared with those of $BeF_3^-$-free $E_1Ca^{2+}$ and of $E_1Ca^{2+}\cdot BeF_3^-$ that is formed and stabilized perfectly under the previously identified most appropriate conditions, i.e. at pH 7.0 with 0.7 mM $Ca^{2+}$ and 15 mM $Mg^{2+}$ in 0.1 M $K^+$ in the absence or presence of A23187. In these states, p81/p83 fragments are produced due to cleavage at Thr242 (p83) and Ala746 (p81) without production of the p95-fragment (Fig. 6b). In C12E8 and $Ca^{2+}$ (Fig. 6a), $E_2\cdot BeF_3^-$ both without and with 30 mM $Mg^{2+}$ is degraded slowly as compared with $E_1Ca^{2+}$, producing the stable p95 fragment as seen with $E_2\cdot BeF_3^-$ in C12E8 without $Ca^{2+}$ (cf. Fig. 3) and a small amount of p81/p83 fragments, which degrade rapidly as the $BeF_3^-$-free $E_1Ca^{2+}$ state. Note also that the 110-kDa ATPase chain degradation is much slower and formation of the rapidly degrading p81/p83 fragments is much less in 30 mM $Mg^{2+}$ than without $Mg^{2+}$. The results show that $E_2\cdot BeF_3^-$ in C12E8 and $Ca^{2+}$ is resistant to the luminal $Ca^{2+}$-induced reverse conversion to $E_1Ca^{2+}\cdot BeF_3^-$, which can be interpreted as very slow $Ca^{2+}$ binding to luminal transport sites and what slow conversion occurs is markedly retarded by 30 mM $Mg^{2+}$. These results accord with those using superfluorescence as the indicator in Fig. 5. In the presence of A23187, as seen in Fig. 6a, formation of the p81/p83 fragments from $E_2\cdot BeF_3^-$ in $Ca^{2+}$ occurs without any p95 fragment, as with $E_1Ca^{2+}$ and $E_1Ca^{2+}\cdot BeF_3^-$ in A23187 (cf. Fig. 6b) indicating a fast conversion of $E_2\cdot BeF_3^-$ to $E_1Ca^{2+}\cdot BeF_3^-$ without the detergent and with the ionophore. These results together with the retardation by $Mg^{2+}$ of loss of TNP-AMP superfluorescence (Fig. 5) indicate that $E_1Ca^{2+}\cdot BeF_3^-$ is formed from $E_2\cdot BeF_3^-$ without detergent on luminal $Ca^{2+}$ binding and further decomposed to $E_1Ca^{2+}$, and that $Mg^{2+}$ at a high concentration.

Figure 4. Hydrophobic property at nucleotide/catalytic site in $E_2\cdot BeF_3^-$ revealed by TNP-AMP superfluorescence. $E_2\cdot BeF_3^-$ or the $BeF_3^-$-free $Ca^{2+}$-ATPase ($E_2$) in SR vesicles (0.06 mg protein/ml) were incubated at 25 °C for 3 min in 0.5 mM EGTA, 30 mM MES/Tris (pH 6.0) or MOPS/Tris (pH 7.4), 0.1 M KCl or LiCl, and 0–60 mM MgCl2 with or without 0.02 mg/ml C12E8 and/or 2.5 μM A23187, as indicated in the figure. Subsequently, TNP-AMP at saturating 4 μM was added. The fluorescence intensity was obtained by subtracting the protein background level without TNP-AMP and the level of 4 μM TNP-AMP without SR vesicles, and plotted versus $Mg^{2+}$ concentration.
The concentration retards the decomposition of E1Ca2·BeF3− to E1Ca2 probably by inhibiting the Ca2+-replacement of Mg2+ at the unoccluded catalytic subsite17.

**Forward conversion of E1Ca2·BeF3− to E2·BeF3− is favored in C12E8.** Also in Fig. 6b, it can be seen that under conditions where E1Ca2·BeF3− is perfectly stable in A2318717, the addition of C12E8 in place of A23187 produces the same proteolytic pattern as developed with E2·BeF3− in C12E8 and C2±. The results reveal that the E2·BeF3− state is produced and stabilized in C12E8 even under conditions that perfectly stabilize E1Ca2·BeF3− in the absence of C12E8. This was further verified by superfluorescence development and loss upon TNP-AMP addition in Fig. 6c, which was performed on the basis of previous findings17 that E1Ca2·BeF3− rapidly decomposes to the non-fluorescent E1Ca2 state upon TNP-AMP binding whereas E2·BeF3− with bound TNP-AMP is stable, and also that the superfluorescence intensity is greater in E2·BeF3− than in E1Ca2·BeF3− (by approximately 25%). In Fig. 6c, E1Ca2·BeF3− was first formed under the conditions in Fig. 6b without A23187 and C12E8, and then A23187 or C12E8 added. After 10 s, superfluorescence upon TNP-AMP addition was recorded. In A23187 or in its absence, superfluorescence development is followed by its rapid loss, which is due to E1Ca2·BeF3− decomposition to E1Ca2 on TNP-AMP binding17. In C12E8, greater superfluorescence develops and its loss is considerably slower than in A23187. The results show again that in C12E8, E2·BeF3− is formed even under conditions that perfectly stabilize E1Ca2·BeF3− (although E2·BeF3− is slowly decomposed to the non-fluorescent E1Ca2 state via E1Ca2·BeF3− in high Ca2+ and decomposition by TNP-AMP).

**E2P hydrolysis.** In Fig. 7, the effects of C12E8, K+, and Mg2+ on the forward E2P hydrolysis rate were examined at pH 7.4 and 6.0. Here E2P was first formed in the reverse reaction of hydrolysis from the Ca2+-deprived E2 state and 32P1 in 7 mM Mg2+ without or with C12E8 (or with A23187) in 20% (v/v) Me2SO, conditions that favor E2P formation. Then hydrolysis was initiated by a 20-fold dilution in non-radioactive P1, various concentrations of Mg2+, and 0.1 M K+ (Fig. 7a) or Li+ (Fig. 7b) at the desired pH. In K+ at pH 7.4, C12E8 markedly retards hydrolysis as found previously at pH 7.537, and increasing Mg2+ concentration in C12E8 hardly affects the rate (perhaps a slight increase), but the cation decreases the rate in the absence of C12E8. Because this decrease is observed both without and with A23187 (an ionophore for Ca2+ and Mg2+) and because Me2SO (used for the P1-induced E2P formation) does not permeabilize the SR membrane, the hydrolysis reaction rate itself is likely affected by Mg2+ at the cytoplasmic side. At pH 6.0 in K+, hydrolysis is much slower than at pH 7.4, as is well known38, and C12E8 and Mg2+ have almost no effect on the slowed rate.

In the absence of K+ (Fig. 7b), E2P hydrolysis at both pH 7.4 and 6.0 is much slower than in 0.1 M K+ (by ~10-fold at the respective pH), in agreement with the well-known acceleration of hydrolysis by specific K+ binding on the P domain39,21. In the absence of K+, hydrolysis in C12E8 is only slightly slower than that without C12E8. Mg2+ at ~10 mM somewhat increases the rate although the rate is still much slower than that in the presence of K+. In summary, induction of the detergent-stabilized state strongly inhibits hydrolysis at pH 7.4, but not following protonation of the transport sites at pH 6.0, and only in the presence of K+.

**Discussion**

Ca2+ transport by Ca2+-ATPase includes phosphorylated intermediates where Ca2+ is occluded at the transport sites and then released to the lumen, i.e. E1P[Ca2+] → E2P + Ca2+. During this process the A domain swings
around and engages with the P domain and neck region of the protein at the cytoplasmic part of M2 (Fig. 1). The A-domain rotation inclines the P-domain by pulling an A/M1′-link, pushing M4 down towards the lumen to release the Ca2+18,19. There is evidence that the gathering and interaction of A and P domains at the cytoplasmic part of M2 occurs progressively. Namely, changes, which are linked to deocclusion and opening of the luminal access channel with an affinity reduction, are followed by constrictions to limit access, protonation, and finally closure, and all these changes are synchronized with catalytic site preparations for hydrolysis 4,13,15,17–19. Part of the development is seen with the Leu119 prtK cleavage site, being exposed in E2PCa2, hidden in E2P, E2~P‡ and E2·Pi, and exposed again in E24,18,19. We found here that non-solubilizing concentrations of C12E8 uncovers the Leu119 prtK site of E2P, as depicted in its analog E2·BeF3−. This indicates that membrane perturbation drives the

---

Figure 6. Luminal Ca2+-effect on E2-BeF3− in C2E2 (a) and formation and stabilization of E2-BeF3− in forward conversion from E1Ca2-BeF3− in C2E2 (b,c). (a) E2-BeF3− in SR vesicles was incubated without or with 0.15 mg/ml C2E2 or with 15 μM A23187 at 25°C for 3 min in 30 mM MOPS/Tris (pH 7.4), 0.1 M LiCl, 0.5 mM EGTA, and 0 (upper panel) or 30 mM MgCl2 (lower panel), then Ca2+ was added to give 0.5 mM free concentration. After 10 s, prtK was added at 0.5 mg/ml and incubated for indicated times. As a control, the BeF3−-free Ca2+-ATPase in SR vesicles (“E1Ca2”) was subjected to the proteolysis in 0.5 mM free Ca2+. (b) The prtK proteolysis was performed under the conditions that produce and perfectly stabilize E1Ca2·BeF3−17, i.e. 30 mM MOPS/Tris (pH 7.0), 0.1 M KCl, 15 mM MgCl2, and 0.7 mM CaCl2 in the presence of 100 μM BeCl2 and 2 mM KF without and with 15 μM A23187, and the effect of C2E2 was examined by including C2E2 without A23187, otherwise as in (a). The BeF3−-free Ca2+-ATPase (“E1Ca2”) in A23187 and in C2E2 was subjected to proteolysis otherwise as above. Note that the slow decomposition of E2-BeF3− in Ca2+ in the absence of A23187 and C2E2 (a) is probably due to slow Ca2+ permeation into the SR vesicles lumen17. (c) E1Ca2-BeF3− was produced by incubating SR vesicles for 30 min with 100 μM BeCl2 and 2 mM KF in the absence of A23187 and C2E2 otherwise as in (b), then C2E2 or A23187 was added to give 0.02 mg/ml and 2.5 μM, respectively. At 10 s after this addition, TNP-AMP was added to give a saturating 4 μM, and the fluorescence monitored; trace b, without C2E2 and A23187; traces c and d, in A23187 and in C2E2, respectively. Trace e, the fluorescence monitored with E2-BeF3− in the presence of 2 mM EGTA without adding Ca2+. Trace a, the non-superfluorescent E1Ca2 level (BeF3−-free Ca2+-ATPase) in 4 μM TNP-AMP.
intermediate towards one more like that with bound Ca\(^{2+}\), and points to an earlier catalytic intermediate with a looser arrangement in the head region, as expected for early engagement of the rotated A domain. The responsiveness of E2P to membrane perturbation and the detergent-induced state to ligand binding (Ca\(^{2+}\), Mg\(^{2+}\), K\(^{+}\), H\(^{+}\), and TG) through changes in exposure of the Leu\(^{119}\) prtK site at the cytoplasmic part of M2 points to flexible and rather unstable forms. These properties are due most probably not only to its unoccupied transport sites and associated circle of negative charges, but also to a loose meeting of domains and neck region with largely unsecured interactions at the cytoplasmic part of M2. The downward thrust of M4 (by a full turn of an \(\alpha\)-helix\(^{8}\)), and associated circle of negative charges, but also to a loose meeting of domains and neck region with largely unsecured interactions at the cytoplasmic part of M2. The downward thrust of M4 (by a full turn of an \(\alpha\)-helix\(^{8}\)), together with M3, is probably partially stabilized by surrounding phospholipids and insertion of non-ionic detergent between them could be disruptive. In the head region the interactions at Leu\(^{119}\) involve the formation of Y122-HC, a hydrophobic interaction network of Tyr122/Leu119 with the A and P domains and A/M3-linker involving seven residues (Fig. 2). As mentioned above, the interactions are likely progressive, loose at first as the A domain engages followed by incremental tightening in E2P to the fully stabilized state in E2P-\(\text{P}_{\alpha}\) and E2-P\(^{2+}\). Indeed, in the E2-BeF\(^{-}\) crystal structures (formed in the presence of C\(_{12}\)E\(_{8}\)) with the bound Mg\(^{2+}\) or with protonation without the Mg\(^{2+}\), Leu\(^{119}/\text{Tyr}^{122}\) on M2 are close but not yet associated with the five other gathered residues involved in Y122-HC formation. The knitting of Leu\(^{119}\) and Tyr\(^{122}\) with the other residues is seen in the crystal structures of analogs of the next intermediates, E2-P\(^{2+}\) and E2-P\(^{-}\). Accumulating interactions fit perfectly with the staggered changes at the luminal transport sites, from closed to open to closed again.

Stabilization of the early detergent-induced state is seen in the forward direction of catalysis coming from E1PCa\(_{2}\), (E1PCa\(_{2}\)-BeF\(^{3-}\)) and in the backward direction with Ca\(^{2+}\) binding to the luminal sites of E2P (E2-BeF\(^{3-}\)), using both TNP-AMP superfluorescence and the prtK sites as probes. Our results suggest that the E2-BeF\(^{3-}\) structural state favored in C\(_{12}\)E\(_{8}\) and stabilized by Mg\(^{2+}\) represents one between E1PCa\(_{2}\) and Ca\(^{2+}\)-released E2P, i.e. the transient E2P state immediately following Ca\(^{2+}\) release denoted as E2P\(^{−}\) with luminaly open and vacant low affinity transport sites (E2P\(^{−}\)Ca\(_{2}\rightarrow\)E2P\(^{−}\) in Fig. 8). C\(_{12}\)E\(_{8}\) stabilizes the E2P\(^{−}\) state and thereby retards both the luminal Ca\(^{2+}\)-induced reverse conversion and the forward hydrolysis of E2P at pH 7.4. Mg\(^{2+}\) binding probably prevents luminal Ca\(^{2+}\)-access and consequent reverse conversion (Figs 3, 5 and 6). This Mg\(^{2+}\) is likely at or near the luminaly open Ca\(^{2+}\) transport sites (in addition to Mg\(^{2+}\) occluded at the catalytic subsite in E2-BeF\(^{3-}\) and E2P) as actually seen in the E2-BeF\(^{3-}\) crystal produced in a high concentration of Mg\(^{2+}\). The Mg\(^{2+}\) probably

Figure 7. Effects of C\(_{12}\)E\(_{8}\) and various factors on E2P hydrolysis. SR vesicles were phosphorylated with 0.1 mM \(\text{P}_{\alpha}\), at 25°C for 10 min in 5 μl of a mixture containing 0.3 mg protein/ml with or without 3 μM A23187 as indicated, 1 mM EGTA, 7 mM MgCl\(_{2}\), 30 mM MOPS/Tris (pH 7.4) or MES/Tris (pH 6.0), and 20% (v/v) Me\(_{2}\)SO. The mixture was then cooled, and a small volume of C\(_{12}\)E\(_{8}\) was added to give 0.1 mg/ml (1/3 (w/w) of luminal Ca\(^{2+}\) 0.1 mM \(\text{P}_{\alpha}\) at 25 °C for 10 min in 5 μl of a mixture containing 0.3 mg protein/ml with or without 3 μM A23187 as indicated, 1 mM EGTA, 7 mM MgCl\(_{2}\), 30 mM MOPS/Tris (pH 7.4) or MES/Tris (pH 6.0), and 20% (v/v) Me\(_{2}\)SO. The mixture was then cooled, and a small volume of C\(_{12}\)E\(_{8}\) was added to give 0.1 mg/ml (1/3 (w/w) of the protein) to the indicated samples. Subsequently, the samples were diluted at 0 °C by the addition of 95 μl of a mixture containing 0.1 mM non-radioactive \(\text{P}_{\alpha}\), 105 mM KCl (Fig. 7a) or LiCl (Fig. 7b), 1 mM EGTA, 1–30 mM MgCl\(_{2}\), and 50 mM MOPS/Tris (pH 7.4) or MES/Tris (pH 6.0), as indicated with different symbols. The E2P hydrolysis rate was determined as described under “METHODS” and plotted versus Mg\(^{2+}\) concentration. Note the difference in the scale of the ordinate in (a) and (b).
manifests itself in the competitive inhibition by Mg\(^{2+}\) of luminal Ca\(^{2+}\)-induced reverse isomerization E2P + 2Ca\(^{2+}\) → E1PCa\(_2\)\(^{29}\). Notably also, the dephosphorylated E1 state is able to accommodate one Mg\(^{2+}\) at the transport sites and forms E1-Mg, which favors high affinity Ca\(^{2+}\)-binding resulting in a rapid E2 → E1-Mg → E1Ca\(_2\) transition\(^{10,11}\) (Fig. 1). Thus it seems that Mg\(^{2+}\) binds at the empty transport sites both in the unphosphorylated and phosphorylated states and modifies transport function.

The E2-BeF\(_3\)\(^{-}\) structures revealed in C\(_{12}\)E\(_8\) and in A23187 at pH 7.4 reflect E2P\(^{+}\) and E2P respectively in Fig. 8 on the basis ofprtK-resistance. Analysis of the Mg\(^{2+}\) inhibition of luminal Ca\(^{2+}\)-induced reverse conversion of E2-BeF\(_3\) in Figs 5 and 6 indicates that Mg\(^{2+}\) accesses E2P\(^{+}\) with a much higher affinity than E2P. Thus the transport sites appear more open and accessible to Mg\(^{2+}\) on the luminal side in the Leu\(^{119}\)-site cleavable E2P\(^{+}\) state than in the prtK-resistant E2P ground state. In fact, in the E2-BeF\(_3\) crystal with bound Mg\(^{2+}\) at the transport sites, the sites are actually more open to the lumen than in the structure without Mg\(^{2+}\) (Fig. 2). Note also that in E2-AlF\(_3\) and E2-MgF\(_2\)\(^{2+}\) (E2-P\(^{−}\) and E2-P) and in E2-BeF\(_3\) with bound TG, Ca\(^{2+}\) cannot bind as the luminal gate is tightly closed\(^{3,4}\), and the Leu\(^{119}\)-site is completely resistant to prtK regardless of the presence of C\(_{12}\)E\(_8\) (Fig. 3).

These findings suggest that the structural change reflected by prtK resistance at Leu\(^{119}\) is associated with luminal gating, supporting the above conclusion that substantial luminal gate closure occurs in E2P → E2P, which probably involves gathering of Leu\(^{119}\)/Tyr\(^{122}\) on M2 with engaged A and P domains.

At pH 6.0 in which the transport sites are protonated, the Leu\(^{119}\)-site is completely resistant to prtK regardless of the presence of C\(_{12}\)E\(_8\) and the E2P hydrolysis rate is not affected by C\(_{12}\)E\(_8\). In Fig. 8, the protonated structural state with the prtK-resistant state in C\(_{12}\)E\(_8\) is denoted as E2P\(^{+}\)(*) to be discriminated from the prtK-cleavable E2P\(^{+}\) state without protonation. Protonation neutralizes charges at the Ca\(^{2+}\)-binding sites and stabilizes the arrangement of transmembrane helices via a hydrogen bonding network\(^5\), which lowers Ca\(^{2+}\)-accessibility (without out completely closing the gate as seen in the E2-BeF\(_3\) crystal formed at pH 5.7\(^{9}\)). The protonated state proceeds promptly to subsequent hydrolysis with tight gate closure E2P + H\(_2\)O → E2 - P\(^{−}\) (E2-BeF\(_3\) → E2-AlF\(_3\)), as indicated previously by kinetic analysis of E2P hydrolysis\(^5\).

K\(^{+}\) in the presence of C\(_{12}\)E\(_8\) accelerates both forward E2P hydrolysis and luminal Ca\(^{2+}\)-induced reverse conversion of E2-BeF\(_3\) (Figs 5 and 7). These findings are in complete agreement with the known role of specific K\(^{+}\) binding on the P domain in accelerating both forward hydrolysis\(^{20,21}\) and luminal Ca\(^{2+}\)-induced reverse conversion of E2P\(^{+}\)\(^{14}\). K\(^{+}\) binding likely destabilizes both E2P and E2P\(^{+}\) in Fig. 8, thus promoting rapid transport.
Finally, induction of the detergent-stabilized state, an early intermediate to ground state E2P, shows how phospholipids are intimately involved in the latter's stabilization. Membrane perturbation effects during the transport cycle may be under-appreciated as fundamental to the mechanism.

Methods

Preparation of SR vesicles and treatment with BeF$_2$, AlF$_3$, and MgF$_2$. SR vesicles were prepared from rabbit skeletal muscle as described$^{12-13}$, in which all the methods were carried out in accordance with institutional laws and regulations of the Asahikawa Medical University and the experimental protocols were approved by the Animal Experimentation Ethics Committee of the Asahikawa Medical University (license number 16006). The content of the phosphorylation site in the vesicles and the Ca$^{2+}$-dependent ATPase activity were determined as described$^{12,13}$. E$_2$-BeF$_2$-, E$_2$-AlF$_3$-, and E$_2$-MgF$_2$ were produced by incubating the SR vesicles with the respective metal fluoride and by washing the unbound ligands as described previously$^4$.

**Formation and hydrolysis of E2P.** The SR vesicles were phosphorylated with 0.1 mM $^{32}$P, at 25°C for 10 min in 20% (v/v) Me$_2$SO in the absence of Ca$^{2+}$, after which the samples were cooled and diluted 20-fold by a solution containing 2.1 mM non-radioactive P$_i$ to initiate the hydrolysis of $^{32}$P-labeled E2P, otherwise as described in detail in the legend to Fig. 7. The reaction was quenched with ice-cold trichloroacetic acid containing P$_i$. The precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn$^{14}$. The radioactivity associated with the separated Ca$^{2+}$-ATPase was quantified by digital autoradiography as described$^{15}$. Rapid kinetics measurement of hydrolysis was performed with a handmade rapid mixing apparatus and the rate of hydrolysis was determined with the least-squares fit to a single exponential, as described$^{16}$.

**Proteolytic analysis.** SR vesicles (0.45 mg/ml protein) were subjected to proteolysis at 25°C by addition of trypsin (0.3 mg/ml, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) or proteinase K (prtK, at 0.1 mg/ml, Sigma) as described previously$^{16,17}$, otherwise as indicated in the figure legends. The proteolysis was terminated by trichloroacetic acid, and the samples were subjected to Laemmli SDS-polyacrylamide gel electrophoresis$^{36}$, and densitometric analyses of the gels stained with Coomassie Brilliant Blue R-250, as described$^{6,16}$. The degradation rate of 110-kDa ATPase chain with prtK was determined by least-squares fit of a single exponential, as described in detail in the legend to Fig. 7. The reaction was quenched with ice-cold trichloroacetic acid containing P$_i$. The precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn$^{14}$. The radioactivity associated with the separated Ca$^{2+}$-ATPase was quantified by digital autoradiography as described$^{15}$. Rapid kinetics measurement of hydrolysis was performed with a handmade rapid mixing apparatus and the rate of hydrolysis was determined with the least-squares fit to a single exponential, as described$^{16}$.

**Fluorescence measurements.** The TNP-AMP fluorescence of the Ca$^{2+}$-ATPase (0.06 mg/ml protein, TNP-AMP from Molecular Probes® Life Technologies) was measured on a RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths 408 and 540 nm (with band widths 5 and 10 nm), as described previously$^4$.

**Miscellaneous.** Protein concentrations were determined by the method of Lowry et al.$^{37}$ with bovine serum albumin as a standard. Three-dimensional models of the enzyme were reproduced by the program VMD$^{38}$. The values presented are the mean ± s.d. (n = 3–4).

References

1. Toyoshima, C. Structural aspects of ion pumping by Ca$^{2+}$-ATPase of sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 476, 3–11 (2008).
2. Toyoshima, C. How Ca$^{2+}$-ATPase pumps ions across the sarcoplasmic reticulum membrane. *Biochim. Biophys. Acta* 1793, 941–946 (2009).
3. Moller, J. V., Olesen, C., Winther, A.-M. L. & Nissen, P. The sarcoplasmic Ca$^{2+}$-ATPase: design of a perfect chemiosmotic pump. *Q. Rev. Biophys.* 43, 501–566 (2010).
4. Danko, S., Yamasaki, K., Daiho, T. & Suzuki, H. Distinct natures of beryllium fluoride-bound, aluminum fluoride-bound, and magnesium fluoride-bound stable analogues of an ADP-insensitive phosphoenzyme intermediate of sarcoplasmic reticulum Ca$^{2+}$-ATPase. *J. Biol. Chem.* 279, 14991–14998 (2004).
5. Seekoe, T., Peali, S. & McIntosh, D. B. Thapsigargin and dimethyl sulfoxide activate medium Pi-HOH oxygen exchange catalyzed by sarcoplasmic reticulum Ca$^{2+}$-ATPase. *J. Biol. Chem.* 276, 46737–46744 (2001).
6. Danko, S., Yamasaki, K., Daiho, T., Suzuki, H. & Toyoshima, C. Organization of cytoplasmic domains of sarcoplasmic reticulum Ca$^{2+}$-ATPase in E$_2$P and E$_2$ATP states: a limited proteolysis study. *FEBS Lett.* 505, 129–135 (2001).
7. Toyoshima, C., Nomura, H. & Tsuda, T. Luminal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. *Nature* 432, 361–368 (2004).
8. Toyoshima, C., Norimatsu, Y., Iwasawa, S., Tsuda, T. & Ogawa, H. How processing of aspartylphosphate is coupled to lumenal gating of the ion pathway in the calcium pump. *Proc. Natl. Acad. Sci. USA* 104, 19831–19836 (2007).
9. Daiho, T., Yamasaki, K., Danko, S. & Suzuki, H. Second transmembrane helix (M2) and long range coupling in Ca$^{2+}$-ATPase. *J. Biol. Chem.* 288, 35241–35252 (2014).
10. Olesen, C., Sorensen, T. L., Nielsen, R. C., Moller, J. V. & Nissen, P. Dephosphorylation of the calcium pump coupled to counterion occlusion. *Science* 306, 2251–2255 (2004).
11. Olesen, C. et al. The structural basis of calcium transport by the calcium pump. *Nature* 450, 1036–1042 (2007).
12. Yamasaki, K., Daiho, T., Danko, S. & Suzuki, H. Multiple and distinct effects of mutations of Tyr$^{122}$, Glu$^{131}$, Arg$^{224}$, and Arg$^{234}$ involved in interactions between the top part of second and fourth transmembrane helices in sarcoplasmic reticulum Ca$^{2+}$-ATPase. *J. Biol. Chem.* 279, 2202–2210 (2004).
13. Wang, G., Yamasaki, K., Daiho, T. & Suzuki, H. Critical hydrophobic interactions between phosphorylation and actuator domains of Ca$^{2+}$-ATPase for hydrolysis of phosphorylated intermediate. *J. Biol. Chem.* 280, 26508–26516 (2005).
14. Yamasaki, K., Wang, G., Daiho, T., Danko, S. & Suzuki, H. Roles of Tyrs$^{122}$-hydrophobic cluster and K+ binding in Ca$^{2+}$-releasing process of ADP-insensitive phosphoenzyme of sarcoplasmic reticulum Ca$^{2+}$-ATPase. *J. Biol. Chem.* 283, 29144–29155 (2008).
15. Yamasaki, K., Daiho, T., Danko, S. & Suzuki, H. Assembly of a Tyr$^{122}$ hydrophobic cluster in sarcoplasmic reticulum Ca$^{2+}$-ATPase synchronizes Ca$^{2+}$ affinity reduction and release with phosphoenzyme isomerization. *J. Biol. Chem.* 290, 27858–27879 (2015).
16. Danko et al. ADP-insensitive phosphoenzyme intermediate of sarcoplasmic reticulum Ca$^{2+}$-ATPase has a compact conformation resistant to proteinase K, V8 protease and trypsin. *FEBS Lett.* 489, 277–282 (2001).
Springer Nature remains neutral with regard to jurisdictional claims in published maps and geographic names and does not endorse the views expressed by authors. The publisher does not accept responsibility for statements of fact or opinion by contributors.

How to cite this article: Danko, S. et al. Membrane Perturbation of ADP-insensitive Phosphoenzyme of Ca\(^{2+}\)-ATPase Modifies Gathering of Transmembrane Helix M2 with Cytoplasmic Domains and Luminal Gating. Sci. Rep. 7, 41172; doi: 10.1038/srep41172 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017

Acknowledgements
We thank Dr. David B. McIntosh for improving our manuscript. This work was supported by JSPS KAKENHI Grant Number JP15H04346.

Author Contributions
S.D. and H.S. conceived and coordinated the study and wrote the paper. S.D., K.Y. and H.S. designed, performed and analyzed the experiments. T.D. provided critical discussion. All authors reviewed the results and approved the final version of the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Danko, S. et al. Membrane Perturbation of ADP-insensitive Phosphoenzyme of Ca\(^{2+}\)-ATPase Modifies Gathering of Transmembrane Helix M2 with Cytoplasmic Domains and Luminal Gating. Sci. Rep. 7, 41172; doi: 10.1038/srep41172 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

17. Danko, S., Daiko, T., Yamasaki, K., Liu, X. & Suzuki, H. Formation of the stable structural analog of ADP-sensitive phosphoenzyme of Ca\(^{2+}\)-ATPase with occluded Ca\(^{2+}\) by beryllium fluoride. J. Biol. Chem. 284, 22722–22735 (2009).
18. Daiko, T., Yamasaki, K., Danko, S. & Suzuki, H. Critical role of Glu\(^{40}\)-Ser\(^{48}\) loop linking actuator domain and first transmembrane helix of Ca\(^{2+}\)-ATPase in Ca\(^{2+}\) deocclusion and release from ADP-insensitive phosphoenzyme. J. Biol. Chem. 282, 34429–34447 (2007).
19. Daiko, T., Danko, S., Yamasaki, K. & Suzuki, H. Stable structural analog of Ca\(^{2+}\)-ATPase ADP-insensitive phosphoenzyme with occluded Ca\(^{2+}\) formed by elongation of A-domain/M1’-linker and beryllium fluoride binding. J. Biol. Chem. 285, 24538–24547 (2010).
20. Shigekawa, M. & Pearl, L. I. Activation of calcium transport in skeletal muscle sarcoplasmic reticulum by monovalent cations. J. Biol. Chem. 251, 6947–6952 (1976).
21. Sorensen, T. L. et al. Localization of a K\(^{+}\)-binding site involved in dephosphorylation of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. J. Biol. Chem. 279, 46355–46358 (2004).
22. Inesi, G., Lewis, D., Toyoshima, C., Hirata, A. & de Meis, L. Conformational fluctuations of the Ca\(^{2+}\)-ATPase in the native membrane environment. Effects of pH, temperature, catalytic substrates, and thapsigargin. J. Biol. Chem. 283, 1189–1196 (2008).
23. Toyoshima, C. & Nomura, H. Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 418, 605–611 (2002).
24. Kato, S. et al. Val\(^{200}\) residue in Lys\(^{198}\)–Lys\(^{202}\) outermost loop on the A domain of sarcoplasmic reticulum Ca\(^{2+}\)–ATPase is critical for rapid processing of phosphoenzyme intermediate after loss of ADP sensitivity. J. Biol. Chem. 278, 9624–9629 (2003).
25. Dupont, Y. & Pougeois, E. Evaluation of H\(_2\)O activity in the free or phosphorylated catalytic site of Ca\(^{2+}\)–ATPase. FEBS Lett. 156, 93–98 (1983).
26. Toyoshima, C., Yonekura, S., Tueda, J. & Iwasawa, S. Trinitrophenyl derivatives bind differently from parent adenine nucleotides to Ca\(^{2+}\)-ATPase in the absence of Ca\(^{2+}\). Proc. Natl. Acad. Sci. USA 108, 18338–18338 (2011).
27. Champaigne, P. et al. Kinetic characterization of the normal and detergent–perturbed reaction cycles of the sarcoplasmic reticulum calcium pump. Rate-limiting step(s) under different conditions. J. Biol. Chem. 261, 16372–16384 (1986).
28. Wakabayashi, S., Ogurusu, T. & Shigekawa, M. Modulation of the hydrolysis rate of the ADP-insensitive phosphoenzyme of the sarcoplasmic reticulum ATPase by H\(^{+}\) and Mg\(^{2+}\). J. Biol. Chem. 262, 9121–9129 (1987).
29. Bishop, J. E. & Al-Shawi, M. K. Inhibition of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase by Mg\(^{2+}\) at high pH. J. Biol. Chem. 263, 1886–1892 (1988).
30. Toyoshima, C. et al. Crystal structures of the calcium pump and sarcolipin in the Mg\(^{2+}\)–bound E1 state. Nature 495, 260–264 (2013).
31. Winther, A. M. et al. The sarcolipin-bound calcium pump stabilizes calcium sites exposed to the cytoplasm. Nature 495, 265–269 (2013).
32. Nakamura, S., Suzuki, H. & Kanazawa, T. The ATP-induced change of tryptophan fluorescence reflects a conformational change upon formation of ADP-sensitive phosphoenzyme in the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. Stopped-flow spectrofluorometry and continuous flow–rapid quenching method. J. Biol. Chem. 269, 16015–16019 (1994).
33. Barrabin, H., Scofano, H. M. & Inesi, G. Adenosinetriphosphatase site stoichiometry in sarcoplasmic reticulum vesicles and purified enzyme. Biochemistry 23, 1542–1548 (1984).
34. Weber, K. & Osborn, M. The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4066–4142 (1969).
35. Daiko, T., Suzuki, H., Yamasaki, K., Saino, T. & Kanazawa, T. Mutations of Arg\(^{198}\) in sarcoplasmic reticulum Ca\(^{2+}\)-ATPase cause inhibition of hydrolysis of the phosphoenzyme intermediate formed from inorganic phosphate. FEBS Lett. 444, 54–58 (1999).
36. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970).
37. Lowery, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951).
38. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. J. Mol. Graph. 14, 33–38 (1996).
39. Juul, B. et al. Do transmembrane segments in proteolyzed sarcoplasmic reticulum Ca\(^{2+}\)-ATPase retain their functional Ca\(^{2+}\) binding properties after removal of cytoplasmic fragments by proteinase K? J. Biol. Chem. 270, 20123–20134 (1995).
40. Brandl, C. J., Green, N. M., Korczak, B. & MacLennan, D. H. Two Ca\(^{2+}\)-ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. Cell 44, 597–607 (1986).