Kinetics of Calcium Dissociation from Its High-Affinity Transport Sites on Sarcoplasmic Reticulum ATPase†

Stéphane Orlowski and Philippe Champeil*
SBPh et URA CNRS 1290, Département de Biologie Cellulaire et Moléculaire, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France

Received May 24, 1990; Revised Manuscript Received September 21, 1990

ABSTRACT: We investigated the kinetics of calcium dissociation from its high-affinity transport sites on sarcoplasmic reticulum Ca^{2+}-ATPase by combining fast filtration with stopped-flow fluorescence measurements. At pH 6 and 20 °C, in the absence of potassium and in the presence of 20 mM $MgCl_2$, isotopic exchange of bound calcium exhibited biphasic kinetics, with two phases of equal amplitude, regardless of the initial extent of binding site saturation. The rapidly exchangeable site, whose occupancy by calcium controlled the rate constant of the slow phase, had an apparent affinity for calcium of about 3–6 μM. A similar high affinity was also deduced from measurements of the calcium dependence of the rate constant for ATPase fluorescence changes. This affinity was higher than the overall affinity for calcium deduced from the equilibrium binding measurements (dissociation constant of 15–20 μM); this was consistent with the occurrence of cooperativity (Hill coefficient of 1.6–1.8). The drop in intrinsic fluorescence observed upon chelation of calcium was always slightly faster than the dissociation of calcium itself, although the rates for both this drop in fluorescence and calcium dissociation varied slightly from one preparation to the other. This fluorescence drop was therefore mainly due to dissociation of the bound ions, not to slow transconformation of the ATPase. Dissociation of the two bound calcium ions in a medium containing EGTA exhibited monophasic kinetics in the presence of a calcium ionophore, with a rate constant about half that of the fast phase of isotopic exchange. This particular pattern was observed over a wide range of experimental conditions, including the presence of KCl, dimethyl sulfoxide, 4-nonylphenol, or a nucleotide analogue, at pH 6 or 7, and at various temperatures. The kinetics of calcium dissociation under the above various conditions were not correlated with the ATPase affinity for calcium deduced from equilibrium measurements under the same conditions. These results are consistent with sequential dissociation of calcium from a narrow binding pocket inside which a single calcium ion can move fairly easily. Escape of calcium might be controlled by a structural compartment acting as a gate.

Active calcium transport into the lumen of sarcoplasmic reticulum is mediated by Ca^{2+}-dependent ATPase.[1] This protein, the main constituent of isolated SR fragments, is one of the prototypes of active ion transport ATPases, and its catalytic cycle has been studied in great detail (Hasselbach, 1974; Tada et al., 1978; de Meis & Vianna, 1979; Martonosi & Beeler, 1985; Inesi, 1985). However, little is known about the mechanism of ion transport and the structural features responsible for it. Even the location of the calcium binding sites on the polypeptide chain is still uncertain, as these sites were initially considered to be located on the ATPase N-terminal stalk, easily accessible from the cytoplasmic medium, but more recently to be located closer to the ATPase C-terminus, in a region believed to be mostly membranous (Brandl et al., 1986; Clarke et al., 1989; Green, 1989; le Maire et al., 1990b). Moreover, the structural predictions for ion transport ATPases are not all in agreement with respect to the location of the putative transport sites [see a discussion of this issue in le Maire et al. (1990a)].

In this connection, functional studies of the dynamics of calcium movements to and from the transport sites can probably provide important information about these sites and how they alter during pump turnover. Unfortunately, few tools are available for such an investigation. One of them, the observation of the ATPase fluorescence changes upon calcium binding or dissociation, was already used long ago as a "black box" for this purpose (Dupont et al., 1978; Guillain et al., 1980; Champeil et al., 1983). Time-resolved indirect measurements of calcium binding and dissociation were also recently made through multimixing experiments (Petithory & Jencks, 1988a,b). As opposed to these indirect measurements, direct exploration of dissociation kinetics through filtration experiments was first attempted at very low temperatures (Dupont, 1982), then at 0 °C (Nakamura, 1986, 1987), and finally at room temperature by using a rapid-filtration device (Dupont, 1984; Inesi, 1987; Meszaros & Ikemoto, 1989). In the present work, we reinvestigated the kinetics of calcium dissociation from its high-affinity sites on ATPase by combining rapid filtration and stopped-flow fluorescence under a wide range of experimental conditions. These detailed studies support the suggestion that the calcium ions bound to ATPase are located in a narrow channellike pocket from which these ions escape sequentially (Inesi, 1987; Petithory & Jencks, 1988a). They show that a single rate-limiting step controls the rate of departure of the two bound calcium ions from this pocket and that there is no severe kinetic restriction on the movement of a single ion inside it. A gating machinery of the binding pocket

† Part of this work was presented at the 34th Biophysical Society Meeting in Baltimore, MD [see Orlowski and Champeil (1990)].

[1] Abbreviations: SR, sarcoplasmic reticulum vesicles; ATPase, adenosine triphosphatase; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $\Delta F/F$, relative fluorescence change; k_{obs}, observed rate constant; ATP, adenosine triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)-triphosphate; Me_2SO, dimethyl sulfoxide; A23187, calcimycin; Trp, tryptophan; SDS, sodium dodecyl sulfate.

0006-2960/91/0430-352$02.50/0

might be part of the mechanism through which calcium is taken up during the catalytic cycle.

Experimental Procedures

Sarcoplasmic reticulum vesicles were prepared as previously described from rabbit skeletal muscle (Champeil et al., 1985). The stock SR suspension was adjusted to 40 mg of protein/mL by solubilizing an aliquot in 1% SDS and assaying its protein concentration through optical density measurements at 280 nm, using a correspondence factor previously checked by comparison with a standard Lowry assay (1 mg/mL protein results in an optical density of 1).

Binding of $^{45}Ca^{2+}$ (Amersham, U.K.) was measured in double-labeling filtration experiments exactly as described in Champeil and Guillain (1986) except that [^{3}H]glucose generally replaced [^{3}H]sucrose. Loading of a Millipore HA filter with 0.3 mg of protein preequilibrated with 100 μM $^{45}Ca^{2+}$ resulted in roughly equal amounts of $^{45}Ca^{2+}$ trapped in the filter with the wetting fluid and $^{45}Ca^{2+}$ specifically bound to the protein, so that subtraction of the trapped $^{45}Ca^{2+}$, estimated by ^{3}H labeling, from the total measured $^{45}Ca^{2+}$ was accurate to less than $\pm$1 nmol/mg.

To measure calcium dissociation rates, we used a rapid-filtration apparatus (Biologic, France). Protein (0.3 mg) was layered onto the filter as in the equilibrium binding experiments above and was perfused for various periods with an unlabeled medium, and ^{3}H and ^{45}Ca radioactivities on the filter were counted. Judging from the residual amount of ^{3}H label on the filter (crosses in Figure 5A), more than 90% of the amount of fluid wetting the filter was washed away after 25 ms, so that the subtraction procedure described above to compute the amount of $^{45}Ca^{2+}$ specifically bound to the vesicles was particularly reliable.

Unless otherwise indicated, the medium contained 150 mM Mes-Tris and 20 mM $MgCl_2$, and the temperature was 20 $\pm$ 1 °C. Ca^{2+} was used in the form of $CaCl_2$ solution. For experiments at different temperatures, the pH of the medium was adjusted so that it would be 6.0 at the desired temperature, assuming a change in pH of about 0.05–0.1 pH units every 5 °C, as checked in separate experiments (the lower the temperature, the more alkaline the medium). In the range of values of interest here, we observed in stopped-flow experiments at 20 °C that k_{obs} did vary from 5.7 to 6.6 s^{-1} when pH varied from 5.9 to 6.1.

Equilibrium and stopped-flow fluorescence experiments were respectively performed with a Perkin-Elmer MPF44A or a SLM 4000S fluorometer and Dionex stopped-flow equipment, as already reported (Guillain et al., 1980; Champeil et al., 1983).

For the calculation of free calcium concentrations, the values of Fabiato and Allen for metal–EGTA dissociation constants were chosen [see Guillain et al. (1980) and Champeil et al. (1985)]; the resulting apparent dissociation constants at pH 6.0 were 3.85×10^{-5} M for Ca–EGTA and 3.02×10^{-1} M for Mg–EGTA. In the presence of 20 mM Mg^{2+}, these figures gave a combined apparent dissociation constant for Ca–EGTA of 4.1×10^{-5} M.

Results

Preliminary Equilibrium Measurements. Under the experimental conditions mainly explored in this report (150 mM Mes-Tris buffer with 20 mM Mg^{2+} and no potassium, pH 6.0 and 20 °C), competition between calcium and protons and magnesium shifted the ATPase affinity for calcium to values lower than under physiological conditions (Watanabe et al., 1981; Guillain et al., 1982), but the ATPase cycle remained perfectly coupled to active transport [see, for example, Figure 13A in Champeil et al. (1986)]. Figure 1 illustrates the concentration dependence of calcium binding to its high-affinity transport sites on SR ATPase under these conditions, as deduced either from direct measurements of the amount of radioactive calcium bound to SR membranes after filtration (panel A) or from the effect of calcium concentration on the fluorescence level of the ATPase tryptophans (panel B). When calculated from panel B and other similar measurements, the mid-point calcium concentration, $[Ca]_{1/2}$, was about 15–20 μM, and the well-defined plateau at high concentrations allowed reasonable estimation of the cooperativity of the curve: for three experiments, the Hill coefficient was 1.6–1.8, implying cooperativity of the binding process under these pH conditions [cf. also Figure 6a in Guillain et al. (1982)].

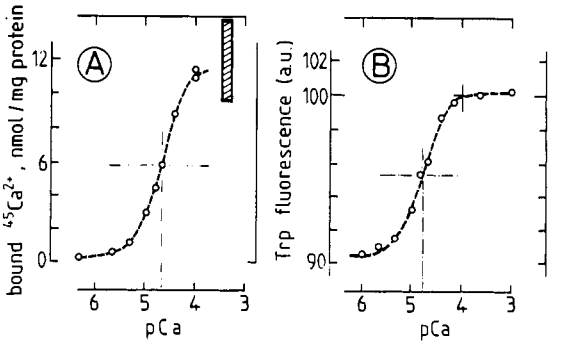

FIGURE 1: $^{45}Ca^{2+}$ binding to SR ATPase (A) and corresponding changes in intrinsic fluorescence (B). For both measurements, SR vesicles were first suspended in a medium containing 150 mM Mes-Tris buffer (pH 6.0), 20 mM $MgCl_2$, and 100 μM added Ca^{2+} (plus $^{45}Ca^{2+}$ tracer and ^{3}H-labeled glucose for panel A). For the filtration experiments, various amounts of EGTA were then added to obtain the desired final free calcium concentration, and 1 mL of the suspension (0.3 mg of protein/mL) was poured onto an HA Millipore filter. For the fluorescence experiments, starting from this pCa 4 medium (100% on panel B, see cross), either EGTA or calcium was added; the protein concentration was 0.1 mg/mL, the excitation wavelength was 290 nm, and here, the emission wavelength was 320 nm (Guillain et al., 1982).

The direct measurements with ^{45}Ca of the amount of calcium bound to the ATPase (Figure 1, panel A) were consistent with the above fluorescence experiments, but data points at very high calcium concentrations were generally not collected because of the large error that would have resulted from the subtraction procedure at these concentrations (see Experimental Procedures), so that a plateau could not be observed. In a few cases, we extended the ^{45}Ca titration curve up to 200 μM Ca^{2+}, and found under our pH conditions no evidence for a significant increase in the amount of bound calcium (not shown). The amount of calcium bound to the SR ATPase at pCa 4 was therefore taken as a measure of the number of transport sites. Figure 1A shows that in one particular experiment the amount of bound $^{45}Ca^{2+}$ at pCa 4 was close to 12 nmol of Ca^{2+}/mg of protein; for this curve, setting the end point at 12 nmol/mg allows us to deduce K_{diss} = 20 μM and n_H = 1.6, consistent with Figure 1B. For all the determinations, the number of sites turned out to range between 9 and 15 nmol of Ca^{2+}/mg of protein (dashed zone in panel A), a range consistent with the relatively high level of phosphoenzyme formed from inorganic phosphate with our SR ATPase [up to 5.5–6.5 nmol/mg; see Table I in Champeil et al. (1985)] and corresponding to two $^{45}Ca^{2+}$ ions bound per ATPase monomer.

Kinetics of $^{45}Ca^{2+}$ Dissociation in a Medium Containing EGTA or $^{40}Ca^{2+}$. Figure 2, panel A, shows the basic experimental situation that will be investigated in detail in this

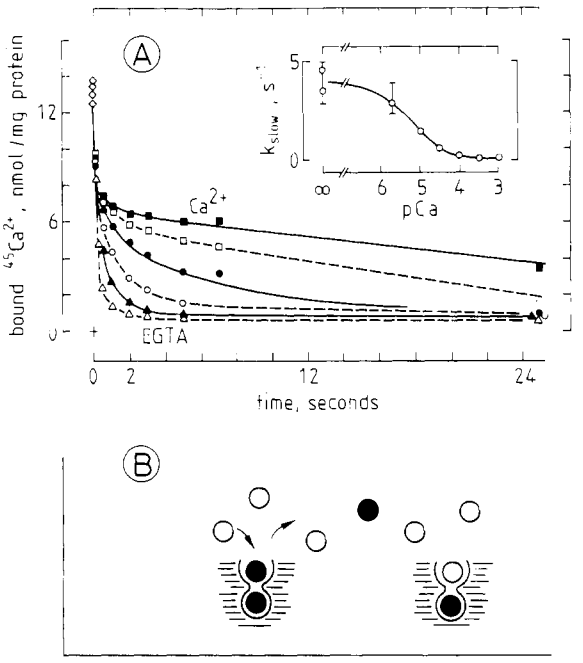

FIGURE 2: Rate of calcium dissociation from SR ATPase high-affinity sites. Panel A: SR vesicles were first equilibrated with 100 μM $^{45}Ca^{2+}$ (and ^{3}H-labeled glucose) in the pH 6 medium described in the legend to Figure 1; 0.3 mg of protein was layered on a filter (diamonds, time zero), and then perfused for various periods (see abscissa) with an unlabeled medium containing either 2mM EGTA (open triangles) or Ca–EGTA buffers resulting in free calcium concentrations of 10 (closed triangles), 30 (open circles), 100 (closed circles), 300 (open squares), or 1000 (closed squares) μM. Inset: Rate constant for dissociation of the second $^{45}Ca^{2+}$ ion, estimated in these experiments as a function of the free calcium concentration in the perfusion solution. Panel B: Schematic model for sequential dissociation of $^{45}Ca^{2+}$ ions (closed circles) from a narrow binding cavity in the presence of unlabeled $^{40}Ca^{2+}$ ions (open circles).

report. To create this situation, SR vesicles equilibrated with radioactive $^{45}Ca^{2+}$ were adsorbed on a cellulose filter and perfused for various periods with an unlabeled medium to extract the bound ions. When the vesicles were perfused with a medium containing EGTA (open triangles), the two $^{45}Ca^{2+}$ ions dissociated rapidly. However, when the perfusion medium contained $^{40}Ca^{2+}$, in other words, when nonradioactive calcium was substituted for radioactive calcium in an exchange experiment, the kinetics of radioactive calcium dissociation became clearly biphasic: half of the bound calcium dissociated rapidly, and the other half, more slowly. The rate of this second phase depended on the concentration of calcium in the perfusion fluid, corresponding to the curves with different symbols in Figure 2A.

To account for these kinetics, an attractive explanation has been suggested [Inesi, 1987, Petithory & Jencks, 1988a; see also Figure 1 in Tanford et al. (1987), from which the diagram in panel B is adapted] that is supported by the results of the present investigation (see Discussion). According to this explanation, the two Ca^{2+} ions bound to the ATPase reside in a relatively narrow pocket or channel, which the superficial ion must leave before the other ion can do so. If unlabeled calcium replaces the first $^{45}Ca^{2+}$ ion just after it leaves the site, the second ion will remain trapped in the deeper site, depending on the degree of occupation of the superficial site.

We found that the dependence on free Ca^{2+} of the rate constant for dissociation of the second ion did reveal that the superficial site had a high affinity for Ca^{2+}. This is shown in the inset to Figure 2A, which indicates that the rate constant for dissociation of the second ion was slowed down by 50% at a concentration of calcium of about 3 μM.[2] At low calcium

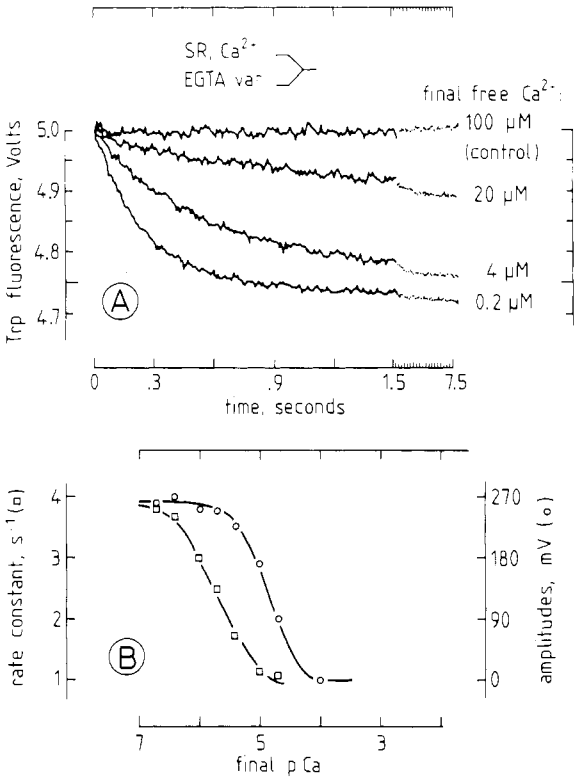

FIGURE 3: Kinetics of the fluorescence drop observed during reequilibration of SR ATPase at various final free calcium concentrations. Panel A: Typical recordings of the fluorescence changes. Panel B: Analysis, as a function of final pCa, of amplitudes (circles) and rate constants (squares) of the observed traces, analyzed as simple exponentials. One syringe of the stopped-flow fluorometer was filled with SR vesicles (0.4 mg of protein/mL) suspended in a medium containing 100 μM Ca^{2+}. The other syringe was filled with the same medium to which various amounts of EGTA up to 40 mM had been added, so that the final free calcium concentration after volume to volume mixing was as desired. The excitation wavelength was 290 nm; the emitted light was analyzed with an interference filter centered at 330 nm. The medium contained 150 mM Mes-Tris and 20 mM Mg^{2+} (pH 6, 20 °C).

concentration, however, the rate constant for dissociation of this second ion was not precisely differentiated from the rate constant for dissociation of the first ion, because the two constants only differed by a moderate factor. Another index of the very high affinity with which calcium may bind to its superficial site was provided by the dependence on the final free calcium concentration of the rate at which ATPase intrinsic fluorescence dropped from its high initial level to a lower level when an EGTA–Ca buffer was added to calcium-equilibrated SR vesicles. Typical traces are shown in Figure 3, panel A, and are analyzed in panel B: the calcium dependence of the observed rate constant for this fluorescence drop was shifted to lower concentrations compared to the calcium dependence of the amplitude of this drop. This agrees with previous stopped-flow observations under different conditions (Guillain et al., 1980, 1981; Dupont, 1982); it is consistent with the above $^{45}Ca^{2+}$ dissociation data in Figure 2A and with published multimixing results indicating a dissociation pathway of very high affinity for calcium (Petithory & Jencks, 1988a). These data are evidence for the presence on the

[2] Analysis of $1/k_{obs}$ as a function of $[Ca^{2+}]$, as suggested by Petithory and Jencks (1988a), results in a straight line for $[Ca^{2+}] > 10$ μM, with a slope of 6×10^{4} s·M^{-1} (not shown); this slope is equal to $k_2/k_{-1}k_{-2}$ [Scheme IV in Petithory and Jencks (1988a)]. Since $k_{-2} = 6$–10 s^{-1}, $k_{-1}/k_2 = 1.5$–3 μM. Since $k_{-1} = k_{-2}/2$ (see below), the true affinity of the superficial calcium site, k_{-2}/k_2, is 3–6 μM.

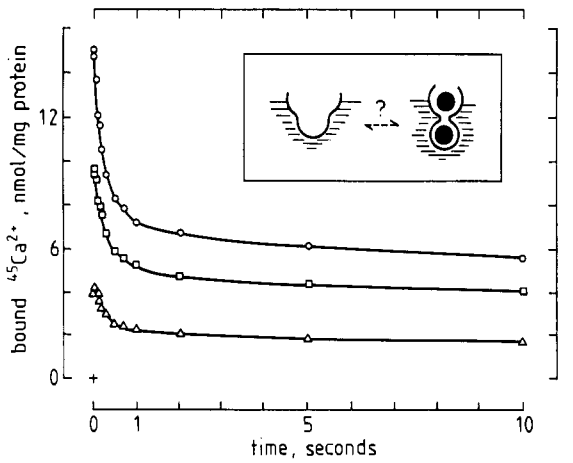

FIGURE 4: Biphasic kinetics of calcium exchange under conditions of submaximal initial binding of calcium. SR vesicles were initially equilibrated with 100 μM $^{45}Ca^{2+}$ (circles), with 100 μM $^{45}Ca^{2+}$ and 190 μM EGTA (25 μM free Ca^{2+}, squares), or with 100 μM $^{45}Ca^{2+}$ and 460 μM EGTA (10 μM free Ca^{2+}, triangles). Here, ionophore was also present (0.04 g of A23187/g of protein; see Figure 5A). The perfusion medium contained 1 mM $^{40}Ca^{2+}$. Other conditions were as for Figure 2. Inset: A model for positive cooperativity, involving the transition from a low-affinity form of calcium-deprived ATPase to a final high-affinity form of calcium-saturated ATPase.

ATPase of a calcium-binding site with an intrinsic affinity higher than the overall affinity revealed by equilibrium measurements, which in turn is consistent with the cooperative nature of the overall process of calcium binding to initially calcium-deprived ATPase (Figure 1 and Discussion).

Figure 4 shows that, regardless of the initial amount of $^{45}Ca^{2+}$ bound to the vesicles, half of it was rapidly exchanged and the other half remained trapped when the vesicles were perfused with 1 mM $^{40}Ca^{2+}$ [see also Nakamura (1986)]. This constant 1:1 ratio between the amplitudes of the two dissociation phases is consistent with the possibility that, under conditions of less than maximal calcium binding, nearly all the ATPase molecules bind either zero or two calcium ions as a result of the above-mentioned positive cooperativity. The inset in Figure 4 shows these two states of the ATPase, the calcium-deprived one and the calcium-saturated one, drawn with different conformations of either low (left) or high (right) affinity.

Detailed Comparison of the Kinetics of Isotopic Superficial Calcium Ion Exchange and of Calcium Ion Dissociation in a Medium Containing EGTA. Figure 5A shows an experiment identical with the one depicted in Figure 2 but on an expanded time scale, in which $^{45}Ca^{2+}$-equilibrated vesicles were perfused with a medium containing EGTA (triangles, equivalent to the triangles in Figure 2). No very fast calcium dissociation phase was observed, but a small fraction of the pool of bound calcium (1–2 nmol/mg of protein) did not dissociate within a few seconds, so that the dissociation curve departed from simple exponential behavior. Note that the fluid wetting the filter, outside the vesicles, was efficiently washed away after a few tens of milliseconds (crosses), so that the subtraction procedure allowing calculation of the bound calcium ions was very reliable under these conditions, making the low residual amount of calcium significant. We suspected that, during preequilibration of the SR vesicles with the radioactive $^{45}Ca^{2+}$ solution, radioactive calcium ions had slowly entered the vesicles and that radioactive ions bound inside the vesicles might thus account for the slowly extractible calcium ion pool. The experiment was therefore repeated in the presence of ionophore (circles), and no slowly extractible pool was observed. When the data were plotted on a semilogarithmic scale, 95% of the

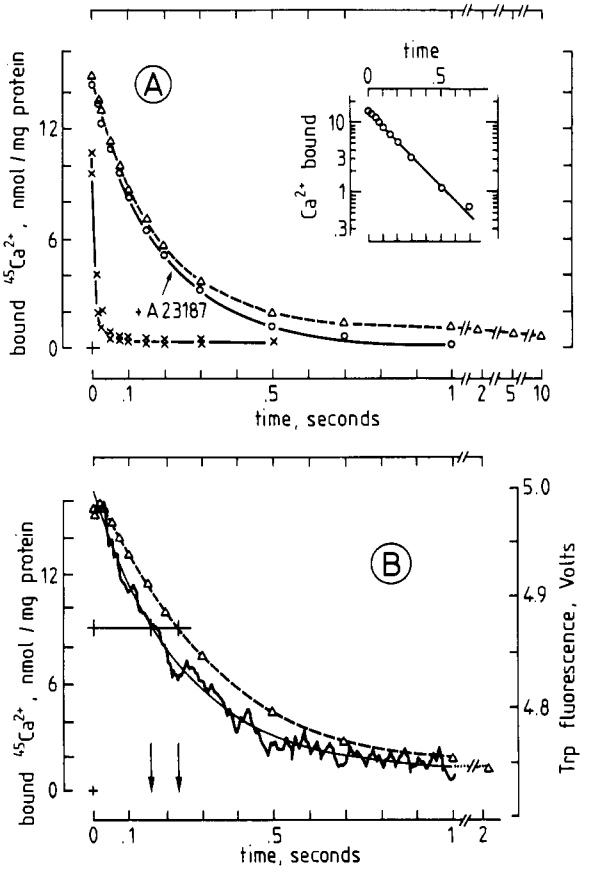

FIGURE 5: EGTA-induced monophasic dissociation of calcium in the presence of ionophore: Comparison with time-resolved fluorescence experiments. Panel A: Dissociation of calcium upon perfusion of SR vesicles with an EGTA-containing medium, as in Figure 2A, was measured with vesicles initially equilibrated in the absence (triangles) or presence (circles) of A23187 [4% (w/w) relative to protein weight]. Crosses: Residual amount of the ^{3}H-labeled marker in the wetting volume (sucrose in this particular experiment). Inset: Semilog plot of the data obtained in the presence of ionophore. Panel B: Comparison between $^{45}Ca^{2+}$ dissociation in the absence of ionophore (triangles, as in panel A) and stopped-flow measurements of the rate of the corresponding fluorescence drop, measured as in Figure 3 (final free Ca^{2+} was 0.2 μM). In both cases the medium contained 150 mM Mes-Tris and 20 mM Mg^{2+} (pH 6); the two experiments were run simultaneously, and the temperature was carefully adjusted to the same value, here 19 °C.

curve for calcium dissociation kinetics proved to be monophasic (see inset). A possible explanation for the nonmonophasic kinetics observed in the absence of ionophore is that calcium binding took place at transport sites located in the small fraction of vesicles in which the ATPases had acquired an inside-out orientation during homogenization of the SR network.

Note that comparison of the kinetics of $^{45}Ca^{2+}$ dissociation in a medium containing EGTA with the kinetics of the fluorescence drop observed when a large concentration of EGTA was added to calcium-equilibrated SR vesicles (Figure 5B) shows that the drop in fluorescence was slightly faster than the kinetics of $^{45}Ca^{2+}$ dissociation. This fluorescence drop and $^{45}Ca^{2+}$ dissociation are clearly related, and the fluorescence decline does not lag behind the calcium dissociation. Comparison of panels A and B in Figure 5, however, reveals that there was some variability in the calcium dissociation rates measured with different SR preparations, as will also be shown below.

The kinetics of $^{45}Ca^{2+}$ dissociation in the presence of EGTA were then compared with the initial portion of the kinetics of this dissociation in the presence of nonradioactive calcium, i.e.,

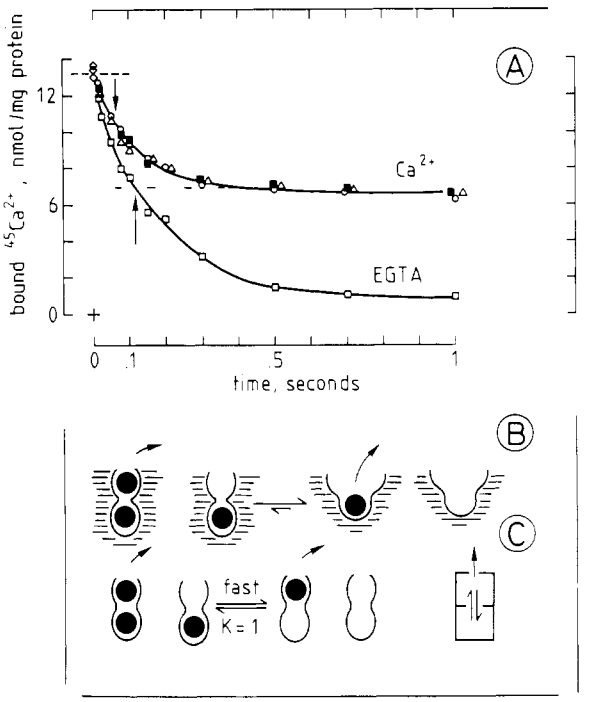

FIGURE 6: Comparison of the kinetics of EGTA-induced dissociation and isotopic exchange. Panel A: The experiment was similar to the one depicted in Figure 2 in the absence of ionophore (hence the residual bound calcium at 1 s); here, the perfusion medium contained either 2 mM EGTA (open squares) or 1 (circles), 10 (closed squares), or 30 (triangles) mM $^{40}Ca^{2+}$. Panels B and C: Two possible models for calcium dissociation from its sites. In Panel B, transition from a high-affinity form to a low-affinity form (drawn with a wider mouth) controls dissociation of the deeply bound calcium ion; in panel C, dissociation of calcium from the superficial site is the only step contributing to rate limitation, and movement of the remaining ion from one subsite to the other in the binding pocket is assumed to be equally fast in both directions. Panel C also shows an analogy of the latter situation in terms of two compartments in rapid equilibrium with each other, slowly emptying through a single leak pathway.

Table I: Effect of Various Modifiers of ATPase Function on the Kinetics of $^{45}Ca^{2+}$ Dissociation[a]

modifier	concn	$t_{1/2}$ in EGTA medium (ms)	$t_{1/2}$ in ^{40}Ca medium (ms)	K_{diss} (μM)
4-nonylphenol	control	150	70	15
	0.33 μmol/mg	310	230	
	0.17 μmol/mg			40
Me_2SO	control	150	75	18
	10% (v/v)	220	120	
	15% (v/v)			25
AMPPCP	control	180	160[b]	20
	250 μM	270	250[b]	12

[a] Half-times for $^{45}Ca^{2+}$ dissociation ($t_{1/2}$) were measured in the presence of ionophore by rapid filtration under conditions similar to those of Figure 6, in the presence of either 2 mM EGTA or 1 mM $^{40}Ca^{2+}$. The dissociation constant for calcium binding to the ATPase at equilibrium (K_{diss}) was deduced either from Trp fluorescence titrations (for nonylphenol) or from $^{45}Ca^{2+}$ measurements; Hill coefficients were similar under all conditions (1.7 ± 0.1). Nonylphenol concentration was expressed as micromoles per milligram of protein because most of it binds to the membrane; for the $^{45}Ca^{2+}$ dissociation measurements, nonylphenol was included in the initial $^{45}Ca^{2+}$-containing SR suspension but not in the perfusion solutions, because nonylphenol dissociated from SR membranes more slowly than the events in which we were interested here. [b] These two measurements were performed in a separate series of experiments, with another SR preparation; therefore, these half-times measured in the presence of calcium should not be compared to the half-times measured in its absence and shown in the preceding column.

with the exchange of $^{45}Ca^{2+}$ from the superficial site (Figure 6). In this experiment, varying the concentrations of $^{40}Ca^{2+}$ from 1 mM (open circles) up to 30 mM (open triangles) had no effect on the exchange kinetics. The half-time for exchange

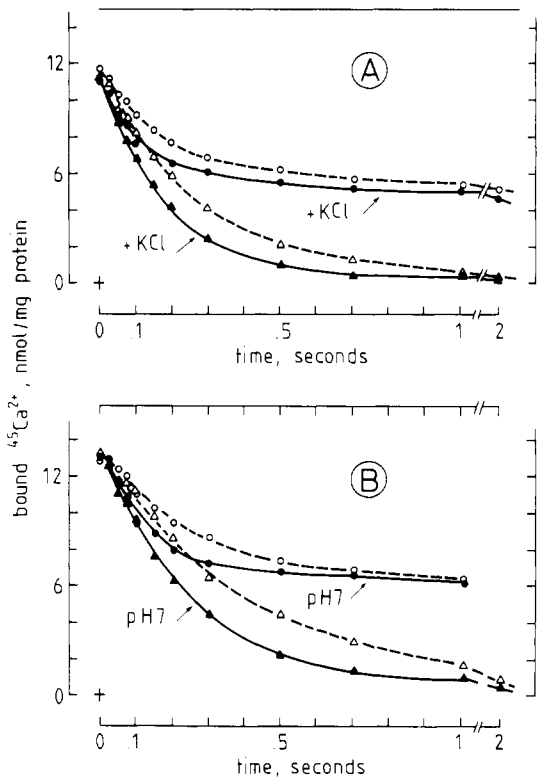

FIGURE 7: Effect of potassium (panel A) and pH (panel B) on the kinetics of $^{45}Ca^{2+}$ dissociation. Panel A: Vesicles were equilibrated with 100 μM $^{45}Ca^{2+}$ and ionophore, either in the standard medium (150 mM Mes-Tris and 20 mM Mg^{2+}, pH 6, open symbols) or in a potassium-containing medium (100 mM KCl, 50 mM Mes-Tris, and 20 mM Mg^{2+}, pH 6, closed symbols). The perfusion medium was identical with the equilibration medium but contained either 2 mM EGTA (triangles) or 1 mM $^{40}Ca^{2+}$ (circles) instead of radioactive calcium. Panel B: Vesicles were equilibrated with 100 μM $^{45}Ca^{2+}$ and ionophore, either in the standard medium (150 mM Mes-Tris and 20 mM Mg^{2+}, pH 6, open symbols) or in a pH 7 medium (150 mM Mops-Tris and 20 mM Mg^{2+}, pH 7, closed symbols). The perfusion medium was identical with the equilibration medium but contained either 2 mM EGTA (triangles) or 1 mM $^{40}Ca^{2+}$ (circles) instead of radioactive calcium.

of the one superficial Ca^{2+} ion in the $^{40}Ca^{2+}$ medium turned out to be roughly twice as short as the half-time for dissociation of the two bound Ca^{2+} ions in the EGTA medium (compare arrows in Figure 6A). Panels B and C in Figure 6 show two possible models accounting for these data, which will be discussed later.

Effects of ATPase Function Modifiers. In an attempt to discriminate between the two models shown in panels B and C of Figure 6, we investigated the kinetics of isotopic exchange and EGTA-induced dissociation in the presence of various agents known to modify the kinetics of some of the transitions involved in the ATPase catalytic cycle. As a first example of the modification of the $^{45}Ca^{2+}$ dissociation kinetics under specific conditions, 100 mM potassium was found to accelerate the rate of this dissociation in the presence of both EGTA and unlabeled calcium (Figure 7, panel A). The kinetics of $^{45}Ca^{2+}$ dissociation in the presence of EGTA, however, remained monophasic. Note that potassium does not greatly change the affinity of ATPase for calcium [compare data in Guillain et al. (1980) and in Guillain et al. (1981)]. Changing the pH from 6 to 7 also accelerated $^{45}Ca^{2+}$ dissociation in either situation (Figure 7, panel B), again leaving the dissociation kinetics in the EGTA medium monophasic, while the affinity of ATPase for calcium is known to be higher at neutral or alkaline pH than at pH 6 [see Watanabe et al. (1981) and Guillain et al. (1982)].

Table I shows that 4-nonylphenol, a very hydrophobic agent known to slow down the rate of the fluorescence changes associated with calcium binding to ATPase and to reduce ATPase affinity for calcium (Michelangeli et al., 1990), distinctly slowed down $^{45}Ca^{2+}$ dissociation in the EGTA medium, although its kinetics remained monophasic. When $^{45}Ca^{2+}$ dissociation in the $^{40}Ca^{2+}$ medium was monitored, nonylphenol again proved to slow down significantly the kinetics of $^{45}Ca^{2+}$ exchange from the superficial site, while half of the bound $^{45}Ca^{2+}$ remained trapped. Table I also shows that 10% Me_2SO [another modifier of ATPase function; see references in de Meis et al. (1980)] slowed down both calcium dissociation in an EGTA medium and calcium exchange from the superficial site; at neutral pH in the presence of potassium, calcium dissociation in an EGTA medium was also slowed down in the presence of Me_2SO concentrations up to 30% (v/v) but remained monophasic (data not shown). Note that Me_2SO only weakly affects the ATPase affinity for calcium (Table I). As a final example, Table I shows that the nucleotide analogue AMPPCP moderately slowed down the rate of $^{45}Ca^{2+}$ dissociation from the ATPase, in the presence of both EGTA and unlabeled calcium, while the ATPase affinity for calcium was enhanced in the presence of AMPPCP [a similar enhancement was previously found by Cable et al. (1985) and Ross and McIntosh (1987)].

It therefore appears that all these modifiers of ATPase function leave $^{45}Ca^{2+}$ dissociation in an EGTA medium monophasic, with a half-time always larger than the half-time for exchange of the superficial $^{45}Ca^{2+}$ ion. Moreover, there is no apparent correlation between the rate of calcium dissociation from the ATPase and the affinity of ATPase for calcium.

Effect of Temperature. Finally, we measured the effect of temperature on the kinetics of calcium dissociation in the presence of either EGTA or unlabeled calcium. The main frame in Figure 8 shows that although rate constants were 5 times slower at 5 °C than at 20 °C (compare time scales in Figures 6 and 8), the same calcium dissociation pattern was observed at both temperatures: it was fairly monophasic in the presence of EGTA, and in the presence of unlabeled calcium superficial calcium exchange was about 2 times faster. Additional data were collected at various temperatures between 5 and 20 °C, and the measured dissociation rates were plotted on an Arrhenius plot in the inset to Figure 8. The rates for complete Ca^{2+} dissociation (open symbols) and for the exchange of only one calcium ion (closed symbols) proved to have the same temperature dependence (circles and squares in this inset refer to two separate experiments). In stopped-flow fluorescence experiments (triangles in the inset) in which EGTA was added to calcium-equilibrated vesicles, as in the experiment illustrated in Figure 5B, the fluorescence drop at all temperatures tested was again slightly faster than the dissociation of calcium in the same experiment (compare triangles and open squares), and an identical temperature dependence of the observed rates was obtained. The temperature sensitivity of these rates was rather high, as between 5 and 25 °C they increased every 10 °C by a factor of 2.5–3, corresponding to an activation energy of about 16 kcal/mol. On the other hand, temperature is known to leave the ATPase affinity for calcium unaffected (Dupont, 1980).

Variability. With regard to the reproducibility of the rate constants we measured during the present series of experiments, the circles and squares in the inset to Figure 8, which correspond to two separate series, illustrate the previously mentioned observation [Figure 5, (A) versus (B); compare also

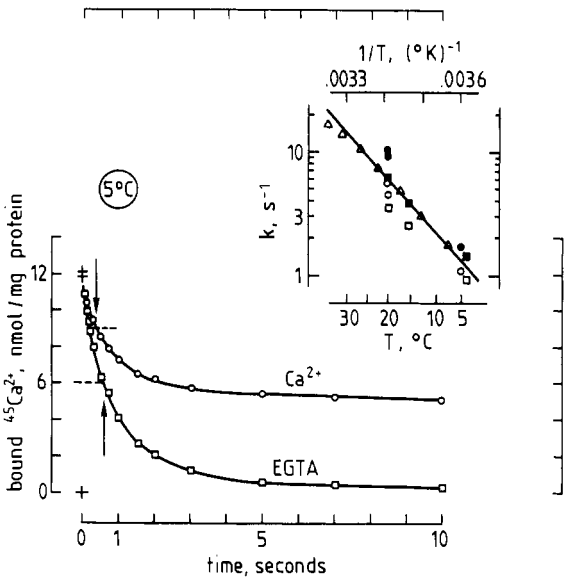

FIGURE 8: Effect of temperature on the kinetics of $^{45}Ca^{2+}$ dissociation from SR ATPase. Main frame: Same experiment as the one shown in Figure 6 but performed in the cold room (5 °C) and in the presence of ionophore. Inset: Arrhenius plot of the rate constants measured at different temperatures. Open and closed symbols stand for experiments performed in the presence of EGTA and calcium, respectively. Circles refer to the experiments shown in the main frame and in Figure 6, which both belong to the same series; squares refer to another series of experiments. Open triangles refer to fluorescence stopped-flow experiments performed in parallel with the experiments illustrated by the squares, as shown in Figure 5B, except that the final pCa was 6.7 and a cutoff filter was used (Schott WG 320; Champeil et al., 1983).

open symbols in Figure 7, (A) and (B)] that individual rate constants can vary by a factor of almost 2 from one series of experiments to another, conducted at an interval of a few weeks or months with another SR preparation. This variability was not restricted to the $^{45}Ca^{2+}$ dissociation experiments, for which temperature control is more difficult than in fluorescence experiments and determination of half-times less reliable, but was also observed in the stopped-flow fluorescence experiments, which give no grounds for concern on either of these points. We observed it in experiments performed on the same day with the same solutions but with different SR preparations. Consequently, experimental results were always compared to the results of simultaneously run controls. ATPase activity variability has been mentioned before (Rooney & Lee, 1983). On the basis of our limited experience, it does not necessarily seem to be due to the aging of the preparation; a speculative candidate responsible for it might be an uncontrolled state of lipids, e.g., phosphorylation of the phosphatidylinositol phospholipids located close to the ATPase molecule (Varsanyi et al., 1983).

Discussion

Cooperative Equilibrium Binding of Two Calcium Ions per ATPase Molecule. Our measurements of calcium binding at equilibrium (Figure 1) call for a few initial comments. First, their dependence on the free calcium concentration proved cooperative (Figure 1B), as observed earlier under conventional experimental conditions [see, for instance, Inesi et al. (1980)] and later also at pH 6 [Guillain et al., 1982; Hill & Inesi, 1982; Fernandez-Belda et al., 1984; note that the initial finding by Watanabe et al. (1981) that cooperativity was lower at pH 6 than at pH 7 was not confirmed in subsequent reports]. Under slightly acidic conditions, the free calcium concentration can be precisely buffered to any desired final value because at acidic pH the affinity of EGTA for calcium is relatively

low, and the estimation of cooperativity under these conditions is therefore reliable.

Second, the total number of calcium sites found here (Figure 1A) was slightly larger than generally reported for native SR, although it was consistent with the amount of ATPase deduced from electrophoresis gels and molecular weight determinations and was in a ratio of 2:1 to the maximum amount of P_i-derived phosphoenzyme that could be measured [see Champeil et al. (1985) and Champeil and Guillain (1986)]. Previous reports have raised the possibility that the smaller numbers of calcium sites found with some preparations were due to the presence of a subpopulation of irreversibly inactivated ATPases (Barrabin et al., 1984; Gafni & Boyer, 1984). An additional reason for these differences between preparations might be the undetected presence in some of them of a protein component unrelated to the ATPase but with a similar molecular weight, whose existence has recently been recognized (Zaidi et al., 1990). Note also that, depending on the duration of the preequilibration step and on the presence or absence of ionophore, $^{45}Ca^{2+}$ may or may not succeed in reaching the transport sites of the ATPase molecules located on the small fraction of vesicles with an inside-out orientation.

Kinetics of Calcium Dissociation Reveal the Structure of the Transport Sites on the High-Affinity Form of ATPase. The observation of biphasic kinetics for calcium dissociation from the transport sites in the presence of external unlabeled calcium, with a 1:1 ratio of rapidly exchangeable to slowly exchangeable calcium ions (Figures 2, 4, and 6–8), fully agrees with the results of similar previous experiments, performed under different temperature and ionic conditions (Dupont, 1982, 1984; Nakamura, 1986; 1987; Inesi, 1987; Petithory & Jencks, 1988a). Only recently, however, the microscopic mechanism for this apparent interaction between sites was ascribed to obligatory ordered dissociation of the bound ions from a narrow binding pocket (Figure 2B), while similar concepts were developed to account for the kinetics of the dissociation of occluded potassium or rubidium ions from the closely related phosphorylated Na^+,K^+-ATPase (Inesi, 1987; Petithory & Jencks, 1988a; Glynn et al., 1985; Glynn & Richard, 1989; Forbush, 1985, 1987).

In the framework of this ordered dissociation model, which as discussed below our data support, the kinetics of calcium exchange, which are biphasic regardless of the initial amount of bound ions (Figure 4), are consistent with the cooperativity of the overall process of calcium binding to the calcium-deprived ATPase as well as with the fundamental feature of the ATPase transport sites, i.e., the existence of a single binding pocket for the two ions that, when both present, cannot exchange positions. An unambiguous index of cooperativity was manifested by the fact that the binding sites present on the calcium-saturated ATPase (K_{diss} of 3–6 μM; cf. Figure 2A inset, Figure 3B, and footnote 2) displayed an affinity for calcium higher than the overall ATPase affinity deduced from the equilibrium measurements in Figure 1. This was also observed by Petithory and Jencks (1988a). Conversely, we think that these high-affinity sites are absent from calcium-deprived ATPase, which only displays sites with a low affinity for calcium, as observed in direct time-resolved measurements of calcium *binding* to the ATPase [Orlowski et al., in preparation; see also Dupont (1982) and indirect suggestions in Inesi et al. (1980), Guillain et al. (1980), Champeil et al. (1983), and Petithory and Jencks (1988b)].

Kinetics of Calcium Dissociation in a Ca^{2+}-Free Medium Are Monophasic. In the presence of EGTA, the kinetics of calcium dissociation from vesicles made leaky with ionophore were found to be monoexponential under our ionic conditions, as shown, for instance, in Figure 5A. This monoexponential behavior was also observed in a large range of experimental situations that we tested (Figures 7 and 8 and Table I). This is at variance with previous reports that at neutral pH the kinetics of calcium dissociation in the presence of EGTA are biphasic [Dupont, 1984; see also chemical quenching measurements by Ikemoto et al. (1981)] and also with a recent similar observation concerning the kinetics of calcium dissociation from longitudinal "light" SR (Meszaros & Ikemoto, 1989). Under the ionic conditions of the latter report, we were unable to observe biphasic kinetics with our SR fraction in the presence of ionophore (data not shown), but we did observe that a small fraction of the bound calcium was not released within 2 s in the absence of ionophore; this fraction was, however, much smaller than 50% of the amount of calcium initially bound and was insensitive to the presence of ruthenium red, a calcium channel blocker used by Meszaros and Ikemoto to discriminate between light and heavy SR (1989). A possible reason for the appearance of a slow phase of calcium dissociation in these authors' experiments might be the limited permeability to calcium of SR vesicles: this permeability is large enough to allow calcium to enter the vesicles and to bind to internal sites—especially in the absence of magnesium—over a 2-min incubation period but too small to allow easy dissociation of these internal calcium ions within a 1–2-s time period, except in the case of heavy SR vesicles, which are spontaneously permeable because of the presence of calcium channels. The same argument might account for the (small) deviation from monophasicity observed in multimixing experiments in which the amount of calcium bound to the ATPase was measured by indirect assay (Petithory & Jencks, 1988a). In addition, different SR preparations might differ in their proportions of inside-out vesicles, in which ATPases with the unusual orientation contribute to internal sites.

A Common Mechanism Controls the Rate of Calcium Dissociation during either Exchange of One Ion at the Superficial Site or Dissociation of the Two Bound Ions in a Calcium-Free Medium. The monophasic Ca^{2+} release during EGTA perfusion and the twice-as-fast kinetics of calcium exchange at the superficial site compared to dissociation in EGTA medium (Figure 6A) place constraints on the possible models accounting for this release. For instance (panel B in Figure 6), after the departure of the superficial Ca^{2+}, one might imagine that the ATPase is immediately converted into another conformation such as the one with a low affinity for calcium mentioned above, from which the second Ca^{2+} ion would be very rapidly released. In that case, however, the second Ca^{2+} ion would dissociate as soon as the first had done so, and the half-times for dissociation in an EGTA medium and for superficial ion exchange would therefore be identical, which was not the case (Figures 6–8 and Table I). Alternatively, one might then imagine that this conformational conversion to a state from which the second calcium ion is immediately released takes place at a particular, noninstantaneous rate. If that were true, however, it would not be simple to account for the fairly monophasic dissociation kinetics found under a wide range of different conditions, including at different temperatures and the presence of various ATPase modifiers (Figures 6–8 and Table I), because under all these conditions the rate of the conformational transition would have to be exactly balanced with the rate at which the first ion leaves the superficial site, although there is no reason why these rates should depend on the same type of regulation. In fact, for sequential dissociation to result in monoexponential loss of

calcium from the ATPase, the dissociation process must, at least apparently, conform to the scheme

$$Ca_2 \cdot E \xrightarrow{k} Ca \cdot E + Ca \xrightarrow{k/2} E + 2Ca \qquad (1)$$

Another way of fulfilling this condition might be for the ATPase to be immediately converted, after dissociation of the first calcium ion, to a state from which the second calcium ion would have to leave its site at a rate exactly half that of the calcium dissociation from the superficial site. Although not impossible, this is rather unlikely since the 1:2 ratio between the dissociation rates from different sites would again have to be maintained over a wide range of experimental conditions. The same requirement for a constant ratio of dissociation rates from different sites over a wide range of conditions also makes it unlikely that the two calcium ions would be located in two different binding pockets and that, due to some allosteric constraint, the occupancy of one would prevent dissociation from the other.

Consequently, we are left with the possibility that a single rate-limiting leak pathway out of a common binding pocket controls the rate of calcium dissociation during both the isotopic exchange of one ion and the dissociation of the two ions in a calcium-free medium. In that case the 1:2 ratio of the half-times would imply that, after depature of the superficial Ca^{2+}, the residual ion rapidly jumps from its initial location to the one from which it will finally be released, with an equal probability of being at one locus or the other inside the pocket (Figure 6C). Although this result can easily be obtained by writing down the simple kinetic equations describing the system, it can also be understood by considering the exchange of ^{45}Ca for ^{40}Ca at the superficial site as the emptying of a superficial compartment through a small leak pathway, whereas ^{45}Ca dissociation in an EGTA medium would result from the emptying, through the same pathway, of a compartment twice as large, at an identical initial rate but with a resulting apparent rate constant twice as slow (see the diagram on the right in Figure 6, panel C). This in turn would imply that there is no severe kinetic constraint restricting movement of the single Ca^{2+} ion inside the pocket, which is why the neck of the peanut on the diagram in Figure 6C is not very narrow. Strong confirmation of this model is provided by our observation that under all the conditions tested here (Figures 6–8 and Table I) the rates of $^{45}Ca^{2+}$ dissociation in an EGTA medium and of $^{45}Ca^{2+}$ exchange in a $^{40}Ca^{2+}$ medium were affected simultaneously and in the same direction. This argues in favor of the existence of a common mechanism controlling both situations.

Significance of the ATPase Intrinsic Fluorescence Changes. As a consequence of the model just described and shown in Figure 6C, the two calcium ions that dissociate from the ATPase during perfusion with an EGTA medium dissociate from the same high-affinity form of the ATPase. Petithory and Jencks (1988a) reached the same conclusion (see their Scheme IV). Transition to the low-affinity form, which we know to be predominant in the absence of calcium at steady state, only takes place after both the calcium ions have left their sites. Regarding the significance of the accompanying fluorescence changes, the present data show that, at pH 6, the drop in ATPase fluorescence upon addition of EGTA was in fact faster by a factor of 1.5–2 than the observed dissociation of calcium (Figure 5B and Figure 8 inset, triangles versus squares). The true time course of $^{45}Ca^{2+}$ dissociation might in fact be slightly faster, by 20–30% at most, than the observed one, because the small volume initially wetting the filter must be rinsed before any dissociation of $^{45}Ca^{2+}$ from the ATPase into the perfusion solution can occur; however, the conclusion that the fluorescence drop is not slower than $^{45}Ca^{2+}$ dissociation is safe. This implies that the ATPase fluorescence level does *not* disclose the ATPase conformational state but rather the occupancy of the calcium sites. We previously obtained some evidence of this by measuring the biphasic fluorescence changes induced by calcium binding (Champeil et al., 1983). The fluorescence level of the ATPase with a single bound calcium ion (Ca·E) is therefore significantly different from the level of the ATPase with two bound ions (Ca_2·E); the fluorescence change could even be slightly larger for the dissociation of the first than the second calcium ion. This conclusion is not necessarily valid under all pH conditions; work is in progress to elucidate this issue. Note also that, assuming that dissociation of $^{45}Ca^{2+}$ in a calcium-free medium obeys eq 1, the time course of the drop in ATPase fluorescence will not be strictly exponential but rather a combination of exponentials with rate constants k and $k/2$, which probably cannot be distinguished from a single exponential, considering noise level and photolysis.

A related argument supports the claim that fluorescence reflects calcium dissociation rather than slow conformational changes: if calcium dissociation were to precede a putative slower conformational change leading to fluorescence reduction, the drop in fluorescence would show a lag corresponding to the rate of departure from the superficial site. However, it is clear from Figures 3 and 5 and from the bulk of published fluorescence measurements that there is no such lag; the drop in fluorescence therefore starts simultaneously with the departure of the first calcium ion.

Opening of a Gate as the Mechanism Controlling Calcium Dissociation. One striking result of our experiments is that there was no correlation between the ATPase apparent affinity for calcium and the rate at which previously bound calcium dissociated from its sites on the ATPase. Thus, nonylphenol and Me_2SO, which shifted the ATPase to different extents to a form of poorer affinity for calcium, both slowed down the rate of Ca^{2+} dissociation and exchange (Table I). The presence of the ATP analogue AMPPCP, which enhanced the ATPase affinity for calcium, also slowed down the dissociation rates (Table I). Neutral pH accelerated the dissociation kinetics, although it enhanced the affinity of ATPase for calcium (Figure 7B), but potassium also accelerated the dissociation kinetics (Figure 7A), although it did not greatly affect affinity. Many possibilities might account for these observations, especially because the rates of calcium binding to the sites were not systematically measured here and because the conformational changes involved in the cooperative binding mechanism obviously play a role in determining the final apparent affinity. One possibility is that there is some kind of restriction on ion escape from the site, due to protein structure or dynamics, imposing a relatively high activation energy (Figure 8, inset). This restriction cannot be due to the migration of the ion through a long narrow access channel with weak binding properties (Tanford, 1983), since very high calcium concentrations up to 30 mM did not slow down isotopic exchange (Figure 6A).

At this point, it should be emphasized that the process of calcium dissociation from unphosphorylated Ca^{2+}–ATPase toward the cytoplasmic medium, as investigated here and in the indirect study by Petithory and Jencks (1988a), seems very similar to the process of potassium or rubidium dissociation from phosphorylated Na^+,K^+-ATPase described by Glynn and co-workers and Forbush: these authors and ourselves both observed monophasic or biphasic dissociation of labeled ions

depending on the absence or presence of unlabeled congeners, with a 1:2 ratio between half times (Forbush, 1987; Glynn & Richards, 1989, and references therein). Unphosphorylated Ca^{2+}-ATPase appears to behave in a manner similar to phosphorylated Na^+,K^+-ATPase, although, surprisingly, the opposite side of the protein is involved. To describe further the nature of this limiting step, which allows ion leakage out of the binding pocket and controls both the dissociation of the two ions and the exchange of only one of them, Forbush suggested that this dissociation did not occur through some slow permanent leakage pathway but resulted from brief and infrequent opening of the binding pocket to the external medium: this is the "flickering gate" model. This model might be one way of accounting for the absence of correlation between dissociation rates and apparent affinities. The relatively large temperature dependence of the dissociation rate might also be consistent with the idea that leakage results from a temperature-sensitive dynamic equilibrium between closed and open conformations [this sensitivity to temperature is much higher than what one would expect, for instance, for passive ion diffusion through a channel with a fixed structure, when the enhancement factor, Q_{10}, is expected to be close to 1.3 or 1.4, and the corresponding activation energy would be 4–5 kcal/mol (Hille, 1984)]. Whether the binding pocket really flickers open spontaneously from time to time (Forbush, 1987, 1988) or whether ion escape requires some activating interaction between the ion and the protein, the basic idea that dissociation of calcium to the outside-facing side might be controlled by a structural component acting as a molecular gate might well apply to the Ca^{2+}-ATPase, and suggests that the two calcium ions on the ATPase are "occluded" most of the time on unphosphorylated ATPase and not only on phosphorylated ATPase, as generally believed [e.g., see Kurzmack et al. (1977) and Dupont (1980)]. It should be remembered that, in unphosphorylated Na^+,K^+-ATPase, occlusion of K^+ is an undisputed fact. The transient accessibility of the transport sites to the external compartment might be just another example of large-scale internal movements of proteins and be in agreement with the view, now established thanks to patch-clamp techniques, that ion channels themselves are structures that oscillate between open and closed conformations on the millisecond time scale.

In the framework of the present search for the location of the amino acids responsible for calcium binding to Ca^{2+}-ATPase, the suggestion that the binding pocket is temporarily occluded is also especially attractive because calcium binding sites, originally believed to be located in the stalk region of the ATPase (Brandl et al., 1986), have more recently been suspected to reside in a C-terminal region up to now considered to be embedded in the membrane (Clarke et al., 1989; Green, 1989; le Maire et al., 1990b). This issue, however, is far from being completely resolved, due, in particular, to the uncertainties that persist about the folding of the ATPase [see a discussion in le Maire et al. (1990a)].

Conclusion: Implications for the Complete Catalytic Cycle. First, we deduced from the kinetics of calcium dissociation under various conditions that a common activation step controlled the rate of calcium dissociation during exchange in a $^{40}Ca^{2+}$ medium and during dissociation in an EGTA medium and that, after the departure of the superficial calcium ion, the remaining single ion could migrate freely from one locus to the other in the binding pocket of unphosphorylated ATPase. The equal probability that the remaining ion binds to either one or the other subsite in the binding pocket (we concluded that the equilibrium constant was close to one) is puzzling, and the structural features responsible for this are not yet clear. On the other hand, with regard to the rapid migration of this single ion inside the binding pocket of unphosphorylated ATPase, it is relevant to mention experiments in our laboratory which showed that the kinetics of calcium dissociation from phosphorylated ATPase and the resulting internalization of calcium in the SR lumen were also fairly monophasic (Orlowski et al., manuscript in preparation); our results did not indicate that migration inside the binding pocket of the phosphorylated ATPase was rate-limiting. Neither did Jencks' group observe any two-step pattern for calcium internalization into empty vesicles [Khananshvili and Jencks (1988) and personal communication]. However, as different results were reported for the migration of the remaining ion (Inesi, 1987), future work will have to clarify this key feature of the calcium translocation mechanism.

Second, we suggest that the dynamic view of the binding sites as a structure only transiently accessible to the external medium is a helpful image for the description of active transport. In this connection, a tentative complement to the above dynamic gate model is to consider the ion-binding sites as a pocket shielded from the medium not only by one but by two gates, one of them oriented toward the cytoplasmic medium and the other toward the lumenal medium. In the unphosphorylated state, the cytoplasmic gate might open fast but infrequently, thus allowing Ca^{2+} to come and go, but the lumenal gate would remain closed. What phosphorylation does is to change the various vibration modes of the ATPase chain and block the opening of the cytoplasmic gate. The binding of the substrate analogue AMPPCP might do part of this job by slowing down[3] the rate of opening of this cytoplasmic gate (Table I). With phosphorylated ATPase, this new dynamic mode would also loosen the constraints on the movement of the lumenal gate, so that it could now open. Provided this opening is accompanied by a slight conformational change of the ion-binding pocket and a corresponding reduction in the affinity for calcium, calcium would then be able to dissociate toward the sarcoplasmic reticulum lumen. According to this view, the ATPase does not use a simple channel to transport calcium, or a channel with a conventional single gate: ion-transporting ATPase is a device with a shielded or occluded pocket and two gates whose mutually exclusive opening toward one or the other of the water compartments is controlled by phosphorylation.

ACKNOWLEDGMENTS

We are indebted to M. Dreyfus for her help in editing the manuscript. It is also a pleasure to acknowledge the constant day-to-day collaboration with F. Guillain and our colleagues in Saclay and the lively discussions we had at different stages of this work with a large number of scientists, including A. G. Lee, N. M. Green, M. le Maire, J. V. Møller, and, at the 34th Biophysical Society Meeting in Baltimore, K. Collins, G. Inesi, M. Berman, W. Jencks, and B. Forbush.

Registry No. ATPase, 9000-83-3; EGTA, 67-42-5; AMPPCP, 3469-78-1; A23187, 52665-69-7; Me_2SO, 67-68-5; Ca, 7440-70-2; K, 7440-09-7; 4-nonylphenol, 104-40-5.

REFERENCES

Barrabin, H., Scofano, H., & Inesi, G. (1984) *Biochemistry 23*, 1542–1548.

Brandl, C. J., Green, N. M., Korczak, B., & Mac Lennan, D. H. (1986) *Cell 44*, 597–607.

[3] Note, however, that ATP was suggested to accelerate rather than slow down calcium dissociation, by a factor of 1.6 (Petithory & Jencks, 1986). We have no explanation for these different findings.

Cable, M. B., Feher, J. J., & Briggs, F. N. (1985) *Biochemistry 24*, 5612–5619.
Champeil, P., & Guillain, F. (1986) *Biochemistry 25*, 7623–7633.
Champeil, P., Gingold, M. P., Guillain, F., & Inesi, G. (1983) *J. Biol. Chem. 258*, 4453–4458.
Champeil, P., Guillain, F., Vénien, C., & Gingold, M. P. (1985) *Biochemistry 24*, 69–81.
Champeil, P., le Maire, M., Andersen, J. P., Guillain, F., Gingold, M., Lund, S., & Møller, J. V. (1986) *J. Biol. Chem. 261*, 16372–16384.
Clarke, D. M., Loo, T. W., Inesi, G., & Mac Lennan, D. H. (1989) *Nature 339*, 476–478.
de Meis, L., & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.
de Meis, L., Martins, O. B., & Alves, E. W. (1980) *Biochemistry 19*, 4252–4261.
Dupont, Y. (1980) *Eur. J. Biochem. 109*, 231–238.
Dupont, Y. (1982) *Biochim. Biophys. Acta 688*, 75–87.
Dupont, Y. (1984) *Anal. Biochem. 142*, 505–510.
Fernandez-Belda, F., Kurzmack, M., & Inesi, G. (1984) *J. Biol. Chem. 259*, 9687–9698.
Forbush, B. (1985) in *The Sodium Pump* (Glynn, I., & Ellory, C., Eds.), pp 599–611, Company of Biologists, Cambridge, U.K.
Forbush, B. (1987) *J. Biol. Chem. 262*, 11116–11127.
Forbush, B. (1988) in *The Na^+,K^+-Pump. Part A: Molecular Aspects* (Skou, J. C., Nørby, J. G., Maunsbach, A. B., & Esmann, M., Eds.) Progress in Clinical and Biological Research 268A, pp 229–248, Alan R. Liss, Inc., New York.
Gafni, A., & Boyer, P. D. (1984) *Biochemistry 23*, 4362–4367.
Glynn, I. M., & Richards, D. E. (1989) *J. Physiol. 408*, 57–66.
Glynn, I. M., Howland, J. L., & Richards, D. E. (1985) *J. Physiol. 368*, 453–469.
Green, N. M. (1989) *Nature 339*, 424–425.
Guillain, F., Gingold, M. P., Büschlen, S., & Champeil, P. (1980) *J. Biol. Chem. 255*, 2072–2076.
Guillain, F., Champeil, P., Lacapère, J. J., & Gingold, M. P. (1981) *J. Biol. Chem. 256*, 6140–6147.
Guillain, F., Gingold, M., & Champeil, P. (1982) *J. Biol. Chem. 257*, 7366–7371.
Hasselbach, W. (1974) in *The Enzymes, 3rd Ed.* (Boyer, P. D., Ed.) pp 432–467, Academic Press, New York.
Hill, T. L., & Inesi, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3978–3982.
Hille, B. (1984) *Ionic Channels of Excitable Membranes*, Sinauer Associates Inc. Publishers, Sunderland, MA.
Ikemoto, N., Garcia, A. M., Kurobe, Y., & Scott, T. L. (1981) *J. Biol. Chem. 256*, 8593–8601.
Inesi, G. (1985) *Annu. Rev. Physiol. 47*, 573–601.
Inesi, G. (1987) *J. Biol. Chem. 262*, 16338–16342.
Inesi, G., Kurzmack, M., Coan, C., & Lewis, D. E. (1980) *J. Biol. Chem. 255*, 3025–3031.
Khananshvili, D., & Jencks, W. P. (1988) *Biochemistry 27*, 2943–2952.
Kurzmack, M., Verjovski-Almeida, S., & Inesi, G. (1977) *Biochem. Biophys. Res. Commun. 78*, 772–776.
le Maire, M., Champeil, P., & Møller, J. V. (1990a) *Nature 346*, 617.
le Maire, M., Lund, S., Viel, A., Champeil, P., & Møller, J. V. (1990b) *J. Biol. Chem. 265*, 1111–1123.
Martonosi, A. N., & Beeler, T. J. (1985) in *Handbook of Physiology, 10, Skeletal Muscle* (Peachy, L. D., Adrian, R. H., & Geiger, S. R., Eds.) pp 417–485, American Physiological Society, Bethesda, MD.
Meszaros, L. G., & Ikemoto, N. (1989) *Eur. J. Biochem. 186*, 677–681.
Michelangeli, F., Orlowski, S., Champeil, P., East, J. M., & Lee, A. G. (1990) *Biochemistry 29*, 3091–3101.
Nakamura, J. (1986) *Biochim. Biophys. Acta 870*, 495–501.
Nakamura, J. (1987) *J. Biol. Chem. 262*, 14492–14497.
Orlowski, S., & Champeil, P. (1990) *Biophys. J. 57*, 205a.
Petithory, J. R., & Jencks, W. P. (1986) *Biochemistry 25*, 4493–4497.
Petithory, J. R., & Jencks, W. P. (1988a) *Biochemistry 27*, 5553–5564.
Petithory, J. R., & Jencks, W. P. (1988b) *Biochemistry 27*, 8626–8535.
Rooney, E. K., & Lee, A. G. (1983) *Biochim. Biophys. Acta 732*, 428–440.
Ross, D. C., & McIntosh, D. B. (1987) *J. Biol. Chem. 262*, 12977–12983.
Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol. Rev. 58*, 1–79.
Tanford, C. (1983) *Annu. Rev. Biochem. 52*, 379–409.
Tanford, C., Reynolds, J. A., & Johnson, E. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7094–7098.
Varsanyi, M., Tölle, H. G., Heilmeyer, L. M. G., Dawson, R. M. C., & Irvine, R. F. (1983) *EMBO J. 2*, 1543–1548.
Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C., & Inesi, G. (1981) Biochemistry *20*, 6617–6625.
Zaidi, N. F., Lagenaur, R. J., Hilkert, R. J., Abramson, J. J., & Salama, G. (1990) *Biophys. J. 57*, 276a.