Ca\(^{2+}\) Release to Lumen from ADP-sensitive Phosphoenzyme E1P\(\text{Ca}_2\) without Bound K\(^+\) of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase*>

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During Ca\(^{2+}\) transport by sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, the conformational change of ADP-sensitive phosphoenzyme (E1P\(\text{Ca}_2\)) to ADP-insensitive phosphoenzyme (E2P\(\text{Ca}_2\)) is followed by rapid Ca\(^{2+}\) release into the lumen. Here, we find that in the absence of K\(^+\), Ca\(^{2+}\) release occurs considerably faster than E1P\(\text{Ca}_2\) to E2P\(\text{Ca}_2\) conformation change. Therefore, the lumenal Ca\(^{2+}\) release pathway is open to some extent in the K\(^+\)-free E1P\(\text{Ca}_2\) structure. The Ca\(^{2+}\) affinity of this E1P is as high as that of the unphosphorylated ATPase (E1), indicating the Ca\(^{2+}\) binding sites are not disrupted. Thus, bound K\(^+\) stabilizes the E1P\(\text{Ca}_2\) structure with occluded Ca\(^{2+}\), keeping the Ca\(^{2+}\) pathway to the lumen closed. We found previously (Yamasaki, K., Wang, G., Daiho, T., Danko, S., and Suzuki, H. (2008) J. Biol. Chem. 283, 29144–29155) that the K\(^+\) binding in E2P reduces the Ca\(^{2+}\) affinity essential for achieving the high physiological Ca\(^{2+}\) gradient and to fully open the lumenal Ca\(^{2+}\) gate for rapid Ca\(^{2+}\) release (E2P\(\text{Ca}_2\) \(\rightarrow\) E2P + 2Ca\(^{2+}\)). These findings show that bound K\(^+\) is critical for stabilizing both E1P\(\text{Ca}_2\) and E2P structures, thereby contributing to the structural changes that efficiently couple phosphoenzyme processing and Ca\(^{2+}\) handling.

Sarcoplasmic reticulum (SR)\(^2\) Ca\(^{2+}\)-ATPase (SERCA1a) catalyzes Ca\(^{2+}\) transport coupled with ATP hydrolysis against an ~10,000-fold concentration gradient (1–9). The ATPase is first activated by the binding of two cytoplasmic Ca\(^{2+}\) ions at the transport sites with a submicromolar high affinity (E2 to E1\(\text{Ca}_\alpha\), see step 1 in Fig. 1) and then autophosphorylated at Asp\(^{351}\) by ATP to form a phosphoenzyme intermediate (E\(P\)) (step 2). This E\(P\) is “ADP-sensitive” (E1P) because it is rapidly dephosphorylated by ADP in the reverse reaction. Upon E1P formation, the bound Ca\(^{2+}\) ions are occluded in the transport sites (E1P\(\text{Ca}_2\)). Subsequently, E1P\(\text{Ca}_2\) undergoes its isomeric transition to an ADP-insensitive form (E2P), i.e. loss of ADP sensitivity, which results in a large reduction of Ca\(^{2+}\) affinity and opening of the lumenal release gate, i.e. Ca\(^{2+}\) deocclusion and release (steps 3–4). Ca\(^{2+}\) release in step 4 is very rapid, so that an E2P\(\text{Ca}_2\) intermediate state does not accumulate and in fact had never been found until we recently established its existence (10–13) and successfully trapped it for the first time (14). Finally, E2P is hydrolyzed back to the inactive E2 form (step 5).

In E1P\(\text{Ca}_2\) \(\rightarrow\) E2P + 2Ca\(^{2+}\), the A domain rotates parallel to the membrane plane and the P domain inclines to the A domain, thereby associating with each other to produce a compactly organized and inclined headpiece (15–27). This tight structure is stabilized by critical interaction networks between the A and P domains at three regions (10–14) (see Fig. 9 for details). The rotation and inclination of the domains result in motions and rearrangements of the transmembrane helices thereby disrupting the Ca\(^{2+}\) sites and opening the luminal gate. In the P domain, there is a specific K\(^+\) binding site (28); K\(^+\) binding here is crucial for rapid hydrolysis of E2P (28–30). Recently, we further found (13) that the K\(^+\) in E2P is critical for reducing the luminal Ca\(^{2+}\) affinity that is required to achieve the high physiological Ca\(^{2+}\) gradient and for rapid Ca\(^{2+}\) release (E2P\(\text{Ca}_2\) \(\rightarrow\) E2P + 2Ca\(^{2+}\)). Thus, bound K\(^+\) contributes to stabilization of the compactly organized and inclined E2P structure with its disrupted Ca\(^{2+}\) sites and fully opened luminal gate, probably by cross-linking the P domain with the A domain/M3-linker (13).

Despite these findings on the Ca\(^{2+}\) release process and E2P, a possible role for K\(^+\) in E1P\(\text{Ca}_2\) has not been explored. The K\(^+\) site is situated at the bottom of the P-domain near the cytoplasmic ends of the transmembrane helices. Therefore, the lack of K\(^+\) binding might have a serious effect on the stability of the helices and Ca\(^{2+}\) handling in E1P\(\text{Ca}_2\). The E2-E1\(\text{Ca}_\alpha\) transition is markedly retarded, and its equilibrium is affected by the absence of K\(^+\) (31–33).

In this study, we explore a possible role of K\(^+\) in E1P\(\text{Ca}_2\) especially in regard to Ca\(^{2+}\) occlusion. Results reveal that K\(^+\)-free E1P\(\text{Ca}_2\) has an open Ca\(^{2+}\) pathway to the lumen. Thus, the Ca\(^{2+}\) binding sites face the lumen, and Ca\(^{2+}\) can be released. The absence of K\(^+\) does not reduce the high Ca\(^{2+}\) affinity (Ca\(^{2+}\) site coordination probably unchanged), and yet the cytoplasmic gate is closed, and the luminal gate is open. These changes probably do not involve large motions of the cytoplasmic domains and transmembrane helices. Therefore, bound K\(^+\) likely stabilizes the Ca\(^{2+}\) occluded structure of E1P\(\text{Ca}_2\) by simply keeping the luminal Ca\(^{2+}\) pathway closed.

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2 The abbreviations used are: SR, sarcoplasmic reticulum; EP, phosphoenzyme; E1P, ADP-sensitive phosphoenzyme; E2P, ADP-insensitive phosphoenzyme; HC, hydrophobic interaction cluster; MOPS, 3-(N-morpholino)propanesulfonic acid.
The structural role of K\(^+\) in \(E1\)Ca\(_2\) is discussed in detail using crystal structures of Ca\(^{2+}\)-ATPase with bound K\(^+\).

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles—SR vesicles were prepared from rabbit skeletal muscle as described (34). The phosphorylation site content in the vesicles determined according to Barrabin et al. (35) was 4.49 ± 0.22 nmol/mg vesicle protein (n = 5).

Determination of EP—SR vesicles were phosphorylated with \([\gamma-\sp{32}P]ATP\) as described in the legends to Figs. 2–6. In the experiments performed in Figs. 2, aliquots of the reaction mixture were spotted on the HAWP membrane filter (Millipore) and washed continuously with a chasing solution for the periods indicated. At the end of chase, the reaction was terminated by washing with 0.1 M HCl. To determine the amount of \(E2P\) in the phosphorylation mixture, the membrane was washed with an ADP solution for 1 s and then with 0.1 M HCl. The membrane was dried, and the radioactivity was measured by digital autoradiography. In Figs. 3 and 6, total EP was measured by quenching the phosphorylation reaction (in a test tube) with 5% (v/v) ice-cold trichloroacetic acid containing \(P_i\), whereas for \(E2P\) determination, the reaction was chased with ADP for 1 s and quenched by addition of the trichloroacetic acid. The precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (36). The radioactivity associated with the separated Ca\(^{2+}\)-ATPase was quantitated by digital autoradiography (37). Rapid kinetic measurements in Fig. 3 were performed with a handmade rapid mixing apparatus (38).

Determination of Bound Ca\(^{2+}\)—In the experiments performed in Figs. 2 and 6, SR vesicles were incubated with \(46\)CaCl\(_2\) as per the figure legends, and an aliquot of reaction mixture was spotted on the HAWP membrane filter (Millipore). Then, the membrane was washed with a chasing solution for indicated time periods using a rapid filtration apparatus RFS-4 (Bio-Logic, Claix, France). To estimate nonspecific \(46\)Ca\(^{2+}\) binding, the same experiments were done in the presence of 1 \(\mu\)M thapsigargin. Specific \(46\)Ca\(^{2+}\) binding was obtained after subtracting this nonspecific binding.

Ca\(^{2+}\) Uptake into SR Vesicles in a Single Turnover of EP—In the experiments performed in Figs. 4 and 5, SR vesicles were incubated with \(46\)Ca\(^{2+}\), and a single turnover of EP was initiated by adding ATP and excess EGTA using the handmade rapid mixing apparatus. After chasing the reaction, the mixture was spotted on the membrane filter and washed for \(\sim 10\) s by an EGTA solution, as described in the figure legends. The background level of \(46\)Ca\(^{2+}\) was determined without ATP and subtracted. This background level was \(< 3\%\) of the maximum Ca\(^{2+}\) uptake level.

Miscellaneous—All of the reactions were performed at 4 °C in 7 mM MgCl\(_2\) and 50 mM MOPS/Tris (pH 7.3). Protein concentrations were determined by the method of Lowry et al. (39) with bovine serum albumin as a standard. Free Ca\(^{2+}\) concentrations were calculated by the Calcon program. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc., Northampton, MA). Three-dimensional models of the enzyme were produced by the program VMD (40).

RESULTS

Time Courses of EP Decay and Ca\(^{2+}\) Release—The Ca\(^{2+}\)-ATPase in SR vesicles was phosphorylated with MgATP in the presence of 0.1 M K\(^+\), 10 \(\mu\)M Ca\(^{2+}\), and Ca\(^{2+}\)-ionophore A23187 (Fig. 2, A and B). The reaction reaches steady state within a few seconds, and almost all of the Ca\(^{2+}\)-ATPase is in the ADP-sensitive form of EP (E1P) because of the rate-limiting E1P to E2P transition followed by rapid E2P hydrolysis in the presence of K\(^+\) (29, 30).

When the reaction was chased with excess EGTA in 0.1 M K\(^+\), the amount of EP decreases in a single exponential time course, and the EP during the decay is almost all ADP sensitive (Fig. 2A). The bound Ca\(^{2+}\) decreases concomitantly with E1P decay, i.e. E1PCa\(_2\) to E2P transition. The result agrees with the established mechanism that the two Ca\(^{2+}\) ions are occluded in E1PCa\(_2\), and Ca\(^{2+}\) release into the lumen occurs very rapidly after the rate-limiting E1PCa\(_2\) to E2PCa\(_2\) transition, E1PCa\(_2\) → E2PCa\(_2\) → E2P + 2Ca\(^{2+}\) (11–14). Thus, the EP transition and Ca\(^{2+}\) release are tightly coupled in the presence of K\(^+\).

Surprisingly, when E1PCa\(_2\) formed as above in 0.1 M K\(^+\), and A23187 was chased with excess EGTA in the absence of K\(^+\), the Ca\(^{2+}\) release is considerably (\(-3\times\)) faster than the E1P decay via its transition to E2P (Fig. 2B). The result shows that in the absence of K\(^+\), there is an E1P species without bound Ca\(^{2+}\) and that the Ca\(^{2+}\) ions are released from E1PCa\(_2\). We found essentially the same results in the presence of choline chloride in place of LiCl without K\(^+\) (data not shown).

\(46\)Ca\(^{2+}\) Uptake in Single Turnover of E1PCa\(_2\)—We then examined whether this rapid Ca\(^{2+}\) release from E1PCa\(_2\) in the absence of K\(^+\) upon the EGTA chase occurs to the lumenal side or cytoplasmic side of the membrane. For this purpose, we performed a \(46\)Ca\(^{2+}\) uptake assay in a single turnover of E1PCa\(_2\) in the absence of ionophore, i.e. with sealed SR vesicles. In Fig. 3, for the single turnover of E1PCa\(_2\), the Ca\(^{2+}\)-ATPase in the vesicles in 10 \(\mu\)M Ca\(^{2+}\) was phosphorylated by a simultaneous addition of \([\gamma-\sp{32}P]ATP\) and excess EGTA in either the presence or the absence of 0.1 M K\(^+\). Approximately half of the ATPase is phosphorylated rapidly to form E1PCa\(_2\) both in the presence and absence of K\(^+\), and then EP decays slowly, in contrast to the full phosphorylation achieved without the removal of Ca\(^{2+}\). In sealed vesicles (without A23187), EP decays at the same rate in the presence or absence of K\(^+\). In the presence of K\(^+\), nearly all EP is E1P (ADP-
Ca\(^{2+}\) Release from E1PCa\(_2\) of Ca\(^{2+}\)-ATPase

**A**

![Image](image_url)

**B**

![Image](image_url)

**FIGURE 2.** Time courses of EP decay and Ca\(^{2+}\) release in the presence or absence of 0.1 m K\(^+\). A, the Ca\(^{2+}\)-ATPase in SR vesicles (20 ¼g protein/ml) was phosphorylated for 10 s with 100 ¼M [\(^{32}\)P\]ATP in 10 ¼M nonradioactive CaCl\(_2\) (closed circles) or with 100 ¼M nonradioactive ATP in 10 ¼M 45CaCl\(_2\) (open circles), 0.1 m KCl, 3 m A23187. Then, 50 ¼I of the reaction mixture was spotted on the membrane and washed for the time periods on the abscissa with a chasing solution containing 1 mM EGTA, 0.1 m KCl, 3 m A23187. The amount of bound 45Ca\(^{2+}\) (open circles) and the total amount of EP (open triangles in inset) were determined. The amounts of E2P (closed triangles in inset) were determined by a subsequent washing by a solution containing 1 mM ADP, 1 mM EGTA, and 0.1 m KCl. The amount of E1P (closed circles) was calculated by subtracting the amount of E2P from total amount of EP. B, the Ca\(^{2+}\)-ATPase in SR vesicles was phosphorylated in the presence of 0.1 m KCl as in A and spotted on the filter. Then, the filter was washed with the EGTA solution (and subsequently with the ADP solution for the E2P determination) containing 0.1 m LiCl instead of KCl, otherwise as in A. Solid lines in A and B show the least squares fit to a single exponential. The rates (s\(^{-1}\)) for E2P decay and the Ca\(^{2+}\) release were 0.66 and 0.58 (A) and 0.21 and 0.81 (B), respectively.

**FIGURE 3.** EP formation and decay in a single turnover. All of the solutions contained 0.1 m KCl (closed symbols) or LiCl (open symbols). SR vesicles (20 ¼g/ml) were incubated in 10 ¼M CaCl\(_2\), and EP formation was initiated by mixing with an equal volume of a solution containing 20 ¼M [\(^{32}\)P\]ATP and 10 mM EGTA (triangles and squares) or 10 mM CaCl\(_2\) (circles). The total amount of EP was determined with the addition of trichloroacetic acid (circles and triangles). To determine the amount of E2P (squares), the phosphorylated sample was mixed with an equal volume of a solution containing 2 mM ADP and 5 mM EGTA, and then the reaction was terminated by trichloroacetic acid at 1 s after the ADP addition.

**FIGURE 4.** Uptake—In Fig. 4 (open circles), we assessed at each time point during the single turnover of E1PCa\(_2\) the amount of 45Ca\(^{2+}\) remaining on the filter with the vesicles. For this purpose, we chased the reaction with ADP and excess EGTA at each time point, i.e. dephosphorylating to E1Ca\(_2\) very rapidly in the reverse reaction and removing 45Ca\(^{2+}\) released to the cytoplasmic side. Both in the presence and absence of K\(^+\), at 0.1 s (first time point) immediately after the ATP/EGTA addition, nearly maximum EP is already formed (all E1PCa\(_2\), Fig. 3), and all of the bound 45Ca\(^{2+}\) is removed by the ADP chase. Then, in the presence of 0.1 m K\(^+\) (A), the ADP-insensitive fraction and the amount of 45Ca\(^{2+}\) released into the lumen increased exponentially due to the forward E1PCa\(_2\) decay via its transition to E2P with Ca\(^{2+}\) release as expected from the established transport mechanism. In fact, the time course agreed with that of EP decay via the rate-limiting E1PCa\(_2\) to E2P transition (Fig. 3, closed triangles).

On the other hand, in the absence of K\(^+\) (B), 45Ca\(^{2+}\), and the ADP-insensitive fraction increase very rapidly (within the initial ~0.5 s) and suddenly slow, showing a clear biphasic time course. The second slow phase occurs at nearly the same...
Ca²⁺ Release from E1P Ca²⁺-ATPase

The rate as the EP decay via the E1P to E2P transition (Fig. 3, open triangles) and the single exponential ⁴⁵Ca²⁺ uptake in the presence of K⁺ (A), i.e. the normal transport process E1P → E₂P, occurs at a significantly faster rate and to a higher extent than in E2P formation (Fig. 3) and therefore cannot be accounted for simply by formation of E2P. Actually, the initial phase is even faster than the Ca²⁺ release from K⁺-free E1P, revealed upon excess EGTA addition (without ADP) in A23187 in Fig. 2. The results suggest that, in K⁺-free E1P, different types of Ca²⁺-sites are produced in the initial rapid phase; the Ca²⁺ ions are not released to the cytoplasmic side even upon ADP-induced reverse dephosphorylation.

Behavior of ⁴⁵Ca²⁺ at Site I in E1P.—We examined whether the above observed biphasic kinetics revealed by the ADP chase is related to the heterogeneity of the Ca²⁺-sites I and II in E1P. In E2P, Ca²⁺ bound at site II is rapidly exchanged with the cytoplasmic Ca²⁺, and the Ca²⁺ bound at the deeper site I can be released to the cytoplasm only when site II is vacant (15, 41–44). Therefore, we first labeled site I with ⁴⁵Ca²⁺ by exchanging the site II-bound ⁴⁵Ca²⁺ with nonradioactive Ca²⁺ (supplemental Fig. S1). In Fig. 5B, we clearly observed a biphasic ⁴⁵Ca²⁺ increase in the ADP-insensitive fraction in the absence of K⁺ as in Fig. 4B. The only difference is that, as expected, the total amount of ⁴⁵Ca²⁺ uptake (0.8 – 1.0 Ca²⁺ per EP) is half of that in Fig. 4 in which both sites I and II are labeled by ⁴⁵Ca²⁺. The results show that the heterogeneity of the two Ca²⁺-sites I and II in E1P is not related to the biphasic ⁴⁵Ca²⁺ increase revealed by the ADP chase in Fig. 4B. Furthermore, we observed a nonsequential release of two Ca²⁺ ions from E1P, to the lumenal side upon removal of free Ca²⁺ in the presence of A23187 without ADP, which therefore is not related to the biphasic ⁴⁵Ca²⁺ increase in Fig. 4B (supplemental Fig. S2) (58, 59).

These results show that there are two different types of E1P, i.e. the normal Ca²⁺-occluded E1P, and another E1P species that possesses lumen-facing Ca²⁺ binding sites (opened lumenal pathway) and a closed cytoplasmic gate. The results further indicate that in the absence of K⁺, the E1P species with the lumen-facing Ca²⁺ binding sites is rapidly produced from normal E1P, and this process is revealed by the ADP chase as the initial rapid phase in Fig. 4B (see more in “Discussion” and a schematic model in Fig. 7).

Affinity of E1P for Luminal Ca²⁺ in Absence of K⁺.—In Fig. 4, we assessed the Ca²⁺ affinity of the transport sites exposed to the lumen in K⁺-free E1P by determining the Ca²⁺ binding to E1P in steady state in the presence of A23187. In Fig. 6A, the total amount of EP increased with increasing Ca²⁺ concentration and reached its maximum level at ~0.5 μM Ca²⁺ due to high affinity Ca²⁺ binding at the transport sites (E2 to E1P transition). The total amount of EP at saturating Ca²⁺ was half of the maximum Ca²⁺ binding in E1P (B); therefore, all Ca²⁺-ATPases are phosphorylated at saturating Ca²⁺. As replotted in Fig. 6C, ~60% of the maximum total amount of EP was E1P in steady state at saturating Ca²⁺ under these conditions.

In Fig. 6B, the amount of bound Ca²⁺ in steady state in the presence of A23187 was determined without washing the filter so as not to alter the equilibrium. As replotted in Fig. 6C with % values relative to the maximum Ca²⁺ binding in E1P, the bound Ca²⁺ under the phosphorylating condition without K⁺ increases concomitantly with an increase in E1P, and their relative values are nearly the same. Note that if the affinity of the lumen-facing Ca²⁺ sites of E1P without K⁺ is significantly lower than that of the high Ca²⁺ affinity in E1 for the phosphorylation, the Ca²⁺ binding curve would be shifted.
Ca$^{2+}$ Release from E1PCA$_2$ of Ca$^{2+}$-ATPase

significant to higher Ca$^{2+}$ concentrations, and the relative value of the bound Ca$^{2+}$ would become significantly smaller than that of E1P in the 0.1–10 μM range. However, this is obviously not the case. We conclude that the affinity of the lumen-facing Ca$^{2+}$ sites of K$^+$-free E1P is as high as the cytoplasmic Ca$^{2+}$ affinity in E1.

DISCUSSION

Ca$^{2+}$ Release from E1PCA$_2$ in Absence of K$^+$—Our studies show that in the absence of K$^+$, Ca$^{2+}$ is released from E1PCA$_2$ to the luminal side. This Ca$^{2+}$ release obviously precedes the conversion of the ADP-sensitive EP (E1P) to ADP-insensi-

![FIGURE 5. Uptake of site I-bound 45Ca$^{2+}$ in a single turnover of EP with and without K$^+$. SR vesicles were incubated with 10 μM 45Ca$^{2+}$ as in Fig. 4 and diluted by an equal volume of a solution containing 2 mM nonradioactive CaCl$_2$ and 0.1 M KCl (A) or LiCl (B), and further incubated for 10 s. By this incubation, site I of the two Ca$^{2+}$ sites (I, II) is labeled with 45Ca$^{2+}$ due to Ca$^{2+}$ exchange with site II (see supplemental Fig. S1) (42–44). Then, 45Ca$^{2+}$ uptake assay in a single turnover was performed as in Fig. 4. In A, the time course obtained with the ADP-chase was best described by a single exponential Ca$^{2+}$ uptake (solid line) with a rate constant of 0.45 s$^{-1}$ and maximum Ca$^{2+}$/EP value of 0.75. In B, it was best described by a double exponential increase (broken line) with a rate constant and maximum value of 6.0 s$^{-1}$ and 0.34 for the fast phase and 0.41 s$^{-1}$ and 0.56 for the slow phase (but not described by a single exponential increase shown as solid line with the rate constant of 1.36 s$^{-1}$ and the maximum value of 0.80).

![FIGURE 6. Ca$^{2+}$ dependence of E1P accumulation and Ca$^{2+}$ binding in steady state. A, SR vesicles (200 μg/ml) were phosphorylated at 4 °C for 30 s with 100 μM [γ$^{32}$P]ATP in 30 mM A23187, 0.1 M LiCl, and 20 μM CaCl$_2$ with various concentrations of EGTA to give the indicated free Ca$^{2+}$ concentrations. The total amount of EP (closed circles) and amount of E1P (open circles) were determined as described in Fig. 3. Solid lines show the least squares fit to the Hill equation. The maximum, K$_{50}$, and Hill coefficient for the total amount of EP were 3.67 nmol/mg, 0.10 μM, and 2.6, respectively, and those for E1P were 2.12 nmol/mg, 0.11 μM, and 2.5, respectively. B, SR vesicles were phosphorylated with 100 μM ATP (open squares) or incubated without ATP (closed squares and open triangles) in 20 μM 45CaCl$_2$ with various concentrations of EGTA and 0.1 M LiCl (squares) or KCl (triangles), otherwise as described in A. Then, 50 μl of the reaction mixture was spotted on the membrane, and the amount of 45Ca$^{2+}$ specifically bound to the ATPase was determined. Solid lines show the least squares fit to the Hill equation. The maximum, K$_{50}$, and Hill coefficient were 8.54 nmol/mg, 0.18 μM, and 2.1 (open triangles), 7.82 nmol/mg, 0.20 μM, and 2.3 (closed squares), and 4.14 nmol/mg, 0.15 μM, and 1.5 (open squares). C, the amount of E1P (open circles) in A and that of bound 45Ca$^{2+}$ under the phosphorylating condition (open squares) in B in the absence of K$^+$ are replotted after normalization to the maximum total amount of EP and to the maximum 45Ca$^{2+}$ binding under the nonphosphorylating condition (E1) in the absence of K$^+$, respectively, and shown as % values. Solid lines show the least squares fit to the Hill equation, and the maximum values were 58% for E1P and 52% for bound 45Ca$^{2+}$, respectively.

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Thus, the Ca$^{2+}$ ion change. The observation also means that such a Ca$^{2+}$-dependent ATPase (28), therefore plays a critical role in lowering the lumenal Ca$^{2+}$ concentration. The Mg$^{2+}$-ATPase species cannot be involved in producing a Ca$^{2+}$-dependent ATPase species with high affinity (Fig. 6). sE1Ca$_2$ is in rapid equilibrium with the normal E1Ca$_2$. Here, s denotes silent because this species is apparently absent in the presence of K$^+$ and also because the bound Ca$^{2+}$ ions are not released to the cytoplasmic side even upon ADP-induced reverse dephosphorylation (to sE1Ca$_2$) in contrast to the normal E1Ca$_2$ reverse dephosphorylation. Actual active Ca$^{2+}$ transport is achieved by a large reduction of the Ca$^{2+}$ affinity during the normal sequence E1Ca$_2$ \( \rightarrow \) E2PCa$_2$ \( \rightarrow \) E2P + 2Ca$^{2+}$ (blue arrows). The schematic is based on crystal structural models for the ADP-sensitive and -insensitive EP states and E1Ca$_2$, with the positions of the cytoplasmic N, P, and A domains, and membrane (orange layer) being approximate. The Ca$^{2+}$ sites in the transmembrane domain are depicted as occluded (closed cytoplasmic and lumenal gates) in normal E1Ca$_2$, as lumen-facing and high Ca$^{2+}$ affinity with the closed cytoplasmic gate in sE1Ca$_2$, and as lumennally opened with reduced Ca$^{2+}$ affinity in E2P and E2PCa$_2$, immediately before the Ca$^{2+}$ release.

The fast initial phase may be accounted for by the rapid formation of E1Ca$_2$, with the positions of the cytoplasmic N, P, and A domains, and membrane (orange layer) being approximate. The Ca$^{2+}$ sites in the transmembrane domain are depicted as occluded (closed cytoplasmic and lumenal gates) in normal E1Ca$_2$, as lumen-facing and high Ca$^{2+}$ affinity with the closed cytoplasmic gate in sE1Ca$_2$, and as lumennally opened with reduced Ca$^{2+}$ affinity in E2P and E2PCa$_2$, immediately before the Ca$^{2+}$ release.

Thus, bound K$^+$ stabilizes both the Ca$^{2+}$-occluded structure of E1Ca$_2$ and the Ca$^{2+}$-released structure of E2P. Thereby, K$^+$ critically contributes to the successive structural changes and ensures strict and efficient coupling for EP processing and Ca$^{2+}$ handling in E1Ca$_2$ \( \rightarrow \) E2PCa$_2$ \( \rightarrow \) E2P + 2Ca$^{2+}$, key events for Ca$^{2+}$ transport. Also notable is the fact that the K$^+$ bound in the P domain is crucial for producing a catalytic site structure in E2P appropriate for its accelerated hydrolysis (28–30).

**Biphasic Ca$^{2+}$ Release in ADP Chase of Single Turnover of E1Ca$_2$ without K$^+$** —In Fig. 7, we provide a schematic model to show the roles of K$^+$ in the Ca$^{2+}$ transport and to account for the biphasic Ca$^{2+}$ release from K$^+$-free E1Ca$_2$, following an ADP chase during a single turnover (Fig. 4B, open circles). The fast initial phase may be accounted for by the rapid formation of sE1Ca$_2$, with lumen-facing, high affinity Ca$^{2+}$ binding sites, in rapid equilibrium with normal E1Ca$_2$. The bound Ca$^{2+}$ ions cannot be released to the cytoplasmic side even upon ADP-induced reverse dephosphorylation (sE1Ca$_2$) but only to the luminal side (yellow arrow). Because sE1P has high affinity, Ca$^{2+}$ rebinding occurs at low luminal

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**FIGURE 7. Schematic model for roles of K$^+$ in EP processing and Ca$^{2+}$ handling in Ca$^{2+}$ transport.** sE1Ca$_2$ is an E1Ca$_2$ species formed without K$^+$ possessing a closed cytoplasmic gate and lumen-facing Ca$^{2+}$ binding sites (an opened lumenal pathway) with high Ca$^{2+}$ affinity (Fig. 6). sE1Ca$_2$ is in rapid equilibrium with the normal E1Ca$_2$. Here, s denotes silent because this species is apparently absent in the presence of K$^+$ and also because the bound Ca$^{2+}$ ions are not released to the cytoplasmic side even upon ADP-induced reverse dephosphorylation (to sE1Ca$_2$) in contrast to the normal E1Ca$_2$ reverse dephosphorylation. Actual active Ca$^{2+}$ transport is achieved by a large reduction of the Ca$^{2+}$ affinity during the normal sequence E1Ca$_2$ \( \rightarrow \) E2PCa$_2$ \( \rightarrow \) E2P + 2Ca$^{2+}$ (blue arrows). The schematic is based on crystal structural models for the ADP-sensitive and -insensitive EP states and E1Ca$_2$, with the positions of the cytoplasmic N, P, and A domains, and membrane (orange layer) being approximate. The Ca$^{2+}$ sites in the transmembrane domain are depicted as occluded (closed cytoplasmic and lumenal gates) in normal E1Ca$_2$, as lumen-facing and high Ca$^{2+}$ affinity with the closed cytoplasmic gate in sE1Ca$_2$, and as lumennally opened with reduced Ca$^{2+}$ affinity in E2P and E2PCa$_2$, immediately before the Ca$^{2+}$ release.

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Ca\(^{2+}\) Release from E1PCa\(_2\) of Ca\(^{2+}\)-ATPase

![Diagram of Ca\(^{2+}\) Release from E1PCa\(_2\) of Ca\(^{2+}\)-ATPase](image_url)

FIGURE 8. Structure of E1PCa\(_2\) with bound K\(^+\). The crystal structure E1PCa\(_2\)-AMPPN (Protein Data Bank code 3BA6) (22) is shown. Panel a, a space-filling model with K\(^-\) (blue), M3 (yellow), M4 (orange), L6–7 (lime), Po1 (pink), Po6/Po7 (light purple), Glu\(^{732}\) (red and cyan), and A/M3-linker (dark gray), b, a schematic model with the view from the same direction as in a. K\(^-\) and Ca\(^{2+}\) are blue and red Van der Waals spheres, respectively. M3, M4, and M5 are yellow, orange, and light purple, respectively. The lower panels in a and b are the enlarged views of the areas surrounded by red broken line. In b, the coordination of K\(^-\) is shown by broken green lines, c, the helices for Ca\(^{2+}\) binding (M4, M5, M6, and M8) and K\(^-\) binding (Po6/Po7) and adjacent components (M3, M7, Po1, and A/M3-linker) are depicted. A large red arrow suggests a possible motion of M3/M4L at the luminal end to open the Ca\(^{2+}\) pathway. The residues involved in coordination of K\(^-\) and Ca\(^{2+}\) are depicted in ball and stick representation. Residues possibly forming interactions at the luminal end (Tyr\(^{289}\), Tyr\(^{295}\), Lys\(^{297}\), and Glu\(^{785}\)) are also depicted. The lower panel in c shows the view from the luminal side.

concentrations\(^3\) and inhibits flux through this pathway. The slow second phase (Fig. 4B) most probably reflects the E1PCa\(_2\) to E2P transition as in the single exponential Ca\(^{2+}\) uptake in 0.1 M K\(^+\) (Fig. 4A and Fig. 7, blue arrows). The formation of sE1PCa\(_2\), in rapid equilibrium with occluded E1PCa\(_2\), necessarily lowers the steady state level of the latter species and hence Ca\(^{2+}\) transport through the normal route. Thus, although progression to sE1PCa\(_2\) is relatively fast, this pathway cannot contribute to gradient formation and ultimately slows normal transport. It is concluded that K\(^+\) ensures the normal structural process for Ca\(^{2+}\) transport (blue arrows) by stabilizing the Ca\(^{2+}\)-occluded structure of E1PCa\(_2\) and disallowing opening of a luminal Ca\(^{2+}\) pathway (this study), and by stabilizing the E2P structure with disrupted Ca\(^{2+}\) sites (greatly reduced affinity) and a fully opened luminal gate (13).

Structural Role of Bound K\(^-\) in E1PCa\(_2\)—The crystal structures provide a likely structural role of bound K\(^+\) in E1PCa\(_2\). In structures analogous to K\(^+\)-bound E1PCa\(_2\) (E1PCa\(_2\)-AMPPN (22) and E1Ca\(_2\)-AlF\(_{4}\) -ADP as well as E1Ca\(_2\)-AMPPCP (17)), K\(^+\) is specifically bound at the bottom part of the P domain and coordinated by the backbone carbonyl oxygens of Leu\(^{711}\), Lys\(^{712}\), and Ala\(^{714}\) on Po6 (sixth P-domain α-helix) (near the catalytic Mg\(^{2+}\) site Asp\(^{703}\)/Asp\(^{707}\) on Po5 of this region) and by the Glu\(^{732}\) side chain oxygen on Po7 (Fig. 8). The importance of Glu\(^{732}\) in the K\(^+\)-induced acceleration of E2P hydrolysis was shown through mutations (28).

The K\(^+\) ion and these ligands are distant from and not in direct contact with the transport sites from which Ca\(^{2+}\) release occurs. On the other hand, adjacent to the K\(^+\) binding site on Po6/Po7 is Po1, which is directly linked with the cytoplasmic end of M4 within the P domain. Po6, Po7, and Po1 constitute the bottom part of one-half of the P domain and move together as a body during the transport cycle (7, 18). Furthermore, Po1 forms a hydrogen-bonding network with L6–7 (a cytoplasmic short loop linking M6 and M7) and top parts of M3/M5. This interaction network is critical for proper arrangement of the transmembrane helices (48–50). In fact, disruption of this network by mutations causes a marked retardation of the E2-E1 transition (48, 49).

Because the bound K\(^+\) is deeply embedded and ligated within this part of the P domain (Fig. 8a), its absence would allow more flexibility of the structural components, such as

Note that the intravesicular volume of SR vesicles has been estimated to be in the range of 2–10 μl/mg protein (45, 46), and therefore, the release of Ca\(^{2+}\) bound in EP (~8 mol/mg protein) into the lumen in a single turnover might increase the luminal Ca\(^{2+}\) to ~0.8–4 mm. Although a fair amount of luminal free Ca\(^{2+}\) may be removed by low affinity Ca\(^{2+}\) buffers such as calsequestrin (47), even a small rise in the luminal Ca\(^{2+}\) level might result in rebinding of luminal Ca\(^{2+}\) to sE1P because of its high affinity revealed in Fig. 6 (yellow arrow in Fig. 7).
segmental fluctuations or wobbling, which in turn would impinge on the cytoplasmic regions of the transmembrane helices and probably destabilize the interaction network Pα1/L6–7/M3/M5. The absence of K+ in fact markedly retards the E2 to E1 transition (31, 32), and, as noted above, disruption of the Pα1/M3/M5/L6–7 interaction network markedly retards the E1–E2 transition and also the E1P to E2P conformation change (48–50). Opening of the luminal pathway and Ca2+ release from E1PCa2 may be caused by such structural perturbations in the absence of bound K+.

As shown in the view from the lumen of the helices M4/M5/M6/M8 ligation Ca2+ in Fig. 8c, the space surrounded by these helices seems to be the only possible Ca2+ exit pathway. M3 is in close contact at the luminal end with the luminal part of M4 (M4L), and they are connected by a short luminal loop (L3–4). During the EP conformation change and subsequent Ca2+ release (E1PCa2 → E2P + 2Ca2+), M3 and M4L incline together and move outward, thereby opening the putative Ca2+ release pathway (luminal gate) (19). The M3/M4L motion is produced by the large rotation and inclination of the A and P domains and by the consequent significant motions and rearrangements of the helices M1–6, in which M1/M2 as a rigid body pushes M4L to open the Ca2+ release gate (Fig. 9) (19). The large motions concomitantly disrupt the Ca2+ binding sites and reduce the Ca2+ affinity (19). In K+-free E1PCa2 (ADP sensitive), these domain motions have not yet taken place, and the Ca2+ sites are not disrupted and maintain a high affinity. Here, these motions are likely much less prominent and opening of the release pathway is simply the result of fluctuations and wobbling of the relevant helices, in particular M3/M4L.

The unique Ca2+ coordination and particular make up of the M3 and M4 helices lend themselves to creating a release pathway while maintaining a high affinity. The Ca2+ sites with properly positioned ligands are located at an unwound portion of the M4 helix creating intrinsic flexibility (Fig. 8). On the other hand, M3 is a continuous helix from the cytoplasmic to the luminal end and is located at the periphery of the transmembrane domain and is not closely associated with other helices including M1/M2 (except for M4L at the luminal end). Thus, in the crystal structures analogous to E1PCa2, M3 seems not to have much steric restriction against possible outward movement, a shift that would open the Ca2+ pathway. Therefore, if the cytoplasmic region of M3 is not fixed as occurs in the absence of bound K+, its luminal part and the associated M4L may become more mobile. Wobbling here would allow the Ca2+ pathway to fluctuate between a closed and open state. The Ca2+ site is not necessarily disrupted because of the flexibility of the unwound structure of M4 and because the large motions of the A–P domains do not occur. (These are the motions that disrupt the Ca2+ sites by inclining the cytoplasmic region of M4/M5.) Also, M3 is not involved directly in the Ca2+ ligation.

Interestingly, at the luminal end of M4L (Fig. 8c), there are bulky and hydrophobic residues (Tyr294/Tyr295/Lys297), which may form hydrogen bonds, e.g. Tyr294/Tyr295 with Glu785 on L5–6. Lys297 seems to seal the Ca2+ channel (51). Tyr295 is important for Ca2+ transport activity and stabilizing E2 relative to E1 (52). These residues may possibly function as the luminal plug, and M3/M4L wobbling may destabilize their interactions helping to open the Ca2+ pathway in K+-free E1PCa2.

Importantly, in the crystal structures of analogues of E1PCa2, the cytoplasmic Ca2+ gate is closed by the Ca2+ ligand Glu309 because Leu65 on M1 locks the Glu309 side chain configuration by van der Waals contact (8, 9, 18, 53). Our observation shows that this cytoplasmic gate is closed in E1PCa2 even without bound K+, and therefore, the Glu309-gating with Leu65 has not been affected.

**Movement of K+ Binding Site during E1PCa2 → E2P + 2Ca2+**

Movement of K+ Binding Site during E1PCa2 → E2P + 2Ca2+ may be used as a model for the overall change in E1PCa2 → E2P + 2Ca2+ (Fig. 9). Hence, the P domain inclines to the A domain that also rotates and inclines (curved arrows), thus producing the A–P domain association in the most compactly organized and inclined headpiece structure, the Ca2+-released E2P. With this change, the cytoplasmic...
region of M4/M5 in the P domain inclines and disrupts the Ca\(^{2+}\) sites (19). M2 inclines with the A domain motion and consequently M1, which forms a rigid V-shaped body with M2, pushes against the lumenal part of M4, and opens the lumenal gate (19).

In these structural changes, the K\(^+\) site with bound K\(^+\) on the P domain moves down to the Gln244 region on the A/M3-linker (blue arrow) and brings in the Gln244 side chain (or neighboring residues) as an additional coordination ligand. Thus bound K\(^+\) likely cross-links the bottom part of the P domain and the A/M3-linker. This cross-link must contribute to the stabilization of the compactly organized and inclined E2P structure with disrupted Ca\(^{2+}\) sites and fully opened lumenal gate (13).

The A/M1’-linker of correct length has a critical function in inclining and compacting the E2P structure (14, 27). The structure is stabilized by three critical interaction networks; at the Tyr122 HC (hydrophobic interaction cluster involving the inclining and compacting the menal gate (13). Thus, bound K\(^+\) following disruption of the Ca\(^{2+}\) site with bound K\(^+\) in E1P\(_{Ca2}\) is produced upon processing and Ca\(^{2+}\) containing a high affinity. Thus, in the natural E1P\(_{Ca2}\) structure, bound K\(^+\) stabilizes the Ca\(^{2+}\) in an occluded form by not allowing the pathway to open. Bound K\(^+\) also stabilizes E2P following disruption of the Ca\(^{2+}\) sites and full opening of the lumenal gate (13). Thus, bound K\(^+\) has a crucial role in E\(_{P}\) processing and Ca\(^{2+}\) occlusion and release to the lumen in the sequence E1P\(_{Ca2}\) → E2P\(_{Ca2}\) → E2P + 2Ca\(^{2+}\).

REFERENCES
1. Hasselbach, W., and Makino, M. (1961) Biochim. Biophys. Acta 280, 518–528
2. Ebashi, S., and Lipmann, F. (1962) J. Cell Biol. 14, 389–400
3. Inesi, G., Sumbilla, C., and Kirtley, M. E. (1990) Physiol. Rev. 70, 749–760
4. Moller, J. V., Juul, B., and le Maire, M. (1996) Biochim. Biophys. Acta 1286, 1–51
5. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28815–28818
6. McIntosh, D. B. (1998) Adv. Mol. Cell. Biol. 23A, 33–99
7. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
8. Toyoshima, C. (2008) Arch. Biochem. Biophys. 476, 3–11
9. Toyoshima, C. (2009) Biochim. Biophys. Acta 1793, 941–946
10. Kato, S., Kamidochi, M., Daiho, T., Yasumaki, K., Gouli, W., and Suzuki, H. (2003) J. Biol. Chem. 278, 9624–9629
11. Yasumaki, K., Daiho, T., Danko, S., and Suzuki, H. (2004) J. Biol. Chem. 279, 2202–2210
12. Wang, G., Yasumaki, K., Daiho, T., and Suzuki, H. (2005) J. Biol. Chem. 280, 26508–26516
13. Yasumaki, K., Wang, G., Daiho, T., Danko, S., and Suzuki, H. (2008) J. Biol. Chem. 283, 34429–34447
14. Daiho, T., Yasumaki, K., Danko, S., and Suzuki, H. (2007) J. Biol. Chem. 282, 34429–34447
15. Toyoshima, C., Nakashiro, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
16. Toyoshima, C., and Nomura, H. (2002) Nature 418, 605–611
17. Sorensen, T. L., Møller, J. V., and Nissen, P. (2004) Science 304, 1672–1675
18. Toyoshima, C., and Mizutani, T. (2004) Nature 430, 529–535
19. Toyoshima, C., Nomura, H., and Tsuda, T. (2004) Nature 432, 361–368
20. Olesen, C., Sorensen, T. L., Nielsen, R. C., Møller, J. V., and Nissen, P. (2004) Science 306, 2251–2255
21. Toyoshima, C., Nomiratsu, Y., Isawa, S., Tsuda, T., and Ogawa, H. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 19831–19836
22. Olesen, C., Picard, M., Winther, A. M., Gyrup, C., Morth, J. P., Oxvig, C., Møller, J. V., and Nissen, P. (2007) Nature 450, 1036–1042
23. Danko, S., Daiho, T., Yasumaki, K., Kamidochi, M., Suzuki, H., and Toyoshima, C. (2001) FEBS Lett. 489, 277–282
24. Danko, S., Yasumaki, K., Daiho, T., Suzuki, H., and Toyoshima, C. (2001) FEBS Lett. 505, 129–135
25. Danko, S., Yasumaki, K., Daiho, T., and Suzuki, H. (2004) J. Biol. Chem. 279, 14991–14998
26. Danko, S., Daiho, T., Yasumaki, K., Liu, X., and Suzuki, H. (2009) J. Biol. Chem. 284, 22722–22735
27. Daiho, T., Danko, S., Yasumaki, K., Siroky, K., and Suzuki, H. (2010) J. Biol. Chem. 285, 24538–24547
28. Sorensen, T. L., Clausen, J. D., Jensen, A. M., Vilsen, B., Møller, J. V., Andersen, J. P., and Nissen, P. (2004) J. Biol. Chem. 279, 46355–46358
29. Shigekawa, M., and Pear, L. J. (1976) J. Biol. Chem. 251, 6947–6952
30. Shigekawa, M., and Dougherty, J. P. (1978) J. Biol. Chem. 253, 1451–1457
31. Medda, P., Fassold, E., and Hasselbach, W. (1987) Eur. J. Biochem. 165, 251–259
32. Lee, A. G., Baker, K., Khan, Y. M., and East, J. M. (1995) Biochem. J. 305, 225–231
33. Chamep, P., Henao, F., and de Foresta, B. (1997) Biochemistry 36, 12383–12393
34. Nakamura, S., Suzuki, H., and Kanazawa, T. (1994) J. Biol. Chem. 269, 16015–16019
35. Barrabin, H., Scofano, H. M., and Inesi, G. (1984) Biochemistry 23, 1542–1548
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36. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
37. Daiho, T., Suzuki, H., Yamasaki, K., Saino, T., and Kanazawa, T. (1999) *FEBS Lett.* 444, 54–58
38. Kanazawa, T., Saito, M., and Tonomura, Y. (1970) *J. Biochem.* 67, 693–711
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
40. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Mol. Graph.* 14, 33–38
41. Nakamura, J. (1989) *J. Biol. Chem.* 264, 17029–17031
42. Inesi, G. (1987) *J. Biol. Chem.* 262, 16338–16342
43. Petithory, J. R., and Jencks, W. P. (1988) *Biochemistry* 27, 8626–8635
44. Orlowski, S., and Champeil, P. (1991) *Biochemistry* 30, 352–361
45. Malan, N. T., Sabbadini, R., Scales, D., and Inesi, G. (1975) *FEBS Lett.* 60, 122–125
46. Duggan, P. F., and Martonosi, A. (1970) *J. Gen. Physiol.* 56, 147–167
47. Fleischer, S., and Inui, M. (1989) *Annu. Rev. Biophys. Biophys. Chem.* 18, 333–364
48. Clausen, J. D., and Andersen, J. P. (2004) *J. Biol. Chem.* 279, 54426–54437
49. Lenoir, G., Picard, M., Møller, J. V., le Maire, M., Champeil, P., and Falcon, P. (2004) *J. Biol. Chem.* 279, 32125–32133
50. Zhang, Z., Lewis, D., Sumbilla, C., Inesi, G., and Toyoshima, C. (2001) *J. Biol. Chem.* 276, 15232–15239
51. Chen, L., Sumbilla, C., Lewis, D., Zhong, L., Strock, C., Kirtley, M. E., and Inesi, G. (1996) *J. Biol. Chem.* 271, 10745–10752
52. Lee, A. G. (2002) *Biochim. Biophys. Acta* 1565, 246–266
53. Einholm, A. P., Vilsen, B., and Andersen, J. P. (2004) *J. Biol. Chem.* 279, 15888–15896
54. de Meis, L. (2001) *J. Biol. Chem.* 276, 25078–25087
55. Reis, M., Farage, M., de Souza, A. C., and de Meis, L. (2001) *J. Biol. Chem.* 276, 42793–42800
56. Barata, H., and de Meis, L. (2002) *J. Biol. Chem.* 277, 16868–16872
57. Yu, X., and Inesi, G. (1995) *J. Biol. Chem.* 270, 4361–4367
58. Hanke, A. M., and Jencks, W. P. (1991) *Biochemistry* 30, 11320–11330
59. Orlowski, S., and Champeil, P. (1991) *Biochemistry* 30, 11331–11342