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Simultaneous Internalization and Binding of Calcium during the Initial Phase of Calcium Uptake by the Sarcoplasmic Reticulum Ca Pump†

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ABSTRACT: The kinetics of Ca^{2+} transport mediated by the sarcoplasmic reticulum (SR) Ca-ATPase were investigated by rapid kinetic techniques that either measure the disappearance of Ca^{2+} from the medium [stopped-flow photometry of Ca^{2+} indicators or rapid filtration (method 1)] or directly detect the changes in the accessibility of Ca^{2+} to the exterior of the membrane, i.e., occlusion of Ca^{2+} within the Ca pump and Ca^{2+} transport into the lumen of SR vesicles [EGTA quench (method 2)]. SR vesicles were preincubated in micromolar Ca^{2+} to form the $E{\cdot}2Ca_{cyt}$ intermediate of the Ca-ATPase, and then Ca^{2+} transport was initiated by addition of ATP. It was found that Ca^{2+} uptake measured by method 1 began with no lag phase, in spite of the prediction of kinetic models of the Ca-ATPase. Instead, the time course of Ca^{2+} uptake was found to have two components: a fast and a slow phase, similar to that obtained using method 2, although the rate constant of the fast phase determined by method 1 was considerably lower than that measured by method 2. The fast phase of Ca^{2+} uptake measured by method 1 was not influenced by either Ca^{2+} ionophore or detergent treatment, whereas the slow phase was diminished. These findings indicate that, upon formation of the phosphorylated intermediate (EP) of the Ca-ATPase, two events occurring simultaneously contribute to Ca^{2+} transport: (1) the internalization of Ca^{2+} that is detectable by method 2, but not by method 1, i.e., Ca^{2+} occlusion within the interior of the Ca-ATPase polypeptide and the following translocation of Ca^{2+} from occluded to internally oriented sites and (2) the binding of Ca^{2+} that is detected by method 1, but not by method 2, to the newly available high-affinity sites that are vacated by Ca^{2+}, as event 1 proceeds. This suggests that, during the Ca-ATPase reaction cycle, Ca^{2+} sites do not convert from high to low affinity, i.e., from outward- to inward-oriented sites; Ca^{2+} instead moves through the membrane from site to site.

The mechanism of Ca^{2+} transport by the sarcoplasmic reticulum (SR)[1] Ca-ATPase (Ca pump) is usually described by a cycle of sequential reaction steps, as first formalized by deMeis and Vianna (1979), with two major states of the enzyme, E_1 and E_2 [for reviews, see Inesi (1985) and Tanford (1984)]. According to this model (E_1–E_2 model), the movement of Ca^{2+} against its concentration gradient is achieved by the simultaneous alteration of both the affinity and the location of a set of Ca-binding sites on the pump. The affinity of the binding sites for Ca^{2+} is high when they are exposed to the cytoplasmic surface of the enzyme (E_1) but low when they face the lumenal side (E_2). Both the translocation and the affinity change of the Ca^{2+} binding sites are driven by a

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid. E·Ca and EP, Ca-loaded and phosphorylated, respectively, intermediates of the Ca-ATPase.

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decrease in the chemical potential of the phosphoryl group of the phosphorylated pump-intermediate during the conversion of the enzyme from one state into the other: $E_1 \sim P \cdot 2Ca_{cyt} - E_2P \cdot 2Ca_{lum}$. Since the same set of Ca-binding sites is transferred from one side of the membrane to the other, a crucial implication of the E_1–E_2 model is that the Ca-binding sites are exclusively in either the high- or low-affinity configuration (Tanford, 1984). There is also evidence (Dupont, 1980; Takisawa & Makinose, 1981, 1983) which indicates that, upon EP formation from $E_1 \cdot 2Ca_{cyt}$ (plus ATP), calcium ions (or, according to the E_1–E_2 model, the Ca sites) become occluded in a state in which they are not accessible from either side of the membrane.

According to kinetic schemes so far formalized to describe the sequence of elementary steps of the Ca-ATPase reaction cycle, the time course of Ca^{2+} uptake initiated by the addition of ATP to the preformed $E_1 \cdot 2Ca_{cyt}$ intermediate should begin with a considerable lag phase [see the detailed kinetic study by Fernandez-Belda et al. (1984)], if it is followed by measuring the disappearance of Ca^{2+} from the medium (by photometry of Ca^{2+} indicators, for instance). However, it has been reported (Pierce et al., 1983; Mészáros & Ikemoto, 1985a,b) that the time course of Ca^{2+} uptake measured by detecting the absorbance changes of the Ca^{2+} indicator arsenazo III begins with an initial fast phase, which is rather contradictory to the E_1–E_2 model. On the other hand, recent reports (Riollet & Champeil, 1987; Beeler, 1990) suggest that arsenazo III photometry might detect artifactual absorbance changes that are not related to actual Ca^{2+} movement. Therefore, it is important to reinvestigate the kinetics of Ca^{2+} uptake by using and comparing a variety of methods. Here we report that, upon addition of ATP to SR vesicles preexposed to micromolar Ca^{2+} to form $E_1 \cdot 2Ca_{cyt}$, the time course of Ca^{2+} uptake was found to have two components, a fast and a slow phase, and showed no initial lag phase, regardless of whether Ca^{2+} uptake was followed by stopped-flow photometry of Ca^{2+} indicators of both arsenazo III and murexide, rapid filtration, or an EGTA-quench technique. The fast phase was not influenced, whereas the slow phase was diminished by either the addition of Ca^{2+} ionophores or detergent treatment. These findings taken together suggest that during the fast phase of Ca^{2+} uptake two events must occur simultaneously: (1) the internalization (occlusion and translocation) and (2) the binding of Ca^{2+} to newly available high-affinity sites that are vacated due to the movement of Ca^{2+} from the cytoplasmic to internal sites. A reaction scheme that accounts for these findings is discussed.

Experimental Procedures

Preparation of SR. SR vesicles from rabbit fast-twitch skeletal muscle were prepared by differential centrifugation (Ikemoto et al., 1989). The final pellets were resuspended in 200 mM sucrose, 75 mM KCl, and 20 mM MOPS, pH 6.8, containing a mixture of protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, and 0.5 μg/mL antipain). SR stock solutions, 20–30 mg of protein/mL as determined by the method of Lowry et al. (1951), were stored at −80 °C until use. Calcium contamination of the preparations were determined by flame photometry.

Quench-Flow Measurements. A Froehlich-Berger rapid mixer was used in a three-syringe setup. Syringe a contained the SR vesicles (0.2 mg/mL) in 150 mM KCl, 20 mM MOPS, pH 6.8 (KCl–MOPS), 5 mM $MgCl_2$, and 50 μM $CaCl_2$ ($^{45}CaCl_2$ for Ca^{2+} uptake). Syringe b delivered 20 μM ATP ([γ-^{32}P]ATP for EP formation) in KCl–MOPS containing 5 mM $MgCl_2$ and 50 μM $CaCl_2$ ($^{45}Ca^{2+}$ for Ca^{2+} uptake). The reaction was started by mixing aliquots from syringes a and b in a 1:1 volume ratio and terminated by delivering the quenching solution from syringe c, which was either 30% TCA (EP formation) or 30 mM EGTA in KCl–MOPS containing 10 mM $MgCl_2$ (Ca^{2+} uptake) as described in Verjovski-Almeida et al. (1978). Quenched aliquots (0.4 mL) were filtered through 0.45-μm Millipore filters and washed with ice-cold solutions containing either 5% TCA and 10 mM P_i (EP formation) or 10 mM EGTA and 5 mM $MgCl_2$ in KCl–MOPS ($^{45}Ca^{2+}$ uptake). Radioactivity remaining on the filter was determined by liquid scintillation counting. Blanks (points at $t = 0$) were determined by reversing the mixing sequence of syringes b and c.

Dual-Wavelength Stopped-Flow Photometry of Extravesicular Ca^{2+}. The changes in extravesicular Ca^{2+} concentration were monitored by using Ca indicators: the absorbance of either arsenazo III (10 μM) or murexide (100 μM) was recorded at 650–680 or 510–550 nm, respectively. The absorbance vs [Ca^{2+}] relationship was determined in EGTA/Ca^{2+} buffers: $CaCl_2$, in total concentrations that resulted in a given concentration of Ca^{2+}, were added to 5 mM EGTA in KCl–MOPS that also contained $MgCl_2$ (see figure legends). The decrease in pH due to the mixing EGTA with Ca^{2+} was compensated for by adding KOH. Ca^{2+} uptake was initiated by mixing the contents of syringes a and b (in a 1:1 volume ratio), which were SR and ATP, respectively, in KCl–MOPS containing either arsenazo III or murexide. Ca^{2+} and Mg^{2+}, when added, were present in both syringes in equal concentrations. The dead time of the apparatus, which was less than 2 ms, was determined by following the absorbance changes that resulted from the reaction of Fe^{3+} with SCN^- in diluted sulfuric acid. The off rate of Ca^{2+} dissociation from arsenazo III was determined by mixing preformed arsenazo III–Ca^{2+} complex with EGTA in KCl–MOPS buffer. Traces obtained in the absence of ATP showed small optical density changes due to the changes in light scattering and were subtracted from those measured in the presence of ATP.

Rapid Filtration. Experiments were carried out with a Bio-Logic (Grenoble, France) rapid-filtration apparatus (Dupont, 1984; Mészáros & Ikemoto, 1989). SR (0.1 mg) was equilibrated in KCl–MOPS buffer containing $^{45}CaCl_2$ and $MgCl_2$ in various concentrations (buffer a) for 30 s, and then layered on a 0.6-μm Millipore filter placed on the filter holder of the apparatus. The solution (buffer b) delivered from the syringe of the apparatus contained ATP in buffer a. Ca^{2+} uptake was initiated by actuating the flow of buffer b through the filter and terminated by discontinuing it. Different flow rates were set for different duration times (reaction times), according to the recommendations of the manufacturer. The vacuum applied was gradually reduced as the preset duration time was increased, in order to minimize the variation of the volume retained by the filter. Such variation (28–33 μL with SR on the filter) was taken into account when the actual Ca^{2+} uptake data points were calculated: measurements without ATP were run and the volume retained by the filter for each measured point was determined. After subtracting the radioactivity retained by the filter (usually 6000–8000 cpm; blanks obtained in the absence of SR), the radioactivity (usually 9000–11 000 cpm) measured in the presence of SR, but in the absence of ATP, was used to calculate the amount of Ca^{2+} bound at $t = 0$.

Filtration experiments of manual mixing were carried out by addition of ATP to SR vesicles in KCl–MOPS to initiate transport, and then aliquots were filtered through a 0.45-μm Millipore filter to terminate $^{45}Ca^{2+}$ uptake. The uncertainty

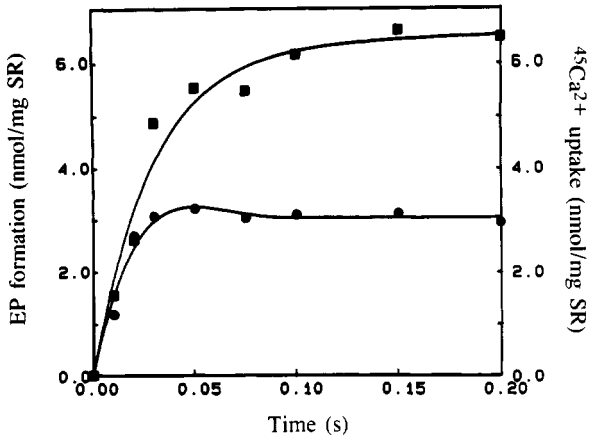

FIGURE 1: Time course of EP formation (●) and the initial phase of $^{45}Ca^{2+}$ (■) uptake determined by quench-flow techniques. Measurements were carried out in a KCl–MOPS buffer also containing 5 mM $MgCl_2$, as detailed under Experimental Procedures. SR (0.2 mg/mL) preincubated in the presence of 50 μM Ca^{2+} (or $^{45}Ca^{2+}$) was rapidly mixed with 20 μM ATP (or [γ-^{32}P]ATP) + 50 μM Ca^{2+} (or $^{45}Ca^{2+}$) in a 1:1 volume ratio. The EP formation reaction was quenched with 15% TCA (final concentration) and $^{45}Ca^{2+}$ uptake with 15 mM EGTA + 5 mM $MgCl_2$. Data points represent the average of six independent determinations on three preparations.

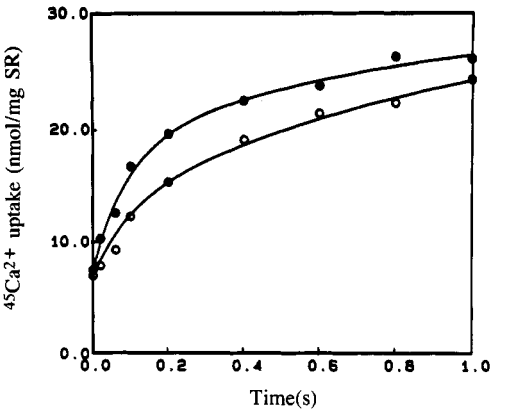

FIGURE 2: Time course of the initial phase of $^{45}Ca^{2+}$ uptake measured by rapid filtration. SR (0.1 mg/mL) in 0.4 mL of KCl–MOPS containing 50 μM $^{45}Ca^{2+}$ (solution a) was layered on the filter, and then, after 30 s, the reaction was initiated by the addition of perfusion solution b containing 10 μM ATP and 50 μM $^{45}Ca^{2+}$ (same specific activity as in solution a) in KCl–MOPS. The reaction was terminated by a sudden interruption of perfusion. The concentration of $MgCl_2$ present in both solutions a and b was 5 mM (●) and 0.25 mM (○). The data points are the average of eight measurements on three preparations.

of the termination time, due to the duration of filtration, was about 1 s.

Equilibrium binding of $^{45}Ca^{2+}$ to SR vesicles was determined in the presence of 80 μM total $^{45}Ca^{2+}$ (taking Ca^{2+} contaminations into account) and various calculated concentrations of EGTA. After 30 s of equilibration, aliquots were Millipore filtered, and the radioactivity retained by the filters was determined.

[γ-^{32}P]ATP with 370 TBq/mol specific activity was purchased from New England Nuclear (NEN), Boston. $^{45}CaCl_2$ (12 TBq/mol) was the product of either Izotōp Intēzet, Budapest, or NEN. Ionomycin from Calbiochem and arsenazo III and murexide from Sigma were used.

Results

Figure 1 illustrates the time courses of both the formation of the phosphorylated intermediate of the Ca pump (EP; circles) and $^{45}Ca^{2+}$ uptake (squares) determined by quench-flow techniques. The E·2Ca_{cyt} intermediate of the Ca-ATPase was preformed by preincubating the SR vesicles in 50 μM Ca^{2+} ($^{45}Ca^{2+}$ to measure Ca^{2+} uptake), and then 10 μM ATP ([γ-^{32}P]ATP to determine EP) was added to initiate EP formation and Ca^{2+} uptake. Similar to that found earlier in many laboratories, a fast phase of $^{45}Ca^{2+}$ uptake (Ca^{2+} burst; Verjovski-Almeida et al., 1978), which progressed in parallel with the formation of EP and led to the internalization of moles of Ca^{2+}/mole of EP formed, was clearly distinguished from a subsequent slow phase. There is kinetic evidence (Dupont, 1980; Takisawa & Makinose, 1983) indicating that, during the internalization step and in parallel with EP formation [for a review, see Inesi (1985)], Ca^{2+} first becomes occluded within the Ca-ATPase polypeptide, resulting in the formation of the intermediate EP·2Ca_{occ}. Thus, the initial burst of Ca^{2+} uptake, i.e., the fast kinetic phase that is detected by using the EGTA-quench technique, most probably represents an isomerization step in which E·2Ca_{cyt} converts into EP·2Ca_{occ}, resulting in the relocation of the Ca sites. Accordingly, a similar initial fast phase of Ca^{2+} uptake would not be detected by using methods which either terminate the reaction by separating the vesicles from the medium (filtration without an EGTA-washing step) or record the changes in the extravesicular Ca^{2+} concentration (photometry of Ca^{2+} indicators). Instead, the time course of Ca^{2+} uptake, if determined by using these methods, should rather begin with a considerable lag phase, due to the fact that the extravesicular Ca^{2+} concentration would remain constant during the above isomerization step, even further, until one cycle of the Ca-ATPase reaction is completed.

Table I: Rate Constants Characteristic of the Fast Phase of Ca^{2+} Uptake Determined by Different Methods[a]

method	[Mg^{2+}] (mM)	k (s^{-1})
quench flow (EGTA quench)[b]	5	36.9 ± 4.7
rapid filtration	5	11.0 ± 2.9
rapid filtration	0.5	5.4 ± 2.3
murexide photometry	5	9.2 ± 1.9
murexide photometry	0.5	2.1 ± 0.7
arsenazo III photometry	5	73.4 ± 10.7
arsenazo III photometry	0.5	2.7 ± 2.1

[a] The rate constants (k) were obtained by fitting the biexponential function to data points of Ca^{2+} uptake time courses determined as described under Experimental Procedures. The values are given as the mean ± SD of at least three independent measurements carried out with at least three SR preparations. [b] As reported earlier (Mészáros & Ikemoto, 1985), the fast phase of the Ca^{2+} uptake time course measured by using EGTA quenching is better approximated by a function which assumes a sequential two-step Ca^{2+} occlusion with rate constants of 78 s^{-1} for each step.

The time course of Ca^{2+} uptake measured by rapid filtration is shown in Figure 2 and that monitored by stopped-flow photometry of murexide is in Figure 3A. Again, after preforming E·2Ca_{cyt} by incubating the SR vesicles in 50 μM Ca^{2+} ($^{45}Ca^{2+}$ for the rapid-filtration experiment), Ca^{2+} uptake was initiated by rapid mixing of ATP with the SR vesicles. [Note that the time course did not begin at the origin (see Figure 2A), due to the fact that at t = 0 E·2Ca_{cyt} already existed.] As shown, instead of a lag phase, the time course of Ca^{2+} uptake measured by either photometry of Ca^{2+} indicators or rapid filtration began with an initial fast phase, of which rate, however, was significantly lower than that obtained with EGTA quenching (Table I). As previously reported by Pierce et al. (1983) and also shown in Table I, the rate of the initial phase measured by using stopped-flow photometry of arsenazo III in the presence of high concentrations (5–10 mM) of Mg^{2+} was significantly higher than that obtained with the murexide technique but was similar to that found with EGTA quenching.

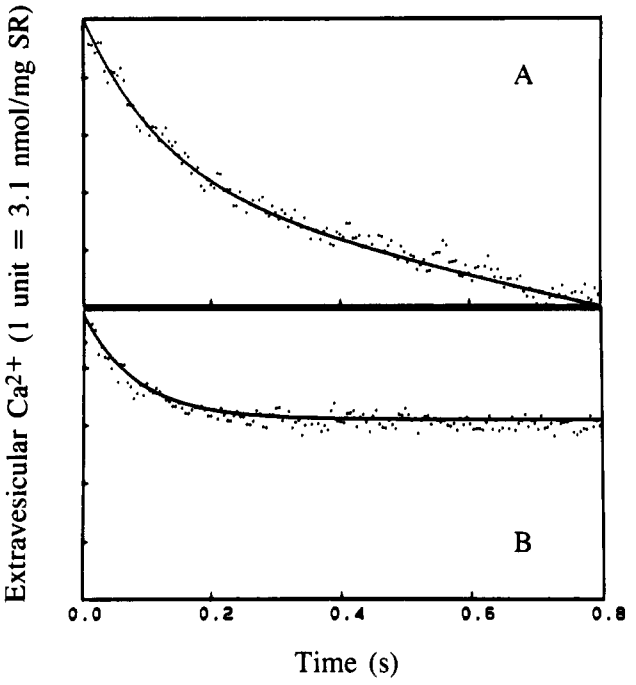

FIGURE 3: Time course of Ca^{2+} uptake determined by stopped-flow photometry of murexide. Both syringes contained 50 μM Ca^{2+}, 5 mM $MgCl_2$, and 100 μM murexide in KCl–MOPS. In panel B, the syringes also contained $C_{12}E_8$ (1 mg/mg of SR protein). SR (0.5 mg/mL in syringe a) was rapidly mixed (in a 1:1 volume ratio) with 20 μM ATP. The traces are the average of 62 (panel A) and 87 (panel B) individual runs, respectively, on two preparations. The optical signal vs [Ca^{2+}] calibration was carried out as described under Experimental Procedures and showed an approximately linear response up to 70 μM free Ca^{2+}. Lines are drawn to bi- (panel A) and monoexponentials (panel B).

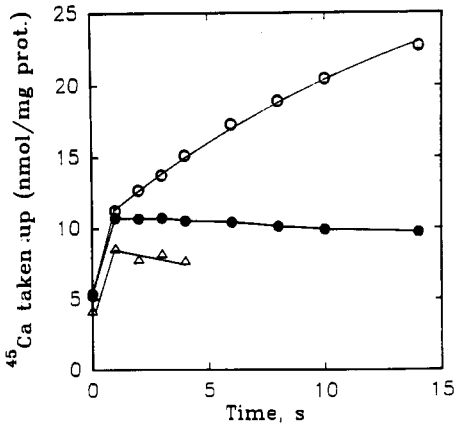

FIGURE 4: $^{45}Ca^{2+}$ uptake measured by Millipore filtration. The effect of ionomycin (●) and $C_{12}E_8$ (▲). The reaction was started by the addition of 0.5 mM ATP to SR vesicles (0.12 mg/mL) preincubated (for 30 s) in the presence of 50 μM $^{45}Ca^{2+}$ and 1.5 mM $MgCl_2$ in KCl–MOPS also containing ionomycin (20 μM) or $C_{12}E_8$ (1 mg/mL SR protein), where indicated. The reaction was stopped by Millipore filtration. Points are the average of four determinations on two preparations. The bars represent the standard deviation.

However, in the presence of low concentrations (0.2–0.5 mM) of Mg^{2+}, the rate in the arsenazo III-based measurements decreased (see Table I and Figure 5A) and became similar to that obtained with either rapid filtration or murexide photometry.

The occurrence of such an initial fast phase of Ca^{2+} uptake measured by methods that do not apply an EGTA quench suggests that, in parallel with the $E{\cdot}2Ca_{cyt}$–$EP{\cdot}2Ca_{occ}$ isomerization step (detected only by EGTA quench), some additional Ca^{2+} movement must occur. Since this additional Ca^{2+}

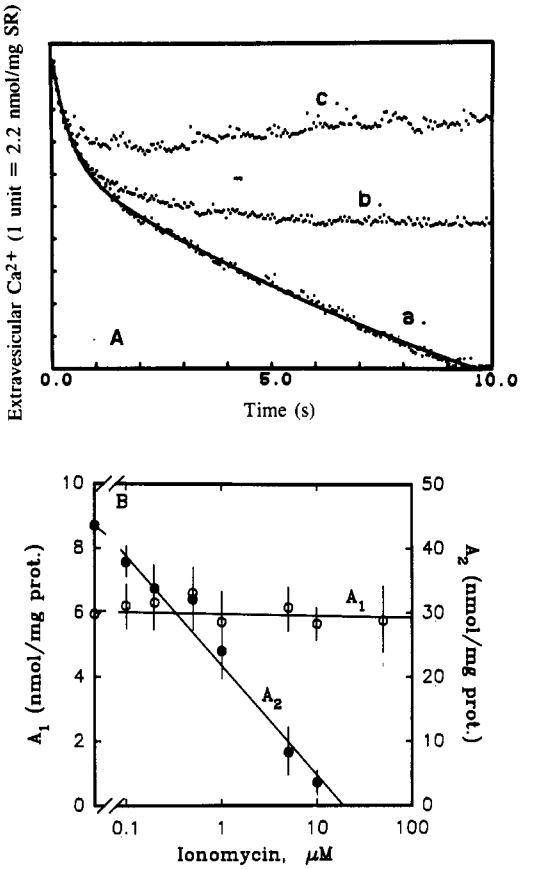

FIGURE 5: Ca^{2+} uptake determined by stopped-flow photometry of arsenazo III. The effect of ionomycin. Syringe a delivered the SR vesicles (0.3 mg/mL) preexposed to 50 μM Ca^{2+} in KCl–MOPS containing 0.5 mM $MgCl_2$, and syringe b contained 0.5 mM ATP in KCl–MOPS with the same total concentrations of Ca^{2+} and $MgCl_2$ as in syringe a. The duration of the deflection in the optical signal due to the mixing of the contents of the syringes with nonequal free Ca^{2+} and Mg^{2+} concentrations (chelation by ATP) was 6–8 ms. (A) Time course of Ca^{2+} uptake. (Traces b and c) 1 and 20 μM ionomycine, respectively, was present in both syringes. The concentrations of aresenazo III added in both syringes were 10 μM. Traces are signal-averaged from 15–20 individual runs on two preparations. (B) Concentration dependence of the effect of ionomycin on the amount of Ca^{2+} taken up during the fast (A_1) and the slow (A_2) phases. Traces of time course measurements carried out at a given ionomycin concentration were averaged, and then a biexponential (or monoexponential) function was fit to the data points to calculate A_1 and A_2.

movement is apparently "invisible" to the EGTA-quench technique, it most likely represents the binding of Ca^{2+}, instead of its internalization (occlusion or transport). Figure 4 depicts time courses of $^{45}Ca^{2+}$ uptake of hand-mixing experiments, in which the uptake reaction was terminated by Millipore filtration (see Experimental Procedures). As shown, in the presence of either the Ca^{2+} ionophore ionomycin (filled circles) or the nonionic detergent $C_{12}E_8$ (triangles), the slow phase of Ca^{2+} uptake diminished, but apparently, a considerable fraction of Ca^{2+} uptake was insensitive to both the ionophore and the detergent treatment (see also Figure 3B). Figure 5 demonstrates the selective removal of the slow phase of Ca^{2+} uptake by the addition of ionomycin: Panel A shows the stopped-flow traces of Ca^{2+} uptake measured by photometry of arsenazo III absorbance in the absence (trace a) and in the presence (traces b and c) of ionomycin, and panel B depicts the concentration dependence of the ionomycin effect. The amount of Ca^{2+} taken up during the fast phase (A_1) was completely ionophore-insensitive, indicating that, during this kinetic phase, Ca^{2+} is either taken up into an ionophore-inaccessible space or Ca^{2+} binding occurs. (The ionophore

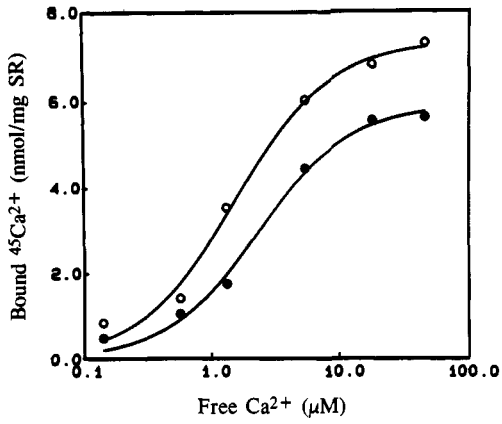

FIGURE 6: Ca^{2+} dependence of the initial $^{45}Ca^{2+}$ uptake phase determined by rapid filtration (●) and that of equilibrium $^{45}Ca^{2+}$ binding (○). The measurements were carried out in the presence of 5 mM $MgCl_2$ in KCl–MOPS as described under Experimental Procedures and in the legend to Figure 2. The total concentration of Ca^{2+} was adjusted to 80 μM, and various concentrations of EGTA were added to establish different concentrations of Ca^{2+}. (●) Time courses were obtained, and the A_1 values (see the legend to Figure 5) were calculated from the data of three time courses obtained on two preparations. (○) The equilibrium binding experiments (no ATP added) with two preparations (one measurement of each) were measured. SR vesicles (0.2 mg/mL) were incubated at various concentrations of Ca^{2+} as indicated for 30 s, and then the vesicles were filtered through 0.45-μm Millipore filters on which the radioactivity retained was determined.

sensitivity of A_2, on the other hand, suggests that during the slow kinetic phase the filling of the lumen of the vesicles with Ca^{2+} takes place.) The ionophore insensitivity of A_1 together with kinetic considerations, i.e., the fact that the rate of the fast phase, when measured by either rapid filtration or stopped-flow photometry, was significantly lower than that determined in EGTA-quench experiments, suggest that the "additional" Ca^{2+} movement represents a binding process (and not identical to the faster Ca^{2+} occlusion step).

As shown in Figure 2 (and Table I), at low concentrations of Mg^{2+} (0.25 mM) the rate of Ca^{2+} uptake during the initial phase measured by rapid filtration [or stopped-flow photometry of murexide (not shown)] considerably decreased (open circles), as compared to that in the presence of 5 mM Mg^{2+} (filled circles). This makes it unlikely that the metal(or Mg^{2+})-binding site of the Ca-ATPase (Ikemoto, 1982), with a similar affinity to Ca^{2+} and Mg^{2+}, would be involved in the additional Ca^{2+} movement (Ca^{2+} binding). Figure 6 (filled circles) depicts the Ca^{2+} dependence of the amount of Ca^{2+} taken up during the initial fast phase of Ca^{2+} uptake measured by rapid filtration. The apparent K_d (5–6 μM, as calculated from data represented by filled circles) that is characteristic of the fast phase, although being slightly higher than that (1.5 μM, see open circles) of "passive" Ca^{2+} binding to SR vesicles (measured in the absence of ATP), suggests that high-affinity Ca sites of the Ca pump are involved in the additional Ca^{2+} binding that occurs during the initial phase of Ca^{2+} uptake, in parallel with the internalization of Ca^{2+}.

Discussion

The kinetic models of the SR Ca-ATPase [see reviews by Ikemoto (1982) and Inesi (1985)] predict a cyclic pathway of elementary reactions in which intermediates of the Ca-ATPase are formed sequentially. By using the EGTA-quench technique together with rapid-kinetic instrumentation to measure the Ca^{2+} uptake initiated by mixing the reactants of ATP and the preformed $E{\cdot}2Ca_{cyt}$ [see Verjovski-Almeida et al. (1978) and Experimental Procedures], a biphasic time course with an initial fast phase (Ca^{2+} burst) that basically parallels the time course of EP formation was observed (Figure 1). Kinetic evidence (Dupont, 1980; Takisawa & Makinose, 1983) indicates that, during this initial fast phase, Ca^{2+} becomes occluded within the Ca pump polypeptide. Thus, the EGTA-quench method, by reporting the accessibility of Ca^{2+} from the cytoplasmic side of the membrane, actually measures the $E{\cdot}2Ca_{cyt}$–$EP{\cdot}2Ca_{occ}$ reaction, as the initial fast phase of Ca^{2+} uptake. It is obvious that such a fast initial phase, i.e., the change in the state of Ca^{2+} (bound vs occluded), must be "invisible" to techniques such as photometry of Ca^{2+} indicators and rapid filtration. Instead, these techniques should reveal a time course with an initial lag phase, since they basically measure the disappearance of Ca^{2+} from the medium, which only occurs after the first cycle is completed, i.e., once the free enzyme (E) reappears (since at $t = 0$ the enzyme is present as $E{\cdot}2Ca_{cyt}$). According to the characteristic rate constants of the elementary steps of the Ca-ATPase reaction cycle [see Fernandez-Belda et al. (1984)], under the experimental conditions of this study E in a significant amount is expected to reaccumulate after a few hundred milliseconds, once the first cycle is completed. Therefore, the time course of Ca^{2+} uptake measured by photometry of Ca^{2+} indicators or filtration must begin with a lag phase that is easily resolvable with the rapid-kinetic techniques used in this study.

As shown here, the time course of Ca^{2+} uptake, even when measured by either photometry of Ca^{2+} indicators (Figure 3) or rapid filtration (Figure 2), began with an initial fast phase, instead of a lag period as expected. The rate constant of the initial fast phase observed by either stopped-flow photometry or rapid filtration, on the other hand, was significantly lower than that detected by EGTA quench (Ca^{2+} occlusion). This clearly indicates that, during the initial fast phase, the Ca^{2+} movements detected by the two different techniques, i.e., EGTA quench and non-EGTA quench, are not identical, but they occur simultaneously.

An important feature of the Ca^{2+} movement detected by filtration and by photometry of Ca^{2+} indicators is that it is virtually unaffected by either detergent treatment of the vesicles (Figures 3B and 4) or the addition of the Ca^{2+} ionophores (Figures 4 and 5). This indicates that it represents a binding process and not an actual Ca^{2+} transport into the vesicles. Furthermore, the finding that the rate of this additional Ca^{2+} movement (binding) increases by elevating the concentration of Mg^{2+} indicates that it does not involve the so-called metal-binding site of the Ca-ATPase (Ikemoto, 1982) that has similar affinity to Ca^{2+} and Mg^{2+}.

The results presented here establish a supernumerary stoichiometry of transport Ca^{2+} sites/EP formed. A model in which a dimer of the Ca-ATPase operates as a functional unit, where one of the monomers is "silent" until the other monomer is phosphorylated, could account for the supernumerary stoichiometry. Although there is kinetic evidence suggesting the occurrence of Ca-ATPase dimers as functional units that operate in such a staggered fashion (Ikemoto et al., 1981; Ikemoto & Nelson, 1984), the fact that virtually the same kinetics of the fast phase (additional Ca^{2+} binding) was observed in the presence of detergents, in which the Ca-ATPase is presumably in its monomeric form (Kosk-Kosicka et al., 1983; Martin et al., 1984) or, at least, the dimeric behavior would be compromised, makes such model less likely. Another possible mechanism is illustrated by

$$E \xrightarrow{2Ca^{2+}} E{\cdot}2Ca_{cyt} \xrightarrow{ATP + 2Ca^{2+}} EP{\cdot}2Ca_{occ/in}{\cdot}2Ca_{cyt}$$

According to this scheme, upon addition of ATP, the Ca^{2+} bound to the Ca pump on the cytoplasmic side ($E{\cdot}2Ca_{cyt}$) first

becomes occluded ($EP{\cdot}2Ca_{occ}$), and then is transferred to the low-affinity lumenal sites ($EP{\cdot}2Ca_{in}$). This process, i.e., the movement of Ca^{2+} away from the high-affinity sites, leaves the high-affinity sites vacant and makes them capable of rapidly binding additional Ca^{2+} ($EP{\cdot}2Ca_{cyt}{\cdot}2Ca_{occ/in}$). Since, as shown in Figure 6, the apparent K_d of the additional Ca^{2+} binding was higher than that of "passive" Ca^{2+} binding, the affinity of the high-affinity Ca^{2+}-binding sites in $EP{\cdot}2Ca_{occ/in}{\cdot}2Ca_{cyt}$ is decreased as compared to that in $E{\cdot}2Ca_{cyt}$. It is to note that such reduction in the affinity of the Ca sites on the operating pump has been reported (Chadwick & Thomas, 1984; Petithory & Jencks, 1988).

Fernandez-Belda et al. (1984) reported a decrease in the intensity of the intrinsic tryptophan fluorescence of the Ca-ATPase upon mixing ATP with $E{\cdot}2Ca_{out}$. On the basis of kinetic arguments, the authors suggested that this change in the tryptophan fluorescence intensity represents a conformational change ascribable to the $E{\sim}P{\cdot}2Ca_{occ}$–$E{-}P{\cdot}2Ca_{in}$ isomerization step. The time course of this type of fluorescence change [as measured also in our laboratory (not shown)] remarkably resembles that of the additional Ca^{2+} binding described here. This suggests that the additional Ca^{2+} binding parallels the formation of $EP{\cdot}2Ca_{in}$ from $EP{\cdot}2Ca_{occ}$. However, the fact that the fluorescence decrease does not show a sigmoidal time course [not shown, but see Fernandez-Belda et al. (1984)] as expected, if it monitors the formation of $E{-}P{\cdot}2Ca_{in}$, might bring this conclusion into question. Whether the fluorescence decrease reports the change in the affinity of the high-affinity Ca^{2+}-binding sites instead will be addressed in the future.

Although future kinetic studies are necessary to precisely pinpoint the elementary step(s) represented by the additional Ca^{2+} binding detected by stopped-flow photometry of Ca^{2+} indicators and by rapid filtration, an important problem should be addressed: One of the crucial implications of the E_1–E_2 model is that the two Ca^{2+}-binding sites of the Ca-ATPase are exclusively in either the high- or low-affinity conformation, i.e., the sites, but not the calcium ions, are transferred from one side of the membrane to the other (Tanford, 1984). The model proposed here would instead support the transfer of calcium ions through a channel-type structure.

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