The Two Calcium Ions Initially Bound to Nonphosphorylated Sarcoplasmic Reticulum Ca^{2+}-ATPase Can No Longer Be Kinetically Distinguished When They Dissociate from Phosphorylated ATPase toward the Lumen

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ABSTRACT: Using rapid filtration, we investigated the kinetics of release toward the lumen of sarcoplasmic reticulum vesicles of the two Ca^{2+} ions transported by the Ca^{2+}-dependent ATPase of these vesicles. Release rates at 20 °C were measured by three methods, with vesicles previously made leaky with an ionophore. First, we measured the rate at which $^{45}Ca^{2+}$ bound to ATPase approached its steady-state level after addition of ATP to the $^{45}Ca^{2+}$-equilibrated ATPase. At pH 6 in the absence of potassium, the observed kinetics did not reveal any very fast phase of $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase. Second, we measured the kinetics of $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase in a "chase" experiment, by isotopic dilution of calcium under turnover conditions in the presence of potassium. We found that these kinetics were essentially monophasic. Moreover, when they were measured in the presence of a high concentration of calcium, designed to saturate the low-affinity calcium transport sites on the lumenal side of the ATPase, they only departed slightly from monophasic behavior, irrespective of the experimental pH (pH 6, 7, or 9). This small perturbation by high calcium concentrations of the observed dissociation kinetics was attributed to ADP-facilitated rapid exchange of $^{40}Ca^{2+}$ for Mg^{2+} at the catalytic site of phosphorylated ATPase. The third method was based on the fact that phosphorylation-induced $^{45}Ca^{2+}$ occlusion occurred faster than $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPase: here, we measured the rate of $^{45}Ca^{2+}$ internalization on addition to $^{45}Ca^{2+}$-saturated ATPase of an unlabeled ATP-containing medium. This method allowed separate observation of the dissociation kinetics of each of the two $^{45}Ca^{2+}$ ions bound to phosphorylated ATPase, after either one or the other had been labeled by a preliminary partial isotopic exchange in the nonphosphorylated state of the ATPase. We found that after ATP-induced phosphorylation, the two $^{45}Ca^{2+}$ ions dissociated toward the lumenal medium with virtually identical rate constants; this was observed under different ionic and pH conditions and also in the presence of a high Ca^{2+} concentration. As a control, the same partial isotopic exchange procedure allowed us to confirm that, in contrast, when ATP was absent from the final dissociation medium, the two $^{45}Ca^{2+}$ ions dissociated from nonphosphorylated ATPase toward the cytoplasmic medium at different rates, the one bound more deeply only dissociating after a lag period corresponding to dissociation of the superficial one. Our results suggest that, after sequential binding to the nonphosphorylated ATPase, the two Ca^{2+} ions transported by the phosphorylated ATPase become kinetically indistinguishable when they are released toward the lumenal side of the SR vesicles.

Sarcoplasmic reticulum Ca^{2+}-ATPase (SR ATPase)[1] is the membranous enzyme responsible for the active transport of calcium from the cytoplasm of muscle cells toward the lumen of their calcium storage compartments (Hasselbach, 1974; Tada et al., 1978; de Meis & Vianna, 1979; Ikemoto, 1982; Møller et al., 1982; Tanford, 1984; Martonosi & Beeler, 1985; Inesi, 1985; Andersen, 1989; Jencks, 1989). In the absence of ATP, high-affinity binding of two calcium ions per monomer of ATPase can be demonstrated (Inesi et al., 1980; Dupont, 1980; Barrabin et al., 1984; Gafni & Boyer, 1984). In the presence of ATP, phosphorylation of the ATPase occurs, eventually leading to a reduction in the affinity of the binding sites for calcium, and to their reorientation toward the SR lumen, in which the bound calcium ions may thus accumulate after their dissociation (Makinose, 1973; Ikemoto, 1975; Watanabe et al., 1981).

The kinetics of calcium binding to nonphosphorylated ATPase were suggested to be biphasic under certain conditions (Inesi et al., 1980; Ikemoto et al., 1981; Dupont, 1982; Champeil et al., 1983; Tanford et al., 1987; Petithory & Jencks, 1988a; Nakamura, 1989). Independent experiments also demonstrated that the chase of bound $^{45}Ca^{2+}$ by the calcium isotope $^{40}Ca^{2+}$ comprised two distinct phases: in the first, one calcium ion dissociated rapidly from nonphosphorylated ATPase and could thus exchange with the externally added isotope, whereas, in the second phase, the second bound ion dissociated much more slowly, at a rate that depended on the extent to which the rapidly exchangeable site was occupied (Dupont, 1982, 1984; Nakamura, 1986, 1987; Inesi, 1987; Petithory & Jencks, 1988b; Orlowski & Champeil, 1991). The latter observation was accounted for by the suggestion that the two Ca^{2+} ions bound to nonphosphorylated ATPase resided in a relatively narrow channel, or pocket, which the superficial ion had to leave before the other ion could do so, resulting in ordered sequential Ca^{2+} dissociation (Inesi, 1987; Petithory & Jencks, 1988b; Orlowski & Champeil, 1991).

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[1] Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; A23187, calcimycin.

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This attractive view of the calcium-binding domain as a channel-like structure was further strengthened by results suggesting that the two ATPase-bound Ca^{2+} ions were sequentially internalized in the SR lumen during turnover, since dissociation of these ions from the ATPase after ATPase phosphorylation was found to comprise both a very rapid initial phase and a subsequent slower phase. Taking advantage of the possibility of differentiating between the two pools of bound calcium through isotopic exchange, it was even suggested that the pool of deeply bound calcium, undergoing slower exchange with the cytoplasmic medium in nonphosphorylated ATPase, was the one dissociating first, very rapidly, toward the lumenal side of the SR during the active transport cycle, according to a "first-in–first-out" mechanism (Inesi, 1987). In a different report, the experimental results of which have been withdrawn but in which the rationale is perfectly valid, it was suggested that, according to a simple model of sequential ion dissociation, internalization of the pool of superficially bound Ca^{2+} could well be blocked in the presence of high concentrations of calcium in the lumenal compartment (Khananshvili & Jencks, 1988, 1990). In both studies, the rate of calcium dissociation from the ATPase toward the lumenal side was obtained from indirect multimixing measurements of the amount of calcium that remained trapped in SR vesicles after an (EGTA + ADP) quench.

Using rapid filtration techniques to directly measure the amount of Ca^{2+} bound to the ATPase and taking our previous results concerning the kinetics of calcium dissociation from nonphosphorylated ATPase as conceptual and experimental background (Orlowski & Champeil, 1991), we investigated here the kinetics of calcium dissociation from phosphorylated ATPase in leaky SR vesicles, corresponding to internalization of the Ca^{2+} ions. Our results do not support biphasic internalization of calcium but rather suggest that the two previously bound ions are kinetically indistinguishable when they are released toward the lumenal side of the SR membrane. The structural corollaries of this observation are discussed.

Experimental Procedures

Sarcoplasmic reticulum vesicles were prepared as previously described from rabbit skeletal muscle (Champeil et al., 1985). Binding of $^{45}Ca^{2+}$ (Amersham, U.K.) was measured in double-labeling filtration experiments as described in Champeil and Guillain (1986) except that [^{3}H]glucose generally replaced [^{3}H]sucrose as a marker of the amount of fluid wetting the filter. $^{45}Ca^{2+}$ dissociation rates were measured with a rapid filtration apparatus (Orlowski & Champeil, 1991). The temperature was 20 °C in all experiments. Unless otherwise indicated, SR vesicles were preincubated in a medium containing 1 mM [^{3}H]glucose and 100 μM $^{45}Ca^{2+}$ (in the form of a $CaCl_2$ solution), in the presence or absence of 0.04 g of ionophore A23187 (Calbiochem) per gram of protein. Next, 0.3 mg of protein was layered onto a Millipore HA filter, and, in some cases, 1 mL of a different medium was flushed through the filter. After that, the filter was perfused for various electronically controlled periods with the final dissociation medium, and the ^{3}H and ^{45}Ca radioactivities on the filter were counted by liquid scintillation. For instance, the perfusion rate was 3 mL/s for a 100-ms perfusion period, which means that 300 μL of fluid was flushed through the filter, i.e., about 8–10 times the volume of fluid wetting the filter (30–40 μL). More than 90% of the amount of fluid wetting the filter was washed away after only 25 ms, as indicated by the residual amount of ^{3}H label on the filter, so that the subtraction procedure allowing the computation of the amount of $^{45}Ca^{2+}$ specifically bound to the vesicles was particularly reliable when the final dissociation medium was devoid of $^{45}Ca^{2+}$. In the presence of 0.04 g of ionophore per gram protein, the vesicle passive permeability to calcium was high enough to ensure that calcium in the vesicles' lumen reequilibrated with the external medium rapidly, compared to the rate at which calcium was released from the ATPase toward the internal side of the vesicles (see Discussion). As noted previously concerning rates of Ca^{2+} dissociation from nonphosphorylated ATPase (Orlowski & Champeil, 1991), the rates of Ca^{2+} dissociation from phosphorylated ATPase measured varied somewhat from one SR preparation to another, almost within a factor of 2, so that all our experiments are shown together with appropriate controls performed in the same series of experiments. For instance, the preparation used for Figure 1A was one with relatively fast rates, and those for Figures 5A and 6A were preparations with slightly slower rates. The preparation used for Figures 1B and 2A was one with slow rates.

Results

Rate of Calcium Internalization during the Transport Cycle, Measured Using Leaky Vesicles. Using SR vesicles rendered leaky by the A23187 ionophore, preequilibrated with $^{45}Ca^{2+}$ under our standard conditions, in the absence of K^+ and in the presence of 150 mM Mes-Tris (pH 6, 20 °C), 20 mM Mg^{2+}, and 0.1 mM $^{45}Ca^{2+}$, and adsorbed on a cellulose filter, perfusion of these vesicles with a medium containing the same concentration of $^{45}Ca^{2+}$ and 2 mM Mg·ATP was first chosen to trigger the ATPase catalytic cycle. The initial rate at which the amount of ATPase-bound $^{45}Ca^{2+}$ dropped after triggering turnover (circles in Figure 1A) reflected the rate of $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase toward the lumenal side of the membrane (step 2 in the inset to Figure 1A), since phosphorylation itself (step 1) was much faster (close to 100 s^{-1}, as measured in separate multimixing experiments). This procedure thus allowed evaluation of the rate constant of the transition which, with tight vesicles, corresponds to the rate of calcium internalization toward the lumenal medium during the transport cycle (Champeil & Guillain, 1986). This rate constant, derived from the *initial rate* of $^{45}Ca^{2+}$ dissociation (see the dashed line drawn down to the intercept of the abscissa axis), was found to be 3–3.5 s^{-1} in this experiment. On the other hand, the amount of $^{45}Ca^{2+}$ remaining bound to the leaky vesicle ATPase during turnover reflected the proportion of calcium-containing and calcium-free ATPase forms at steady state under these conditions (the latter forms were predominant here). A control experiment performed in the absence of ionophore only revealed the expected ATP-induced uptake of $^{45}Ca^{2+}$ in the sealed vesicles (squares in Figure 1A). In agreement with our previous measurements (Champeil & Guillain, 1986), this experiment provided no evidence in favor of rapid dissociation toward the lumenal side of one of the two previously bound $^{45}Ca^{2+}$ ions, unlike the results of a recent experiment performed at neutral pH and in the presence of potassium (Inesi, 1987).

We then aimed at repeating our measurements in the presence of potassium. However, the difference between the amounts of $^{45}Ca^{2+}$ bound to leaky vesicle ATPase in the absence of ATP, and at steady state in the presence of ATP, is much smaller in the presence of potassium than in the absence of potassium (Champeil & Guillain, 1986). This is because at steady state K^+ greatly shifts the balance between the calcium-containing and calcium-free forms of ATPase in favor of the calcium-containing form due, in particular, to K^+-induced stimulation of steps 3 and 4 in the catalytic cycle (inset to Figure 1), which allows the ADP-sensitive Ca_2E_1P form to accumulate to a larger extent [see references in Champeil and

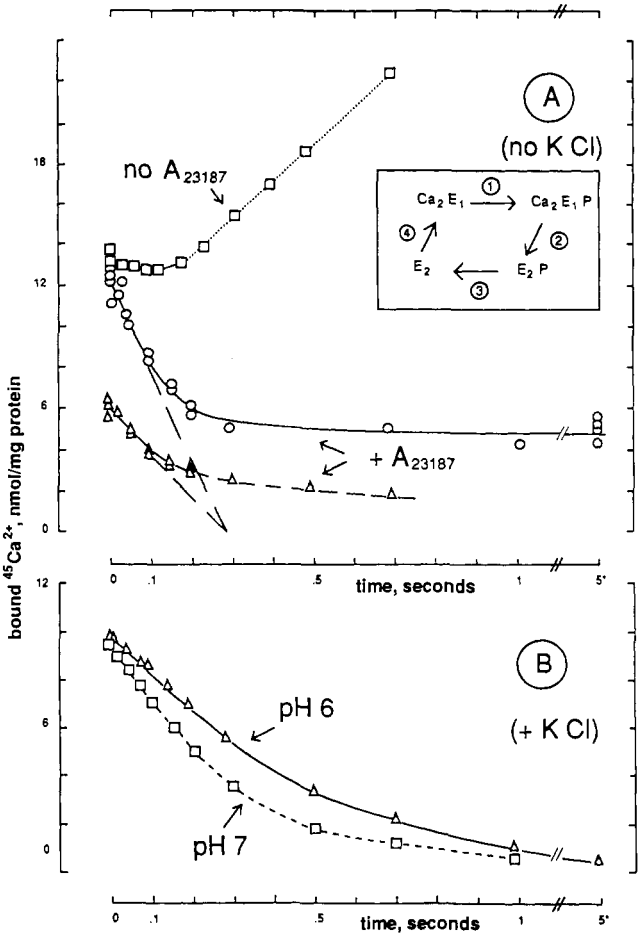

FIGURE 1: Phosphorylation-dependent $^{45}Ca^{2+}$ dissociation from ATPase toward the lumenal side during the transport cycle in leaky SR vesicles. SR vesicles (0.3 mg of protein/mL) were first equilibrated in a pH 6 medium containing 0.012 mg/mL A23187 ionophore (i.e., 4% w/w), 0.1 mM $^{45}Ca^{2+}$, 1 mM [^{3}H]sucrose, 20 mM Mg^{2+}, and either 150 mM Mes-Tris (panel A) or 50 mM Mes-Tris and 100 mM KCl (panel B); 0.3 mg of protein was then layered onto Millipore HA filters. In the experiment illustrated by circles in panel A, the loaded filters were directly perfused for various electronically controlled periods with a pH 6 solution containing 0.1 mM $^{45}Ca^{2+}$, 1 mM [^{3}H]sucrose, 20 mM Mg^{2+}, 150 mM Mes-Tris, and 2 mM Mg·ATP. In the control experiment illustrated by squares in panel A, ionophore was omitted. Alternatively, the loaded filters were manually perfused with 0.5 mL of a pH 6 solution containing 0.1 mM $^{45}Ca^{2+}$, 1 mM [^{3}H]sucrose, 20 mM Mg^{2+}, 2 mM Mg·ATP, and either 150 mM Mes-Tris (triangles in panel A) or 50 mM Mes-Tris and 100 mM KCl (panel B), and within 5 s submitted to the electronically controlled perfusion period, with a medium containing 0.1 mM $^{40}Ca^{2+}$ instead of $^{45}Ca^{2+}$, 2 mM ATP, and one of the following: 20 mM Mg^{2+} and 150 mM Mes-Tris at pH 6 (triangles in panel A), 20 mM Mg^{2+}, 50 mM Mes-Tris and 100 mM KCl at pH 6 (triangles in panel B), or 5 mM Mg^{2+}, 50 mM Mops-Tris and 100 mM KCl at pH 7 (squares in panel B). The temperature was 20 °C throughout. The 5-s perfusion (5*) was performed manually. (Inset) Simplified scheme of the ATPase catalytic cycle. Under our conditions, step 1 is fast; in the absence of K^+ at high ATP concentration, step 3 is the slowest in the cycle, and, at steady-state, the calcium-free E_2P species accumulates to a significant extent; this is not the case in the presence of K^+ [see Champeil and Guillain (1986), and references therein].

Guillain (1986)]. Consequently, a time-resolved experiment in the presence of K^+ similar to the one performed in its absence was not very informative because of the poor amplitude of the observed drop (data not shown).

For this reason, we decided to use a protocol described by Wakabayashi et al. (1986), which we initially tested in the absence of K^+ to compare it with our previous protocol. Here, steady state was attained first through manual flushing of previously adsorbed $^{45}Ca^{2+}$-equilibrated leaky vesicles with a solution containing both $^{45}Ca^{2+}$ and ATP. Only then was the filter perfused for various periods with a solution containing $^{40}Ca^{2+}$ and ATP at the same concentrations. Under these conditions (Figure 1A, triangles), the amount of $^{45}Ca^{2+}$ bound to the ATPase at time zero, corresponding to the steady state (compare with circles in Figure 1A at time ≥0.7 s), was less than two ions per ATPase monomer, but the rate constant of $^{45}Ca^{2+}$ dissociation could equally well be measured. The results obtained with this method (observed rate constant of 3–3.5 s^{-1}) were consistent with those obtained with the preceding method (compare initial rates in the curves illustrated by circles and triangles in Figure 1A). The advantage of this pulse–chase experiment is that the observed $^{45}Ca^{2+}$ dissociation directly monitors the transition form Ca_2E_1P to E_2P (step 2 in the inset to Figure 1A), since $^{45}Ca^{2+}$ bound at steady state (time zero of the experiment) is mainly bound to Ca_2E_1P.

This second protocol was well suited for an experiment in the presence of potassium, as shown in Figure 1B. The triangles in Figure 1B show that at pH 6, in the presence of 100 mM K^+, the amount of $^{45}Ca^{2+}$ bound to the ATPase at steady state, corresponding to time zero of the experiment, was higher than in the absence of K^+, as expected. Under these conditions, again, $^{45}Ca^{2+}$ dissociation from Ca_2E_1P gave no indication of a very fast initial dissociation of one of the two bound calcium ions. In these experiments, the rate constant for Ca^{2+} dissociation could be evaluated from either its initial rate or its half time. In the experiment illustrated by the triangles in panel B, it was about 2 s^{-1}, i.e., slower than the one measured in the absence of potassium (panel A), partly because a different SR preparation was used and partly because K^+ is known to slow down the rate of the Ca_2E_1P to E_2P transition, thus contributing to the higher proportion of ADP-sensitive phosphoenzyme found at steady state in the presence of potassium [Yamaguchi & Kanazawa, 1985; Champeil et al., 1986; see also Shigekawa and Akowitz (1979)].

This protocol could not be directly extended to experiments at neutral pH, because under these conditions the ATPase activity is high and the adsorbed ATPase keeps hydrolyzing ATP on the filter after the manual flushing period, so that ATP would be exhausted before the final perfusion is initiated. In the original experiments by Wakabayashi et al. (1986), the authors had lowered the temperature to reduce ATPase activity. To overcome the problem raised by the high ATPase activity at neutral pH and room temperature, we combined these experiments with a pH jump, as also used in some cases by Wakabayashi et al. (1986). For the experiments illustrated by the squares in Figure 1B, the steady state in the presence of K^+ at pH 6 was reached first, and dissociation of $^{45}Ca^{2+}$ from phosphorylated ATPase was monitored with a perfusion fluid which was buffered to pH 7 with 50 mM Mops-Tris and also contained 100 mM KCl, 2 mM Mg·ATP, 0.1 mM $^{40}Ca^{2+}$, and 5 mM Mg^{2+} (free Ca^{2+} was about 0.085 mM). We found calcium dissociation to be faster at pH 7 than at pH 6, and again monophasic, in agreement with results previously obtained at low temperature (Wakabayashi et al., 1986).

Effect of a High Calcium Concentration on $^{45}Ca^{2+}$ Dissociation from Phosphorylated ATPase in Leaky Vesicles. If the transport pathway were indeed a channel-like single-file structure (see the introduction), the monophasic $^{45}Ca^{2+}$ dissociation observed at pCa 4 could be expected to turn into biphasic dissociation in the presence of a high concentration of calcium on the lumenal side during the dissociation process (Khananshvili & Jencks, 1988). The most straightforward way of testing this possibility was to combine the protocol just described with a calcium jump during the perfusion step. This

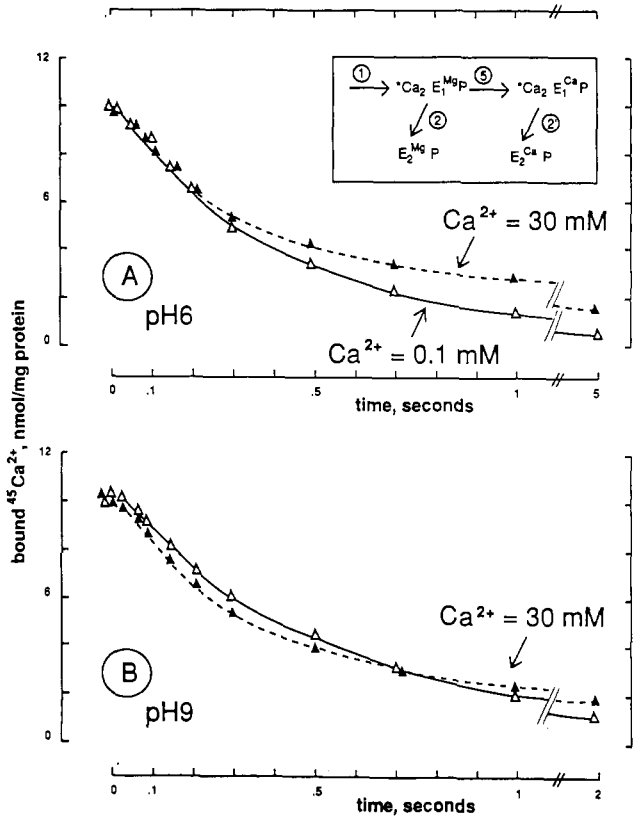

FIGURE 2: Effect of a high $^{40}Ca^{2+}$ concentration on $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase at pH 6 (panel A) or 9 (panel B) in the presence of K^+. SR vesicles were first equilibrated in a medium containing 0.1 mM $^{45}Ca^{2+}$, 1 mM [^{3}H]glucose, 20 mM Mg^{2+}, 100 mM KCl, and 50 mM Mes-Tris at pH 6 (20 °C) in the presence of 4% (w/w) ionophore A23187, and 0.3 mg of protein was then layered onto HA filters. Next, the loaded filters were manually flushed with a solution containing 0.1 mM $^{45}Ca^{2+}$, 1 mM [^{3}H]glucose, 20 mM Mg^{2+}, 100 mM KCl, 50 mM Mes-Tris at pH 6, and 2 mM Mg·ATP, and then, within 5 s, submitted to perfusion for electronically controlled periods. In the experiments illustrated in panel A, the final perfusion solution contained 2 mM ATP, 100 mM KCl, 20 mM Mg^{2+}, 50 mM Mes-Tris at pH 6, and either 0.1 mM (open symbols) or 30 mM (closed symbols) $^{40}Ca^{2+}$. In the experiments illustrated in panel B, the final perfusion solution contained 2 mM ATP, 100 mM KCl, 20 mM Mg^{2+}, 50 mM Tris-HCl at pH 9, and either 2 mM EGTA (open symbols) or 30 mM $^{40}Ca^{2+}$ (closed symbols). (Inset) Diagram illustrating $^{45}Ca^{2+}$ dissociation from the transport sites of phosphorylated ATPase with either magnesium or calcium at the catalytic site (steps 2 and 2′, respectively).

is shown in Figure 2. In the same way as for the experiment illustrated in Figure 1B, steady state was reached first in the presence of $^{45}Ca^{2+}$, K^+, and ATP, at pH 6. For the experiments illustrated in panel A of Figure 2, $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase was measured when the final perfusion medium contained either 0.1 mM $^{40}Ca^{2+}$ and ATP, as in Figure 1B (open symbols), or 30 mM $^{40}Ca^{2+}$ and ATP (closed symbols), at pH 6. The presence of a high $^{40}Ca^{2+}$ concentration in the perfusion fluid did modify the time course of $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase (Figure 2A), but only slightly. We wondered whether this small effect of $^{40}Ca^{2+}$ in the perfusion fluid could be due to poor affinity of the phosphorylated ATPase for Ca^{2+} at pH 6. However, when we repeated the experiment with a perfusion medium buffered at pH 7 as in Figure 1B, the same pattern was observed (data not shown), i.e., the high $^{40}Ca^{2+}$ concentration of 30 mM only exerted a small effect on the kinetics of $^{45}Ca^{2+}$ dissociation, in contrast with what was previously conjectured (Khananshvili & Jencks, 1988). We then repeated the experiment at very alkaline pH, a condition known to enhance markedly the affinity for calcium of the ATPase transport sites in both the nonphosphorylated and phosphorylated states (Verjovski-Almeida & de Meis, 1977), with the expectation that if ordered sequential dissociation of $^{45}Ca^{2+}$ from phosphorylated ATPase did occur, 30 mM $^{40}Ca^{2+}$ at pH 9 would completely saturate the lumenal dissociation site and would therefore render the dissociation of the second ion virtually impossible and the $^{45}Ca^{2+}$ dissociation kinetics clearly biphasic, with a one-to-one stoichiometry between a rapid and a very slow phase. However, contrarily to this expectation, Figure 2B shows that the high calcium concentration (closed symbols) only moderately slowed down the dissociation of 30–40%, at most, of the pool of bound $^{45}Ca^{2+}$.

We therefore considered the possibility that this pH-independent nonmonophasic $^{45}Ca^{2+}$ dissociation pattern at high $^{40}Ca^{2+}$ concentration was due to the exchange of $^{40}Ca^{2+}$ for Mg^{2+} at the catalytic site of phosphorylated ATPase (step 5 in the diagram forming the inset to Figure 2), leading to the subsequent slowing down of the $Ca_2E_1P \rightarrow E_2P$ transition and therefore of $^{45}Ca^{2+}$ dissociation from the transport sites of phosphorylated ATPase. As a matter of fact, calcium dissociation from the transport sites of a phosphoenzyme with calcium at the catalytic site (step 2′ in the inset to Figure 2) is known to be slower than calcium dissociation from the transport sites of a phosphoenzyme with magnesium at the catalytic site (step 2) (Nakamura, 1984; Yamada, et al., 1986; Lund & Møller, 1988; Orlowski et al., 1988). Under our conditions, a major reason for metal exchange could have been the presence of ADP, which sustains active ATP–ADP exchange even at low concentrations (Orlowski et al., 1988; Soler et al., 1990). We therefore repeated our experiments in the presence of 0.5 mM phosphoenolpyruvate and 0.01 mg/mL pyruvate kinase, which was not sufficient to compete with the high rate of ADP production by the ATPase on the filter but which did allow the removal of contaminating ADP from the perfusion medium (1–2% of 2 mM ATP already hydrolyzed in the perfusion medium results in an ADP concentration of 20–40 μM). The presence of the ATP-regenerating system at this low concentration more than halved the difference between the dissociation curves observed in the presence of 0.1 and 30 mM $^{40}Ca^{2+}$, at both pH 6 and 7 (data not shown). This supports the suggestion that the calcium-induced deviation from monophasic behavior observed in our experiments could be attributed to ADP-dependent exchange of $^{40}Ca^{2+}$ for Mg^{2+} at the catalytic site of phosphorylated ATPase and not to ordered sequential $^{45}Ca^{2+}$ dissociation from the ATPase transport sites.

Measurements of $^{45}Ca^{2+}$ Internalization on Addition of $^{40}Ca^{2+}$ and ATP to $^{45}Ca^{2+}$-Saturated ATPase. It is also possible to measure the rate of calcium dissociation from phosphorylated ATPase in leaky vesicles by performing an experiment related to the one illustrated by the circles in Figure 1A, i.e., starting from ATPase preequilibrated with $^{45}Ca^{2+}$ in the absence of ATP, but now perfusing the ATPase with $^{40}Ca^{2+}$ together with ATP. The only requirement for this purpose is that ATPase phosphorylation from ATP must be much faster than dissociation of $^{45}Ca^{2+}$ from the transport sites of nonphosphorylated ATPase, which takes place toward the external medium. Since ATPase phosphorylation is known to render the bound $^{45}Ca^{2+}$ unable to exchange with external calcium (Kurzmack et al., 1977; Dupont, 1980), subsequent ATPase turnover will permit $^{45}Ca^{2+}$ dissociation on the lumenal side only (see diagrams in Figure 3B).

As controls for this protocol, SR vesicles in the absence of ionophore were preequilibrated with 0.1 mM $^{45}Ca^{2+}$ at pH 6,

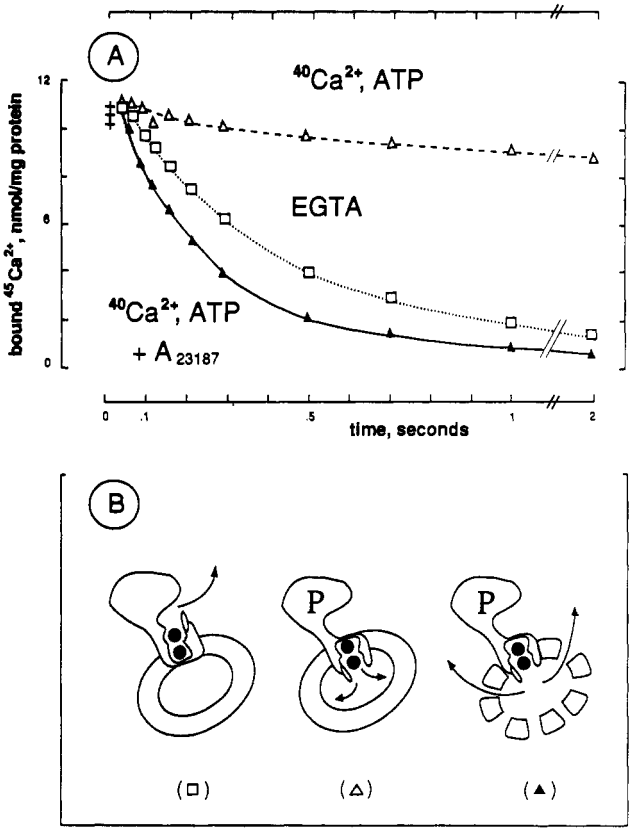

FIGURE 3: Phosphorylation-induced $^{45}Ca^{2+}$ internalization in sealed SR vesicles or dissociation toward the lumenal side of leaky vesicles, upon addition of ATP and $^{40}Ca^{2+}$ to vesicles equilibrated with $^{45}Ca^{2+}$, as compared to $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPase. (Panel A) SR vesicles were first equilibrated with 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose in the same medium as for the experiment illustrated in Figure 1A, either in the absence of A23187 ionophore (squares and open triangles) or in its presence (closed triangles), and layered onto a filter. They were then perfused at 20 °C for various periods with a medium containing 0.1 mM $^{40}Ca^{2+}$ and 2 mM ATP (open and closed triangles). Alternatively, sealed vesicles were perfused with a medium containing 2 mM EGTA (squares). (Panel B) Diagram of $^{45}Ca^{2+}$ movements in the above experiments.

in the absence of K^+, and then perfused for various periods with a solution containing either EGTA alone (open squares in Figure 3A) or 0.1 mM nonradioactive calcium together with 2 mM ATP (open triangles in Figure 3A). Under the latter conditions, most of the initially bound $^{45}Ca^{2+}$ remained bound to the vesicles on the subsecond time scale and was obviously transported into the SR internal compartment. ATP-induced phosphorylation of ATPase and occlusion of $^{45}Ca^{2+}$ therefore occurred much faster than the dissociation of calcium from nonphosphorylated ATPase. In separate multimixing experiments, we indeed measured identical phosphorylation time courses and levels (with a half-time of about 5 ms) when calcium-equilibrated SR vesicles were mixed with solutions containing [γ-^{32}P]ATP plus either calcium or a high concentration of EGTA (unpublished results). The latter observation even suggests that the major reason for loss of part of the bound $^{45}Ca^{2+}$ ions when ATP was added to the SR vesicles in the absence of ionophore (open triangles) was the slowly proceeding exchange of $^{40}Ca^{2+}$ for previously accumulated $^{45}Ca^{2+}$ which is coupled to ATP–ADP exchange [e.g., Soler et al. (1990)], plus the possible presence in our vesicle preparation of a small population of spontaneously leaky SR membrane fragments.

The same experiment was then repeated using vesicles which had been preequilibrated with $^{45}Ca^{2+}$ in the presence of enough ionophore to make them completely leaky to calcium (Champeil & Guillain, 1986). When these vesicles were perfused with the medium containing 2 mM ATP and 0.1 mM nonradioactive calcium, $^{45}Ca^{2+}$ did dissociate from the phosphorylated ATPase (closed triangles in Figure 3A). This dissociation therefore took place on the lumenal side of the leaky vesicles, as illustrated in the diagram on the right of panel B of Figure 3. The rate measured was consistent with the one found using the two previous protocols (3–3.5 s^{-1}), and again there was no indication of very fast dissociation of part of the $^{45}Ca^{2+}$ pool toward the lumenal medium. Note that, in this case, the rate of $^{45}Ca^{2+}$ internalization was slightly faster than the rate of calcium dissociation in the direction of the external medium (compare closed triangles and open squares). Note also that, as long as the high ATP concentration used ensured that ATPase phosphorylation was faster than $^{45}Ca^{2+}$ dissociation toward the external medium, any concentration of free calcium could be used in the perfusion solution. Similar dissociation rates were in fact measured when the perfusion solution contained ATP and 2 mM EGTA instead of ATP and 0.1 mM $^{40}Ca^{2+}$ (open squares in Figures 5A and 6A, and data not shown).

This protocol was used to define the order in which the ATPase internalizes the two $^{45}Ca^{2+}$ ions bound to it. As a preliminary experiment, we first showed that these two ions could be differentiated through their rate of dissociation from nonphosphorylated ATPase to the cytoplasmic medium.

Differentiation of the Two Pools of Bound Ca^{2+} during Ca^{2+} Dissociation from Nonphosphorylated ATPase toward the Cytoplasmic (i.e., External) Side of SR Vesicles. The inset to Figure 4A recalls the basic experiment [taken from Orlowski and Champeil (1991); see references therein] which made possible differentiation of two pools of bound calcium through isotopic exchange experiments (Inesi, 1987). When SR vesicles previously equilibrated with $^{45}Ca^{2+}$ are adsorbed on a cellulose filter and perfused for various periods with a medium devoid of radioactive tracer, the kinetics of $^{45}Ca^{2+}$ dissociation are fairly monophasic when the perfusion medium contains a calcium chelator such as EGTA (solid line); however, these kinetics become clearly biphasic when the perfusion medium contains nonradioactive calcium, in other words when $^{45}Ca^{2+}$ is replaced by $^{40}Ca^{2+}$ rather than simply extracted. In that case, one of the two bound $^{45}Ca^{2+}$ ions is exchanged rapidly from what was called the "superficial" site; the higher the concentration of free $^{40}Ca^{2+}$ in the perfusion medium, the slower the rate at which the other $^{45}Ca^{2+}$ ion dissociates from the "deeper" site (compare the dotted line obtained at pCa 4 and the dashed line obtained at pCa 3). When the $^{40}Ca^{2+}$ concentration in the perfusion medium is sufficiently high, the deeply bound calcium ion remains trapped on the ATPase for several seconds. This allowed us to perform experiments in which the superficial $^{45}Ca^{2+}$ ion was exchanged with $^{40}Ca^{2+}$ during a preliminary manually performed perfusion step, before the adsorbed ATPase, together with the residual deeper $^{45}Ca^{2+}$ bound to it, was submitted to an electronically controlled rapid filtration step using the desired perfusion solution.

Here, the application of this procedure allowed us to distinguish between the kinetic properties of the two bound $^{45}Ca^{2+}$ ions during their dissociation from nonphosphorylated ATPase toward the external (i.e., cytoplasmic) side of the SR vesicles (see Figure 4B). Experimental results are shown in the main frame of Figure 4A. In the control experiment (squares), the vesicles were equilibrated with 0.1 mM $^{45}Ca^{2+}$ and then perfused for various periods with the rapid filtration device, using a medium containing 2 mM EGTA: the resulting ki-

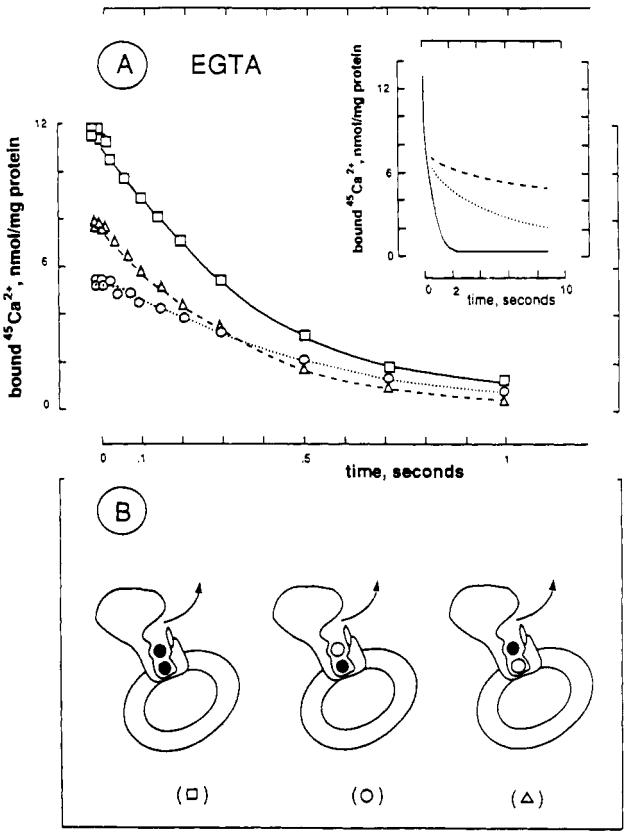

FIGURE 4: Sequential dissociation toward the cytoplasmic side of the two pools of $^{45}Ca^{2+}$ ions bound to nonphosphorylated ATPase, at pH 6. (Panel A, inset) Biphasic dissociation of $^{45}Ca^{2+}$ from nonphosphorylated SR ATPase, during isotopic exchange in the absence of ATP. In these experiments, vesicles equilibrated with $^{45}Ca^{2+}$ were perfused with a medium containing either EGTA (continuous line) or $^{40}Ca^{2+}$, at a concentration of 0.1 mM (dotted line) or 1 mM (dashed line) [from Orlowski and Champeil (1991)]. (Panel A, main frame) For the control experiment illustrated by the squares, vesicles were first equilibrated with 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose and then perfused for predetermined periods with a solution containing 2 mM EGTA. For the experiment illustrated by the circles, vesicles were first equilibrated with 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose, subsequently flushed manually with a solution containing 2 mM $^{40}Ca^{2+}$ to allow partial isotopic exchange, and then, within 2–3 s, perfused for predetermined periods with a solution containing 2 mM EGTA. For the experiment illustrated by the triangles, vesicles were first equilibrated with 0.2 mM $^{40}Ca^{2+}$, perfused manually with a solution containing 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose, and then, within 2–3 s, submitted to perfusion for predetermined periods with a solution containing 2 mM EGTA. The medium also contained 20 mM Mg^{2+} and 150 mM Mes-Tris at pH 6. The temperature was 20 °C. Although ionophore was not necessary in these experiments, it was always included (4% w/w relative to protein), in order to make them exactly comparable to those illustrated in Figure 5, performed in the presence of ATP. (Panel B) Diagram of the pools of calcium labeled in these experiments. Closed symbols represent radioactive $^{45}Ca^{2+}$ ions, and open symbols, unlabeled $^{40}Ca^{2+}$ ions.

netics of $^{45}Ca^{2+}$ dissociation in this experiment had a half-time close to 250 ms. In the experiment illustrated by the circles, preequilibration of the vesicles with 0.1 mM $^{45}Ca^{2+}$ was followed first by manual perfusion with a medium containing 2 mM $^{40}Ca^{2+}$ to exchange the superficial $^{45}Ca^{2+}$ ion and then, within 2–3 s, by electronically controlled perfusion with the same EGTA-containing medium as the one used in the control experiment. The amount of $^{45}Ca^{2+}$ initially bound to the ATPase was 11–12 nmol/mg of protein, corresponding to two $^{45}Ca^{2+}$ ions bound per mole of ATPase monomer, and the residual amount bound to the ATPase after the manual perfusion, at the start of the electronically controlled perfusion, was about 5 nmol/mg of protein. This implies that the rapidly exchangeable pool of calcium plus a small fraction of the slowly exchangeable pool of calcium was replaced by $^{40}Ca^{2+}$ during the few seconds of the manual perfusion before the electronically controlled perfusion started. It is clear from Figure 4A (circles) that the initial rate of dissociation in the EGTA medium of the slowly exchangeable pool of $^{45}Ca^{2+}$ bound at the "deeper" site was slow and that a lag (see also the circles in Figure 7A) preceded its complete dissociation, as expected (Petithory & Jencks, 1988a; Orlowski & Champeil, 1991). The overall half-time for dissociation was close to 350 ms, i.e., longer than in the control experiment.

The complementary experiment was also performed (triangles in Figure 4) in which SR vesicles were preequilibrated with saturating nonradioactive calcium, subsequently manually perfused for a few seconds with a solution containing 0.1 mM $^{45}Ca^{2+}$, and then submitted, as for the other experiments, to rapid filtration with the EGTA-containing medium. In this case, during the few seconds of manual perfusion, the rapidly exchangeable pool must have been completely replaced by $^{45}Ca^{2+}$, plus, again, a small fraction of the slowly exchangeable pool (hence the initial amount of bound $^{45}Ca^{2+}$ measured was 7–8 nmol/mg of protein). In this experiment, there was no delay in $^{45}Ca^{2+}$ dissociation during the perfusion with EGTA, and the half-time for the overall process was close to 200 ms, which was slighly faster than in the control experiment. Similar results were observed repeatedly (see also the triangles in Figure 7A).

The calcium dissociation rates illustrated in Figure 4A were obtained with SR vesicles made leaky with ionophore, to make them exactly comparable to the ones to be described in the next paragraph. However, similar results would be expected with sealed vesicles [see Figures 2 and 6 versus 4 and 5 in Orlowski and Champeil (1991)], because in the absence of ATP, the calcium dissociation from nonphosphorylated ATPase took place toward the external, i.e., cytoplasmic, side of the SR vesicles. These results showed that it was possible to distinguish, after partial isotopic exchange, the two pools of calcium ions bound to nonphosphorylated ATPase, on the basis of the rate at which each pool dissociated from the ATPase toward the external medium in the presence of EGTA.

Attempts To Differentiate the Two Pools of Bound Ca^{2+} during Ca^{2+} Dissociation from Phosphorylated ATPase toward the Lumenal (i.e., Internal) Side of SR Vesicles. The procedures described above and illustrated in Figures 4 and 3 allowed us to test each of the bound calcium ions to see if after ATPase phosphorylation they were released toward the lumenal side in an ordered way, as expected for a linear single-file channel-like structure allowing transmembrane passage of the ions (see diagrams in Figure 5B). An experiment similar to the one shown in Figure 4A was therefore performed, except that the leaky vesicles adsorbed on a filter were now perfused with a solution containing ATP and EGTA (Figure 5A). The symbols are similar to those used in Figure 4A. Contrarily to what would be expected for dissociation from a simple linear channel-like structure, the pool of "deeply" bound calcium ions, i.e., those that had remained trapped on nonphosphorylated ATPase after isotopic exchange of the superficial ions (circles in Figure 5A), did not dissociate from phosphorylated ATPase toward the lumenal side faster than the pool of superficially bound ions (triangles), and no clear-cut lag was observed for either kinetics. In a separate experiment, the leaky vesicles were perfused with a solution containing ATP and 0.1 mM nonradioactive calcium, a concentration which is expected to trigger steady-state cycling without having any effect on the lumenal side of the ATPase (Figure 6A). As

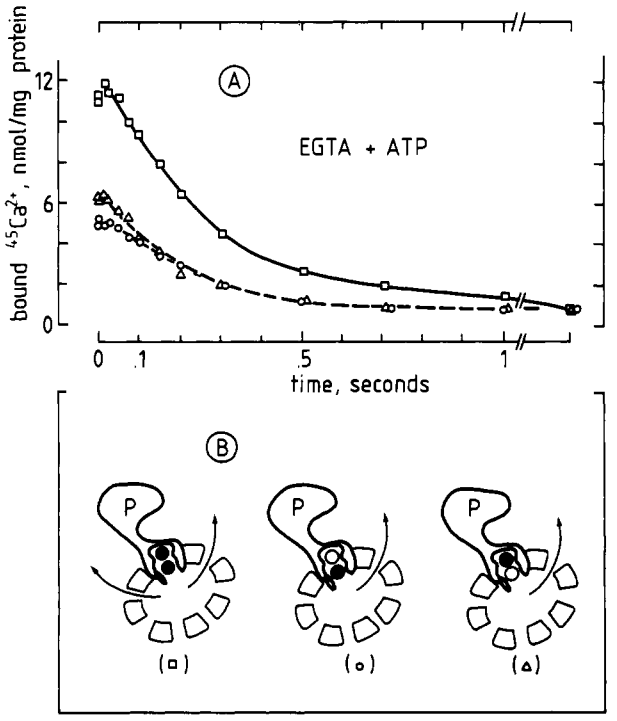

FIGURE 5: Phosphorylation-induced dissociation toward the lumenal side of the two pools of bound $^{45}Ca^{2+}$, at pH 6. (Panel A) The experiments here were similar to those shown in Figure 4A, except that the perfusion medium contained 2 mM ATP together with 2 mM EGTA. The medium also contained 20 mM Mg^{2+} and 150 mM Mes-Tris at pH 6. The symbols are the same as for Figure 4. (Panel B) Diagram of the pools of calcium labeled in these experiments. Closed symbols represent $^{45}Ca^{2+}$ ions, open symbols, $^{40}Ca^{2+}$ ions.

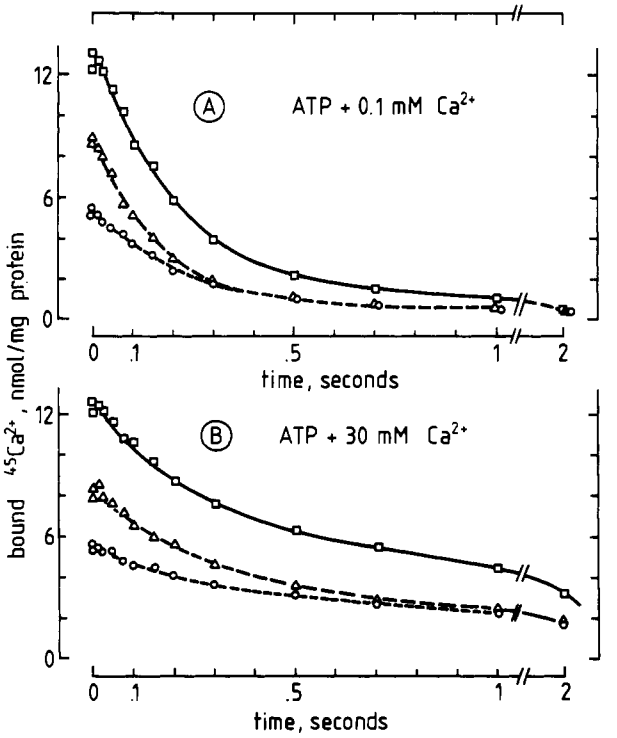

FIGURE 6: Phosphorylation-induced dissociation toward the lumenal side of the two pools of bound $^{45}Ca^{2+}$, in the presence of 0.1 mM (A) or 30 mM (B) $^{40}Ca^{2+}$, at pH 6. The experiments and symbols were similar to those shown in Figure 5, except that the perfusion medium contained 2 mM ATP together with either 0.1 mM $^{40}Ca^{2+}$ (panel A) or 30 mM $^{40}Ca^{2+}$ (panel B). In the former case, the concentration of $^{45}Ca^{2+}$ for preliminary labeling of the Ca^{2+} pools was 0.2 mM instead of 0.1 mM; the slightly different labeling conditions in this particular experiment also allowed distinction of the two calcium pools when dissociation to the cytoplasmic side in the absence of ATP was examined in experiments similar to those in Figure 4 (data not shown).

mentioned above, the measured kinetics of overall $^{45}Ca^{2+}$ dissociation were virtually the same in the single-turnover experiment in the presence of EGTA and ATP (Figure 5A) and in the experiment where steady-state cycling was triggered by adding 0.1 mM $^{40}Ca^{2+}$ and ATP (Figure 6A). Similar results were also obtained as regards the apparently identical rates of dissociation of the superficially and deeply bound Ca^{2+} ions. From these observations we conclude that, during active transport and dissociation from the phosphorylated ATPase to the lumenal medium, the pool of deeply bound calcium that undergoes slower exchange with the cytoplasmic medium when bound to nonphosphorylated ATPase cannot be kinetically discriminated from the pool of superficially bound calcium.

The above experiments were also repeated when, together with ATP, the perfusion solution contained a very high concentration of $^{40}Ca^{2+}$ (30 mM) designed, in those experiments with leaky vesicles, to ensure a high concentration of Ca^{2+} on the lumenal side of the ATPase (Figure 6B). Under these conditions, the phosphorylation-induced occlusion of $^{45}Ca^{2+}$ was again faster than its dissociation toward the external medium, as shown by control experiments performed with tight vesicles in the absence of ionophore, the results of which were similar to those illustrated by the triangles in Figure 3A. Using vesicles made leaky with ionophore, the measured initial rates of dissociation toward the lumenal side for both the deeply and the superficially bound $^{45}Ca^{2+}$ ions were slower under these conditions (Figure 6B) than after addition of EGTA + ATP (Figure 5A) or 0.1 mM Ca^{2+} + ATP (Figure 6A), and the dissociation time courses were biphasic. Evidently, this is because, for a significant fraction of the ATPases, $^{45}Ca^{2+}$ dissociated from a phosphoenzyme with $^{40}Ca^{2+}$ at the substrate site (step 2′ in the diagram of Figure 2A), as Ca·ATP was present in significant amounts under these conditions. Nevertheless, the data in Figure 6B clearly demonstrate that the presence of 30 mM Ca^{2+} with free access to the SR lumenal side failed to block or even specifically slow down internalization of the superficially bound Ca^{2+} ion (triangles), compared to that of the deeply bound Ca^{2+} ion (circles). The two transported kinetically equivalent $^{45}Ca^{2+}$ ions cannot be discriminated through their sensitivity to high lumenal Ca^{2+}.

We also repeated these $^{45}Ca^{2+}$ dissociation experiments at pH 9 in the presence of 100 mM KCl. A pH jump was used to reach pH 9, as in the experiments illustrated in Figure 2B. SR vesicles were first preequilibrated at pH 6 in the presence of KCl; for the experiments after partial isotopic exchange, illustrated by triangles and circles in Figure 7, partial exchange was manually performed at pH 6 in the presence of KCl (symbols as for Figures 4 and 5). The SR-loaded filters were then submitted to the electronically controlled rapid perfusion, with a solution buffered at pH 9 and containing either EGTA plus ATP, to measure $^{45}Ca^{2+}$ dissociation toward the lumenal side (Figure 7B), or EGTA only, to measure this dissociation toward the cytoplasmic side (Figure 7A). By showing that in the absence of ionophore, as in the experiments illustrated in Figure 3A, most of the $^{45}Ca^{2+}$ ions bound to the ATPase remained trapped in the vesicles when the latter were perfused with EGTA plus ATP, we confirmed that, under the present conditions, the prerequisite for our experiment was also verified, i.e., ATP-induced occlusion was faster than $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPase (data not shown). The experiment was then performed in the presence of ionophore: panel B in Figure 7 shows that, as previously found at pH 6 (Figure 5A), the pool of deeply bound calcium

ions (circles) did not dissociate toward the lumenal medium after phosphorylation with ATP more rapidly than the superficial pool (compare circles to triangles). As a control, panel A shows that, in the absence of ATP, the relative rates of dissociation toward the cytoplasmic side for these two pools displayed the usual pattern; the distinction between these dissociation kinetics was even clearer at pH 9 than at pH 6, $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPase being biphasic at pH 9.[2] Note that, in contrast with the situation prevailing at pH 6, $^{45}Ca^{2+}$ dissociation at pH 9 occurred faster in the absence of ATP than in its presence (compare squares in panels A and B). All these measurements of $^{45}Ca^{2+}$ dissociation at pH 9 therefore fully confirmed the conclusions drawn from the experiments performed at pH 6.

Discussion

Using rapid filtration techniques, the kinetics of calcium dissociation from phosphorylated ATPase toward the lumenal side of leaky vesicles were explored by three methods in the present work; this dissociation corresponded to ion internalization during pump turnover in tight vesicles. The first method was perfusion of $^{45}Ca^{2+}$-equilibrated leaky vesicles with a solution containing $^{45}Ca^{2+}$ and ATP and measurement of the approach to steady state [circles in Figure 1A; see also Champeil and Guillain (1986)]. The second method was perfusion with $^{40}Ca^{2+}$ (or EGTA) and ATP of leaky SR vesicle ATPase which had previously reached steady state in the presence of $^{45}Ca^{2+}$ and ATP [squares in Figure 1B, triangles in Figure 1, and Figure 2; see also Wakabayashi et al. (1986)]. The third procedure consisted of perfusing $^{45}Ca^{2+}$-equilibrated leaky vesicles with a solution containing ATP and either $^{40}Ca^{2+}$ or EGTA (Figures 3, 5, 6, and 7B); the trapping of bound $^{45}Ca^{2+}$ which resulted, and which was also used in recent single-turnover studies (Inesi, 1987; Petithory & Jencks, 1988a,b), occurred because phosphorylation was much faster than calcium dissociation toward the cytoplasmic side, as previously shown by many authors (Rauch et al., 1978; Sumida et al., 1978).

Taking into account the variations between individual SR preparations [see Experimental Procedures and Orlowski and Champeil (1991)], all three methods gave rate constants for $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase consistent with one another (about 3 s^{-1} at pH 6, 20 °C, in the absence of KCl and in the presence of 20 mM Mg^{2+}) and with the overall ATPase activity of leaky SR or purified ATPase under the same conditions [about 6 nmol/(mg·s), see Champeil et al. (1986); this hydrolysis rate is much lower than the one measured under more usual conditions, for instance at pH 7, 25 °C, and in the presence of KCl]. In agreement both with

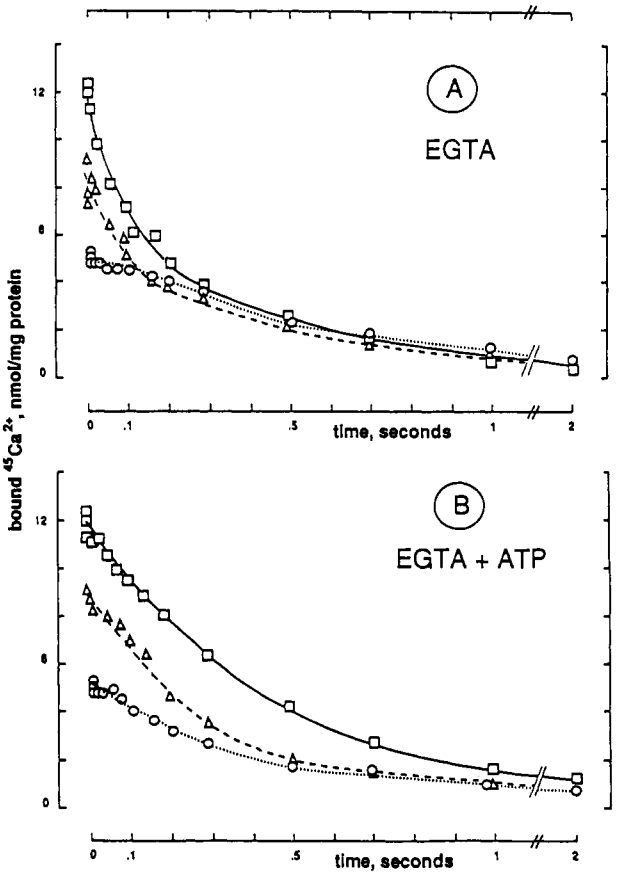

FIGURE 7: Comparison, at pH 9 in the presence of KCl, of the rates of $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPase (panel A) and phosphorylated ATPase (panel B) for the two pools of bound calcium. SR vesicles were first equilibrated in a medium containing 20 mM Mg^{2+}, 100 mM KCl, 50 mM Mes-Tris at pH 6, 4% A23187, and either 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose (squares and circles) or 0.2 mM $^{40}Ca^{2+}$ (triangles), and 0.3 mg of protein was then layered onto HA filters. For partial isotopic exchange (circles and triangles), the loaded filters were manually perfused for a few seconds with a solution containing 20 mM Mg^{2+}, 100 mM KCl, 50 mM Mes-Tris at pH 6, and either 2 mM $^{40}Ca^{2+}$ (circles) or 0.1 mM $^{45}Ca^{2+}$ and 1 mM [^{3}H]glucose (triangles). The filters were then immediately perfused for various periods with a solution containing 50 mM Tris-HCl at pH 9, 100 mM KCl, 20 mM Mg^{2+}, and 2 mM EGTA, either in the presence of 2 mM Mg·ATP (panel B) or in its absence (panel A). For the control experiments (squares), the loaded filters were directly perfused with the same final perfusion solutions without prior isotopic exchange.

our previous findings and with those of other filtration studies performed at low temperature and neutral pH (Champeil & Guillain, 1986; Wakabayashi et al., 1986), all three methods failed to reveal any very fast phase in the time course of $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase. These results contrast with those of a recent multimixing study, in which $^{45}Ca^{2+}$ internalization was found to comprise an initial rapid phase followed by a slower rate-limiting one; in that study, a single enzyme cycle was observed by adding $^{40}Ca^{2+}$ + ATP to $^{45}Ca^{2+}$-equilibrated sealed SR vesicles, followed by an ADP + EGTA quench (Inesi, 1987).

A critical question about our experiments is whether, in the presence of 0.04 g of A23187 ionophore per gram of protein, the vesicle passive permeability to calcium was high enough to ensure that calcium in the vesicle lumen reequilibrated with the external medium rapidly, compared to the rate at which calcium was released from the phosphorylated ATPase toward the internal side of the vesicles. We think that this was the case, because (i) neither transient accumulation nor any delay in the reduction of vesicle-associated $^{45}Ca^{2+}$ was observed upon

[2] Calcium dissociation toward the cytoplasmic side at pH 9 in the presence of EGTA and Mg^{2+} (squares in Figure 7A) was not monophasic, in contrast with what was previously observed at pH 6 or 7 [see Figure 4A and Orlowski and Champeil (1991)]; this was also observed in separate experiments performed at pH 8 in the presence of 20 mM magnesium. However, in that case, removal of magnesium from the alkaline perfusion medium accelerated the dissociation kinetics and made them virtually monophasic once again (data not shown). Since magnesium is known to reduce the ATPase apparent affinity for calcium, possibly by binding to a calcium site (Guillain et al., 1982; Loomis et al., 1982; Champeil et al., 1983), one may speculate that, under these alkaline conditions, magnesium binding to the superficial Ca^{2+} site after departure of the first radioactive calcium ion was strong enough to partially slow down dissociation of the second radioactive calcium ion, just as unlabeled calcium itself would have done. This would also explain why the distinction between the dissociation kinetics of the two pools of bound calcium was clearer at pH 9 (Figure 7A) than at pH 6 (Figure 4A).

perfusion of ionophore-treated vesicles with ATP + $^{45}Ca^{2+}$ (see Figure 1A, open circles), which implies that the half-time for equilibration of Ca^{2+} between lumen and medium was *much* shorter than the half-time for enzyme turnover—in relation to that, 4% w/w A23187 was *several-fold higher* than the amount of A23187 ionophore required to fully release inhibition of ATPase activity by accumulated Ca^{2+} (data not shown)—and (ii) using membranes of purified ATPase which are spontaneously leaky to calcium and devoid of any internal or external compartimentalization, fast dissociation of calcium from phosphorylated purified ATPase was not detected either (Champeil et al., 1986; Wakabayashi et al., 1986); moreover, in those experiments as well as in experiments performed with A23187-treated SR, the rate of $^{45}Ca^{2+}$ dissociation from phosphorylated membranes was modulated by ATP (data not shown and the above references). These results exclude that the observed rate reflected the ionophore-mediated membrane permeability instead of the true rate of Ca^{2+} dissociation from phosphoenzyme. As an additional argument, experiments with detergent-solubilized ATPase in which the changes in medium Ca^{2+} were monitored with murexide failed to reveal very fast dissociation of a fraction of the bound Ca^{2+} (Andersen, 1989).

The above facts therefore make it unlikely that too slow ionophore-mediated equilibration between internal and external calcium in our perfusion experiments, or any other artifact due to the presence of the ionophore, could alter our conclusion that Ca^{2+} dissociation from phosphorylated ATPase is monophasic. We have no explanation for this experimental discrepancy with the previous report that very fast dissociation of one Ca^{2+} toward the lumen occurs after ATPase phosphorylation (Inesi, 1987). The dead time of the filtration equipment prevented us from exploring exactly the same experimental conditions as in the latter work, in which fast multimixing equipment was used as opposed to our measurements in which the amount of bound $^{45}Ca^{2+}$ was measured directly. However, our data are consistent with other recent measurements which also failed to detect rapid dissociation of one of the two Ca^{2+} ions [Hanel and Jencks (1991) and the preceding paper in this issue].

On the cytoplasmic side of SR vesicles, dissociation of $^{45}Ca^{2+}$ from nonphosphorylated ATPase, which is usually monophasic in the presence of EGTA (Petithory & Jencks, 1988a; Orlowski & Champeil, 1991), is known to become markedly biphasic when $^{40}Ca^{2+}$ is present in the cytoplasmic medium [inset to Figure 4A; see Dupont (1982, 1984), Nakamura (1986, 1987), Inesi (1987), Petithory and Jencks (1988b), and Orlowski and Champeil (1991)]. This was the basis for the suggestion that the two Ca^{2+} ions bound to nonphosphorylated ATPase reside in a narrow pocket, or channel, from which they can only dissociate in an ordered sequential way toward the cytoplasmic medium (Inesi, 1987; Petithory & Jencks, 1988a; Orlowski & Champeil, 1991). Khananshvili and Jencks (1988) suggested that the pattern could be the same on the lumenal side when Ca^{2+} ions dissociate from the phosphorylated ATPase. We therefore aimed at confirming this, but we were unable to do so.

In our experiments, we did observe a deviation from monophasic kinetics when $^{45}Ca^{2+}$ dissociation from Ca_2E_1P was monitored in the presence of 30 mM $^{40}Ca^{2+}$ (Figure 2), but we attributed it to progressive substitution of $^{40}Ca^{2+}$ for Mg^{2+} at the phosphorylation site (step 5 in the inset to Figure 2A) rather than to the action of calcium at the transport sites on the lumenal side of the phosphorylated ATPase. The main reason for rejecting the latter possibility was that, at pH 9, the large pH-induced enhancement expected in the affinity for calcium of the transport sites (Verjovski-Almeida & de Meis, 1977) should have made any obligatory ordered sequential dissociation from phosphorylated ATPase result in markedly biphasic kinetics, with a one-to-one ratio between the amplitudes of the two phases, whereas this was not the case in our experiments (closed triangles in Figure 2B). Similarly, in experiments previously performed at pH 9 and low temperature, the effect of 10 mM $^{40}Ca^{2+}$ was found to be only moderate (Wakabayashi et al., 1986). The nonmonophasic pattern observed in both studies was consistent with a gradual slowing down of the calcium dissociation rate resulting from the substitution of calcium for magnesium at the phosphorylated ATPase catalytic site, because it was found that the rate of metal exchange at the catalytic site (step 5 in the diagram of the inset to Figure 2A) was of the same order of magnitude as the rate of calcium dissociation from transport sites on Mg^{2+}-containing phosphoenzyme (step 2 of this diagram), especially in the presence of low amounts of ADP (Yamada et al., 1986; Wakabayashi & Shigekawa, 1987; Lund & Møller, 1988; Orlowski et al., 1988). In fact, in our experiments, a high concentration of $^{40}Ca^{2+}$ was even less efficient in slowing down $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase when a regenerating system was included in the perfusion medium to reduce the concentration of contaminating ADP (see Results). Consequently, the data favor the conclusion that a high lumenal calcium concentration per se does not inhibit dissociation of any of the two ions transported. It is fair to say, however, that metal exchange at the catalytic site makes the results of this type of experiment with leaky vesicles less clear-cut than measurements of the rate of calcium internalization into sealed vesicles with a well-controlled internal Ca^{2+} concentration. Nevertheless, the same conclusion was recently reached by A. Hanel and W. P. Jencks after a repetition of direct internalization measurements [Hanel and Jencks (1991) and the preceding paper in this issue].

As an independent means of checking whether the monophasic calcium dissociation from phosphorylated ATPase took place through an ordered sequential mechanism or not, we decided to measure separately the kinetics of this dissociation toward the lumenal side for each of the two ATPase-bound calcium ions. The two pools of calcium bound to nonphosphorylated ATPase could be labeled specifically, on the basis of the biphasic kinetics of isotopic exchange which was previously observed[3] (Dupont, 1982, 1984; Nakamura, 1986, 1987; Inesi, 1987; Petithory & Jencks, 1988b; Orlowski & Champeil, 1991). We first checked the kinetics of dissociation toward the cytoplasmic side for each pool and found that the two pools did dissociate from nonphosphorylated ATPase at different rates during perfusion with EGTA and that, for the pool of deeply bound $^{45}Ca^{2+}$ ions, dissociation was preceded by a lag (Figures 4A and 7A). A similar lag was also reported by Petithory and Jencks [Figure 8 in Petithory and Jencks (1988a)]. The different dissociation kinetics measured for the two calcium pools are perfectly consistent with the ordered sequential dissociation of the two bound calcium ions from a single binding pocket with a narrow channel-like structure, as illustrated in the diagram of Figure 4B [see also Orlowski

[3] Previous authors have considered the possibility that the asymmetry between the two Ca^{2+} sites might not be real but rather result from the interaction between two otherwise identical protein subunits (Ikemoto et al., 1981; Dupont, 1982). This hypothesis can in fact not account for biphasic isotopic exchange, because, if the fast escape of $^{45}Ca^{2+}$ from one protomer were prevented because of the occupation of the other protomer by $^{40}Ca^{2+}$, the initial fast escape of one $^{45}Ca^{2+}$ ion from the putative dimer would never be observed. The two calcium-binding sites in nonphosphorylated ATPase are therefore intrinsically different.

and Champeil (1991)]. Consequently, for the simplest single-file channel-like structure, it would be reasonable to expect the deeper Ca^{2+} ion, which dissociates slowly toward the cytoplasmic or external side of the SR vesicles, to be the first to dissociate toward the lumenal or internal side during pump turnover (see the center diagram in Figure 5B). However, Figures 5A, 6A, and 7B show that such was not the case, because the superficial $^{45}Ca^{2+}$ ion (triangles) never dissociated more slowly than the deeply bound ion (circles), even in the presence of a very high concentration of $^{40}Ca^{2+}$. The two ions, which are distinguishable in the nonphosphorylated ATPase, can no longer be kinetically distinguished when they are released toward the lumen.

More careful examination of the data is required here. We already pointed out that Figures 5, 6, and 7B show that the superficial $^{45}Ca^{2+}$ ion (triangles) never dissociated from phosphorylated ATPase more slowly than the deeply bound ion (circles). In some of these curves, it might even seem that the overall half-time for complete dissociation of the deeply bound calcium pool (circles) was slightly longer than the half-time for dissociation of the superficially bound calcium pool (triangles), so that, on this basis, a "first-in–last-out" mechanism could have been suggested instead of "first-in–first-out" (since the two ions bound to nonphosphorylated ATPase cannot exchange position, the deeply bound ion must be the one which has bound first). However, we think that this slight difference between overall half-times, if any, was not due to the presence of such a mechanism. In fact, some of these dissociation curves comprise a slow component of small amplitude (e.g., squares in Figures 5A and 6A), which complicates the detailed analysis. As to the origin of this slower component of small amplitude, one could conjecture that the delayed dissociation of a fraction of the calcium pool evidenced by this component was due to the insertion of a fraction of the ATPases into vesicles which were formed inside-out during the SR homogenization procedure: in the experiments in which $^{45}Ca^{2+}$-equilibrated vesicles were perfused with an ATP-containing medium, the ATPases in the inside-out vesicles were probably not immediately phosphorylated by the impermeant ATP, as has been previously observed in some cases with reconstituted ATPase (Inesi et al., 1983). The existence of this small, ill-controlled proportion of ATPases inserted in vesicles with an unusual orientation was previously invoked to explain small deviations from monoexponential behavior in the kinetics of $^{45}Ca^{2+}$ dissociation from nonphosphorylated ATPases in the absence of calcium ionophore (Orlowski & Champeil, 1991).

Taking these restrictions into account, we therefore consider that our data (Figure 5, 6, and 7B) should be interpreted as indicating that the two pools of bound calcium are in fact released toward the lumenal side of the SR vesicles with virtually identical rate constants. In addition, these two pools cannot be distinguished on the basis of their sensitivity to a high lumenal calcium concentration (Figure 6B). In recent experiments, Hanel and Jencks also measured the rate of $^{45}Ca^{2+}$ internalization after selectively labeling the ions bound to the "deeper" and "superficial" calcium sites, respectively, and concluded that the two ions became kinetically indistinguishable after ATPase phosphorylation [Hanel and Jencks (1991) and the preceding paper in this issue]. The experimental results of that work and those of the present report therefore concur in excluding the possibility of distinguishing which ion is released first toward the SR lumen.

At this point, one should also recall that related lines of research have been developed by Forbush to characterize deocclusion of K^+ ions in Na^+,K^+-ATPase (Forbush, 1987). In those experiments, the two K^+ sites were identified by a double incubation sequence under conditions of phosphorylation from P_i. Although the two sites differed with respect to the rate at which the bound ions were released in the presence of P_i, no kinetic difference between them was observed when deocclusion was promoted by Na^+ and ATP addition and consequent return to the nonphosphorylated so-called E_1 form. Beyond apparent differences in these dissociation kinetics, it appears that a common feature of the Ca^{2+}-ATPase and the Na^+,K^+-ATPase is that dissociation of the transported ions is ordered sequential under conditions where these ions bind to their transport sites with high affinity, whereas the two ions can no longer be kinetically distinguished under conditions where they bind to the ATPase with low affinity.

These findings can now be discussed in relation to their possible structural implications. Our results might be tentatively interpreted in terms of independent but virtually simultaneous direct dissociation of the two Ca^{2+} ions from the phosphorylated ATPase toward the SR lumen. If the observed curves did reflect true rates of $^{45}Ca^{2+}$ dissociation from their binding sites, the virtually identical rates of dissociation of the two ions and the absence of inhibition by lumenal calcium of any of these rates would imply that once the transport sites are reoriented toward the lumenal side of the vesicles, the calcium-binding pocket is no longer narrow enough to impose *ordered* dissociation of the two bound ions. Conceivably, the putative transmembrane helices which create the narrow binding pocket in nonphosphorylated ATPase could part after phosphorylation, so that the binding pocket would become wider, permitting the two Ca^{2+} ions to rapidly "mix" on the phosphoenzyme (Hanel & Jencks, 1991) before sequential dissociation, or even permitting independent dissociation of the two ions toward the lumen.[4] The widening of the Ca^{2+}-binding pocket would presumably simultaneously reduce its affinity for calcium, as required for active transport (Tanford et al., 1987). Significant structural rearrangement of the transmembrane section of the ATPase has been suggested to occur on phosphorylation from P_i [e.g., see de Foresta et al. (1990), and references therein], and a less compact arrangement of the bundle of transmembrane helices forming the putative transport sites might account for the remarkable instability of the solubilized and delipidated ATPase in its phosphorylated E_2P conformation (Lund et al., 1989). Anyhow, such speculations would imply significant phosphorylation-induced changes in the structure of the Ca^{2+}-binding pocket, making doubtful its close resemblance with the interior of a single-file channel.

Alternatively, it is also possible that the observed rate of $^{45}Ca^{2+}$ dissociation does not reflect the rate of Ca^{2+} dissociation per se but instead reflects the rate of some preliminary slow reorganization of the protein structure, leading to a conformation and an accessibility of the Ca^{2+} sites allowing fast dissociation of Ca^{2+} from these sites toward the lumen. To interpret the simultaneous intracellular deocclusion of the two K^+ ions observed on addition of Na^+ and ATP to Na^+,K^+-ATPase, Forbush already suggested that a conformational change of the whole protein, the so-called "E_2 to E_1" transition, was rate limiting in this process (Forbush, 1987). If such a

[4] Note that, at pH 9 and low temperature, Wakabayashi et al. (1986) found that $^{45}Ca^{2+}$ dissociation from phosphorylated ATPase in a medium containing EGTA somehow deviated from monoexponential behavior. The exact location of the lumenal gate with respect to the bound ions might allow to account for such small differences in the dissociation rates for the two ions.

preliminary slow conformational rearrangement precedes the actual fast dissociation of Ca^{2+} from phosphorylated ATPase, it places limitations on the previous discussion about the putative changes occurring in the Ca^{2+}-binding pocket as a result of phosphorylation. For instance, 30 mM Ca^{2+} might not be high enough to saturate the potentially blocking lumenal subsite of this binding pocket, because we do not know the true affinity of this subsite from which Ca^{2+} dissociates toward the lumen (we only know the apparent affinity with which lumenal Ca^{2+} drives the catalytic cycle backward, which combines true dissociation constants and the equilibrium constant of the conformational rearrangement). In addition, if the actual dissociation rate of both ions is very fast, we do not know whether the slowing down of the rate of dissociation of the second ion induced by the rebinding of one calcium at the lumenal subsite will be large enough to affect in a detectable way the overall rate of calcium dissociation, which, according to this hypothesis, is mainly limited by the rate of the preliminary reorganization. Therefore, from our data, we cannot completely exclude that sequential dissociation from phosphorylated ATPase still occurs. Finally, in terms of models where the ion-binding pocket is "occluded" most of the time, and only transiently accessible to the medium (Forbush, 1987; Orlowski & Champeil, 1991), it might be that, in phosphorylated ATPase, the lumenal gate which limits access to the binding pocket remains open long enough for *both* ions to leave the pocket, in contrast with nonphosphorylated ATPase in which the cytoplasmic gate closes very rapidly after its opening, thus only allowing exit of one of the two bound ions at a time. A different balance between gate opening rates and closure rates in phosphorylated versus nonphosphorylated ATPase could also account for part of the difference in the apparent ATPase affinity for Ca^{2+} of these two ATPase states without requiring changes in the true Ca^{2+}-binding strength within the pocket.

Anyhow, it seems likely that dissociation of the two Ca^{2+} ions indeed comprises some preliminary reorganization of the protein structure. The reason for this view is the documented existence of several experimental situations where, by using Cr·ATP as the substrate (Vilsen & Andersen, 1986), by cross-linking the active site with glutaraldehyde (McIntosh et al., 1991), or by mutating the ATPase at positions critical for conformational rearrangements [e.g., Gly 233 or Pro 312, see Andersen et al. (1989) and Vilsen et al. (1989)], the ATPase can be phosphorylated in a stable state from which the bound calcium ions can probably no longer dissociate toward the lumenal medium. The existence of a state in which the Ca^{2+} ions are occluded from the medium on both sides of the ATPase is therefore likely. This strictly occluded state would be the fundamental state of the "Ca_2E_1P" (sometimes called "$Ca_2E\sim P$") ATPase. The above-mentioned reorganization of the protein structure allowing dissociation of the two ions might then comprise the transient opening toward the lumenal medium of the calcium-binding pocket. However, it is not yet clear how fast this reorganization is, and whether it should be called a "Ca_2E_1P to Ca_2E_2P" transition (Pickart & Jencks, 1984). As regards the latter issue, it seems that the large-scale protein movements comprising the so-called "E_2 to Ca_2E_1" transition and those allowing the calcium-binding sites to become exposed to the bulk water medium on the lumenal side of the membrane do involve different molecular mechanisms, as these two events are affected in different ways by various modifiers of ATPase function like dimethyl sulfoxide, nonylphenol, perturbing detergents, or cross-linking agent at the active site (unpublished data). Anyhow, the ATPase state from which Ca^{2+} ions would dissociate toward the lumenal side (let us call it "$Ca_2E\#P$", with no subscript) would only be transient.

Finally, we have to address the question of how dissociation of two Ca^{2+} ions from the open "$Ca_2E\#P$" state can be reconciled with the report that lumenal calcium inhibits ATPase activity with a Hill coefficient close to 1 (Khananshvili et al., 1990; Hanel & Jencks, 1991; and the preceding paper in this issue). In our view, this result could imply that binding to phosphoenzyme of only one calcium on the lumenal side is sufficient to slow down hydrolysis, or, in other words, that the phosphoenzyme is not able to react with water and be hydrolyzed as long as there is one calcium left on the transport sites ($Ca_2E\#P$ or $CaE\#P$). In fact, this would ensure a constant ratio of 2 between transported Ca^{2+} ions and hydrolyzed ATP molecules. The putative conformational signal from the transport sites to the catalytic site responsible for chemical specificity (resulting in formation of the true E_2P species) would therefore be transmitted *after* both ions have dissociated ($E\#P$ to E_2P). Similarly, we previously suggested that transition of nonphosphorylated ATPase from its form with high affinity for Ca^{2+} to its form with low affinity for Ca^{2+} only takes place after both Ca^{2+} ions have left their sites (Orlowski & Champeil, 1991).

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References

Andersen, J. P. (1989) *Biochim. Biophys. Acta 988*, 47–72.
Andersen, J. P., Vilsen, B., Leberer, E., & MacLennan, D. H. (1989) *J. Biol. Chem. 264*, 21018–21023.
Barrabin, H., Scofano, H., & Inesi, G. (1984) *Biochemistry 23*, 1542–1548.
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. P., Lund, S., & Møller, J. V. (1986) *J. Biol. Chem. 261*, 16372–16384.
de Foresta, B., Champeil, P., & le Maire, M. (1990) *Eur. J. Biochem. 194*, 383–388.
de Meis, L., & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.
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.
Forbush, B. (1987) *J. Biol. Chem. 262*, 11116–11127.
Gafni, A., & Boyer, P. D. (1984) *Biochemistry 23*, 4362–4367.
Guillain, F., Gingold, M. P., & Champeil, P. (1982) *J. Biol. Chem. 257*, 7366–7371.
Hanel, A., & Jencks, W. P. (1991) *Biophys. J. 59*, 561a.
Hasselbach, W. (1974) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., pp 432–467, Academic Press, New York.
Ikemoto, N. (1975) *J. Biol. Chem. 250*, 7219–7224.
Ikemoto, N. (1982) *Annu. Rev. Physiol. 44*, 297–317.
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.
Inesi, G., Nakamoto, R., Hymel, L., & Fleischer, S. (1983) *J. Biol. Chem. 258*, 14804–14809.
Jencks, W. P. (1989) *J. Biol. Chem. 264*, 18855–18858.
Khananshvili, D., & Jencks, W. P. (1988) *Biochemistry 27*, 2943–2952.
Khananshvili, D., & Jencks, W. P. (1990) *Biochemistry 29*, 4030.
Khananshvili, D., Myung, J., Kolouch, R., & Jencks, W. P. (1990) *FEBS Lett. 260*, 83–84.
Kurzmack, M., Verjovski-Almeida, S., & Inesi, G. (1977) *Biochem. Biophys. Res. Commun. 78*, 772–776.
Loomis, C. R., Markin, D. W., MacCaslier, D. R., & Tanford, C. (1982) *Biochemistry 21*, 151–156.
Lund, S., & Møller, J. V. (1988) *J. Biol. Chem. 263*, 1654–1664.
Lund, S., Orlowski, S., de Foresta, B., Champeil, P., le Maire, M., & Møller, J. V. (1989) *J. Biol. Chem. 264*, 4907–4915.
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.
Makinose, M. (1973) *FEBS Lett. 37*, 140–143.
McIntosh, D. B., Ross, D. C., Champeil, P., & Guillain, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6437–6441.
Møller, J. V., Andersen, J. P., & le Maire, M. (1982) *Mol. Cell. Biochem. 42*, 83–107.
Nakamura, J. (1986) *Biochim. Biophys. Acta 870*, 495–501.
Nakamura, J. (1987) *J. Biol. Chem. 262*, 14492–14497.
Nakamura, J. (1989) *J. Biol. Chem. 264*, 17029–17031.
Nakamura, Y. (1984) *J. Biol. Chem. 259*, 8183–8189.
Orlowski, S., & Champeil, P. (1991) *Biochemistry 30*, 352–361.
Orlowski, S., Lund, S., Møller, J. V., & Champeil, P. (1988) *J. Biol. Chem. 263*, 17576–17583.
Petithory, J. R., & Jencks, W. P. (1988a) *Biochemistry 27*, 5553–5564.
Petithory, J. R., & Jencks, W. P. (1988b) *Biochemistry 27*, 8626–8535.
Pickart, C. M., & Jencks, W. P. (1984) *J. Biol. Chem. 259*, 1629–1643.
Rauch, B., von Chak, D., & Hasselbach, W. (1978) *FEBS Lett. 93*, 65–68.
Shigekawa, M., & Akowitz, A. A. (1979) *J. Biol. Chem. 254*, 4726–4730.
Soler, F., Teruel, F. A., Fernandez-Belda, F., & Gomez-Fernandez, J. C. (1990) *Eur. J. Biochem. 192*, 347–354.
Sumida, M., Wang, T., Mandel, F., Froehlich, J. P., & Schwartz, A. (1978) *J. Biol. Chem. 253*, 8772–8777.
Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol. Rev. 58*, 1–79.
Tanford, C. (1984) *CRC Crit. Rev. Biochem. 17*, 123–151.
Tanford, C., Reynolds, J. A., & Johnson, E. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7094–7098.
Verjovski-Almeida, S., & de Meis, L. (1977) *Biochemistry 16*, 329–334.
Vilsen, B., & Andersen, J. P. (1986) *Biochim. Biophys. Acta 855*, 429–439.
Vilsen, B., Andersen, J. P., Clarke, D. M., & MacLennan, D. H. (1989) *J. Biol. Chem. 264*, 21024–21031.
Wakabayashi, S., & Shigekawa, M. (1987) *J. Biol. Chem. 262*, 11524–11531.
Wakabayashi, S., Ogurusu, T., & Shigekawa, M. (1986) *J. Biol. Chem. 261*, 9762–9769.
Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C., & Inesi, G. (1981) *Biochemistry 20*, 6617–6625.
Yamada, S., Fujii, J., & Katayama, H. (1986) *J. Biochem. (Tokyo) 100*, 1329–1342.
Yamaguchi, M., & Kanazawa, T. (1985) *J. Biol. Chem. 259*, 9526–9531.