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Dissociation of Calcium from the Phosphorylated Calcium-Transporting Adenosine Triphosphatase of Sarcoplasmic Reticulum: Kinetic Equivalence of the Calcium Ions Bound to the Phosphorylated Enzyme†

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ABSTRACT: The internalization of ^{45}Ca by the calcium-transporting ATPase into sarcoplasmic reticulum vesicles from rabbit muscle was measured during a single turnover of the enzyme by using a quench of 7 mM ADP and EGTA (25 °C, 5 mM $MgCl_2$, 100 mM KCl, 40 mM MOPS·Tris, pH 7.0). Intact vesicles containing either 10–20 μM or 20 mM Ca^{2+} were preincubated with ^{45}Ca for ~20 s and then mixed with 0.20–0.25 mM ATP and excess EGTA to give 70% phosphorylation of E_{tot} with the rate constant k = 300 s^{-1}. The two ^{45}Ca ions bound to the phosphoenzyme (EP) become insensitive to the quench with ADP as they are internalized in a first-order reaction with a rate constant of k = ~30 s^{-1}. The first and second Ca^{2+} ions that bind to the free enzyme were selectively labeled by mixing the enzyme and ^{45}Ca with excess ^{40}Ca, or by mixing the enzyme and ^{40}Ca with ^{45}Ca, for 50 ms prior to the addition of ATP and EGTA. The internalization of each ion into loaded or empty vesicles follows first-order kinetics with k = ~30 s^{-1}; there is no indication of biphasic kinetics or an induction period for the internalization of either Ca^{2+} ion. The presence of 20 mM Ca^{2+} inside the vesicles has no effect on the kinetics or the extent of internalization of either or both of the individual ions. The Ca^{2+} ions bound to the phosphoenzyme are kinetically equivalent. A first-order reaction for the internalization of the individual Ca^{2+} ions is consistent with a rate-limiting conformational change of the phosphoenzyme with k_c = 30 s^{-1}, followed by rapid dissociation of the Ca^{2+} ions from separate independent binding sites on E~P·Ca_2; lumenal calcium does not inhibit the dissociation of calcium from these sites. Alternatively, the Ca^{2+} ions may dissociate sequentially from E~P·Ca_2 following a rate-limiting conformational change. However, the order of dissociation of the individual ions can not be distinguished. An ordered-sequential mechanism for dissociation requires that the ions dissociate much faster ($k \geq 10^5$ s^{-1}) than the forward and reverse reactions for the conformational change (k_{-c} = ~3000 s^{-1}). Finally, the Ca^{2+} ions may exchange their positions rapidly on the phosphoenzyme ($k_{mix} \geq 10^5$ s^{-1}) before dissociating. A Hill slope of n_H = 1.0–1.2, with $K_{0.5}$ = 0.8–0.9 mM, for the inhibition of turnover by binding of Ca^{2+} to the low-affinity transport sites of the phosphoenzyme was obtained from rate measurements at six different concentrations of Mg^{2+}.

The calcium-transporting ATPase of sarcoplasmic reticulum (SR)[1] couples the chemical free energy released from the hydrolysis of ATP to the vectorial transport of two calcium ions into the lumen of the SR (de Meis & Vianna, 1979; Jencks, 1980; Inesi, 1985). Scheme I describes a simple catalytic cycle for the coupled reaction in which the different states of the enzyme are defined by their chemical composition, rather by numbers or signs (Makinose, 1973; Pickart & Jencks, 1984).

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[1] Abbreviations: CaATPase, calcium-transporting ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EP, phosphoenzyme; MOPS, 4-morpholinepropanesulfonic acid; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles.

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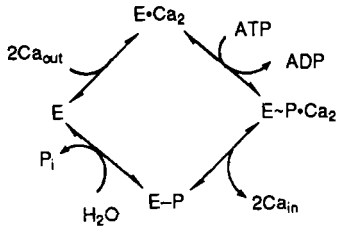
Scheme I



The coupling between the chemical reaction of ATP hydrolysis and the vectorial reaction of calcium transport is brought about by alternating changes in chemical and vectorial specificity, so that neither ATP hydrolysis nor the transport of calcium can occur unless the other process also occurs. Thus, $E{\cdot}Ca_2$ reacts reversibly with ATP and E reacts reversibly with inorganic phosphate, while E binds and dissociates calcium only at the cytoplasmic side and E–P binds and dissociates calcium only at the lumenal side of the membrane, as shown in Scheme I. If these specificity rules are followed, the transport of two Ca^{2+} ions and the hydrolysis or synthesis of ATP will be fully coupled; if any of the rules are not followed, the reaction will be completely uncoupled.

Two calcium ions bind to the nonphosphorylated and to the phosphorylated enzyme at temperatures near 25 °C. Tonomura and co-workers (Yamada et al., 1972) observed that, during the synthesis of ATP from P_i and ADP by the enzyme, two calcium ions are transported out of intact vesicles for each molecule of ATP that is synthesized. This result is consistent with the requirement that two calcium ions must bind to the phosphorylated enzyme in order for the reaction with ADP to occur. The equilibrium binding of two calcium ions to the free enzyme, E, is cooperative (Dupont & Leigh, 1978; Ikemoto et al., 1978; Guillain et al., 1980; Inesi et al., 1980; Champeil et al., 1983; Fernandez-Belda et al., 1984; Petithory & Jencks, 1988b). The binding of calcium to the phosphorylated enzyme is difficult to measure directly, but the amount of phosphoenzyme that is formed at equilibrium from P_i at 5 and 20 °C has been shown to increase when the concentration of calcium inside the vesicle is raised (Yamada et al., 1972; Prager et al., 1979). Suko and co-workers (Prager et al., 1979) reported that the increase in EP is sigmoidal, not hyperbolic, with respect to interior $[Ca^{2+}]$ and can be described by a Hill slope of $n_H = 1.72$. Coan and Inesi (1979) showed that the inhibition of turnover and the dependence of the change in the ESR signal of spin-labeled CaATPase in the presence of 5 mM ATP and increasing concentrations of calcium is also sigmoidal. Steady-state turnover of the unmodified enzyme in the presence of 0.1–1.5 mM ATP is inhibited by Ca^{2+}; however, the inhibition follows a Hill slope of $n_H = 0.9$–1.3 (Bodley & Jencks, 1987; Khananshvili et al., 1990; and this work).

Several investigators have reported that two calcium ions bind to and dissociate from the nonphosphorylated enzyme by an ordered-sequential mechanism (Dupont, 1982; Nakamura, 1986; Inesi, 1987; Petithory & Jencks, 1988a,b; Nakamura, 1989; Orlowski & Champeil, 1991). The important observation that led to this conclusion is that high concentrations of unlabeled calcium in the medium prevent the dissociation of half of the labeled calcium ions that are bound to the species of enzyme, $E{\cdot}^{45}Ca{\cdot}^{45}Ca$. This fact allowed Inesi to selectively label the individual calcium ions bound to the nonphosphorylated enzyme and measure their transport into intact SR vesicles (Inesi, 1987). He observed that the first calcium ion that binds to the free enzyme was internalized rapidly while the second calcium ion was internalized more slowly. Inesi proposed that the dissociation of two calcium ions from the phosphorylated enzyme is sequential and that the order of binding of the two ions to the free enzyme is the same as the order of their dissociation from the phosphorylated enzyme. A previous report from this laboratory also stated that the dissociation of calcium from the phosphorylated enzyme is ordered and sequential (Khananshvili & Jencks, 1988). This conclusion was based primarily on the reported observation that high concentrations of intravesicular calcium prevented the dissociation of one of the calcium ions that is bound to the phosphorylated enzyme. However, we have not been able to reproduce this result, and the conclusions of that report have been withdrawn (Khananshvili & Jencks, 1990).

This paper reports the results of a reinvestigation of the transport of calcium ions into empty and loaded vesicles by the CaATPase during a single turnover of the enzyme. The uptake of radiolabeled calcium was initiated by the simultaneous addition of saturating concentrations of ATP and excess EGTA to the stable form of the enzyme with two bound calcium ions, $^cE{\cdot}Ca_2$, which gives 70% phosphorylation of the total enzyme and prevents further binding of calcium (Petithory & Jencks, 1986). The phosphoenzyme with two bound calcium ions reacts with ADP to form ATP, which changes the side of exposure of the transport sites for calcium so that they face the outside medium; the phosphoenzyme ceases to react with ADP as the calcium ions are transported into the lumen of the vesicle (Scheme I). The internalization of calcium during the transport reaction was quenched by the addition of 7 mM ADP in the presence of EGTA, which chelates the calcium that dissociates into the outside medium from enzyme that has reacted with ADP to form ATP.

We have found that loading the vesicles with 20 mM $CaCl_2$ has no effect on the rate at which the enzyme loses its reactivity with ADP as the two calcium ions that are bound to the phosphorylated enzyme are internalized. The same rate constant, within experimental error, is measured for the internalization of both calcium ions and for each of the individual ions. There is no indication that the internalization of the calcium ions is biphasic, in contrast to previous reports (Inesi, 1987; Khananshvili & Jencks, 1988). Inhibition of turnover of the enzyme in the steady state by added calcium was found to follow a Hill slope of $n_H = 1.2$. The data are consistent with mechanisms in which a conformational change is rate limiting for the dissociation of calcium from the phosphorylated enzyme. The calcium ions bound to the phosphoenzyme could then dissociate rapidly from separate independent binding sites. Alternatively, the Ca^{2+} ions could dissociate sequentially from a single site; models in which sequential dissociation of the two Ca^{2+} ions occurs very rapidly after a rate-limiting conformational change or the two Ca^{2+} ions rapidly exchange their positions on the enzyme before dissociating can account for the kinetic equivalence of the two ions. Rapid filtration experiments carried out independently by P. Champeil and S. Orlowski at 20 °C with leaky vesicles show that high concentrations of calcium in the medium do not inhibit the dissociation of calcium from the phosphorylated enzyme, as described in the following paper in this issue. Furthermore, their measurements show that the individual Ca^{2+} ions dissociate from the phosphorylated enzyme with approximately the same rate constant; biphasic kinetics are not observed for the dissociation of the individual ions.

Materials and Methods

Materials. Disodium ATP ("Sonderqualitat"), NADH, phosphoenolpyruvate, pyruvate kinase (glycerol suspension), lactate dehydrogenase (glycerol suspension), and Tris base

were purchased from Boehringer Mannheim. ADP (grade VIII, potassium salt) was obtained from Sigma. $MgCl_2$ (Gold Label) and $CaCl_2$ (Gold Label) were obtained from Aldrich. The sodium salt and free acid of MOPS (Ultrex Grade) and the calcium ionophore A23187 were purchased from Calbiochem. KCl and EGTA were obtained from Fluka. [γ-^{32}P]-ATP (>99% pure) and $^{45}CaCl_2$ were purchased from New England Nuclear. All solutions were made with Milli-Q grade water (Millipore Co.) and stored in polypropylene containers (Nalgene Co.).

Sarcoplasmic reticulum vesicles were prepared as described by Khananshvili and Jencks (1988). The preparations were stored at −80 °C until they were used. Enzyme solutions (0.2–0.5 mL) were dialyzed at 4 °C overnight against 1 L of 100 mM KCl, 40 mM MOPS·Tris buffer, pH 7.0, and 0.2 M sucrose to lower the concentration of calcium inside the vesicles to ambient levels (10–20 μM). SRV were loaded passively with calcium by dialyzing enzyme solutions against 100 mM KCl, 40 mM MOPS·Tris, pH 7.0, 0.2 M sucrose, and 20 mM $CaCl_2$ overnight at 4 °C. The steady-state ATPase activities of the preparations used in this work were 2–4 μmol(min·mg of total protein)$^{-1}$ after the vesicles were made permeable to calcium by the addition of A23187 in ethanol (final concentrations: 2 μM A23187 and 0.1% ethanol).

Vesicles were also made permeable to calcium by treatment with the chelating reagent Chelating Sepharose (Pharmacia) at alkaline pH (Fujimori & Jencks, 1990). The pH of SRV solutions (40 mg/mL) was raised to 9.5 by the addition of solid Tris base and mixed with Chelating Sepharose (0.2 g/0.2 mL SRV solution) that had been previously washed with 10 mM EGTA and 0.2 mM sucrose, pH 12, followed by 10 mM Tris·HCl and 0.2 mM sucrose, pH 9.5. The mixture was kept at room temperature, and aliquots were withdrawn at 15-min intervals and assayed for activity in the absence and presence of A23187. Vesicles were considered 100% leaky when the addition of A23187 caused no increase in the measured rate of turnover. The mixture was centrifuged through glass wool to remove the Chelating Sepharose, and the pH was lowered immediately to 7.0 by the addition of solid MOPS (free acid). Typically, <25% of the initial enzyme activity was lost during this treatment.

Methods. Steady-state hydrolysis of ATP was measured spectrophotometrically by coupling the formation of ADP to the oxidation of NADH with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard conditions for the assays were 25 °C, 100 mM KCl, 40 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 0.40 mM EGTA, 0.42 mM $CaCl_2$ (26 μM free Ca^{2+}), 0.20–0.25 mM ATP, 1.5 mM PEP, 0.15 mM NADH, and 0.05 mg each of pyruvate kinase and lactate dehydrogenase, in a total volume of 2.0 mL. Protein concentrations were estimated by using the method of Lowry et al. (1951) with bovine serum albumin as the protein standard.

Rapid Mix–Quench Experiments. The uptake of ^{45}Ca into intact vesicles and the formation of the labeled phosphoenzyme were measured at 25 °C using a thermostated rapid-mixing chemical-quench apparatus as described previously (Stahl & Jencks, 1984; Petithory & Jencks, 1988a). The instrument was fit with either three or four syringes (0.89 mL/syringe), so that either a single reaction or two consecutive reactions could be followed. All syringes contained 100 mM KCl and 40 mM MOPS·Tris, pH 7.0.

Determination of ^{45}Ca Accumulation in Vesicles. The transport of two labeled calcium ions into tightly sealed SR vesicles was initiated with the rapid-mixing apparatus by mixing the enzyme and labeled calcium with solutions containing a large excess of ATP and enough EGTA to lower the concentration of free Ca^{2+} to less than 0.1 μM. In separate experiments, the "inner" and "outer" calcium ions bound to the enzyme were selectively labeled by first incubating the enzyme with either ^{40}Ca or ^{45}Ca and then mixing with ^{45}Ca or ^{40}Ca, respectively, for 50 ms (Petithory & Jencks, 1988a). Transport reactions were initiated immediately thereafter, as described above. Calcium transport reactions were quenched by the addition of 7 mM ADP (final concentration) in the presence of 5 or 10 mM EGTA. Reaction samples were filtered within 5 s over nitrocellulose filters (Millipore HAW/P 0.45 μm) that had been washed with ice-cold 10 mM $CaCl_2$, 100 mM KCl, and 40 mM MOPS·Tris, pH 7.0. The filters were then washed with three 5-mL portions of ice-cold 5 mM EGTA, 100 mM KCl, and 40 mM MOPS·Tris, pH 7.0, and dried for ~20 s by using the vacuum of the aspirator. The labeled calcium on the dried filters was measured by liquid scintillation counting in glass vials that contained ~7 mL of Aquasol 2 (New England Nuclear).

Determination of [^{32}P]Phosphoenzyme. The amount of radiolabeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. (1978). Phosphorylation reactions that measured total phosphoenzyme were initiated by mixing the enzyme and calcium with [γ-^{32}P]ATP or [γ-^{32}P]ATP and EGTA and were quenched 12 ms later by the addition of $HClO_4$ and KH_2PO_4 to give final concentrations of 0.125 M and 2 mM, respectively. Bovine serum albumin was added to the quenched reaction mixtures to give a final concentration of 0.25 mg/mL. The samples were put on ice for ~1 h and then centrifuged at 1500*g* for 25 min at 4 °C. The supernatant fluid was decanted, and the pellets were resuspended in 5 mL of ice-cold 5% trichloroacetic acid that contained 10 mM KH_2PO_4. The protein was collected by vacuum filtration with Whatman GF/C glass fiber filters. The sample collection tubes were rinsed with three 5-mL portions of the resuspension solution, and the amount of labeled protein was determined by liquid scintillation counting, as described above. The total amount of phosphoenzyme for each of the three preparations that were used in this study was found to be 2–3 nmol(mg of total protein)$^{-1}$ after phosphorylation for 12 ms.

Computer Analysis. Rate constants were estimated on an IBM compatible computer by using a kinetic analysis program, ENZFITTER (Elsevier Biosoft, 68 Hill Rd., Cambridge, U.K. CB2 1LA), that allowed weighted nonlinear least-squares fitting of the kinetic data and the modeling of kinetic equations (Capellos & Bielski, 1972). The program TUTSIM (Applied i, 200 California Ave, no. 212, Palo Alto, CA 94306) was used for kinetic simulations. The computer spread sheet Lotus 1-2-3 (Lotus Development Corporation) was used for calculating the concentrations of the different forms of the enzyme at equilibrium and the rates of turnover of the enzyme in the steady state.

RESULTS

Quench by ADP and EGTA. The phosphorylated CaATPase with two bound calcium ions reacts rapidly with ADP to synthesize ATP on the enzyme; reversal of the phosphorylation reaction by ADP changes the side of exposure of the calcium transport sites so that the sites face the outside medium. The reactivity of the phosphoenzyme with ADP is lost when the calcium ions are internalized into the lumen of the vesicle. The addition of ADP plus EGTA to reaction mixtures blocks further internalization of calcium ions bound to the phosphoenzyme by converting E~P·Ca_2 to E·ATP·Ca_2 and then E·ATP, which does not react further.

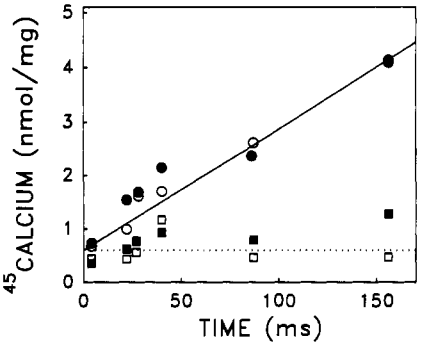


FIGURE 1: Uptake of calcium into SRV in the presence of 7 mM ADP and 2 mM ATP. All syringes of the rapid-mixing apparatus contained 40 mM MOPS·Tris, pH 7.0, 100 mM KCl, and 5 mM $MgCl_2$ (buffer A) at 25 °C. Empty (○, □) or loaded (20 mM Ca^{2+}, ●, ■) vesicles were diluted into buffer A containing 0.40 mM EGTA and 0.42 mM ^{45}Ca (26 μM $^{45}Ca_{free}$) and loaded into syringe A of the rapid-mixing apparatus. The final protein concentration was 0.27 mg/mL, and the specific activity of the ^{45}Ca following dilution of the vesicles was 8.5 and 6.8 μCi/μmol, respectively, for empty and loaded vesicles. Reactions were initiated within 20 s by mixing the diluted enzyme with 4 mM ATP and 14 mM ADP (○, ●) or 4 mM ATP, 14 mM ADP, and 10 mM EGTA (□, ■) in syringe B. The reactions were quenched at the indicated times by the addition of 7 mM ADP and 15 mM EGTA (○, ●) or 7 mM ADP and 5 mM EGTA (□, ■) contained in syringe C. Blanks were measured by mixing the diluted enzyme in buffer A containing ^{45}Ca and EGTA with 10 mM EGTA from syringe B and 5 mM EGTA from syringe C. The lines are calculated for rates of calcium uptake of 1.4 μmol/(min·mg) (solid line) and 0 μmol/(min·mg) (dotted line).

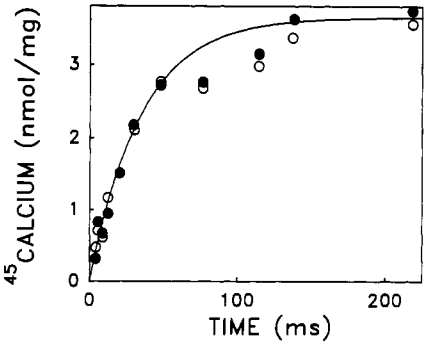


FIGURE 2: Internalization of both calcium ions into empty and loaded vesicles during a single turnover. All syringes of the rapid mixing apparatus contained 40 mM MOPS·Tris, pH 7.0, 100 mM KCl, and 5 mM $MgCl_2$ (buffer A) at 25 °C. Empty vesicles (○) or vesicles loaded with 20 mM ^{40}Ca (●) were diluted 180-fold into 0.42 mM ^{45}Ca and 0.40 mM EGTA (26 μM $^{45}Ca_{free}$) and loaded into syringe A of the rapid mixer. The concentration of protein following dilution was 0.27 mg/mL, and the final specific activity of the ^{45}Ca was 8.5 and 6.8 μCi/μmol for empty and loaded vesicles, respectively. The enzyme was mixed with 0.50 mM ATP and 10 mM EGTA contained in syringe B, and the reactions were quenched at the indicated times with 21 mM ADP and 5 mM EGTA from syringe C. Blanks were measured by mixing the diluted enzyme in buffer A containing ^{45}Ca and EGTA with 4 mM ATP, 14 mM ADP, and 10 mM EGTA contained in syringe B, followed by mixing with 7 mM ADP and 5 mM EGTA from syringe C at the indicated times to quench the reaction. The observed values for the blanks did not increase with time. The solid line is calculated for a rate constant of 29 s^{-1} and an endpoint of 3.65 nmol/mg of Ca.

Scheme II

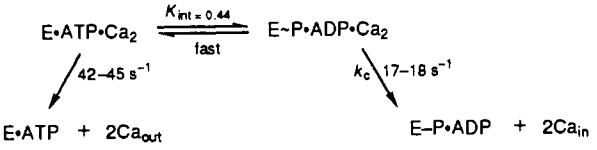


Figure 1 shows that the quench of 7 mM ADP plus EGTA blocks ~85% of the internalization of calcium (squares) into vesicles containing ambient concentrations of calcium (0.01–0.02 mM Ca^{2+}) (empty symbols) and 20 mM Ca^{2+} (filled symbols) during a single turnover of the enzyme (40 mM MOPS, 100 mM KCl, 5 mM $MgCl_2$, pH 7.0, at 25 °C). The dotted line shows that the simultaneous addition of 7 mM ADP, 2 mM ATP, and 5 mM EGTA results in the transport of 0.6 nmol/mg Ca^{2+} within 30 ms after mixing; further transport of calcium at times up to 160 ms is not detected. Petithory and Jencks (1986) established for similar experimental conditions that the simultaneous addition of ATP and EGTA alone to the stable form of the enzyme with two bound calcium ions, °E·Ca_2, results in the phosphorylation of 70% of the total enzyme with a rate constant k = 300 s^{-1}. Furthermore, two Ca^{2+} ions are transported for each mole of phosphoenzyme that is formed during a single turnover (Petithory & Jencks, 1988a,b). This corresponds to 4 nmol/mg of Ca^{2+} as the maximal amount of calcium that can be internalized by the preparation of enzyme used in the experiments described by Figures 1 and 2 (E_{tot} = 2.8 nmol/mg) when the internalization of calcium is initiated by the addition of ATP plus EGTA.

The addition of ADP plus EGTA blocks the internalization of calcium because ADP rapidly reverses the phosphorylation of the enzyme by ATP and forms a mixture of phosphorylated and nonphosphorylated enzyme at equilibrium, which is described by the equilibrium constant K_{int} = 0.44 (Pickart & Jencks, 1982; Hanel & Jencks, 1990) (Scheme II). Calcium that is bound to the phosphorylated enzyme is internalized into the lumen of the vesicle with the rate constant k_c, while calcium that is bound to the nonphosphorylated enzyme dissociates irreversibly into the outside medium; the presence of EGTA prevents the rebinding of calcium to the nonphosphorylated enzyme and further phosphorylation by ATP. The irreversible dissociation of calcium from the nonphosphorylated enzyme following the addition of saturating concentrations of ADP [K_s(ADP) = 0.72 mM] (Pickart & Jencks, 1984) plus EGTA occurs with the rate constant k = 42–45 s^{-1} (Stahl & Jencks, 1987). The rate constant k_c = 17–18 s^{-1} for the internalization of calcium by E~P·ADP·Ca_2 was calculated from the value of 0.6 nmol/mg that is measured for the amount of Ca^{2+} that is internalized after the simultaneous addition of ATP, ADP, and EGTA, when 70% of the total enzyme reacts as shown in Scheme II. However, it will be shown later that the rate constant k_c for the internalization of Ca^{2+} from E~P·Ca_2 in the absence of ADP is ~30 s^{-1}.

The time course for the uptake of calcium following the addition of 2 mM ATP and 7 mM ADP is shown by the circles in Figure 1. The reactions were quenched by the addition of 5 mM EGTA, which chelates the calcium in the outside medium and prevents further binding of calcium and phosphorylation of the enzyme by ATP. The solid line is drawn for a rate of calcium transport of 1.4 μmol(min·mg of total protein)$^{-1}$, which is consistent with a rate constant of k = ~4 s^{-1} for turnover of the enzyme (E_{tot} = 2.8 nmol/mg) in the presence of 7 mM ADP. This result shows that the enzyme is capable of internalizing calcium in the presence of saturating concentrations of ADP. Turnover numbers for the uninhibited reaction of k = ~17 s^{-1} are typically measured for the conditions of the experiment in the absence of ADP (Pickart & Jencks, 1984; Stahl & Jencks, 1987). The inhibition by ADP results from binding of ADP to the nonphosphorylated enzyme (K_i = 0.09 mM) (Pickart & Jencks, 1984) and from a decrease in the concentration of E~P·Ca_2, which results from the reaction of E~P·Ca_2 with ADP to form E·ATP·Ca_2 (Scheme II).

Internalization of Two Ca^{2+} Ions. The rate constant for the loss of reactivity of the phosphoenzyme with ADP as the result of the internalization of two Ca^{2+} ions bound to the phosphoenzyme during a single turnover was measured by using a quench of 7 mM ADP and 5 mM EGTA. Figure 2 shows that two calcium ions are internalized into empty vesicles (open circles) in a first-order reaction that is described by a rate constant of $k = 29\ s^{-1}$. The same rate constant describes the transport of two Ca^{2+} ions into vesicles loaded with 20 mM Ca^{2+} (closed circles). The reactions were initiated by the simultaneous addition of 0.2 mM ATP and 5 mM EGTA to $^{c}E{\cdot}Ca_2$, the stable form of the enzyme with two bound calcium ions; this gives 70% phosphorylation of E_{tot} (2.8 nmol/mg) with $k = 300\ s^{-1}$, while the remaining 30% of E_{tot} dissociates Ca^{2+} irreversibly and is not phosphorylated (Petithory & Jencks, 1986). The data were corrected for the uptake of 0.6 nmol/mg Ca^{2+} that occurs after the addition of the quench (Figure 1). The endpoint of 3.7 nmol/mg ^{45}Ca is equal, within experimental error, to the endpoint of 4.0 nmol/mg that is expected for the internalization of two calcium ions when 70% of the enzyme is phosphorylated.

A total of 23 experiments, which measured the internalization of calcium using a quench with ADP, were performed over seven months with three different enzyme preparations. Values for the rate constant describing the internalization reaction were between 24 and 35 s^{-1} for 17 experiments; the maximal range for the rate constant was 17–53 s^{-1}. There were no significant trends in the variations of the rate constant with either the time of storage or the preparation of enzyme that was used. Fluctuations up to 2-fold in the magnitude of the rate constant that describes the release of Na^+ or K^+ from the Na,K-ATPase in the presence of NaMgATP have also been reported (Forbush, 1987a).

The data of Figure 2 show that loading the vesicles with 20 mM Ca^{2+} (closed circles) has no effect on the rate constant for the internalization of Ca^{2+} and the number of calcium ions that are transported during a single turnover of the enzyme. These results differ from those in a previous report from this laboratory (Khananshvili & Jencks, 1988), which indicated that only one calcium ion was transported into loaded vesicles and that the rate constant that describes the transport of one calcium ion into loaded vesicles was approximately twice the rate constant for the transport of two calcium ions into empty vesicles.

Transport of the Individual Ions. The transport of the individual calcium ions bound to $^{c}E{\cdot}Ca_2$ into empty and loaded SR vesicles during a single turnover is shown in Figure 3A,B. It has been shown that two calcium ions bind to and dissociate from the high-affinity calcium transport sites of the nonphosphorylated enzyme by an ordered-sequential mechanism (Dupont, 1982; Nakamura, 1986; Inesi, 1987; Petithory & Jencks, 1988a,b; Nakamura, 1989; Orlowski & Champeil, 1991). Petithory and Jencks (1988a) found that under the experimental conditions used in this study, the "outer" or rapidly exchanging calcium ion dissociates from $^{c}E{\cdot}Ca_2$ with a rate constant of 60 s^{-1} and that 0.6 μM calcium in the medium decreases the observed rate constant for the dissociation of the "inner" calcium ion by 50%.

The enzyme with either the "inner" or "outer" calcium ion labeled with ^{45}Ca was formed by diluting SR vesicles into buffer containing labeled or unlabeled calcium, respectively, for 20 s and was loaded into syringe A of the rapid mixing apparatus. The enzyme with bound labeled calcium was then mixed with 0.4 mM (empty vesicles) or 0.65 mM (loaded vesicles) unlabeled calcium delivered from syringe B for t_1 = 50 ms to form the species $^{c}E{\cdot}^{45}Ca{\cdot}^{40}Ca$, the form of the enzyme with the "inner" ion labeled (eq 1). During the 50-ms time period, only the outer, fast-exchanging labeled calcium ion is replaced with an unlabeled calcium ion from the medium. Similarly, enzyme with bound unlabeled calcium was mixed with 0.65 mM labeled calcium for 50 ms to form $^{c}E{\cdot}^{40}Ca{\cdot}^{45}Ca$ (eq 2). Transport reactions were initiated immediately thereafter by the addition of 0.25 mM ATP and 10 mM EGTA. The uptake of calcium was quenched by the addition

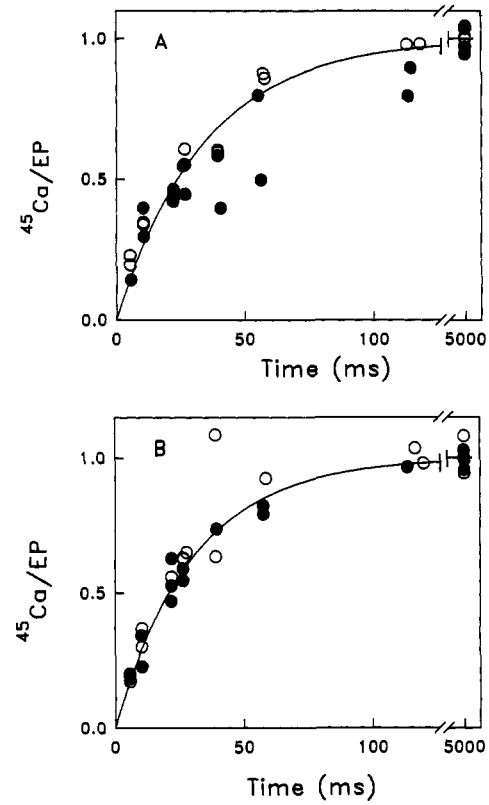


FIGURE 3: Transport into empty or loaded intact SR vesicles of the individual calcium ions initially bound to the nonphosphorylated CaATPase during a single turnover. (Panel A, top) All syringes of the rapid-mixing apparatus contained 40 mM MOPS·Tris, 100 mM KCl, pH 7.0 (buffer A) at 25 °C. The inner calcium ion was selectively labeled by first diluting empty vesicles (○) or vesicles loaded with 20 mM ^{40}Ca (●) 180-fold into buffer A containing either 0.04 mM ^{45}Ca (20 $\mu Ci/\mu mol$) (○) or 0.02 mM ^{45}Ca (131 $\mu Ci/\mu mol$) (●). The final concentration of protein and the specific activity of ^{45}Ca following dilution were 0.39 mg/mL and 20 $\mu Ci/\mu mol$, respectively, for both loaded and empty vesicles. The diluted enzyme was then loaded into syringe A of the rapid-mixing apparatus, and exchange of the outer calcium ion was initiated within 20 s by mixing the contents of syringe A for t_1 = 50–52 ms with 0.8 mM (○) or 1.3 mM (●) ^{40}Ca contained in syringe B. The enzyme was then mixed for t_2 = 5–120 ms with 0.75 mM ATP, 15 mM $MgCl_2$, and 30 mM EGTA contained in syringe C. The reactions were quenched by the addition of 28 mM ADP and 10 mM EGTA delivered from syringe D. Endpoints for the reactions were measured by manually adding the contents of syringe D at 5 s. Reaction blanks were obtained by mixing the enzyme after t_1 with 0.75 mM ATP, 21 mM ADP, and 30 mM EGTA from syringe C for t_2 = 19 ms, followed by the addition of 7 mM ADP and 10 mM EGTA from syringe D. (Panel B, bottom) The outer calcium ion was selectively labeled by first diluting empty (○) or loaded (●) vesicles into buffer A containing 0.13 mM (○) or 0.02 mM (●) ^{40}Ca to give final concentrations of protein = 0.39 mg/mL and ^{40}Ca = 0.13 mM and then loading this mixture into syringe A of the rapid-mixing apparatus. Exchange of the outer calcium ion was initiated within 20 s by mixing the contents of syringe A with 0.13 mM ^{45}Ca (40 $\mu Ci/\mu mol$) contained in syringe B for t_1 = 50–52 ms (○, ●). All other reaction conditions were the same as those specified above for panel A. The solid lines of panels A and B were calculated for rate constants of $k = 29\ s^{-1}$ and $k = 33\ s^{-1}$ and endpoints of 1 $^{45}Ca/EP$ corresponding to 1.9 and 2.1 nmol/mg ^{45}Ca, respectively.

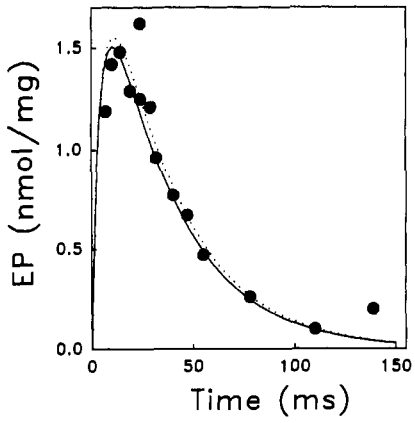


FIGURE 4: Formation and decay of the total phosphoenzyme with leaky vesicles in the presence of EGTA. The rapid-mixing apparatus was used in the three-syringe configuration containing 40 mM MOPS·Tris, pH 7.0, 100 mM KCl, and 5 mM $MgCl_2$ in syringes A and B at 25 °C. Syringe A also contained 0.43 mg/mL base-treated leaky vesicles, 0.4 mM EGTA, and 0.42 mM $CaCl_2$ (26 μM free Ca^{2+}). Phosphorylation was initiated by mixing the contents of syringe A with 0.5 mM [γ-^{32}P]ATP, 10 mM EGTA, 1.5 mM phosphocreatine, and 0.05 mg/mL creatine kinase in syringe B. The reactions were quenched at the times indicated in the figure by the addition of 0.38 M $HClO_4$ and 1.5 mM KH_2PO_4 in syringe C. An endpoint value of 0.07 nmol/mg EP was measured at 5 s by adding the quench solution manually and subtracted from the measured values of EP. The total EP for the preparation was 2.4 nmol/mg. The solid line is calculated for the formation and disappearance of the phosphoenzyme according to the reaction: $E{\cdot}Ca_2 + ATP \rightarrow E{\sim}P{\cdot}Ca_2 + ADP \rightarrow E{-}P + 2\ Ca^{2+} \rightarrow E + P_i$ with rate constants of 300, 30, and 115 s^{-1} for the formation of $E{\sim}P{\cdot}Ca_2$, E–P, and E, respectively. The dotted line is calculated for the reaction sequence $E{\cdot}Ca_2 + ATP \rightarrow E{\sim}P{\cdot}Ca_2 + ADP \rightarrow {}_{in}E{-}P{\cdot}Ca + Ca^{2+} \rightarrow E{-}P + Ca^{2+} \rightarrow E + P_i$ with rate constants of 300, 30, 400, and 115 s^{-1} for the formation of $E{\sim}P{\cdot}Ca_2$, ${}_{in}E{-}P{\cdot}Ca$, E–P, and E, respectively.

of 7 mM ADP at the times specified at the bottom of the figures.

$$E + {}^{45}Ca \xrightarrow{20\ s} E{\cdot}{}^{45}Ca_{inner}{\cdot}{}^{45}Ca_{outer} \xrightarrow[50\ ms]{{}^{40}Ca} E{\cdot}{}^{45}Ca_{inner}{\cdot}{}^{40}Ca_{outer} \quad (1)$$

$$E + {}^{40}Ca \xrightarrow{20\ s} E{\cdot}{}^{40}Ca_{inner}{\cdot}{}^{40}Ca_{outer} \xrightarrow[50\ ms]{{}^{45}Ca} E{\cdot}{}^{40}Ca_{inner}{\cdot}{}^{45}Ca_{outer} \quad (2)$$

Figure 3A shows that the first calcium ion to bind to the free enzyme, the "inner" calcium ion, is transported into empty (○) and loaded (●) vesicles in a reaction that is consistent with a first-order rate constant of $k = 29\ s^{-1}$, as shown by the solid line. Loading the vesicles with 20 mM Ca^{2+} does not affect either the rate constant or the endpoint for the reaction. The endpoints of 1.9 nmol/mg ^{45}Ca that were measured at 5 s correspond to 63% of the total amount of enzyme of the preparation that was used (3 nmol/mg), which is equal, within experimental error, to the expected endpoint of 2.1 nmol/mg, corresponding to 70% of E_{tot}. The data of Figure 3A show that the rate constant of 29 s^{-1} for the transport of the first calcium ion that binds to the free enzyme is the same as the rate constant for the transport of two calcium ions that was measured with a different preparation of enzyme.

Similar experiments that measure the rate of transport of the "outer" calcium ion in a single turnover are shown in Figure 3B. The solid line is drawn for a rate constant of $k = 33\ s^{-1}$, which describes a first-order reaction for transport of the "outer" calcium ion into the lumen of both empty vesicles (empty circles) and vesicles loaded with 20 mM Ca^{2+} (filled circles). No indication of an induction period or a biphasic reaction was observed for the reaction with either empty or loaded vesicles. The observed endpoints of 2.1 nmol/mg ^{45}Ca correspond to 70% of E_{tot} and represent the transport of one labeled calcium ion during a single turnover for each molecule of phosphoenzyme that is formed. The rate constant of $k = 33\ s^{-1}$ for the transport of the second calcium ion does not differ significantly from the rate constant of $k = 29\ s^{-1}$ that was measured for the transport of two calcium ions into empty and loaded vesicles (Figure 2).

Scheme III

$$^{c}E{\cdot}Ca_2 + ATP \xrightarrow{k_p} E{\sim}P{\cdot}Ca_2 + ADP \xrightarrow{k_{Ca}}$$

$$E{-}P + 2Ca^{2+} \xrightarrow{k_h} E + P_i$$

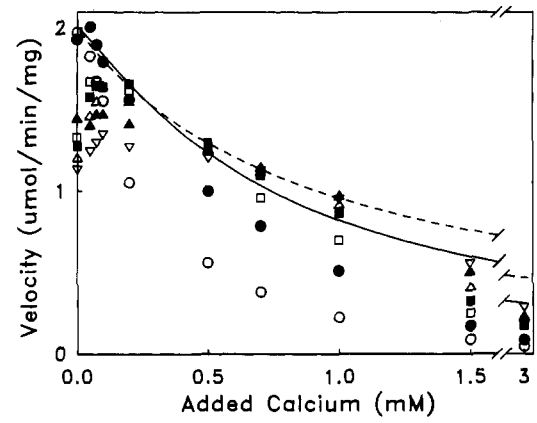


FIGURE 5: Inhibition by calcium of the hydrolysis of ATP catalyzed by the CaATPase during turnover in the steady state at 25 °C. The reaction mixtures contained 0.014 mg of base-treated leaky vesicles, 40 mM MOPS·Tris, pH 7.0, 100 mM KCl, 0.20 mM ATP, 1.5 mM PEP, 0.15 mM NADH, 0.05 mg of pyruvate kinase, 0.05 mg of lactate dehydrogenase, 0.5% glycerol, 1 (○), 3 (●), 5 (□), 7 (■), 10 (△), 15 (▲), or 20 (▽) mM $MgCl_2$, and the indicated amount of added calcium in a 1.0-mL total volume. The maximal velocity at each concentration of calcium was obtained by inspection of plots of velocity against $[Mg^{2+}]$ at that calcium concentration. The highest observed velocity was 2.0 μmol/(min·mg). The lines drawn correspond to fits of the data taken from plots of log $[V/(V_{max} - V)]$ against log $[Ca^{2+}]$ and were calculated for values of $K_{0.5} = 0.76$ mM and 0.91 mM $[Ca^{2+}]$ and Hill slopes with $n_H = 1.2$ (solid line) and $n_H = 1.0$ (dashed line), respectively.

Formation and Decay of the Phosphoenzyme during a Single Turnover. Figure 4 shows the time course for the formation and decay of the phosphoenzyme during a single turnover with vesicles that were made permeable to calcium by treatment with immobilized EGTA at alkaline pH, as described under Materials and Methods. The phosphoenzyme was formed by the addition of labeled ATP and excess EGTA to $^{c}E{\cdot}Ca_2$, which gives 70% phosphorylation of the total enzyme with a rate constant of $k = 300\ s^{-1}$ (Petithory & Jencks, 1986). Further phosphorylation is prevented by the presence of EGTA, which lowers the concentration of free calcium in the medium to <0.1 μM. The time course for the formation and hydrolysis of the phosphoenzyme is consistent with the rate constants $k_p = 300\ s^{-1}$, $k_{Ca} = 30\ s^{-1}$, and $k_h = 115\ s^{-1}$, as shown by the calculated solid line for a single turnover of the enzyme according to Scheme III. There is no indication of two kinetically significant steps for dissociation of the calcium ions. The dotted line is calculated for a sequence of reactions similar to Scheme III but with two first-order reaction steps with rate constants of 30 and 400 s^{-1} for dissociation of the two Ca^{2+} ions from $E{\sim}P{\cdot}Ca_2$ to give E–P. The value of 400 s^{-1} for the rate constant of the fast step is a lower limit; a detectable delay in the disappearance of the phosphoenzyme would be observed for a rate constant less than 400 s^{-1}.

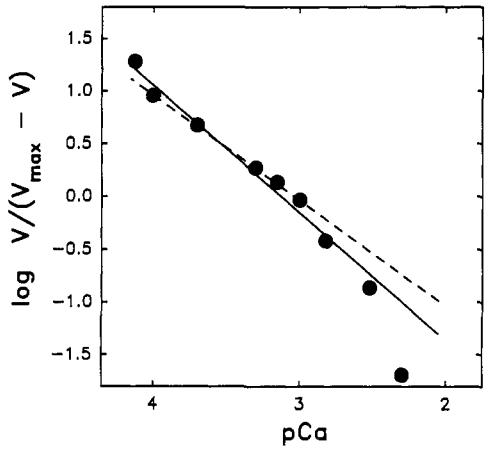


FIGURE 6: Hill plot for the inhibition of CaATPase activity by calcium, from the data shown in Figure 5. The lines are drawn with slopes of 1.2 (solid line) and 1.0 (dashed line) and values of $K_{0.5}$ = 0.76 and 0.9 mM Ca^{2+}, respectively. The values of $K_{0.5}$ = 0.76 mM and n_H = 1.2 were determined from a linear regression analysis of the data. The maximal velocity for the uninhibited reaction is 2.0 μmol/(min·mg).

Inhibition of Turnover by Calcium. The inhibition of steady-state turnover of the CaATPase by added calcium is shown in Figure 5. The enzyme was made permeable to calcium by treatment with an immobilized chelating agent at alkaline pH and reaction velocities were determined in the presence of six different concentrations of Mg^{2+}, in the range of 1–20 mM.

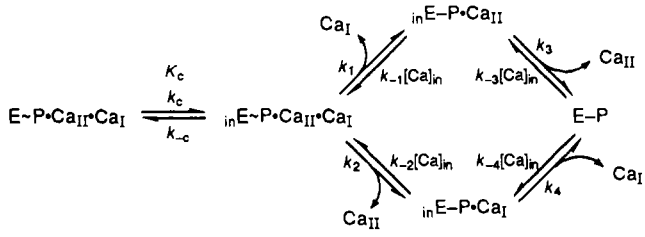
The rate of turnover with 200 μM ATP is inhibited by concentrations of calcium greater than ~70 μM. Some of the observed inhibition by calcium can be overcome by increasing the concentration of magnesium in the assay. This is because significant amounts of Ca·ATP are formed when the ratio of $[Ca^{2+}]$ to $[Mg^{2+}]$ in the assay is increased and Ca·ATP inhibits turnover of the enzyme (Yamada & Tonomura, 1972; Vianna, 1975; Shigekawa et al., 1983; Orlowski et al., 1988; Lacapère & Guillain, 1990). This inhibition arises from the slow formation and hydrolysis of phosphorylated enzyme with calcium in the catalytic site (Yamada & Ikemoto, 1980; Shigekawa et al., 1983; Wakabayashi & Shigekawa, 1987; Hanel & Jencks, 1990). At low concentrations of calcium there is inhibition by Mg^{2+}, which has been attributed to the binding of Mg^{2+} at the calcium transport sites of the nonphosphorylated enzyme (Yamada & Tonomura, 1972; Vianna, 1975). The optimal reaction velocity at each concentration of calcium was obtained from plots of the reaction velocity against Mg^{2+} concentration (not shown).

A Hill plot of the optimal reaction velocity at different concentrations of calcium is shown in Figure 6. The data give a best fit to a line with a slope of n_H = 1.2 and a value of 0.76 mM Ca^{2+} for half-maximal inhibition by Ca^{2+}, as shown by the solid line. However, there is a relatively large uncertainty of the velocity ratios at the highest and lowest concentrations of calcium, and the data are also consistent with a slope of n_H = 1.0 (dashed line). Hill slopes of 1.3 and 0.9 with values of $K_{0.5}$ = 0.5 mM Ca^{2+} for inhibition of turnover by calcium have been reported previously (Bodley & Jencks, 1987; Khananshvili et al., 1990).

Discussion

Quench with ADP. The rate of internalization of calcium into the lumen of SR vesicles by the phosphorylated CaATPase was measured during a single turnover of the enzyme by using a quench of 7 mM ADP and 5–10 mM EGTA, which lowers the concentration of free calcium in the outside medium to

Scheme IV



<0.1 μM. The binding of ADP to E~P·Ca_2 results in the rapid synthesis of ATP on the enzyme, which causes the calcium transport sites to face the outside medium and initiates the irreversible dissociation of calcium from these sites when EGTA is present with a rate constant of k = 42–45 s^{-1} (Stahl & Jencks, 1987). The simultaneous addition of 7 mM ADP and 5 mM EGTA to E·ATP·Ca_2 prevents the uptake of ~85% of the calcium that would have been internalized by the phosphorylated enzyme during a single turnover of the enzyme in the absence of ADP (Figure 1).

Kinetics of Internalization of Ca^{2+} by E~P·Ca_2. The two calcium ions of E~P·Ca_2 are internalized into empty vesicles in a first-order reaction with a rate constant of k = ~30 s^{-1} (Figure 2, empty circles). The presence of 20 mM Ca^{2+} inside the vesicles does not affect either the rate of internalization of the two Ca^{2+} ions or the total amount of calcium that is transported during a single turnover of the enzyme (Figure 2, filled circles). The individual calcium ions that bind to the nonphosphorylated enzyme, which were selectively labeled, are internalized with the same first-order rate constant of k = ~30 s^{-1}. Loading the vesicles with 20 mM Ca^{2+} has no effect on either the rate or the extent of internalization of either of the individual ions, and there is no indication of an induction period or lag for the uptake of either ion (Figure 3).

The results of Figures 2 and 3 show that the two Ca^{2+} ions of E~P·Ca_2 are kinetically indistinguishable. Both ions become insensitive to the quench during a first-order process that is described by the same rate constant of k = ~30 s^{-1}. Figure 4 shows that the time course for the formation and decay of the phosphorylated enzyme of leaky vesicles in the presence of EGTA, to give the free enzyme and P_i, is consistent with the formation of E~P·Ca_2 with the rate constant k = 300 s^{-1} (Petithory & Jencks, 1986), followed by dissociation of calcium to give the phosphoenzyme E–P with the rate constant k = 30 s^{-1}, and hydrolysis of E–P with the rate constant k = ~115 s^{-1} (Pickart & Jencks, 1984). The dissociation of calcium with k = ~30 s^{-1} is predominantly rate limiting for the hydrolysis of E~P·Ca_2.

Mechanisms for the Dissociation of Ca^{2+} from E~P·Ca_2. The kinetic equivalence of the two Ca^{2+} ions bound to the phosphoenzyme and the absence of inhibition by lumenal calcium of the dissociation of the individual ions can be accounted for by three possible mechanisms, all of which involve a rate-limiting conformational change that precedes dissociation of the ions. According to the first mechanism, shown in Scheme IV, a conformational change with rate constant k_c = 30 s^{-1} gives $_{in}$E~P·Ca_{II}·Ca_I and exposes the two Ca^{2+} ions to the inside of the vesicle; this is followed by the rapid dissociation of the calcium ions from independent transport sites on the enzyme. There is no difference in the rate of dissociation of the two Ca^{2+} ions from $_{in}$E~P·Ca_{II}·Ca_I. Rapid dissociation of the two calcium ions after the conformational change is consistent with the rate constant of 30 s^{-1} that is observed for the internalization of the Ca^{2+} ions into empty vesicles (Figures 2 and 3, empty symbols) and the biphasic time course for the disappearance of the phosphoenzyme that

is observed with leaky vesicles (Figure 4).

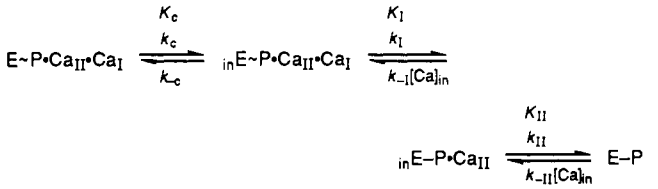
The binding of calcium to independent transport sites on the phosphorylated enzyme can explain why high concentrations of calcium in the lumen do not affect dissociation of the labeled Ca^{2+} ions bound to the phosphorylated enzyme during a single turnover. A first-order rate constant of $k = 30\ s^{-1}$ will be observed for internalization of the individual ions when reaction steps for the binding and dissociation of calcium are fast compared with the formation of $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ from $E\sim P\cdot Ca_{II}\cdot Ca_I$ and the reversal of the conformational change with the rate constant k_{-c}. Lumenal calcium will bind to $_{in}E-P\cdot Ca_I$ or $_{in}E-P\cdot Ca_{II}$ with the rate constants $k_{-2}[Ca]_{in}$ and $k_{-1}[Ca]_{in}$, respectively, to form $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ and block the formation of E–P; however, the dissociation of either Ca^{2+} ion from $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ will not be affected by lumenal calcium.

The conformational change, which follows the rapid phosphorylation of the enzyme by ATP, causes a reorganization of the binding sites for calcium on the phosphorylated enzyme. The species $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ is formed transiently during turnover and does not accumulate appreciably when the reaction cycle is at equilibrium. This assumption is valid if the rate constant for reversal of the conformational change, k_{-c}, is $\sim 3000\ s^{-1}$ and the calcium ions bound to $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ dissociate with rate constants of $\geq 10^5\ s^{-1}$. A lower limit of $k = 10^5\ s^{-1}$ for the dissociation of each calcium ion from $_{in}E\sim P\cdot Ca_2$ is consistent with an apparent equilibrium constant of $K \geq 10^{-3}$ M for the binding of calcium to $_{in}E-P\cdot Ca$ when the binding of calcium occurs with a second-order rate constant of $k = 10^8\ M^{-1}\ s^{-1}$, which is at or near the diffusion limit. Monovalent cations have been shown to bind to the gramicidin A channel with a second-order rate constant of $2 \times 10^8\ M^{-1}\ s^{-1}$ (Andersen, 1983).

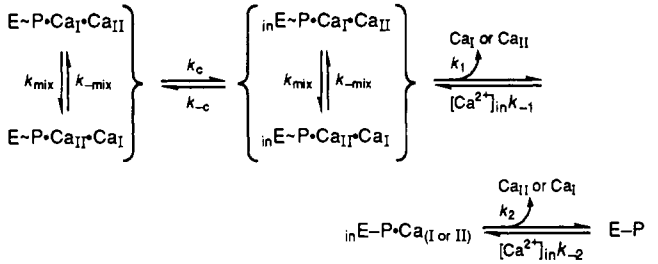
The transient formation of $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$, $_{in}E-P\cdot Ca_I$, or $_{in}E-P\cdot Ca_{II}$ during turnover minimizes the likelihood that these intermediates will react with ADP to give a form of the nonphosphorylated enzyme that has the binding sites for calcium facing the lumen of the SR. The dissociation of calcium from such species of nonphosphorylated enzyme would result in the internalization of two calcium ions without the concomitant hydrolysis of one molecule of ATP; this is not observed.

The equilibrium constants for the binding of calcium to E–P and $_{in}E-P\cdot Ca$ have not been measured directly. Steady-state velocities for turnover at various concentrations of calcium were calculated by the method of net rate constants (Cleland, 1975) using a model in which the phosphoenzyme is formed from ATP with $k = 70\ s^{-1}$ (Stahl & Jencks, 1987) and undergoes hydrolysis with $k = 115\ s^{-1}$ (Pickart & Jencks, 1984); calcium dissociation was described according to Scheme IV with second-order rate constants of $k = 10^8\ M^{-1}\ s^{-1}$ for the binding of calcium to the phosphorylated enzyme. Those calculations showed that the Hill slope of $n_H = 1.2$ for the inhibition of turnover by binding of calcium to the phosphorylated enzyme (Figure 6) is consistent with noncooperative binding of the two Ca^{2+} ions to the phosphoenzyme. Apparent dissociation constants of 0.36 and 1.4 mM for the binding of calcium to E–P and $_{in}E-P\cdot Ca$, respectively (Scheme IV), are consistent with the measured value of $K_{0.5} = 0.8$ mM for the inhibition. The 4-fold difference between the affinities for binding the two Ca^{2+} ions arises from statistical considerations and reflects the facts that the second-order rate constant for the binding of calcium to E–P is twice as large as that for the binding of calcium to $_{in}E-P\cdot Ca$, because two sites are available for binding of calcium to E–P, while the dissociation of one of the two Ca^{2+} ions from $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ to give $_{in}E-P\cdot Ca$ is twice as fast as the dissociation of calcium from $_{in}E-P\cdot Ca$ to give E–P, because calcium can dissociate from either of the two sites that are occupied on $_{in}E\sim P\cdot Ca_2$.

Scheme V



Scheme VI



Alternatively, the dissociation of calcium from $E\sim P\cdot Ca_2$ can be described by the models shown in Schemes V and VI in which a rate-limiting conformational change with $k_c = \sim 30\ s^{-1}$ precedes rapid and sequential dissociation of the bound Ca^{2+} ions. Scheme V describes a model in which the calcium ions dissociate in a specific order after the conformational change. We were unable to determine the order of dissociation, however, because 20 mM calcium in the lumen of the vesicles does not affect the rate of internalization of the individual ions (Figure 3).

A concentration of 20 mM calcium in the lumen of the vesicles does not inhibit the dissociation of calcium from $_{in}E-P\cdot Ca_{II}$, according to Scheme V, because the rate constants k_I and k_{II}, which describe the dissociation of calcium from $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ and $_{in}E-P\cdot Ca_{II}$, respectively, are much larger (k_I, $k_{II} \geq 10^5\ s^{-1}$) than k_c and k_{-c}, the rate constants for the forward and reverse steps of the conformational change. Furthermore, the binding of 20 mM lumenal calcium to $_{in}E-P\cdot Ca_{II}$, which gives $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ and blocks the dissociation of calcium from $_{in}E-P\cdot Ca_{II}$, is unfavorable, $K_I = \sim 0.1$ M. Weak binding of calcium to $_{in}E-P\cdot Ca_{II}$ is balanced by the equilibrium constant $K_c = 0.01$, which favors the formation of $E\sim P\cdot Ca_{II}\cdot Ca_I$ from $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$; values of $K_c = 0.01$ and $K_I = 0.1$ M give a value of $K_{app} = \sim 10^{-3}$ M for the apparent equilibrium constant describing the dissociation of the first Ca^{2+} ion, Ca_I. (eqs 3 and 4). This may explain why

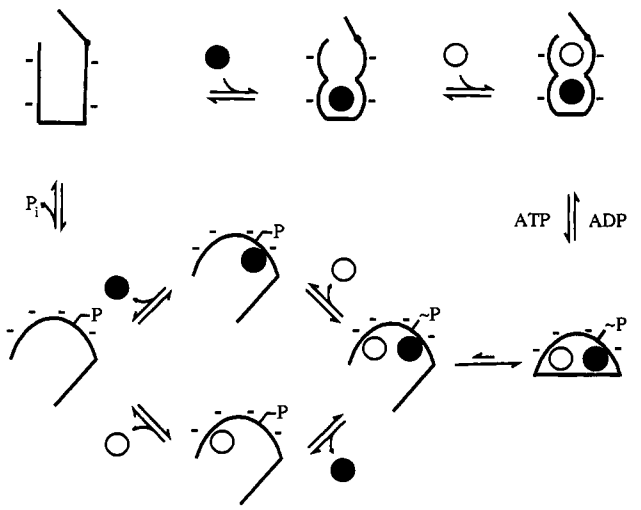
$$K_{app} = \frac{[_{in}E-P\cdot Ca_{II}][Ca]}{[E\sim P\cdot Ca_{II}\cdot Ca_I + {}_{in}E\sim P\cdot Ca_{II}\cdot Ca_I]} \quad (3)$$

$$K_{app} = K_I/(1 + 1/K_c) \quad (4)$$

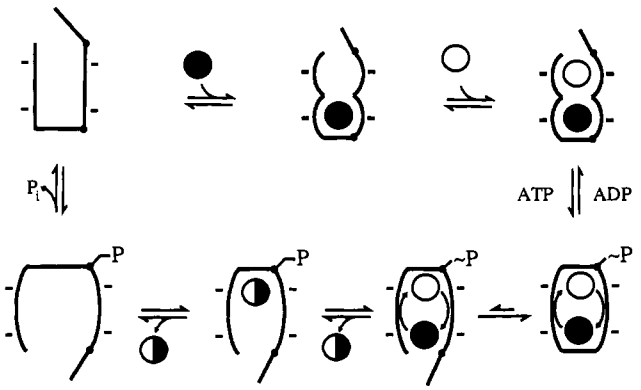
20 mM lumenal calcium is not effective in blocking the dissociation of calcium from $_{in}E-P\cdot Ca_{II}$; significant amounts of $_{in}E\sim P\cdot Ca_{II}\cdot Ca_I$ do not accumulate during a single turnover.

The dissociation of the Ca^{2+} ions bound to the phosphoenzyme may also be described by the model shown in Scheme VI, in which the two Ca^{2+} ions rapidly exchange their positions at the transport sites after phosphorylation of the enzyme by ATP. Mixing of the ions is followed by a rate-limiting conformational change with $k_c = 30\ s^{-1}$, which precedes rapid and sequential dissociation of the bound Ca^{2+} ions from the enzyme. After the first Ca^{2+} ion dissociates with the rate constant k_1, calcium from the lumen will bind to $_{in}E-P\cdot Ca$ with

Scheme VII



Scheme VIII



the rate constant $k = k_{-1}[Ca]_{in}$ and mix rapidly ($k_{mix} \geq 10^5$ s^{-1}) with the second Ca^{2+} ion. A single Ca^{2+} ion will again dissociate as this sequence of reactions is repeated. The binding, mixing, and dissociation reactions for calcium are fast enough so that all of the labeled calcium that is initially bound to the enzyme is replaced by calcium from the lumen before the conformational change reverses, with a rate constant of $k_{-c} \geq 3000\ s^{-1}$. The mechanism of Scheme VI will also account for the fact that 20 mM lumenal calcium does not inhibit the dissociation of either of the Ca^{2+} ions (Figure 3).

The decrease in the affinity of the enzyme for bound calcium upon phosphorylation of $E{\cdot}Ca_2$ by ATP, by a factor of ~1000-fold, can be caused by a decrease in the number and strength of the interactions between the calcium-ligating groups on the enzyme and the bound Ca^{2+} ions. The change in ligation of the Ca^{2+} ions of $E{\sim}P{\cdot}Ca_2$ is presumably caused by a conformational change that is brought about by phosphorylation of the enzyme by ATP. This is depicted in Scheme VII as the formation of a second site from which calcium can dissociate and in Scheme VIII as a widening of the binding sites that allows the ions to mix. One possible mechanism for this conformational change is a twisting of the transmembrane helices that are thought to contain the calcium-binding sites (Clarke et al., 1989), as proposed by Tanford (1982) and Williams (Levine & Williams, 1988).

Kinetic equivalence of bound ions has been observed previously for the dissociation of K^+ or Rb^+ from the Na,K-ATPase. The kinetics for the dissociation of the individual K^+ or Rb^+ ions from the enzyme in the presence of Mg^{2+} and P_i supports an ordered and sequential mechanism (Forbush, 1987b), which is similar to the ordered-sequential mechanism for the dissociation of Ca^{2+} from the nonphosphorylated CaATPase. It was shown that K^+ in the medium blocks the dissociation of the first K^+ or Rb^+ ion that binds to the enzyme; this is analogous to the inhibition of dissociation of the "inner" Ca^{2+} ion from ${}^cE{\cdot}Ca_2$ by Ca^{2+} in the medium. However, in the presence of ATP and P_i both K^+ and Rb^+ ions dissociate from the Na,K-ATPase in a first-order reaction that is not inhibited by concentrations of K^+ in the medium up to 2 M. The absence of inhibition by added K^+ may result from a slow conformational change, which precedes very rapid sequential and ordered dissociation of the ions (Forbush, 1987b). Alternatively, the absence of inhibition may be explained by mechanisms similar to those described here, in which the K^+ or Rb^+ ions either dissociate from separate independent binding sites on the Na,K-ATPase or mix rapidly after ATP binds and initiates sequential dissociation of the two ions.

ATP–ADP-Mediated Calcium Exchange and the Kinetic Equivalence of the Two Ca^{2+} Ions on $E{\sim}P{\cdot}Ca_2$. The CaATPase catalyzes the exchange of labeled Ca^{2+} ions across the SR membrane in the presence of ADP and ATP (Makinose, 1973; Waas & Hasselbach, 1981; Takenaka et al., 1982). Takakuwa and Kanazawa have shown that the rate of this exchange increases with increasing Ca^{2+} concentration on the outside and on the lumenal sides of the membrane, with values of $K_{0.5}$ = 2–3 μM for the outside and 6 mM for the lumenal Ca^{2+} concentrations (Takakuwa & Kanazawa, 1981; Inao & Kanazawa, 1986). This exchange reaction will not occur unless both of the Ca^{2+} ions of $E{\sim}P{\cdot}Ca_2$ can dissociate and mix with Ca^{2+} ions in the lumen. In order for calcium exchange to occur, both of the Ca^{2+} ions must be able to dissociate from the transport sites on one side of the membrane; increasing the concentration of exterior calcium prevents dissociation of one Ca^{2+} because of the ordered-sequential dissociation of these ions. However, dissociation of both calcium ions from ${}_{in}E{\sim}P{\cdot}Ca_2$ into the lumen after the rate-limiting conformational change permits the exchange of labeled calcium into and out of loaded vesicles, regardless of the concentration of Ca^{2+} on the outside and inside of the vesicle.

Cooperativity and the Binding of Two Ca^{2+} Ions to E–P·Mg. The apparent dissociation constants for the binding of lumenal calcium to E–P·Mg and ${}_{in}E$–P·Ca·Mg have not been measured directly; rapid hydrolysis of the phosphoenzyme makes the measurement of these constants difficult. (In this section we include Mg^{2+} in the formula for the phosphoenzyme because we are considering the equilibrium constant for the formation of phosphoenzyme in the presence of Mg^{2+}). Suko and co-workers have reported an approximate 5-fold increase in the equilibrium amount of phosphoenzyme that is formed from inorganic phosphate and Mg^{2+} at 5 °C as the concentration of calcium in the lumen of SR vesicles is increased; the increase in phosphoenzyme formation follows a Hill slope of n_H = 1.72 for the conditions of these experiments (Prager et al., 1979). The magnitude of the Hill slope will depend, in part, on the equilibrium constant for the formation of E–P·Mg in the absence of lumenal calcium because E–P·Mg must be formed before calcium from the lumen can bind to give $E{\sim}P{\cdot}Mg{\cdot}Ca_2$.

Scheme IX describes a sequence of reactions and equilibrium constants for the formation of $E{\sim}P{\cdot}Mg{\cdot}Ca_2$ from P_i; "fraction EP" is the ratio of phosphorylated to nonphosphorylated enzyme at specified concentrations of P_i and Mg^{2+} and no lumenal Ca^{2+} (eq 5). A value of "fraction EP" = 0.25 is applicable for the experimental conditions of Prager et al. (1979). A Hill slope of n_H = 1.6 with $K_{0.5}$ = 5 mM is calculated for the increase in total phosphoenzyme, as de-

Scheme IX

$$E + P_i + Mg^{2+} \rightleftharpoons E{\cdot}P_i{\cdot}Mg \rightleftharpoons E{-}P{\cdot}Mg \overset{Ca^{2+}}{\underset{K_I}{\rightleftharpoons}}$$

$$_{in}E{-}P{\cdot}Mg{\cdot}Ca \overset{Ca^{2+}}{\underset{K_{II}}{\rightleftharpoons}} E{\sim}P{\cdot}Mg{\cdot}Ca_2$$

scribed by Scheme IX, with increasing concentrations of calcium when "fraction EP" = 0.25, K_I = 3 mM, and K_{II} = 3 mM. These values for the dissociation constants describe the binding of a single Ca^{2+} ion to E–P that gives $_{in}$E–P·Mg·Ca but does not increase the affinity of $_{in}$E–P·Mg·Ca for binding of the second Ca^{2+} ion.

$$\text{"fraction EP"} = \left(\frac{[E{-}P{\cdot}Mg]}{[E + E{\cdot}Mg{\cdot}P_i]}\right)_{Ca=0} \quad (5)$$

Thus, the apparent cooperativity for the binding of calcium can be accounted for simply by the unfavorable equilibrium for the formation of phosphoenzyme in the absence of calcium; the magnitude of the Hill slope for total EP formation increases as "fraction EP" decreases. The reported Hill slope of n_H = 1.7 does not differ significantly from n_H = 1.6 and does not provide evidence that two calcium ions bind to the *phosphorylated* enzyme with positive cooperativity. The model for the binding of two Ca^{2+} ions shown in Scheme IX can give cooperativity when the first and second calcium ions bind with equal affinity to E–P·Mg and $_{in}$E–P·Mg·Ca, respectively. Hill slopes that are determined from experiments that do not measure directly the binding of a ligand to a single protein species require careful evaluation; their physical significance can be misinterpreted when postulating mechanisms and dissociation constants for the binding of ligand.

Conformational Change. We have not been able to account for the kinetics of calcium internalization, the absence of inhibition by internal calcium, and the kinetic equivalence of the two Ca^{2+} ions, without postulating a rate-limiting conformational change that exposes the two calcium ions of $E{\sim}P{\cdot}Ca_2$ to the inside of the vesicles in $_{in}E{\sim}P{\cdot}Ca_2$. Such a change has been postulated in many models for the enzyme that invoke species such as E_1 and E_2, E* and E, or E and E′. However, we believe that it is important to describe the mechanism of coupled ion transport by this and other enzymes with models in which the postulated properties of the enzyme species in the model are in agreement with observed properties of these species; this is not the case for many such models. For example, we are not aware of evidence for a species, $E_1{\sim}P{\cdot}Ca_2$, that will dissociate calcium to the cytoplasmic side of the vesicle or for a species, $E_2{-}P{\cdot}Ca_2$, that will not react with ADP to form ATP. All of the evidence of which we are aware is consistent with the simple model of Scheme I in which the direction of calcium binding and dissociation is controlled by the state of phosphorylation of the enzyme, and the chemical specificity of the enzyme for reacting reversibly with ATP or inorganic phosphate is controlled by the presence or absence of calcium at the transport sites.

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