Roles of Phosphorylation and Nucleotide Binding Domains in Calcium Transport by Sarcoplasmic Reticulum Adenosinetriphosphatase†

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ABSTRACT: The roles of the phosphorylation (phosphorylated enzyme intermediate) and nucleotide binding domains in calcium transport were studied by comparing acetyl phosphate and ATP as substrates for the Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles. We found that the maximal level of phosphoenzyme obtained with either substrate is approximately 4 nmol/mg of protein, corresponding to the stoichiometry of catalytic sites in our preparation. The initial burst of phosphoenzyme formation observed in the transient state, following addition of either substrate, is accompanied by internalization of 2 mol of calcium per mole of phosphoenzyme. The internalized calcium is then translocated with a sequential pattern, independent of the substrate used. Following a rate-limiting step, the phosphoenzyme undergoes hydrolytic cleavage and proceeds to the steady-state activity which is soon "back inhibited" by the rise of Ca^{2+} concentration in the lumen of the vesicles. When the "back inhibition" is released by the addition of oxalate, substrate utilization and calcium transport occur with a ratio of 1:2, independent of the substrate and its concentration. When the nucleotide binding site is derivatized with FITP, the enzyme can still utilize acetyl phosphate (but not ATP) for calcium transport. No secondary activation of acetyl phosphate utilization by the FITC–enzyme was obtained with millimolar nucleotide. These observations demonstrate that the basic coupling mechanism of catalysis and calcium transport involves the phosphorylation and calcium binding domains, and not the nucleotide binding domain. On the other hand, occupancy of the FITC-sensitive nucleotide site is involved in kinetic regulation not only with respect to utilization of substrate for the phosphoryl transfer reaction but also for subsequent steps related to calcium translocation and phosphoenzyme turnover. In well-coupled SR vesicles, following the initial burst of phosphoenzyme formation, turnover proceeds with a rate of 1 s^{-1} when the substrate is acetyl phosphate, as opposed to 10 s^{-1} when the substrate is ATP.

The mechanism of calcium transport across the sarcoplasmic reticulum (SR) membrane comprises a number of partial reactions [for a review, see Inesi (1985)], including high-affinity calcium binding to the ATPase, binding and utilization of ATP by transfer of its terminal phosphate onto the enzyme, vectorial translocation and dissociation of the bound calcium against a concentration gradient, and hydrolytic cleavage of the phosphorylated enzyme intermediate, as in

$$E + 2Ca^{2+}_{out} \rightleftharpoons E{\cdot}Ca_2 \quad (1)$$

$$E{\cdot}Ca_2 + ATP \rightleftharpoons ATP{\cdot}E{\cdot}Ca_2 \quad (2)$$

$$ATP{\cdot}E{\cdot}Ca_2 \rightleftharpoons ADP + E{-}P{\cdot}Ca_2 \quad (3)$$

$$E{-}P{\cdot}Ca_2 \rightleftharpoons E{-}P + 2Ca^{2+}_{in} \quad (4)$$

$$E{-}P \rightleftharpoons E{\cdot}P_i \quad (5)$$

$$E{\cdot}P_i \rightleftharpoons E + P_i \quad (6)$$

Kinetic resolution of the first catalytic and transport cycle, following addition of ATP, provided the experimental evidence indicating that a basic coupling step is the phosphoryl transfer from ATP to the enzyme (Inesi et al., 1978). In fact, formation of the phosphorylated intermediate is rapidly followed by (and is required for) internalization and vectorial translocation of 2 mol of bound calcium per mole of enzyme. Furthermore, a reduction of the enzyme affinity for Ca^{2+} is observed when the catalytic site is occupied by vanadate (Dupont & Bennett, 1982; Medda & Hasselbach, 1983) or P_i (de Meis & Inesi, 1985), suggesting that the phosphoryl group, and not the adenosine moiety, is involved in the coupling mechanism. Some degree of uncertainty, however, still remains, since substrates other than ATP were reported to sustain transport with variable and less favorable stoichiometric ratios (Rossi et al., 1979). Furthermore, the stoichiometry of the phosphorylated enzyme intermediate has been reported to be greater when substrates other than ATP were used (Friedman & Makinose, 1970; Pucell & Martonosi, 1971). Finally, a secondary effect of ATP on the enzyme turnover has been observed in early studies on the SR ATPase (Inesi et al., 1967; Yamamoto & Tonomura, 1967).

Information on the functional roles of phosphorylation and nucleotide binding domains is rendered more cogent by the mechanistic hypotheses that were suggested recently, based on the structure of these domains (Green et al., 1986).

Acetyl phosphate is a non-nucleotide phosphorylated substrate of SR ATPase (de Meis, 1969; Friedman & Makinose, 1970; Pucell & Martonosi, 1971) which has been recently the subject of detailed characterization with respect to the kinetics of catalysis (Bodley & Jencks, 1987). We have then conducted a series of experiments in order to characterize the coupling mechanism of catalytic and transport activities when acetyl phosphate, as compared to ATP, is used as the substrate.

Materials and Methods

[^{32}P]P_i, [γ-^{32}P]ATP, and $^{45}CaCl_2$ were purchased from New England Nuclear. The calcium ionophore A23187 was from Calbiochem, and all other reagents were from Sigma.

Sarcoplasmic reticulum vesicles were prepared from rabbit hind leg muscle as described previously (Eletr & Inesi, 1972), and the protein concentration was determined according to the procedure of Lowry et al. (1951) using bovine serum albumin as standard. Leaky vesicles were obtained by addition

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of 10 μM calcium ionophore A23187 to native sarcoplasmic reticulum vesicles.

SR vesicles were labeled with FITC (Pick & Karlish, 1980) as follows: The vesicles (2 mg of protein/mL) were incubated in a medium containing 0.2 M sucrose, 80 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, 25 mM Na-glycine, pH 9.2, 0.1 mM EGTA, and 10 μM FITC (from a daily prepared, 4 mM stock solution in dimethylformamide) for 10 min at room temperature. The labeling reaction was stopped by transferring a 1-mL sample through a Sephadex G-50 column (0.65 × 20 cm) preequilibrated with ice-cold stopping solution containing 20 mM MOPS, pH 6.8, 80 mM KCl, and 5 mM $MgCl_2$. The stoichiometry of labeling was measured in the presence of 1% SDS and 0.1 M NaOH, using $\epsilon_{495} = 8 \times 10^4$ M^{-1} cm^{-1}.

Free calcium concentration was estimated from total calcium and total EGTA concentrations (Fabiato & Fabiato, 1979) taking into account pH and Mg^{2+}, K^+, P_i, and nucleotide concentrations.

Carrier-free [^{32}P]P_i was purified before use as described by de Meis and Tume (1977). Acetyl [^{32}P]phosphate was synthesized according to the method of Kornberg et al. (1956).

All the experiments were carried out at a constant temperature of 25 °C.

Acetyl phosphatase activity was assayed by measuring the unhydrolyzed acetyl phosphate as described by Lipmann and Tuttle (1945). Ca^{2+}-dependent ATPase activity was measured by a colorimetric determination of inorganic phosphate as described by Lin and Morales (1977).

Phosphorylation of sarcoplasmic reticulum vesicles was achieved in a medium containing 50 mM MOPS, pH 6.8, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1.05 mM $CaCl_2$, and acetyl [^{32}P]phosphate or [γ-^{32}P]ATP concentrations as stated in the figures. The phosphorylation reaction was quenched by addition of 0.25 M TCA and 13 mM P_i. The quenched samples were cooled down in ice and washed and centrifuged repeatedly, and the final pellets were dissolved for determination of radioactivity and protein concentration.

Ca^{2+} uptake experiments were performed with a reaction mixture identical with that used for enzyme phosphorylation, but using ^{45}Ca tracer instead of ^{32}P. Quenching of the Ca^{2+} uptake reaction at serial times was obtained by addition of a 10 mM $LaCl_3$, 20 mM MOPS, pH 6.8, and 80 mM KCl solution. An aliquot of the quenched reaction mixture (corresponding to 40–60 μg of protein) was filtered (Millipore, 0.45-μm pore size), and the vesicles remaining on the filters were washed with 4 mL of the quenched solution. The amount of ^{45}Ca in the filter was determined by scintillation counting. Other conditions are described in the figures.

Rapid mixing experiments were carried out with the aid of a Froehlich–Berger chemical quench-flow apparatus, with temperature control set at 25 °C.

Results

Substrate Utilization for Enzyme Phosphorylation. The maximal level of phosphorylated enzyme intermediate obtained with *ATP* as a substrate in the forward direction of the cycle, or with P_i in the reverse direction of the cycle, is approximately 4 nmol/mg of protein in our preparation of SR vesicles. This is in contrast with the higher levels of phosphoenzyme observed in early experiments in which *acetyl phosphate*, rather than *ATP*, was used as the substrate (Friedman & Makinose, 1970; Pucell & Martonosi, 1971). On the other hand, when we used *acetyl phosphate* rather than *ATP*, we found maximal levels varying between 3.0 and 4.0 nmol/mg of protein, in agreement with those obtained with ATP. The highest levels were obtained at relatively high acetyl phosphate concentrations

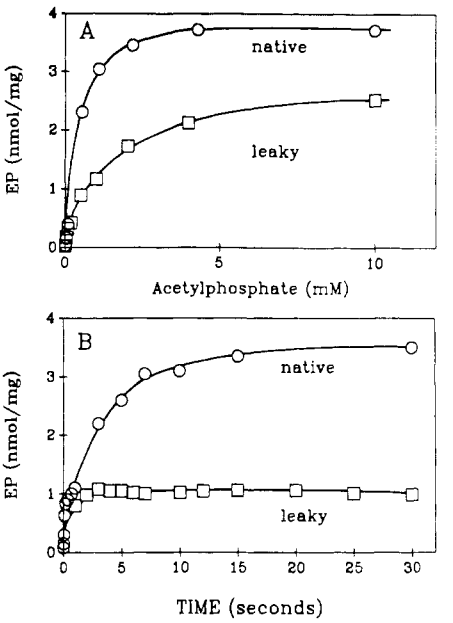

FIGURE 1: Formation of phosphorylated enzyme intermediate with acetyl phosphate as the substrate. Concentration (A) and time (B) dependence. The reaction was carried out with native (○) or leaky (□) vesicles in a medium containing 50 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1.05 mM $CaCl_2$, and 1 mg of SR protein/mL. When indicated (□), the SR vesicles were rendered leaky by the addition of 10 μM A21387 to the medium. The incubation was started by adding various concentrations (A) or 1 mM (B) acetyl [^{32}P]phosphate and carried out for 10 s (A) or variable times (B). Quenching was obtained by the addition of TCA and P_i to final concentrations of 0.25 M and 2 mM, respectively.

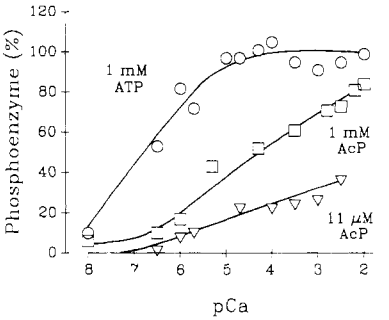

FIGURE 2: Steady-state levels of phosphoenzyme in the presence of various Ca^{2+} concentrations, using 1 mM [γ-^{32}P]ATP (○), 1 mM acetyl [^{32}P]phosphate (□), or 11 μM acetyl [^{32}P]phosphate (▽) as the substrate. The phosphorylation reaction was carried out with leaky SR vesicles, for 10 s, as explained in the legend to Figure 1. The concentration of EGTA was 1 mM, and total calcium was adjusted to yield free Ca^{2+} concentrations indicated in the figure. The maximal (100%) level of phosphoenzyme in this experiment corresponds to 4 nmol/mg of protein.

(Figure 1), when the vesicles were allowed to fill with calcium as a consequence of active transport (Figure 1A,B). If filling was prevented by rendering the vesicles leaky, the phosphoenzyme levels were considerably lower (Figure 1). These findings can be explained with inhibition of phosphoenzyme cleavage by accumulated product (high Ca^{2+} concentration inside the sealed vesicles).

It is shown in Figure 2 that even when leaky vesicles are used, high steady state of phosphoenzyme can be obtained by increasing the concentration of Ca^{2+} in the medium to levels (millimolar) that inhibit its hydrolytic cleavage (see below, Figure 5). This effect is less apparent when ATP (rather than acetyl phosphate) is used at high concentrations, since in this case the velocity of phosphoenzyme formation is very high and compensates for phosphoenzyme cleavage. The effect of

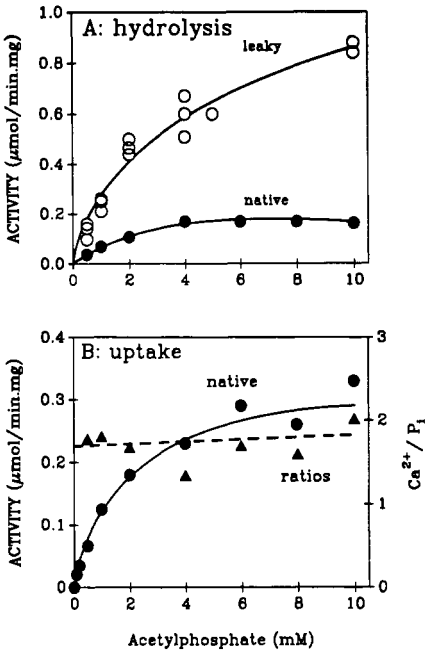

FIGURE 3: Rates of hydrolytic activity (A) and calcium uptake (B) in the presence of various concentrations of acetyl phosphate as the substrate. The reaction was carried out using native (●) or leaky (○) vesicles, in a medium containing 20 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1.05 mM $CaCl_2$, and 0.1 mg of SR protein/mL. When native vesicles were used (●), 5 mM oxalate was also present in the medium. When indicated (○), the SR vesicles were rendered leaky by the addition of 10 μM A23187 to the medium. The ratios between the rates of calcium uptake (B) (●) and acetyl phosphate hydrolysis (A) by native (●) vesicles at various acetyl phosphate concentrations are also shown (▲) in (B).

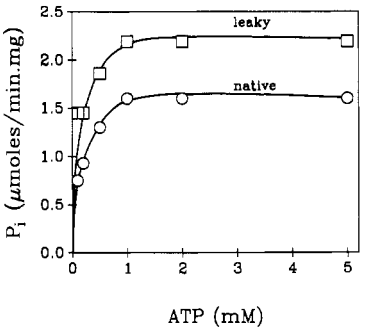

FIGURE 4: Rates of hydrolytic activity in the presence of various concentrations of ATP as the substrate. Experimental conditions for native (○) and leaky (□) SR vesicles are as described in the legend for Figure 3.

millimolar Ca^{2+} is more evident when acetyl phosphate or ATP at low concentrations is used, owing to a lower velocity of phosphoenzyme formation under these conditions.

Catalytic and Transport Activities in Steady State. Steady-state acetyl phosphate hydrolysis (Figure 3A) and calcium uptake (Figure 3B) by native vesicles in the presence of oxalate, as well as acetyl phosphate hydrolysis by leaky vesicles in the absence of oxalate (Figure 3A), were then measured at various acetyl phosphate concentrations. Half-maximal activity was obtained in the presence of 4 mM acetyl phosphate. The maximal velocity of hydrolysis was 1.2 μmol min^{-1} (mg of protein)$^{-1}$ (extrapolation of data in Figure 3A) when leaky vesicles were used. This value is significantly lower than observed with ATP (2.25 μmol mg^{-1} min^{-1}, as shown in Figure 4).

As observed with ATP, the steady-state utilization of acetyl phosphate by native vesicles is low, due to "back inhibition" by accumulated calcium. This inhibition is partially relieved

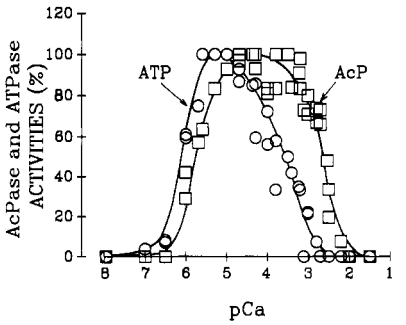

FIGURE 5: Ca^{2+} concentration dependence of hydrolytic activity in the presence of ATP (○) or acetyl phosphate (□) as the substrate. The reaction was carried out in the presence of 50 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, various concentrations of $CaCl_2$ to yield the free Ca^{2+} concentrations indicated in the figure, 10 μM A12387, and 0.1 mg of SR protein (leaky vesicles)/mL. Maximal (100%) values for hydrolytic activity correspond to 2.5 and 0.3 μmol min^{-1} mg^{-1}, in the presence of ATP or acetyl phosphate, respectively.

by oxalate which is known to form calcium–oxalate complex in the lumen of the vesicles (de Meis et al., 1974). In the presence of oxalate, the stoichiometric ratio between calcium transport and hydrolytic activity is approximately 2, throughout a wide range of acetyl phosphate concentrations (Figure 3B).

Ca^{2+} Concentration Dependence. It is well-known the SR ATPase requires activation by Ca^{2+} (micromolar range), while it is inhibited by higher concentrations (millimolar range) of Ca^{2+} (Ikemoto, 1975). We then compared the Ca^{2+} concentration dependencies of enzyme activation and inhibition with ATP or acetyl phosphate as the substrate. We found that when acetyl phosphate is used instead of ATP (Figure 5) both the activation and inactivation curves are displaced to somewhat higher concentrations, with half-maximal activation shifted from pCa 6.1 to pCa 5.9, and half-maximal inhibition shifted from 3.6 to 2.9. Similar results were recently obtained by Bodley and Jencks (1987), who attributed these changes to kinetic effects, related to the extent to which Ca^{2+}-sensitive partial reactions contribute to overall rate limitation when acetyl phosphate, as opposed to ATP, is used as the substrate.

Transient-State Enzyme Phosphorylation and Calcium Translocation. High-affinity binding of 2 mol of calcium per mole of enzyme is a strict requirement for substrate utilization by SR ATPase through phosphoryl transfer from the substrate to the enzyme. Addition of ATP to the calcium-activated enzyme is rapidly followed by phosphoenzyme formation and simultaneous internalization of bound calcium (i.e., the bound calcium becomes unavailable for displacement by quench reagents on the outer surface of the vesicles). Then, after a lag period related to slow Ca^{2+} dissociation inside the vesicles, the phosphoenzyme undergoes hydrolytic cleavage and starts a second cycle (Inesi et al., 1978).

We find now that the same pattern is obtained in the transient state following addition of acetyl phosphate to the calcium-activated enzyme. Extrapolation of the circle and triangle data to zero time in Figure 6 shows that rapid formation of 2 nmol of phosphoenzyme is accompanied by internalization of 4 nmol of calcium, i.e., 2 mol of calcium per mole of phosphorylated enzyme. Further calcium uptake then occurs at a lower rate as the steady state sets in (line A in Figure 6).

It is of interest that if excess nonradioactive calcium isotope is added with acetyl phosphate (Figure 6C, square data points), the steady-state uptake is not observed since the enzyme picks up nonradioactive calcium. We also find that the initial

Table I: Steady-State Velocities [μmol min^{-1} (mg of Protein)$^{-1}$] of Substrate Hydrolysis and Calcium Uptake: Effect of Derivatization with FITC[a]

	ATP		AcP		AcP + AMP-PNP	
SR vesicles	hydrolysis	uptake	hydrolysis	uptake	hydrolysis	uptake
native	0.98	2.00	0.10	0.17		
FITC	0.05	0.00	0.10	0.13		
leaky			0.20		0.00	
FITC–leaky			0.17		0.16	

[a] The reaction medium contained 50 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1.05 mM $CaCl_2$, and 0.1 mg of SR protein/mL. The experiments with native vesicles contained also 5 mM ammonium oxalate and, if calcium uptake was measured, a ^{45}Ca tracer. The concentrations of ATP, acetyl phosphate, and AMP-PNP were 1 mM. When indicated, the SR vesicles were reacted with FITC and/or recorded leaky as described under Materials and Methods. Velocities were derived from two sets of experiments. Each determination was based on five samples taken at sequential time intervals.

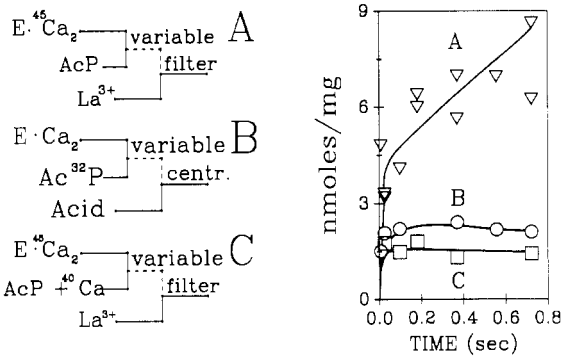

FIGURE 6: Early phase of [^{32}P]phosphoenzyme (B) formation and internalization of bound ^{45}Ca (A and C) following addition of acetyl phosphate to native SR vesicles preincubated with Ca^{2+}. Rapid mixing was obtained with the aid of a Froehlich–Berger chemical quench-flow apparatus, with temperature control set at 25 °C. Syringe A contained 20 mM MOPS (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 30 μM $CaCl_2$ (○) or $^{45}CaCl_2$ (▽, □), and 0.1 mg of SR (native vesicles) protein/mL. Syringe B contained 20 mM MOPS (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, and acetyl [^{32}P]phosphate (○) or acetyl phosphate (△, □). When indicated (□), a third syringe (C) contained 20 mM MOPS (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. For phosphoenzyme formation, quenching was obtained with TCA (0.8 M) and P_i (40 mM). For determination of ^{45}Ca internalization, quenching was obtained with 20 mM MOPS (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, and 10 mM $LaCl_3$, and the reaction mixture was filtered 1 min following quenching.

calcium internalization involves only 1 (rather than 2) mol of radioactive calcium per mole of phosphorylated enzyme. This is due to the rather slow enzyme phosphorylation with acetyl phosphate [as compared with ATP; see Boland and Jencks (1987); also see discussion below], which allows exchange of 1 of the 2 mol of radioactive calcium initially bound to the enzyme with the nonradioactive calcium added with acetyl phosphate. In fact, it was previously shown that calcium binding to SR ATPase occurs with a sequential mechanism (Inesi, 1987) whereby half of the (high-affinity) calcium bound to the enzyme exchanges rapidly with an isotope added to the external medium. It is shown in Figure 6 that if the enzyme is first saturated with radioactive calcium, and then the rapidly exchangeable half of the bound calcium is exchanged with nonradioactive calcium (C), the remaining (slowly exchangeable) half of the (radioactive) bound calcium is still internalized upon enzyme phosphorylation.

Activity following ATPase Derivatization with FITC. It was originally reported by Pick and Karlish (1980) that FITC can be reacted covalently with a lysine residue of the ATPase nucleotide site, thereby preventing ATP binding (Mitchinson et al., 1982). The FITC-derivatized ATPase can still form a phosphorylated intermediate (Pick & Bassilian, 1981) and sustain a full rate of hydrolytic activity when acetyl phosphate, but not ATP, is the substrate. The FITC-derivatized ATPase can even sustain transport activity at rates nearly as high as the native vesicles when acetyl phosphate, but not ATP, is used as the substrate (Table I). These findings demonstrate that the nucleotide moiety of the substrate is not essential to the coupling mechanism of catalytic and transport activities.

In experiments in which acetyl phosphate hydrolysis was catalyzed by leaky vesicles, we found that addition of 1 mM AMP-PNP caused inhibition by competing for the catalytic site. We reasoned then that if FITC-derivatized ATPase was used, AMP-PNP would not be able to compete with acetyl phosphate owing to FITC occupancy of the portion of catalytic site where the adenosine moiety of the nucleotide would normally bind. This was in fact observed experimentally (Table I). Furthermore, we noted that the nucleotide in millimolar concentrations failed to produce the known secondary activation of hydrolytic activity (Inesi, et al., 1967), indicating that such an activation is due to nucleotide occupancy of the FITC-sensitive site, rather than an additional site.

Calcium-Independent Hydrolytic Activity. A final point of interest pursued in our studies is the hydrolytic activity which is sustained by the enzyme in the absence of calcium. It is well-known that ATP is hydrolyzed by SR vesicles in the absence of calcium at a "basic" low rate (Hasselbach, 1964) with a mechanism that does not include a phosphorylated enzyme intermediate. To what extent the calcium-independent activity is sustained by the Ca^{2+}-ATPase proper, or by a contaminating enzyme, is still an open question.

When acetyl phosphate is used as the substrate, the ratio of calcium-independent to calcium-dependent activity is somewhat higher than observed with ATP. We then carried out experiments in which we compared the activity in the absence of calcium with that sustained by vesicles which were loaded maximally by preincubation with calcium in the presence of acetyl phosphate, and then EGTA was added to the medium to measure calcium-independent activity. In the latter case, most of the Ca^{2+}-ATPase was kept in the form of phosphorylated intermediate, and we reasoned it would be prevented from participating in further catalytic activity. It is shown in Figure 7 that in fact the phosphorylated intermediate was maintained for the duration of our measurements. Following chelation of Ca^{2+} by addition of EGTA to the medium, however, the hydrolytic activity sustained by these vesicles was nearly equal to that of control vesicles in which the Ca^{2+}-ATPase was not engaged in the phosphorylated state. This suggests that the calcium-independent acetyl phosphate hydrolysis is catalyzed by a contaminating phosphatase.

Discussion

The aim of our experiments was to characterize the mechanism of acetyl phosphate utilization for calcium transport by the SR ATPase. We thought that comparing a simple phosphoryl donor such as acetyl phosphate to ATP would unveil any specific role of the nucleotide moiety in the coupling mechanism of catalysis and active transport.

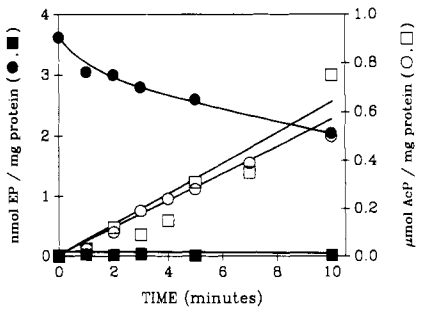

FIGURE 7: Hydrolysis of acetyl phosphate in the absence of Ca^{2+} in the medium, using SR vesicles containing high lumenal Ca^{2+} and phosphorylated ATPase (○, ●) or SR vesicles containing non-phosphorylated ATPase and no Ca^{2+} in the lumen (□, ■). Native SR vesicles (2 mg/mL) were first added to a medium containing 20 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1.05 mM $CaCl_2$, and 1 mM (○, ●) or 10 mM (□, ■) EGTA. In half of the reaction mixtures (○, ●), acetyl [^{32}P]phosphate (10 mM) was added 2 min *before* EGTA to phosphorylate the enzyme and produce calcium loading. In the other half of the reaction mixtures (□, ■), the acetyl phosphate was added *after* EGTA. Serial samples for determination of acetyl phosphate hydrolysis (○, □) and phosphoenzyme levels (●, ■) were collected thereafter.

The Catalytic Mechanism. We first established that the stoichiometry of the phosphorylated intermediate formed by phosphoryl transfer from acetyl phosphate to the enzyme is identical with that obtained with ATP (i.e., approximately 4 nmol/mg of protein in our preparation of SR vesicles). These findings are in agreement with those of Bodley and Jencks (1987) and in disagreement with the higher stoichiometries observed in earlier studies (Friedman & Makinose, 1970; Pucell & Martonosi, 1971). In this connection, it was demonstrated by Barrabin and de Meis (1983) that this type of discrepancy is most commonly due to inaccurate assessment of radioactive substrate-specific activity.

When acetyl phosphate (rather than ATP) is the substrate, maintenance of high levels of phosphorylated enzyme intermediate requires a more careful optimization of the ratio of phosphoenzyme formation and phosphoenzyme cleavage velocities. This is due to a relatively high K_m [4.3 mM vs 15 μM for ATP; see Boland and Jencks (1987) and Fernandez-Belda and Inesi (1986)] and a relatively low rate constant [32 vs 100–300 s^{-1} for ATP; see Boland and Jencks (1987), Froehlich and Taylor (1965), and Fernandez-Belda and Inesi (1986)] for the phosphoryl transfer reaction. For this reason, high steady-state levels of phosphoenzyme are observed only if the concentration of acetyl phosphate is sufficiently high and/or the hydrolytic cleavage of the enzyme is inhibited by high concentrations of Ca^{2+} in the lumen of the vesicles (Figures 1 and 2).

The Ca^{2+} concentration dependence of acetyl phosphate utilization by SR ATPase is quite similar to that of ATP, exhibiting stimulation in the micromolar range and inhibition in the millimolar range (Figure 5). Inhibition is produced when the Ca^{2+} concentration in the lumen of the vesicle is high, even if the Ca^{2+} concentration in the medium is low (Figure 1B). Therefore, the bell-shaped effect of Ca^{2+} can be explained quite well with enzyme activation by binding to high-affinity sites on the outer surface of the vesicles, and enzyme activation by binding to low-affinity sites exposed to the lumen of the vesicles, as outlined by eq 1–6 for the mechanism of ATP utilization by SR ATPase.

The Coupling Mechanism. It was originally demonstrated by rapid quench experiments that enzyme phosphorylation by ATP is immediately followed by internalization (i.e., inability to be displaced by quench reagents) of 2 mol of calcium bound per mole of enzyme (Inesi et al., 1978). We now find an identical pattern (Figure 6) when acetyl phosphate is used as the phosphoryl donor. Following this initial burst of calcium internalization, steady-state transport sets in (Figure 6) at a lower rate, with a molar ratio approximately of 2:1 between calcium transport and acetyl phosphate utilization (Figure 3).

A basic feature of calcium binding to, and translocation by, the SR ATPase is a sequential mechanism whereby half of the calcium involved in a single enzyme cycle binds first and the other half binds thereafter (Inesi, 1987). The first half undergoes very slow exchange with calcium isotope added to the medium, while the second half undergoes rapid exchange. While all the bound calcium (both halves) is internalized upon enzyme phosphorylation, the first half to bind is also the first to be released into the lumen of the vesicles; the second half is translocated more slowly (Khananshvili & Jencks, 1987; Inesi, 1987). It is of interest that these sequential events occur also when acetyl phosphate is used as the substrate. It is shown in Figure 6 that half (the second half) of the bound (radioactive) calcium undergoes rapid exchange with nonradioactive calcium, while the other (slowly exchangeable) half is still internalized upon enzyme phosphorylation with acetyl phosphate.

The experiments described above indicate that coupling of catalysis and active transport includes the same mechanistic features, whether ATP or acetyl phosphate is used as substrate. This is consistent with the idea that occupancy of the phosphorylation site is the essential event required for vectorial translocation of the bound calcium (Jencks, 1980; Tanford, 1982). Furthermore, acetyl phosphate sustains calcium transport even when the nucleotide binding site is derivatized with FITC (Table I) which prevents ATP binding and utilization all together. This demonstrates that the ATP nucleotide moiety is not required for active transport. It also suggests that the nucleotide binding domain of the enzyme is not involved in the actual coupling mechanism, since coupling occurs in the presence of chemical derivatization of that domain.

Kinetic Regulation. Although the coupling mechanism is essentially the same, kinetic regulation appears to be different when acetyl phosphate, as opposed to ATP, is used as the substrate. Firstly, the K_m is higher and the rate constant for the phosphoryl transfer reaction is lower when acetyl phosphate is the substrate (Bodley & Jencks, 1987). Furthermore, the V_{max} for hydrolytic activity (measured with vesicles rendered leaky to avoid inhibition by the lumenal Ca^{2+} rise) is somewhat lower (1.2 vs 2.25 μmol min^{-1} mg^{-1}) when acetyl phosphate, as opposed to ATP, is the substrate (Figures 3A and 4). Finally, the enhancement of phosphoenzyme turnover and steady-state velocity, which is produced by high ATP concentrations, is not observed with acetyl phosphate (Bodley & Jencks, 1987).

The slower kinetics of acetyl phosphate (as compared to ATP) utilization are much more pronounced when activity is measured with native vesicles (compare Figures 3 and 4) rather than leaky vesicles (also see Figures 3 and 4). Of particular interest is the much slower phosphoenzyme turnover observed in rapid quench experiments, following the initial burst of calcium internalization. In the experiment shown in Figure 6, the phosphoenzyme turnover (assuming 2 mol of calcium per cycle) is only 1 s^{-1}. On the other hand, in comparable experiments with ATP (Inesi, 1987), the phosphoenzyme turnover is 10 s^{-1}.

It should be pointed out that breakdown of the phosphorylated enzyme intermediate is not rate limited by the hydrolytic reaction, which proceeds with a rate constant of 60

s^{-1} (Inesi et al., 1982), but by a transition following calcium internalization and preceding the hydrolytic reaction. It is noteworthy in this connection that the kinetic features of ATP are related not only to its rapid utilization as a substrate in the phosphoryl transfer reaction but also to its stimulation of the phosphoenzyme turnover. This latter effect appears to be produced by occupancy of the nucleotide site following formation of the phosphorylated enzyme intermediate (McIntosh & Boyer, 1983; Gould et al., 1986; Bishop et al., 1987). In fact, we found no secondary activation by millimolar nucleotide, when acetyl phosphate was utilized by FITC–enzyme (Table I).

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