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Two-Step Internalization of Ca^{2+} from a Single E~P·Ca_2 Species by the Ca^{2+}-ATPase†

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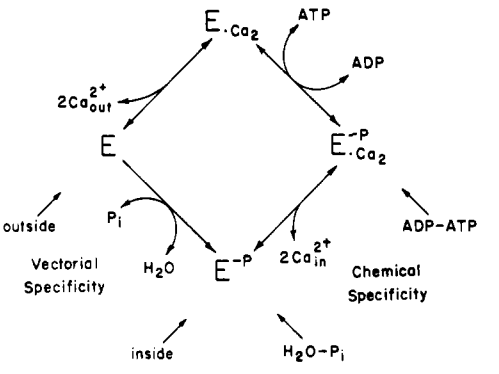
ABSTRACT: Phosphorylation by ATP of E·*Ca_2 (sarcoplasmic reticulum vesicles (SRV) with bound $^{45}Ca^{2+}$) during 5–10 ms leads to the occlusion of 2 *Ca^{2+}/EP_{tot} [quench by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) alone] in both "empty" (10 μM free Ca^{2+}_{in}) or "loaded" SRV (20–40 mM free Ca^{2+}_{in}). The rate of Ca^{2+} "internalization" from the occluded E~P·*Ca_2 was measured by using an ADP + EGTA quench; a *Ca^{2+} ion that is not removed by this quench is defined as internalized. In the presence of 20–40 mM unlabeled Ca^{2+} inside SRV, 1 *Ca^{2+}/EP_{tot} is internalized from ^{45}Ca-labeled E~P·*Ca_2 with a first-order rate constant of $k_1 = 34\ s^{-1}$. Empty SRV take up 2 *Ca^{2+}/EP_{tot} with the same initial rate, but the overall rate constant is $k_{obsd} = 17\ s^{-1}$. The apparent rate constant ($k_b = 17\ s^{-1}$) for internalization of the second *Ca^{2+} is inhibited by $[Ca]_{in}$, with $K_{0.5} \sim 1.3$ mM and a Hill coefficient of $n = 1.1$. These data show that the two Ca^{2+} ions are internalized sequentially, presumably from separate sequential sites in the channel. [^{32}P]EP·Ca_2 obtained by rapid mixing of E·Ca_2 with [γ-^{32}P]ATP and EGTA disappears in a biphasic time course with a lag corresponding to $\sim 34\ s^{-1}$, followed by EP* decay with a rate constant of $\sim 17\ s^{-1}$. This shows that both Ca^{2+} ions must be internalized before the enzyme changes its specificity for catalysis of phosphoryl transfer to water instead of to ADP. Increasing the concentration of ATP from 0.25 to 3 mM accelerates the rate of $^{45}Ca^{2+}$ internalization from 34 to 69 s^{-1} for the first Ca^{2+} and from 17 to 34 s^{-1} for the second Ca^{2+}. High [ATP] also accelerates both phases of [^{32}P]EP·Ca_2 disappearance by the same factor. The data are consistent with a single form of ADP-sensitive E~P·Ca_2 that sequentially internalizes two ions. The intravesicular volume was estimated to be 2.0 μL/mg, so that one turnover of the enzyme gives 4 mM internal $[Ca^{2+}]$.

The mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase[1] of sarcoplasmic reticulum may be described by a set of rules that define vectorial and chemical specificity in the coupled process (Jencks, 1980, 1983; Pickart & Jencks, 1984). The rule for vectorial specificity is that phosphorylation of the Asp-COO^- of the Ca^{2+}-ATPase changes the direction of the Ca^{2+} dissociation–binding to the enzyme (Verjovsky-Almeida et al., 1978; Dupont, 1980; Takisawa & Makinose, 1981, 1983), acting as a vectorial switch that tells the enzyme whether to react with calcium outside or inside (Scheme I). Before phosphorylation Ca^{2+} dissociates outside but not inside (Ikemoto, 1975; Inesi et al., 1980), whereas after phosphorylation it dissociates inside from the "occluded" EP·Ca_2 but not outside (Dupont, 1980; Takisawa & Makinose, 1981, 1983; Champeil, et al., 1986). The rule for chemical specificity is that dissociation–binding of Ca^{2+} acts as a chemical "switch" that tells the enzyme whether to react with ATP or P_i: In the absence of Ca^{2+} the enzyme can be phosphorylated by P_i but not by ATP, and E·Ca_2 can be phosphorylated by ATP but not by P_i (Sumida et al., 1978; Scofano et al., 1979; de Meis, 1981; Martonosi & Beeler, 1985). After phosphorylation of E·Ca_2 by ATP,

Scheme I



all of the EP·Ca_2 is ADP sensitive but not H_2O sensitive (Pickart & Jencks, 1982; Stahl & Jencks, 1984, 1987; Fernandez-Belda & Inesi, 1986). After calcium comes off EP·Ca_2, the covalently bound phosphate can be transferred

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[1] Abbreviations: E or Ca^{2+}-ATPase, calcium adenosinetriphosphatase; SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; TNP, trinitrophenyl.

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to H_2O but not to ADP (Knowles & Racker, 1975; Ikemoto, 1975; Punzengruber et al., 1978; Suko et al., 1981; de Meis, 1981; Pickart & Jencks, 1984).

The Ca^{2+}-ATPase must overcome two difficult thermodynamic problems: (a) the conversion of the "low-energy" phosphate of EP (which can be transferred to H_2O) into "high-energy" phosphate (which can be transferred to ADP) and (b) the conversion of "low-energy" calcium (which is bound at high-affinity Ca^{2+}-translocating sites that are exposed to the cytoplasm) into "high-energy" calcium (which is bound at low-affinity sites so that it can dissociate into a high concentration of calcium inside the SRV). The enzyme solves these problems by mutual destabilization of calcium and covalently bound phosphate in EP·Ca_2, which contains sequestered calcium that is not directly accessible to either the inside or the outside of the SRV (Jencks, 1983; Pickart & Jencks, 1984). The intrinsic binding energy of phosphate alone is expressed to drive the spontaneous formation of the acyl phosphate E–P, while the intrinsic binding energy of calcium alone is expressed in the strong binding of Ca^{2+} to the high-affinity site of the enzyme (Jencks, 1980; Hill & Eisenberg, 1981; Tanford, 1981). However, when both species are present together in E$\sim$P·Ca_2, there is a mutual destabilization of the calcium and phosphate by $\Delta G_D \simeq 8.0$ kcal/mol, an interaction energy that increases the escaping tendency of both ligands (Jencks, 1983; Pickart & Jencks, 1984). There is similar mutual destabilization of sodium and phosphate in the Na^+,K^+-ATPase, with $\Delta G_D = 10$ kcal/mol (Taniguchi & Post, 1975). Both the covalently bound phosphate and the calcium are activated for transfer: phosphate to ADP, but not to H_2O; calcium to the inside of SRV, but not to the outside. An important question is whether this interaction energy and destabilization involves a single species of E$\sim$P·Ca_2, in which both the phosphate and calcium are destabilized, or two interconvertable species in a "flip-flop" mechanism with destabilization of phosphate in one species and Ca^{2+} in the other, as might be expected in some "E_1–E_2" or E–E* models (Jencks, 1980; Hill & Eisenberg, 1981; Tanford, 1981).

We describe here our initial attempts to investigate this problem by measuring the properties of the calcium internalization reaction. We find that "internalization", as measured by loss of sensitivity to an EGTA–ADP quench, proceeds through a stepwise reaction in which internalization of the second, but not the first, Ca^{2+} ion is inhibited by internal Ca^{2+}. A preliminary report of this work has been presented (Khananshvili & Jencks, 1987). The conclusion that there is sequential internalization of two Ca^{2+} ions has been confirmed by Inesi; both laboratories have found that the ion that binds first from the outside is the first to be internalized (Inesi, 1987, and unpublished experiments).

Materials and Methods

Materials. Highest purity reagents were used without further purification. Na_2ATP (sonderqualitat) and coupled enzymes for ATPase assay (Rossi et al., 1979) were obtained from Boehringer Mannheim; NaADP (grade III) was purchased from Sigma; [γ-^{32}P]ATP (>99% pure) and $^{45}CaCl_2$ were from New England Nuclear.

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit white back and hind leg muscle by a slight modification of the procedure of MacLennan (1970). Ground white muscle was homogenized twice at 12000 rpm in a Waring Vortex 16 blender in 5 mM HEPES buffer, pH 7.5, containing 0.15 M NaCl (3 volumes/g of muscle) for 20 s with a 10-s interval. The homogenate was centrifuged at 3800*g* for 10 min. The supernatant was decanted through two layers of cheesecloth and adjusted to pH 7.0 at 4 °C with 1 M NaOH. The pellet was rehomogenized and centrifuged. The supernatant was adjusted to pH 7.0 as described above. The combined supernatants were centrifuged at 12000*g* for 15 min. The supernatant was centrifuged at 106000*g* for 60 min. The pellet was resuspended in 0.4 M sucrose and 5 mM Tris/SO_4, pH 7.0 ($\sim$0.5 mL of buffer/g of starting material) by gentle homogenization with a Dounce homogenizer, and the suspension was centrifuged at 12000*g* for 15 min. The supernatant was centrifuged at 106000*g* for 60 min, and the pellet was resuspended in a minimal amount of sucrose/Tris buffer. The SRV preparations were stored at −80 °C. The SRV preparations hydrolyzed ATP at rates of 4.5–5.0 μmol (mg of total protein)$^{-1}$ min^{-1} when the vesicles were made permeable with the calcium ionophore A23187 (final concentration of 2 μM). SRV as isolated were $\sim$98% sealed, as shown by 50–65-fold increases in the steady-state hydrolysis rate upon addition of ionophore in the standard assay. E_{tot} that was observed for intact or leaky vesicles with $\sim$30 μM free Ca^{2+} and 250 μM [γ-^{32}P]ATP was 3.9–4.1 nmol (mg of total protein)$^{-1}$.

Methods. Ca^{2+}-ATPase activity was assayed spectrophotometrically by coupling ADP production to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard conditions for assay were 40 mM MOPS, 100 mM KCl, 5 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.40 mM EGTA (23 μM free Ca^{2+}), 1.5 mM ATP, pH 7.0, and 25 °C.

Ca^{2+} uptake and formation or decay of phosphoenzyme were followed at 25 °C with a temperature-regulated rapid-mixing apparatus that can be used with either three or four syringes of equal volume, as described previously (Stahl & Jencks, 1984, 1987; Petithory & Jencks, 1986). The reaction times for rapid mixing were calibrated from the rate of 2,4-dinitrophenyl acetate hydrolysis (Barman & Gutfreund, 1964). The basic buffer, 50 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM $MgSO_4$ (buffer A), was used for rapid mixing experiments unless otherwise noted. Concentrations of free calcium were calculated from a dissociation constant of $K_d = 7.4 \times 10^{-7}$ M for CaEGTA (Godt, 1974) and $K_d = 0.61 \times 10^{-3}$ M for calcium citrate in citrate buffer at pH 7.0 (Martell & Calvin, 1956; Perrin, 1974). This value of K_d for CaEGTA was chosen because it was measured directly under conditions identical with ours (Godt, 1974); other values used in the literature are generally smaller (Ogawa, 1968; Guillain et al., 1980). It is necessary to divide the [Ca^{2+}] in this work by 2–4-fold for comparison with results obtained by using the smaller values of K_d. The release of protons from EGTA upon the formation of the CaEGTA complex was neutralized with 1.47 equiv of KOH.

For the Ca^{2+}-uptake assay, SRV (25–30 mg of protein/mL) were passively loaded overnight at 4 °C in the presence of 0.4 M sucrose with buffer A containing 25–40 mM sodium citrate and 0.35–57 mM $CaCl_2$ (0.01–20 mM free Ca^{2+}). Preloaded SRV were diluted 50-fold in the same buffer (without sucrose) containing 0.39 mM EGTA and 0.63–1.25 mM ^{45}Ca (28 μM free Ca^{2+}) and placed in syringe A. Rapid mixing was initiated after 15 s. In order to obtain empty SRV, the intact vesicles ($\sim$30 mg of protein/mL) were dialyzed for 16–24 h at 4 °C against 250 volumes of buffer A containing 0.4 M sucrose and 25–40 mM citrate buffer with 0.35–0.56 mM $CaCl_2$ ($\sim$10 μM free Ca^{2+}). Leaky SRV, for the measurement of EP levels, were obtained by dilution of intact SRV in buffer A containing 0.39 mM EGTA, 0.4 mM $CaCl_2$ (28 μM free Ca^{2+}), and 2 μM ionophore A23187, before rapid mixing as described above.

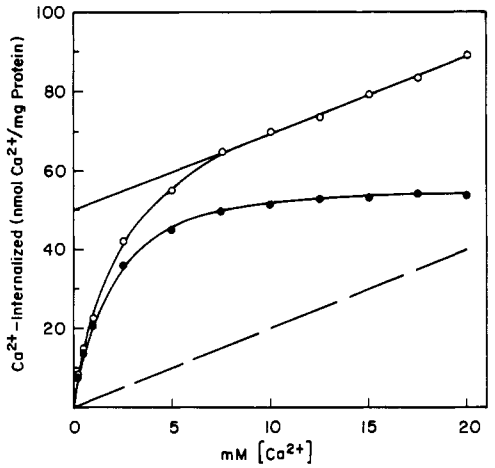


FIGURE 1: Internalization of $^{45}Ca^{2+}$ with passively loaded SRV. SRV (10 mg of protein/mL) were incubated for 48 h at 4 °C with 0.1–20 mM $^{45}CaCl_2$ in buffer A (see Materials and Methods) containing 0.4 M sucrose. The total internalized calcium was determined at 4 °C by diluting 100-μL aliquots 50-fold in buffer A containing 15 mM EGTA and filtering on Millipore filters as described under Materials and Methods. In the control experiments, samples were diluted in the presence of 2 μM A23187. The saturable internalized calcium (Ca^{2+}_{bound}, ●) was obtained by subtraction of the nonsaturable internalized calcium (Ca^{2+}_{free}, ---) from the total internalized calcium (Ca^{2+}_{tot}, ○).

After Ca^{2+} uptake was quenched, internalized Ca^{2+} was measured by filtration of SRV on Millipore filters. Before sample application, the filter was washed with 3 mL of buffer A containing 100 μM $CaCl_2$, followed by 3 mL of buffer A containing 15 mM EGTA. Filtered SRV were washed 3 times with 5 mL of buffer A containing 15 mM EGTA. For the measurement of EP levels the reactions were quenched by trichloracetic acid at a final concentration of 12%; BSA and ATP (pH 7.0) were added to the quenched reaction to give final concentrations of 0.3 mg/mL and 0.3 mM, respectively. The amount of EP was determined as described by Verjovski-Almeida et al. (1978).

Rate constants of biphasic reactions were estimated by a program written for IBM-compatible microcomputers, which calculates the best fit of experimental data by a nonlinear least-squares procedure (Stahl & Jencks, 1987). Simulation of the pre-steady-state kinetics was also carried out by this procedure, which allows input of rate or equilibration expressions and the initial concentrations of each species for any model.

RESULTS

Estimation of Intravesicular Volume and Ca^{2+}-Binding Capacity of SRV. For the accurate measurement of ATP-dependent ^{45}Ca internalization during the first turnover of the enzyme, it was necessary to characterize the internal Ca^{2+}-binding properties of the SRV preparations. In order to estimate the intravesicular volume of SRV that is available to free internal Ca^{2+}, SRV were incubated with 0.1–20 mM $^{45}CaCl_2$ at 4 °C for 48 h, and the total amount of internalized $^{45}Ca^{2+}$ in SRV was assayed by filtration (Figure 1, open circles). The pattern of passively internalized calcium by SRV shows a nonlinear increase up to ~8–9 mM calcium and a linear increase at higher calcium concentrations. Very similar results have been obtained before (Prager et al., 1979). The nonsaturable pool is assumed to represent the internalized free Ca^{2+} (dashed line in Figure 1), and the saturable pool is assigned to the bound internalized Ca^{2+} (solid circles in Figure 1, obtained by subtraction of the nonsaturable line from the total amount of internalized $^{45}Ca^{2+}$). The total amount of internalized calcium (nmol/mg of protein) is the sum of the free and bound calcium inside the SRV.

$$Ca^{2+}_{total} = Ca^{2+}_{free} + Ca^{2+}_{bound} \quad (1)$$

Under equilibrium conditions the activity of free calcium is the same inside and outside the vesicles. Assuming equal concentrations inside and outside at equilibrium, with a known amount of internalized free calcium, Ca^{2+}_{free} (nmol/mg of protein), at a given concentration of calcium, $[Ca^{2+}]_{free}$ (nmol/μL), the internal volume, V, of SRV was calculated according to eq 2. The slope of the linear portion of the curve

$$V = Ca^{2+}_{free}/[Ca^{2+}]_{free} \quad (2)$$

in Figure 1 gives $V = 2.0 \pm 0.2$ μL/mg of protein. This intravesicular volume represents the space that is available for free Ca^{2+}; it does not include space that is occupied by membranes or internal proteins.

The maximal calcium binding inside the SRV (Ca^{max}_{bound}) and the apparent half-saturation ($K^{app}_{0.5}$) were estimated from the saturation curve in Figure 1 (solid circles) as $Ca^{max}_{bound} \simeq 55$–60 nmol/mg of protein and $K^{app}_{0.5} \simeq 1.5$ mM. If the internal volume of SRV is 2 μL/mg of protein and the concentration of active sites is 4 nmol/mg of protein (see Materials and Methods), the total concentration of internalized Ca^{2+} after the first turnover of the enzyme is 4 mM; presumably much of this binds to internal binding proteins.

Since it was not known how fast the internal binding proteins can interact with internalized Ca^{2+}, it was desirable to trap translocated Ca^{2+} during the first turnover of the enzyme. For this purpose the SRV were equilibrated with 25–40 mM sodium citrate buffer at 4 °C for 16–24 h, and citrate was included also in the ATP-dependent Ca^{2+}-uptake assay buffer. Under these conditions, the concentration of free internalized Ca^{2+} cannot exceed ~10 and ~150 μM after internalization of the first and second Ca^{2+}, respectively, in the empty SRV. The actual $[Ca]_{in}$ may be smaller because of binding to the internal proteins.

Internalization of Two Ca^{2+} Ions from E~P·Ca_2 into SRV. In order to characterize intravesicular dissociation of Ca^{2+} from E~P·Ca_2, the ATP-dependent $^{45}Ca^{2+}$ uptake was measured in the presence of different concentrations of $[Ca]_{in}$. Passively loaded (20 mM free Ca^{2+}_{in}) or empty SRV were preincubated (15 s) with 28 μM free exterior $^{45}Ca^{2+}$ and mixed with an equal volume of 500 μM ATP and 28 μM $^{45}Ca^{2+}$ at 25 °C, quenching the reaction by addition of EGTA alone or EGTA and ADP. When Ca^{2+} uptake was terminated by addition of EGTA alone, 2 $^*Ca^{2+}/EP_{tot}$ were occluded during the first 5–20 ms of the reaction with both 10 μM and 20 mM unlabeled $[Ca]_{in}$ (Table I). This shows that up to 20 mM $[Ca]_{in}$ does not alter the stoichiometry of Ca^{2+} occlusion. These data are in good agreement with previous evidence that high $[Ca]_{in}$ does not inhibit the rate of enzyme phosphorylation from ATP (Petithory & Jencks, 1986; Stahl & Jencks, 1987). At longer times there is a slow increase in $^{45}Ca^{2+}$ uptake because of internalization and turnover (see below).

The rate of Ca^{2+} internalization (k_{in}) was measured by rapid mixing of equal volumes of E·$^{45}Ca_2$ with 500 μM ATP and 28 μM free $^{45}Ca^{2+}$, and the reaction was quenched after 3–200 ms by addition of 21 mM ADP and 45 mM EGTA to give final concentrations of 7 mM ADP and 15 mM EGTA. In the presence of 20 mM unlabeled $[Ca]_{free}$ inside the SRV, 1 $^*Ca^{2+}/EP_{tot}$ is internalized in a first-order reaction with $k_{in} = 34 \pm 3$ s^{-1} (Figure 2, solid circles). Identical results were obtained with 20 or 40 mM $[Ca]_{free}$ inside the SRV in the presence or absence of citrate buffer (not shown). For empty SRV, which contain ~10 μM free Ca^{2+}, the same initial rate

Table I: Ca^{2+} Occlusion and Phosphoenzyme Formation in Empty and Loaded SRV Measured by Quenching with EGTA or Acid

	$*Ca^{2+}/EP_{tot}$[a] at t (ms)				EP^*/EP_{tot}[b] at t (ms)			
$[Ca]_{in}$	5	10	15	20	5	10	15	20
10 μM	1.8	1.9	2.1	2.2	0.80	0.92	0.98	1.0
20 mM	1.8	1.9	2.0	2.0	0.75	0.89	0.94	1.0

[a] Empty and loaded SRV were prepared and preincubated with 28 μM free exterior $^{45}Ca^{2+}$ as described in Figure 2. Ca^{2+} internalization was measured by mixing loaded (20 mM free unlabeled Ca^{2+}_{in}) or empty (10 μM free unlabeled Ca^{2+}_{in}) SRV $E{\cdot}^{45}Ca_2$ with an equal volume of 500 μM ATP and 28 μM ^{45}Ca and quenching at the indicated times by addition of 45 mM EGTA to give a final concentration of 15 mM. Occluded $*Ca^{2+}$ was measured by filtration as described under Materials and Methods. All syringes contained 40 mM MOPS, pH 7.0, 100 mM KCl, 25 mM sodium citrate, 15 mM $MgSO_4$ (0.9 mM free Mg^{2+}), and 0.39 mM EGTA. In addition, syringe A contained 0.63 mM $^{45}CaCl_2$ (28 μM free exterior $^{45}Ca^{2+}$) with empty or loaded SRV (1 mg of protein/mL). Syringe B contained 28 μM free $^{45}Ca^{2+}$ and 500 μM ATP. Syringe C contained 45 mM EGTA. [b] Phosphorylation of enzyme was done under the same conditions, but [γ-^{32}P]ATP was used instead of ^{45}Ca and the reaction was quenched by addition of 1 M HCl and 50 mM KH_2PO_4. Labeled EP* was assayed as described under Materials and Methods.

of Ca^{2+} internalization was observed, but the overall rate constant for internalization of 2 $*Ca^{2+}/EP_{tot}$ is 17 ± 2 s^{-1} (Figure 2, open circles), after correction for a slow increase after the first turnover (Figure 2, top, dashed line). The Ca^{2+} internalization after the first turnover was measured by rapid mixing of unlabeled $E{\cdot}Ca_2$ with Ca^{2+} + ATP (t_1 = 15 ms) followed by addition of $^{45}Ca^{2+}$ + ATP. The reaction was quenched with ADP and EGTA at different times (t_2 = 25–200 ms), as shown by the open triangles in Figure 2 (inset). The experimental data are consistent with the sequential internalization of two Ca^{2+} ions from $EP{\cdot}*Ca_2$ into SRV according to eq 3, in which k_1 is the rate constant for inter-

$$EP{\cdot}*Ca_2 \xrightarrow[k_1 = 34\ s^{-1}]{*Ca^{2+}} EP{\cdot}*Ca \xrightarrow[k_b = 17\ s^{-1}]{*Ca^{2+}} EP \qquad (3)$$

nalization of the first Ca^{2+} (observed in the loaded SRV) and k_b is an apparent rate constant for internalization of the second Ca^{2+} (k_b is the difference between the internalization of two Ca^{2+} ions in empty SRV and the first Ca^{2+} in loaded SRV; see Figure 2, bottom). Internalization of the second Ca^{2+} shows a lag phase (Figure 2, squares), which represents dissociation of the first Ca^{2+} and accumulation of $EP{\cdot}*Ca_1$ under pre-steady-state conditions. The rate constant k_b = 17 s^{-1} was calculated to give an optimal fit to the data (see Materials and Methods) as shown by the solid line.

Inhibition of Internalization of the Second Ca^{2+} by $[Ca]_{in}$. In order to characterize the inhibition of internalization of the second Ca^{2+} by unlabeled $[Ca]_{in}$, SRV were preincubated with 40 mM sodium citrate and 1–57 mM unlabeled Ca^{2+} (0.03–20 mM free Ca^{2+}) at 4 °C during 20 h as described under Materials and Methods. SRV were diluted at 25 °C with 28 μM free exterior $^{45}Ca^{2+}$ (final concentration) to give $E{\cdot}^{45}Ca_2$, and rapid mixing was initiated 15 s later. Under these conditions, <5% of $[Ca]_{in}$ is lost during the dilution (measured in control experiments by dilution of SRV, preincubated with 1–57 mM $^{45}Ca^{2+}$ under standard experimental conditions). Preparations of $E{\cdot}*Ca_2$ with different free $[Ca]_{in}$ were mixed with an equal volume of 600 μM ATP and 28 μM $^{45}Ca^{2+}$ at 25 °C, and the reaction was quenched by addition of ADP and EGTA after t = 5–260 ms. In the presence of citrate buffer, the increase in the free $[Ca]_{in}$ does not exceed 20% after internalization of 2 $*Ca^{2+}/EP_{tot}$ in a single turnover of the enzyme at any calcium concentration. The top of Figure 3 shows internal-

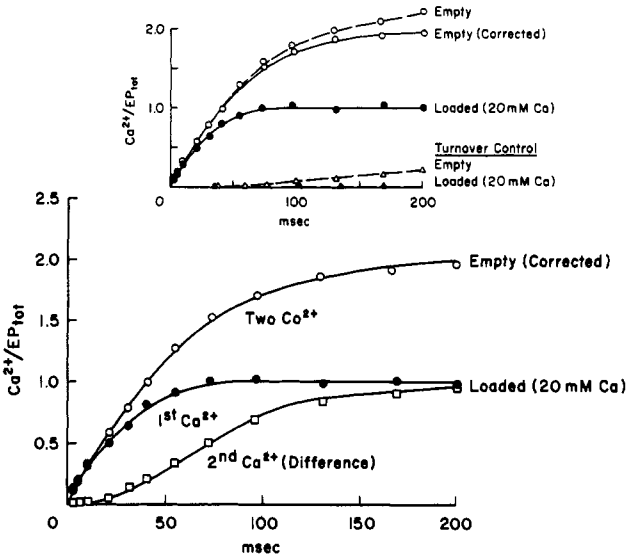


FIGURE 2: ATP-dependent ^{45}Ca internalization in empty and loaded SRV during a single turnover of the enyme. SRV (25 mg of protein/mL) were dialyzed for 24 h at 4 °C in the presence of 0.4 M sucrose, buffer A, and 25 mM sodium citrate (see Materials and Methods) with 0.35 mM Ca^{2+} (~10 μM free Ca^{2+}) or 43 mM $CaCl_2$ (20 mM free Ca^{2+}). Empty or loaded SRV were diluted 50-fold into 40 mM MOPS, pH 7.0, 100 mM KCl, 25 mM sodium citrate, 15 mM $MgSO_4$ (0.9 mM free Mg^{2+}), 0.39 mM EGTA (buffer B), and a final concentration of 0.63 mM $^{45}CaCl_2$ (28 μM free Ca^{2+}). After 15 s, three-syringe rapid mixing was initiated at 25 °C, and the reaction was quenched after t = 3–200 ms by addition of ADP + EGTA. All syringes contained buffer B. In addition, syringe A contained 28 μM free exterior $^{45}Ca^{2+}$ with empty or loaded SRV (0.5 mg of protein/mL). Syringe B contained 28 μM free $^{45}Ca^{2+}$ and 0.5 mM ATP. Syringe C contained 21 mM ADP and 45 mM EGTA. Internalized calcium was determined as described under Materials and Methods. For the assay of Ca^{2+} internalization after the first turnover (turnover controls) a four-syringe configuration was used. Syringes A and B were mixed with t_1 = 15 ms followed by addition of C with t_2 = 25–200 ms. The reaction was quenched by addition of ADP + EGTA in syringe D. Syringe A contained 28 μM free unlabeled Ca^{2+} with 0.5 mg of protein/mL of empty or loaded SRV. Syringe B contained 28 μM free unlabeled Ca^{2+} and 0.5 mM ATP. Syringe C contained 28 mM free $^{45}Ca^{2+}$ and 0.25 mM ATP. Syringe D contained 28 mM ADP and 60 mM EGTA. Top: Observed internalization of $^{45}Ca^{2+}$ into empty (○) and loaded SRV (●) and turnover controls for $^{45}Ca^{2+}$ internalization, after the first turnover with unlabeled Ca^{2+}, into empty (△) and loaded SRV (▲). Bottom: Internalization of the first, second, and two Ca^{2+} ions inside the SRV. Internalization of two Ca^{2+} ions into empty SRV corrected for turnover (○). For internalizatioin of the first Ca^{2+} into loaded SRV (●) the line was calculated for k_1 = 34 s^{-1} and end point 1.03 Ca^{2+}/EP_{tot}. Difference between the internalization of two Ca^{2+} ions (empty SRV) and the first Ca^{2+} (loaded SRV) (□); the line was calculated for k_1 = 34 s^{-1} and k_b = 17 s^{-1} for the second Ca^{2+}.

ization of labeled Ca^{2+} in the presence of different concentrations (0.03–20 mM) of unlabeled free calcium inside the SRV. Controls for the slow increase of Ca^{2+} internalization after the first turnover were measured for each $[Ca]_{in}$ as described above (Figure 3, top, solid points). For the estimation of k_b for internalization of the second Ca^{2+}, the experimental data were corrected for the turnover control at each $[Ca]_{in}$ and the internalization of the first Ca^{2+} (observed in the presence of 20 mM $[Ca]_{in}$; Figure 3, top, dashed line) was subtracted from the corrected data (Figure 3, bottom). The rate constant k_b was calculated to give the best fit to the data with k_1 = 34 s^{-1} (see Materials and Methods).

Free Ca^{2+} inside SRV in the range 0.03–13 mM decreases the rate constant for internalization of the second Ca^{2+} (k_b) from 17 to ~0.8 s^{-1}. Half-maximal decrease for the rate of Ca^{2+} internalization for the second Ca^{2+} (k_b) by $[Ca]_{in}$ is achieved at $K_{0.5}$ = 1.3 mM (Figure 4, bottom). The Hill plot

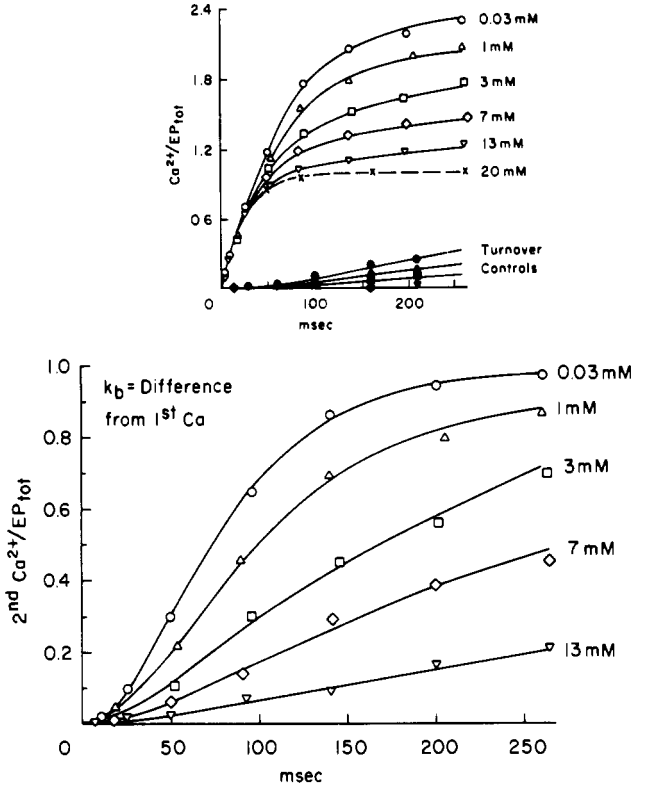


FIGURE 3: Inhibition by internal Ca^{2+} of the internalization of the second Ca^{2+}. SRV (25 mg of protein/mL) in buffer A and 0.4 M sucrose were incubated for 18 h at 4 °C with 40 mM sodium citrate and different concentrations of $CaCl_2$: 2 mM (0.03 mM free Ca^{2+}), 26 mM (1 mM free Ca^{2+}), 35.5 mM (3 mM free Ca^{2+}), 42.5 mM (7 mM free Ca^{2+}), 50 mM (13 mM free Ca^{2+}), and 57 mM (20 mM free Ca^{2+}). The SRV were diluted in buffer B (with 40 mM sodium citrate) to give a final concentration of 1.25 mM $^{45}Ca^{2+}$ (28 μM free Ca^{2+}) as described in Figure 2. In addition to the basic buffer B with 40 mM sodium citrate (Figure 2), syringe A contained 28 μM free $^{45}Ca^{2+}$ and loaded SRV (1.0 mg of protein/mL) containing different $[Ca]_{in}$. Syringe B contained 28 μM free $^{45}Ca^{2+}$ and 0.6 mM ATP. Syringe C contained 21 mM ADP and 45 mM EGTA. Four-syringe experiments for the estimation of turnover controls were done for each $[Ca]_{in}$, as described in Figure 2 with t_1 = 15 ms and t_2 = 15–190 ms. Top: Observed Ca^{2+} internalization in the presence of different $[Ca]_{in}$ (○, △, □, ◇, ▽) and turnover controls [Ca^{2+} internalization after the first turnover in the presence of different $[Ca]_{in}$ (●, ▲, ■, ◆, ▼)]. Bottom: Difference between observed Ca^{2+} internalization in the presence of different $[Ca]_{in}$ (corrected for turnover) and internalization of the first Ca^{2+} [20 mM free $Ca^{2+}{}_{in}$ (---), shown in the top]. The values of k_b for each $[Ca]_{in}$ were calculated according to eq 3 with k_1 = 34 s^{-1} to give an optimal fit to the data (see Materials and Methods). The lines were calculated for k_b = 17 s^{-1} (○), 10.5 s^{-1} (△), 5 s^{-1} (□), 2.8 s^{-1} (◇), and 0.8 s^{-1} (▽).

for the inhibition of k_b by $[Ca]_{in}$ shows a Hill coefficient of n = 1.1 (Figure 4, inset), suggesting that only one low-affinity Ca^{2+}-translocating site is inhibited by $[Ca]_{in}$.

Acceleration of the Internalization of Each Ca^{2+} Ion by High Concentrations of ATP. Figure 5B shows that rapid mixing of $E{\cdot}^{45}Ca_2$ with 0.5 or 6 mM ATP, using SRV loaded with 20 mM Ca^{2+}, leads to the internalization of 1 $*Ca^{2+}/EP_{tot}$ with different rate constants, k_1 = 34 ± 3 s^{-1} with 0.25 mM ATP and k_1 = 69 ± 4 s^{-1} with 3 mM ATP. This shows that high concentrations of ATP increase the rate constant for the internalization of the first Ca^{2+}. Internalization of 2 $*Ca^{2+}/EP_{tot}$ into empty vesicles is also accelerated. SRV with 10 μM unlabeled free internal Ca^{2+} in the presence of increasing concentrations of ATP show k_{obsd} = 17 ± 2 s^{-1} for 0.25 mM ATP and k_{obsd} = 34 ± 3 s^{-1} for 3 mM ATP (Figure 5A). This shows that the apparent rate constant for internalization of both Ca^{2+} ions is also increased ~2-fold in the

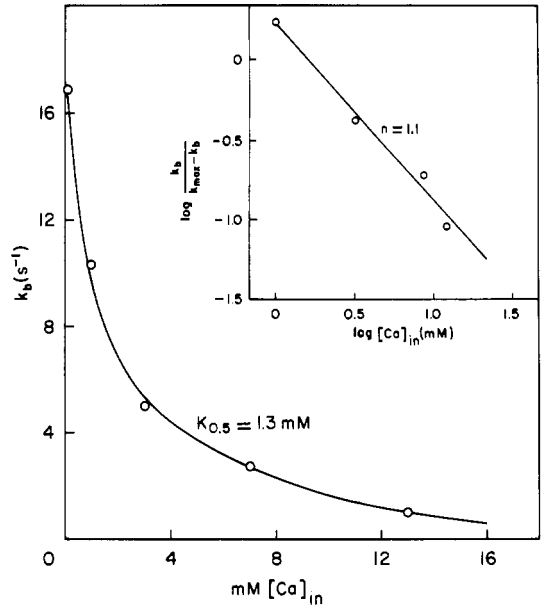


FIGURE 4: $K_{0.5}$ and Hill coefficient for inhibition by $[Ca]_{in}$ of internalization of the second Ca^{2+}. Dependence of k_b on $[Ca]_{in}$. The values of k_b, estimated in Figure 3, were plotted against the concentration of the free $[Ca]_{in}$. Inset: Hill plot for the inhibition of k_b. The value of k_{max} was taken as 17 s^{-1}.

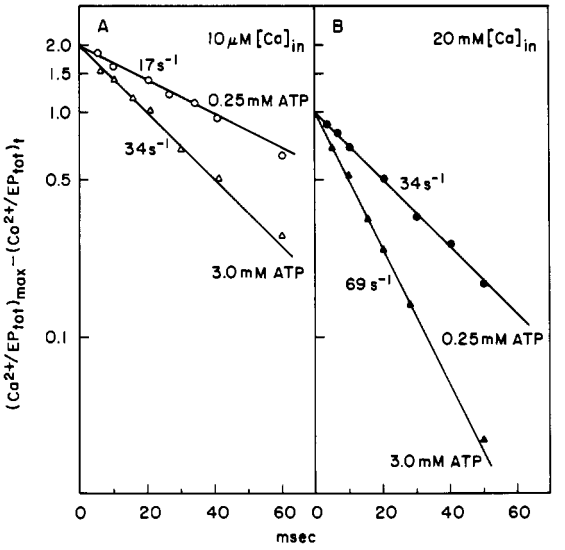


FIGURE 5: Accelerated internalization of each Ca^{2+} ion by a high concentration of ATP during a single turnover of the enzyme. ATP-dependent $^{45}Ca^{2+}$ internalization in the empty (left, A) or loaded SRV (right, B) was measured as described in Figure 2. The only difference was that syringe B (in the three-syringe experiments) contained 0.5 or 6 mM ATP to give 0.25 or 3 mM ATP after mixing. (A) internalization of $^{45}Ca^{2+}$ in the presence of 10 μM free Ca^{2+} inside the SRV with 0.25 mM (○) or 3.0 mM ATP (△). (B) Internalization of $^{45}Ca^{2+}$ in the presence of 20 mM free Ca^{2+} inside the SRV with 0.25 mM (●) or 3.0 mM ATP (▲). The end point for the loaded SRV was $(Ca^{2+}/EP_{tot})_{max}$ = 1.0 and for the empty SRV was $(Ca^{2+}/EP_{tot})_{max}$ = 2.0 (after correction for turnover control; see the legend of Figure 2). The lines are drawn for first-order rate constants of 17 s^{-1} (○), 34 s^{-1} (△), 34 s^{-1} (●), and 69 s^{-1} (▲).

presence of high concentrations of ATP.

The reaction of $E{\cdot}Ca_2$ of intact, unloaded or loaded SRV with ATP and EGTA results in a partial phosphorylation of the enzyme (Ikemoto, 1981; Ikemoto & Nelson, 1984; Petithory & Jencks, 1986). Under standard experimental conditions ~70% of the enzyme is phosphorylated with k_{obsd} = 300 s^{-1} (Petithory & Jencks, 1986). Phosphoenzyme hydrolysis in loaded SRV is slow (k_{obsd} < 2 s^{-1}) because of the high $[Ca]_{in}$.

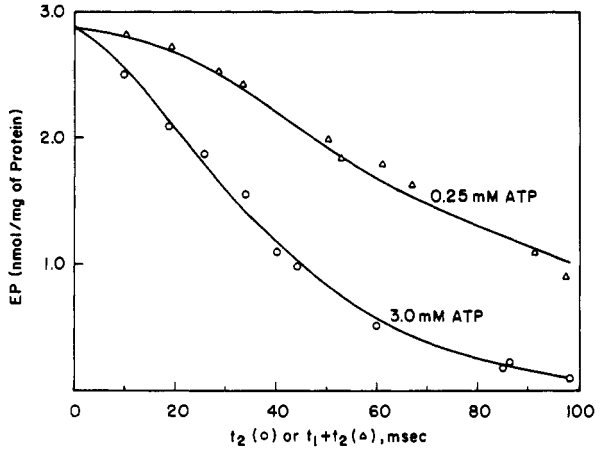


FIGURE 6: Acceleration of the biphasic phosphoenzyme decay in the presence of a high concentration of ATP. The four-syringe system was used in this experiment. All syringes, except D, contained buffer A with 2 μM A23187. Additionally, syringe A contained leaky SRV, E·Ca_2 (1 mg of protein/mL), 0.39 mM EGTA, and 0.4 mM $CaCl_2$ (28 μM free Ca^{2+}), syringe B contained 0.5 mM [γ-^{32}P]ATP (~8000 cpm/nmol) and 30 mM EGTA, and syringe C contained 0.25 mM (△) or 8.5 mM (○) unlabeled ATP and 15 mM EGTA. Syringe D contained 55 mM Na_2HPO_4 and 1.0 M HCl. Syringes A and B were mixed followed by the addition of the contents of syringe C after t_1 = 15 ms and by acid quench from syringe D after t_2 = 10–100 ms. For t_2 = 0 the reaction was quenched with acid after t_1 by reversing the order of addition of the contents of syringes C and D. The value of EP^*_{max} = 2.85 nmol/mg of protein was measured in loaded SRV with t_1 = 25 ms and t_2 = 0 ms. For 0.25 mM ATP the phosphoenzyme levels were plotted against $t_1 + t_2$ (△) ([ATP] = 0.25 mM during both t_1 and t_2). For 3.0 mM ATP the data were corrected for phosphoenzyme hydrolysis during t_1 = 10 ms with 0.25 mM ATP (~0.05 nmol/mg of protein) and plotted against t_2 (○) ([ATP] = 0.25 mM during t_1 and 3.0 mM during t_2). The lines were calculated (see Materials and Methods) according to eq 3, with k_1 = 34 s^{-1}, k_b = 17 s^{-1} (△) and k_1 = 72 s^{-1}, k_b = 34 s^{-1} (○).

In order to follow the disappearance of phosphoenzyme during the sequential intravesicular dissociation of two Ca^{2+} ions in the presence of 0.25 or 3 mM ATP, SRV were made leaky by the addition of 2 μM A23187, a calcium ionophore.

E·Ca_2 of leaky SRV was mixed with 0.5 mM [^{32}P]ATP and 30 mM EGTA. After t_1 = 15 ms, unlabeled ATP to give 0.25 or 3 mM and 15 mM EGTA were added within t_2 = 10–100 ms. As in the case of loaded SRV (Petithory & Jencks, 1986) phosphorylation of the enzyme was complete with leaky SRV after ~15 ms, giving $EP_{max} \simeq 0.7\ EP_{tot}$ (not shown). The disappearance of phosphoenzyme is biphasic at both ATP concentrations. With 0.25 mM ATP the biphasic reaction can be described by $k_1 = 34 \pm 3\ s^{-1}$ and $k_b = 17 \pm 2\ s^{-1}$, while in the presence of 3 mM ATP both rate constants are increased, k_1 to 72 ± 5 s^{-1} and k_b to 34 ± 3 s^{-1}, as shown by the calculated lines in Figure 6. The biphasic reaction does not represent hydrolysis of Ca^{2+}-free EP, because the hydrolysis of EP is much faster than 34 s^{-1} under the conditions of these experiments (Pickart & Jencks, 1984). This suggests that a high concentration of ATP accelerates the rate of internalization for each Ca^{2+} ion, causing an increase of about 2-fold in the overall rate constant for internalization of two Ca^{2+} ions.

Discussion

Intravesicular Volume and Ca^{2+}-Binding Capacity of SRV Preparations. The internal volume of SRV has been estimated for different preparations by using various methods, and vesicular volumes in the range 3–10 μL/mg of protein were reported (Weber et al., 1963; Duggan & Martonosi, 1970; Malan et al., 1975; Herbette, 1981). In this paper we describe a simple method for estimation of the intravesicular volume of SRV that is available to Ca^{2+} by using equilibrated incubation of $^{45}Ca^{2+}$ with SRV preparations and assay of the internal $^{45}Ca^{2+}$. The intravesicular volume of 2.0 μL/mg of protein estimated by this procedure is close to ~2.7 μL/mg of protein estimated by different methods (Herbette et al., 1981). The apparent equilibrium constant of $K^{app}_{0.5}$ ~1.5 mM for the dissociation of bound Ca^{2+} from the internal binding proteins as well as the capacity for Ca^{2+} binding of ~55–60 mmol of Ca^{2+}/mg of protein is in good agreement with previously reported values (Prager et al., 1979). It was shown that SRV preparations usually contain 30–40 nmol of bound Ca^{2+}/mg of protein (Yamada et al., 1972; Prager et al., 1979; Pickart & Jencks, 1984). This means that intact SRV already contain 15–20 mM total internal calcium, assuming that the intravesicular volume is 2 μL/mg of protein; presumably, most of this calcium is bound. Even if it is possible to remove all internal Ca^{2+}, a single turnover of enzyme will result in an internal calcium concentration of 4 mM, based on 4 nmol of active site/mg of protein (see Materials and Methods), an intravesicular volume of 2 μL/mg of protein, and a stoichiometry of 2 Ca^{2+} translocated/ATP hydrolyzed. Translocated Ca^{2+} could bind to the internal binding proteins, but the apparent binding affinity is low and it has not been reported previously how fast binding occurs.

In order to prevent an increase of $[Ca]_{in}$ during the first turnover of the enzyme in the experiments described here, the translocated Ca^{2+} was trapped by sodium citrate buffer inside the SRV. After a single turnover of the enzyme, the free $[Ca]_{in}$ did not exceed 0.15–0.2 mM, even without taking account of the binding capacity of internal proteins.

However, a very similar pattern of ATP-dependent Ca^{2+} internalization was observed when SRV were dialyzed against micromolar concentrations of free Ca^{2+} in the absence (not shown) or presence of citrate buffer (Figure 2). Since the Ca^{2+} concentration reaches ~4 mM during the first turnover, which would give inhibition of the reaction if it were free, this result suggests that Ca^{2+}-free internal binding proteins can bind internalized Ca^{2+} so fast that it does not inhibit internalization of the second Ca^{2+}.

High Concentrations of $[Ca]_{in}$ Do Not Inhibit Occlusion of Two Ca^{2+} Ions in E~P·Ca_2. There is experimental evidence that phosphorylation of the enzyme by ATP in the presence of calcium leads to the immediate occlusion of two Ca^{2+} ions bound to the high-affinity Ca^{2+}-transporting sites so that they cannot dissociate to the outside of the vesicle (Verjovski-Almeida et al., 1978; Dupont, 1980). The quench by EGTA alone shows how many Ca^{2+} ions are occluded per active site but not the rate of Ca^{2+} dissociation to the inside of the vesicle (Sumida et al., 1978; Ikemoto et al., 1981; Inesi & Hill, 1983; Froehlich & Heller, 1985). It was shown in this work that after quenching of ATP-dependent Ca^{2+} internalization by EGTA alone about two Ca^{2+} ions are occluded in empty and loaded SRV during the first turnover of the enzyme (Table I). This shows that up to 20–40 mM $[Ca]_{in}$ does not prevent the rapid occlusion of two Ca^{2+} ions. The reaction is almost complete in both loaded or empty SRV after 15 ms, which is in good agreement with k_{obsd} = 220 s^{-1} for ATP-dependent phosphorylation of E·Ca_2 (Petithory & Jencks, 1986). We conclude that both the rate of phosphorylation by ATP and the occlusion of two bound Ca^{2+} ions are not inhibited by $[Ca]_{in}$ during the first turnover of the enyzme.

Sequential Internalization of Two Ca^{2+} Ions from E~P·Ca_2 into SRV. Various methods have been used to resolve the kinetics of Ca^{2+} internalization into SRV (Sumida et al., 1978;

Chiu & Haynes, 1980; Dupont, 1980; Ikemoto et al., 1981; Takisawa & Makinose, 1981; Inesi & Hill, 1983; Pierce et al., 1983). In order to characterize the mechanism of Ca^{2+} internalization from $E\sim P\cdot Ca_2$, the ATP-dependent $^{45}Ca^{2+}$ internalization was examined during the first turnover of the enzyme in the presence of different concentrations of internal Ca^{2+}. The stoichiometry and the rate of ATP-dependent Ca^{2+} internalization were measured by quenching the reaction with ADP + EGTA (Sumida et al., 1978; Pickart & Jencks, 1982; Inesi & Hill, 1983; Froehlich & Heller, 1985). No burst or lag phase was observed for Ca^{2+} internalization, and the experimental data were extrapolated to zero Ca^{2+} internalization at $t = 0$, suggesting that the quench efficiency of ADP + EGTA is close to 100%.

In the presence of 20–40 mM unlabeled $[Ca]_{in}$, 1 $*Ca^{2+}/EP_{tot}$ is internalized with a first-order rate constant of $k_1 = 34\ s^{-1}$. In the presence of internal citrate buffer, to maintain a low $[Ca^{2+}]$ inside, 2 $*Ca^{2+}/EP_{tot}$ are internalized in empty SRV (corrected for turnover control) with the same initial rate, but the overall rate constant for the internalization of two Ca^{2+} ions is $k_{obsd} = 17\ s^{-1}$ (Figure 2). The first-order internalization of the first Ca^{2+} indicates that the reaction occurs from a single form of phosphoenzyme. This fact is in good agreement with the suggestion that all $EP\cdot Ca_2$ is a single species (Pickart & Jencks, 1982, 1984; Fernandez-Belda & Inesi, 1986; Petithory & Jencks, 1986; Stahl & Jencks, 1987). Inhibition of the internalization of the second Ca^{2+} and the absence of inhibition of the initial rates of Ca^{2+} internalization suggest that the first $*Ca^{2+}$ has to come off its binding site in order to allow dissociation of the second Ca^{2+} and that an internal Ca^{2+} ion can bind to this or another site to inhibit internalization of the second Ca^{2+} (eq 4). The apparent rate constant for inter-

$$\begin{array}{ccccc} & {}^{*}Ca^{2+} & & {}^{*}Ca^{2+} & \\ EP\cdot{}^{*}Ca_2 & \xrightarrow{k_1} & EP\cdot{}^{*}Ca & \xrightarrow{k_b} & EP \\ & k_1 \uparrow\downarrow k'_{-1}[Ca]_{in} & & & \\ & EP\cdot{}^{*}Ca\cdot Ca & & & \end{array} \qquad (4)$$

nalization of the second Ca^{2+} is $k_b \sim 17\ s^{-1}$, with $k_1 = 34\ s^{-1}$ for internalization of the first Ca^{2+} and $k_{obsd} = 17\ s^{-1}$ for the overall internalization of two Ca^{2+} ions into SRV. If we assume that under steady-state conditions two Ca^{2+} ions dissociate sequentially with $k_1 = 34\ s^{-1}$ and $k_b = 17\ s^{-1}$ (eq 3), the overall rate constant for the dissociation of two Ca^{2+} ions can be calculated as $11\ s^{-1}$ [$k_{ov} = k_1k_b/(k_1 + k_b)$; see Stahl and Jencks (1987)]. A turnover number of $12\ s^{-1}$ was observed for steady-state ATP hydrolysis with leaky SRV in the presence of 200 μM ATP and 10–30 μM Ca^{2+} (not shown). A similar turnover number was observed before under similar experimental conditions (Chiesi & Inesi, 1979).

The fact that high $[Ca]_{in}$ does not prevent internalization of the first Ca^{2+}, while it inhibits internalization of the second Ca^{2+}, suggests that the two Ca^{2+} ions are in separate Ca^{2+}-translocating sites in the channel from which the two Ca^{2+} are internalized sequentially. It was shown for the Na^+,K^+-ATPase that addition of K^+ and MgP_i to $E\cdot{}^{*}Rb_2$ or $E\cdot{}^{*}K_2$ releases 50% of the bound radioactive isotope, giving $EP\cdot{}^{*}Rb\cdot K$ or $EP\cdot{}^{*}K\cdot K$ (Glynn et al., 1985; Forbush, 1985, 1987a,b). This suggests that both enzymes undergo stepwise binding–dissociation of transported ions.

Low-Affinity Inhibition of Internalization of the Second Ca^{2+} by $[Ca]_{in}$. It is believed that the internal binding sites of the enzyme have a low affinity for Ca^{2+} (Chaloub et al., 1979; de Meis, 1981; Suko et al., 1981; Tanford, 1984; Pickart & Jencks, 1984; Martonosi & Beeler, 1985); however, the properties of these sites have not yet been thoroughly characterized.

No burst or lag phase was observed in the presence of high $[Ca]_{in}$ when the first Ca^{2+} is internalized with a first-order rate constant of $k_1 = 34\ s^{-1}$ or when two Ca^{2+} ions are internalized into empty vesicles with $k_{obsd} = 17\ s^{-1}$ (Figure 2). A small lag, with $t_{1/2} \sim 3$ ms, is expected from the rate constant of $220\ s^{-1}$ for phosphorylation but was not observed. This may reflect either experimental uncertainty or a small compensating burst, which could arise from a small delay or inefficiency in the ADP quench. Nevertheless, the data show that all or very nearly all of $EP\cdot Ca_2$ is ADP sensitive at $t = 0$, and there is no evidence for the existence of an ADP-insensitive E_2–$P\cdot Ca_2$ species in the presence of either low or high internal $[Ca^{2+}]$ (Figure 2). This result provides additional support for the conclusion that all $EP\cdot Ca_2$ is ADP sensitive (Pickart & Jencks, 1982, 1984; Stahl & Jencks, 1987).

In order to estimate the Ca^{2+} affinity of the enzyme for inhibition of internalization of the second Ca^{2+}, the internalization of Ca^{2+} was assayed after equilibration of the SRV and citrate with different $[Ca]_{in}$, under conditions in which the concentration of free internal Ca^{2+} does not vary more than 20% during the first turnover of the enzyme. Under these conditions, 0.03–13 mM Ca^{2+}_{in} decreases the apparent rate constant k_b for internalization of the second Ca^{2+} from 17 to $\sim 0.8\ s^{-1}$ (Figure 3). Half-maximal inhibition is achieved at $K_{0.5} = 1.3$ mM with a Hill coefficient of $n = 1.1$ (Figure 4), suggesting that inhibition occurs when only one low-affinity Ca^{2+}-binding site is occupied by high $[Ca]_{in}$.

The values of k_b were obtained by optimizing the fit to the experimental data with $k_1 = 34\ s^{-1}$, as shown by the solid lines in Figure 3. These values of k_b were used to obtain the value of $K_{0.5} = 1.3$ mM, as shown in Figure 4. If inhibition occurs at the site that was previously occupied by the Ca^{2+} that dissociated with the rate constant $k_1 = 34\ s^{-1}$, and $K_{diss} = k_1/k_{-1}' = 1.3$ mM, the second-order rate constant for Ca^{2+} binding to this site is $\sim 3 \times 10^4\ M^{-1}\ s^{-1}$, which is far below diffusion limited and corresponds to a considerable barrier for binding. Simulation of the reaction progress with these rate constants according to the mechanism of eq 4 gave calculated lines that agree with the experimental data in Figure 3 within experimental error (not shown).

The dependence of the rate of steady-state ATP hydrolysis on free calcium concentration with leaky SRV or the solubilized enzyme has a bell-shaped pattern on a logarithmic scale (Vianna, 1975; Møller et al., 1980; Pickart & Jencks, 1984; Bodley & Jencks, 1987). A Hill plot of the ascending part of the curve shows $K_{0.5} = 0.5$–2.6 μM and a Hill coefficient of $n = 1.6$–1.9 and presumably corresponds to cooperative binding of two Ca^{2+} ions to the cytoplasmic Ca^{2+}-translocating sites (Inesi et al., 1980; Highsmith, 1982). However, the descending portion for inhibition of turnover shows a smaller Hill coefficient of $n = 1.3$–1.5 and $K_{0.5} = 0.35$–0.50 mM. Although the interpretation of the Hill coefficient may be complex (Tanford et al., 1985), the results described here suggest that under steady-state conditions there will be no inhibition of internalization of the first Ca^{2+} in the presence of high $[Ca]_{in}$ and that the small Hill coefficient for inhibition may reflect inhibition of dissociation of the second Ca^{2+} by a single Ca^{2+} (Figure 4), as well as inhibition of phosphoenzyme hydrolysis by the cooperative binding of two Ca^{2+} to EP; this will give an observed value of n between 1 and 2.

High ATP Concentrations Accelerate Internalization of Both Ca^{2+} Ions. It is well-known that the steady-state hydrolysis of ATP in the presence of Ca^{2+} is accelerated by

increasing concentrations of ATP or ATP analogues (Yamamoto & Tonomura, 1967; Inesi et al., 1967; de Meis et al., 1974; Møller et al., 1980; de Meis, 1981; Dupont, 1985; Champeil et al., 1986). However, there is disagreement with respect to the nature of such activation, and different mechanisms have been suggested (Coan & Keating, 1982; McIntosh & Boyer, 1963; Coll & Murphy, 1985; Dupont et al., 1985; Reynolds et al., 1985; Martonosi & Beeler, 1985; Bishop et al., 1987). It has been demonstrated that high concentrations of ATP accelerate the disappearance of EP·Ca_2, which is largely the rate-limiting step for enzyme turnover in the presence of potassium (Shigekawa & Dougherty, 1977; Shigekawa, 1978, 1983; Dupont, 1980; Ikemoto & Nelson, 1984; Champeil & Guillain, 1986; Champeil et al., 1986; Bodley & Jencks, 1987). ATP-dependent acceleration of Ca^{2+} dissociation from leaky SRV was demonstrated at pH 6.0 by the rapid filtration technique under conditions in which the dissociation of Ca^{2+} and the dephosphorylation step are slow (Champeil & Guillian, 1986; Champeil et al., 1986). It is difficult to detect by this technique in the presence of potassium at neutral pH, where Ca^{2+} dissociation and phosphoenzyme disappearance are faster, but it has been demonstrated at low temperature under these conditions (Wakabayashi et al., 1986).

Direct measurement of ATP-dependent Ca^{2+} internalization shows that the observed rate constant for internalization of 2 $*Ca^{2+}/EP_{tot}$ increases from 17 to 34 s^{-1} with increasing ATP concentration from 0.25 to 3.0 mM (Figure 5A). Two independent observations show that the accelerated internalization of two Ca^{2+} is accounted for by increases in the rate of reaction of each calcium ion.

(1) The first-order rate constant k_1 for internalization of the first Ca^{2+} (in the presence of high Ca^{2+}_{in}) increases from 34 to 69 s^{-1} with increasing ATP concentration (Figure 5B). The apparent rate constant for internalization of the second Ca^{2+}, k_b, is also increased ~2-fold from 17 to 34 s^{-1} in the presence of high [ATP].

(2) A high concentration of ATP changes both phases of E~P*·Ca_2 disappearance (Figure 6). The lag phase of EP decay, which corresponds to internalization of the first Ca^{2+}, is reduced, and the second phase of EP disappearance, corresponding to dissociation of the second Ca^{2+}, is accelerated. These changes correspond to an increase of k_1 from 34 to ~72 s^{-1} and an increase of k_b from 17 to 34 s^{-1}. These results show that occupation of a catalytic or "regulatory" site by ATP increases the rate of internalization of each Ca^{2+} ion by approximately 2-fold. There is evidence that high [ATP] accelerates other steps of catalytic turnover (McIntosh & Boyer, 1963; Reynolds et al., 1985; Bishop et al., 1987), but internalization of each Ca^{2+} ion is the most important step for the "secondary activation" of steady-state ATP hydrolysis by high [ATP] in the presence of potassium. It has been suggested recently that the regulatory nucleotide analogue TNP-AMP binds to the phosphorylated catalytic site, which indicates that a single nucleotide binding site mediates both catalytic and regulatory functions (Bishop et al., 1987). However, other possibilities are not excluded; for example, it was found that millimolar levels of ATP do not overcome product inhibition by added ADP, suggesting that there is an allosteric site for ATP activation (Coll & Murphy, 1985). These data are consistent with the finding that the equilibrium levels of phosphoenzyme formed in the presence of 0.25 mM ADP with 0.5–1 mM ATP are very similar and are approximately half that of the control phosphoenzyme (Stahl & Jencks, 1987). The simplest interpretation of these data is that millimolar concentrations of ATP in the presence of $MgCl_2$ and KCl do not compete with ADP at the active site of EP.

Previous reports from this and other laboratories have described a first-order disappearance of phosphoenzyme formed from E·Ca_2 and ATP with $k \sim 17$ s^{-1} in the presence of KCl at pH 7; however, the fit to a first-order reaction is variable (Chiesi & Inesi, 1979; Inesi et al., 1983; Nelson & Ikemoto, 1984; Pickart & Jencks, 1984; Froechlich & Heller, 1985; Bodley & Jencks, 1987). The experiments described here were carried out by adding ATP and EGTA together to enzyme that was equilibrated with Ca^{2+}. Under these conditions, 70% of the enzyme is phosphorylated rapidly, with $k_{obsd} = 300$ s^{-1}, so that the subsequent reaction course starting with a single species of phosphoenzyme is observed. In the presence of EGTA, 30% of the enzyme from which Ca dissociates is not phosphorylated and there is no further phosphorylation of the enzyme in subsequent turnovers (Petithory & Jencks, 1986). In most of the earlier experiments it is likely that several species of enzyme, including a large fraction of EP·Ca_1, were present at the initiation of the measurement of phosphoenzyme dissapearance so that the observed rate constant represented a composite of the rate constants for the reactions of these species.

Mechanism of the Sequential Internalization of Two Ca^{2+} Ions. The results reported here show that the internalization of the two Ca^{2+} ions of E~P·Ca_2 involves at least two steps and that internalization of the second, but not the first, Ca^{2+} is inhibited by millimolar Ca^{2+} inside the vesicle. These results characterize several aspects of the transport mechanism and exclude several possible mechanisms, but they do not establish the complete reaction mechanism.

The absence of inhibition of internalization of the first Ca^{2+} by 20–40 mM $[Ca^{2+}]_{in}$ shows that the initial step does not involve Ca^{2+} translocation from a high-affinity to a low-affinity site that is exposed to the inside of the vesicle, such as might be expected for some E_1–E_2 models.

As noted above, there is no evidence in this work for two species of EP·Ca_2, one ADP sensitive and the other ADP insensitive. The ADP quench is highly effective at all reaction times and excludes the existence of more than a very small amount of ADP-insensitive EP·Ca_2 (Figure 2). The biphasic disappearance of EP·Ca_2 upon addition of ADP to this species has been attributed to the rapid formation of an equilibrium mixture of EP·Ca_2·ADP and E·Ca_2·ATP, followed by slow dissociation of ATP and/or Ca^{2+}, as shown in eq 5 (Pickart

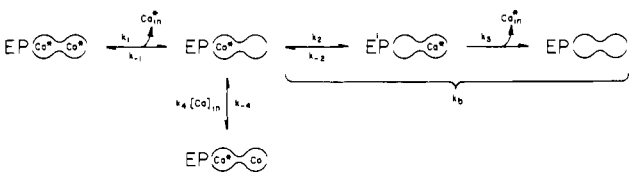
$$\mathrm{EP{\cdot}Ca_2{\cdot}ADP} \underset{K_{in}}{\overset{\text{fast}}{\rightleftharpoons}} \mathrm{E{\cdot}Ca_2{\cdot}ATP} \begin{cases} \nearrow \mathrm{E{\cdot}Ca_2 + ATP} \\ \searrow \mathrm{E{\cdot}ATP + 2Ca^{2+}} \end{cases} \quad (5)$$

& Jencks, 1982). This interpretation has been challenged (Froelich & Heller, 1985; Wang, 1986) but is supported by recent work (Fernandez-Belda & Inesi, 1986; Stahl & Jencks, 1987) as well as by the experiments reported here.

The biphasic disappearance of phosphoenzyme that is formed after the addition of ATP + EGTA to leaky vesicles (Figure 6) is described by the same rate constants of 34 and 17 s^{-1} that are observed for internalization of the two Ca^{2+} ions. The increase in these rate constants to 34 and 72 s^{-1} in the presence of 3.0 mM ATP also agrees with the same rate constants for Ca^{2+} internalization under the same conditions. These results show that the rate constants for the Ca^{2+} internalization steps control the hydrolysis of phosphoenzyme; hydrolysis occurs rapidly only after both Ca^{2+} ions have been internalized.

In particular, the time course of phosphoenzyme hydrolysis in Figure 6 shows that the product of the initial reaction at

Scheme II



34 s^{-1} does not undergo hydrolysis. This is important physiologically, because it ensures that there will be a stoichiometry of 2 Ca^{2+} ions transported for each molecule of ATP hydrolyzed.

Scheme II shows a simple model for the sequential internalization of two Ca^{2+} ions and the conversion of $E\sim P\cdot Ca_2$ (ADP-sensitive and H_2O-insensitive) to the EP (ADP-insensitive and H_2O-sensitive) form. This model depicts two Ca^{2+}-translocating sites in a channel and the movement of one $*Ca^{2+}$ from one site, EP·*Ca, to the other, E'P·*Ca. According to this model, $k_1 = k_{-4} = 34\ s^{-1}$, because the two reactions differ only in the isotopic species of Ca^{2+}. The k_2–k_{-2} step is fast and can be described by the equilibrium constant K_2, because the observed internalization of Ca^{2+} can be described by only two rate constants. The K_b step is then described by eq 6. If the two sites are equivalent, with $K_2 = 1.0$, the rate

$$k_b = k_3K_2/(1 + K_2) \quad (6)$$

constant k_3 is 34 s^{-1}, the same as k_1 and k_{-4}. The value of $K_{0.5} \simeq 1.3$ mM may not represent a simple dissociation constant for Ca^{2+} from a low-affinity Ca^{2+} site on the inside, but it is consistent with low-affinity calcium binding.

However, the simplest interpretation of Scheme II and the low-affinity inhibitory Ca^{2+} site with $K_{0.5} = 1.3$ mM raise a thermodynamic problem. This problem arises if one Ca^{2+} is transported to the interior of the vesicle with $k = 34\ s^{-1}$ and the $EP\cdot Ca_1$ that remains is quenched with ADP, presumably to give ATP. This would lead to the internalization of one Ca^{2+} ion into the lumen of the vesicle against a concentration gradient with no net hydrolysis of ATP. The 34-s^{-1} step may, therefore, represent exchange with internal Ca^{2+} or may be only the first step for internalization of the first Ca^{2+}, by movement to an additional site in the channel or by closing of a gate between the first and second Ca^{2+} sites that prevents dissociation to the cytoplasm after the ADP quench. A somewhat similar gating mechanism has recently been suggested by Tanford (1987). The inhibition by internal Ca^{2+} may represent binding either to a site in the channel or to some "regulatory" site; however, it is not accounted for in a simple way by binding to the interior binding site described by Suko et al. (1981), because this site binds two Ca^{2+} ions and is half-saturated at $\sim$5 mM Ca^{2+}.

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