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Coupling of Calcium Transport with ATP Hydrolysis in Scallop Sarcoplasmic Reticulum[1]

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Previously, we showed that incubation of the scallop sarcoplasmic reticulum (SR) with EGTA at above 37°C resulted in the uncoupling of ATP hydrolysis with Ca^{2+} transport [Nagata *et al.* (1996) *J. Biochem.* 119, 1100–1105]. We have extended this study by comparing the kinetic behavior of Ca^{2+} release and binding to the uncoupled SR with that of intact scallop or rabbit SR. The change in the Ca^{2+} concentration in the reaction medium, as determined as the absorption of APIII, was followed using a stopped flow system. Intact scallop SR was preincubated with Ca^{2+} in the presence of a Ca^{2+} ionophore, A23187, and then ATP was added to initiate the reaction. The Ca^{2+} level in the medium increased to the maximum level in several seconds, and then slowly decreased to the initial low level. The rising and subsequent slow decay phases could be related to the dissociation and reassociation of Ca^{2+} with the Ca-ATPase, respectively. When uncoupled scallop SR vesicles were preincubated with $CaCl_2$ in the absence of A23187 and then the reaction was initiated by the addition of ATP, a remarkable amount of Ca^{2+} was released from the SR vesicles into the cytosolic solution, whereas, with intact scallop or rabbit SR, only a sharp decrease in the Ca^{2+} level was observed. Based on these findings, we concluded that the heat treatment of scallop SR in EGTA may alter the conformation of the Ca-ATPase, thereby causing Ca^{2+} to be released from the enzyme, during the catalytic cycle, at the cytoplasmic surface, but not at the lumenal surface of SR vesicles.

Key words: Ca-ATPase, Ca^{2+} release, phosphorylated enzyme, scallop SR, uncoupling.

The Ca^{2+} pump ATPase of SR catalyzes the active transport of Ca^{2+} across the SR membrane by utilizing energy from ATP hydrolysis. The simplest coupling mechanism, presented in Scheme 1, has been proposed based on a number of kinetic studies (*1–3*).

The scheme is based on the existence of two conformational states of the Ca^{2+} pump ATPase, E1 and E2, which are characterized by the ability to react with ATP and P_i, respectively. The enzyme in the E1 state can bind cooperatively 2 mol of Ca^{2+} in the cytoplasmic medium to form E1•2Ca (*4–7*). The enzyme can then be phosphorylated by ATP to form E1P < 2Ca, in which 2 mol of Ca^{2+} is occluded by the enzyme (*8, 9*). The Ca^{2+} ions are deoccluded and released from the enzyme into the SR lumen as the conformation of the enzyme changes from E1P to E2P. The low energy phosphorylated intermediate E2P is then hydrolyzed to E2, which is recycled to E1.

What remains to be elucidated is the molecular mechanism of the energy-transducing process in the binding and movement of Ca^{2+} through the Ca^{2+} pump protein. Many investigations have been performed for further understanding of the uncoupling between ATP hydrolysis and Ca^{2+} transport in SR by mens of chemical modifications (*10–14*), site-directed mutagenesis (*15–17*), and substrate analogues (*18–21*).

We have previously observed that incubation of scallop SR vesicles at above 37°C in the presence of Ca^{2+} results in complete inactivation of the activities of both ATP hydrolysis and Ca^{2+} transport, whereas the heat treatment of SR vesicles in the presence of EGTA uncoupled ATPase activity from Ca^{2+} transport without increasing the Ca^{2+} permeability of the membrane (*22, 23*).

In this work, we have extended these studies by investigating the effect of heat treatment of scallop SR with EGTA on the Ca^{2+} binding and release from the Ca^{2+} pump ATPase during the catalytic cycle. We found that, in the reaction step of deocclusion of E1P > 2Ca, the uncoupled scallop SR released Ca^{2+} at the cytosolic surface of vesicles, while intact SR released Ca^{2+} into the SR lumen. Based on these observations, we concluded that the heat-treatment of scallop SR in EGTA may alter the vectorial movements of Ca^{2+}, which might account for the lack of accumulation of Ca^{2+} in the SR vesicle.

EXPERIMENTAL PROCEDURES

Preparation of SR—Scallop SR was prepared from the adductor muscle of scallop as reported previously (*24*). Rab-

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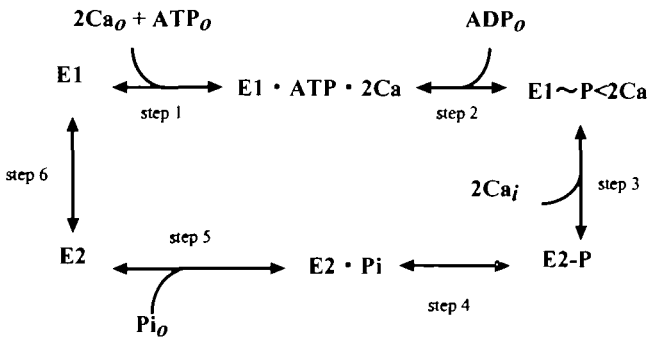
Abbreviations: APIII, antipyrylazo-III; EP, phosphoenzyme; LDH, L-lactate dehydrogenase; PEP, phosphoenolpyruvic acid; PK, pyruvate kinase; SR, sarcoplasmic reticulum; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.



Scheme 1. **Mechanism of coupling of ATP hydrolysis with Ca^{2+} transport across the SR membrane.** E1 and E2 indicate two major conformational states of Ca-ATPase, which are characterized by the ability to react with ATP and P_i, respectively. Suffixes *o* and *i* indicate outer and inner membrane, respectively. <2Ca represents the Ca^{2+} ions occluded by the enzyme.

bit SR was prepared from rabbit back and hind limb white skeletal muscle by a method described previously (*25*). Each SR preparation was suspended at 10–20 mg/ml in a stock solution containing 0.1 M KCl, 10% glycerol, and 5 mM Tris-maleate (pH 7.0). The suspension was divided into small portions, quickly frozen in liquid nitrogen and stored at –80°C.

Reagents—LDH, PK, PEP, and NADH were purchased from Wako. A23187 was obtained from Dojin, and APIII from Nacalai. All other chemicals were of reagent grade. Monovanadate was prepared from V_2O_5 according to Goodno (*26*).

Heat Treatment of Scallop SR Vesicles—SR vesicles at 1/10–1/15 of the total volume were added at 1 mg/ml to the standard medium, which contained 0.1 M KCl, 1–5 mM $MgCl_2$, 10% glycerol, and 20 mM Tris-maleate (pH 7.0), at 38–41°C. At the indicated times, the SR suspension was quickly cooled in ice, and centrifuged to wash SR.

Assaying of ATP Hydrolytic Activity—ATP hydrolysis was measured at 25°C in a medium containing 0.02–0.05 mg/ml SR protein, 5 mM oxalate, 0.1 M KCl, 5 mM $MgCl_2$, 10% glycerol, and 50 mM Tris-HCl (pH 7.0), coupled with a regeneration system (1 mM PEP, 0.2 mM NADH, 96.2 U/ml PK, and 100 U/ml LDH). The reaction was started by the addition of ATP, and the time course of NADH absorption at 340 nm was followed with a spectrophotometer U-3210 (Hitachi, Tokyo).

Assaying of Ca^{2+} Transport—Ca^{2+} transport activity was measured in a reaction mixture containing 0.05 mg/ml SR protein, 50 μM $CaCl_2$, and 0.2 mM APIII in the medium for the ATPase activity. The reaction was started at 25°C by the addition of 0.1–0.2 mM ATP. Ca-uptake into SR vesicles was determined by measuring the absorption at 700 nm in the reaction mixture as described previously (*27*).

Stopped Flow Measurements—Rapid kinetic measurements were performed with a micro-volume stopped flow spectrofluorometer (Applied Photophysics, U.K.). The reaction was started by mixing equal volumes of standard solutions from two syringes, one containing SR vesicles (0.1–0.4 mg/ml) and the other containing various concentrations of ATP and/or Ca^{2+}. The transient phase of Ca^{2+} binding and release from the SR was followed by monitoring the absorption of APIII in the reaction medium at 700 nm. The measurement was repeated 10–20 times. Data were analyzed using the program SX.18MV kinetic measurement software. The Ca^{2+} concentration was estimated from the absorbance by referring to a calibration curve.

RESULTS

Effects of Ca^{2+}, Nucleotides, and Vanadate on Thermal Stabilization of Scallop Ca-ATPase—We previously observed that incubation of scallop SR vesicles at above 37°C in the presence of Ca^{2+} resulted in complete inactivation of both ATP hydrolysis and Ca^{2+} transport (*22, 23*). We further investigated the conditions which contribute to the thermal stability of scallop Ca-ATPase. Our present results, together with previous data (*22*), are summarized in Table I and Fig. 1.

As reported previously, heat treatment of scallop SR vesicles in the presence of EGTA resulted in the uncoupling of ATP hydrolysis from Ca^{2+} transport (*22, 23*). In this work, we observed that ATP at as high as 5 μM also induced the uncoupled reaction, irrespective of the presence or absence of Ca^{2+}. These results are in agreement with our previous studies on the effect of AMPPNP (*22*). These data suggest that ATP binding at a high affinity catalytic site on the enzyme resulted in thermal stabilization of the catalytic site for ATP hydrolysis but not for the Ca^{2+} translocation site.

As shown in Fig. 2 and Table I, vanadate ions at a concentration below 5 μM were found to protect both the ATP hydrolytic and Ca^{2+} transport activities from heat denaturation.

Since these effects of vanadate require the presence of EGTA as well as $MgCl_2$, it is postulated that vanadate ions can bind to the E2 conformation of the Ca-ATPase to form a stable intermediate, whereby the thermal stability of the enzyme increases. In addition, P_i also protects the scallop SR from thermal inhibition (data not shown), although its effective concentration was higher than 5 mM. Judging from these results, stabilization of the enzyme structure in the E2P form may be important for the coupling between the ATP hydrolysis and Ca^{2+} transport reactions.

Binding and Release of Ca^{2+} Coupled to ATP Hydrolysis—As shown in Scheme 1, 2 mol of Ca^{2+} are occluded by the Ca^{2+} pump ATPase when γ-P from ATP is transferred to the 351 Asp residue of the enzyme to yield E1P (Step 2). This reaction is followed by the conversion of E1P to E2P (Step 3), during which 2 mol of Ca^{2+} are deoccluded and dissociated from the enzyme, moving into the vesicle lumen, and the Ca^{2+} binding sites become empty (Step 3), while they become occupied again with cytosolic Ca^{2+} when E2 is converted into E1.

To determine whether or not the scallop Ca^{2+} pump ATPase binds and releases Ca^{2+} during the cycle of ATP hydrolysis in a manner similar to that observed for the Ca^{2+} pump ATPase of rabbit SR, we compared their kinetic properties for the individual stages of the catalytic cycle by measuring the kinetics of the change in the Ca^{2+} level of the reaction medium in the presence of a Ca^{2+}-indicator, APIII. The stopped flow technique enables us to measure partial reactions with short time resolution.

Figure 3 shows the time courses of Ca^{2+} transport by rabbit and scallop SR vesicles after initiation of the reaction by mixing the solution in syringe A, in which 0.1 mg/ml SR was preincubated with 5 μM $CaCl_2$, with an equal volume

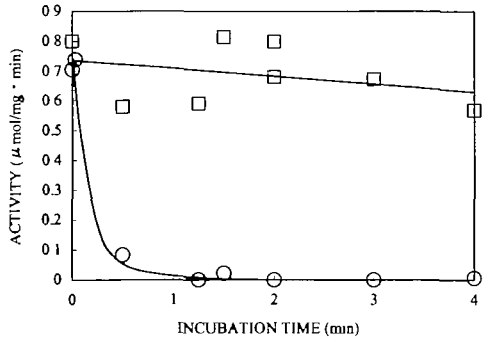


Fig. 1. **Uncoupling of Ca^{2+} transport from ATP hydrolysis after heat treatment of scallop SR in EGTA.** Scallop SR was incubated at 38°C for the indicated times under the conditions given under "EXPERIMENTAL PROCEDURES." The resulting SR was washed by centrifugation and resuspended at 0.02–0.05 mg/ml in the standard solution, which contained 2 μM A23187, 5 mM oxalate, 0.1 M KCl, 5 mM $MgCl_2$, 10% glycerol, and 50 mM Tris-HCl (pH 7.0). ATP hydrolysis (□) was measured by the coupled enzyme method as described under "EXPERIMENTAL PROCEDURES." The reaction was initiated at 25°C by the addition of 50 μM $CaCl_2$ to the reaction mixture. Ca^{2+} transport (○) was measured in a reaction mixture containing 0.05 mg/ml SR protein, 50 μM $CaCl_2$, and 0.2 mM APIII in the standard solution. Ca^{2+} uptake into SR vesicles was determined by measuring the absorption of APIII at 700 nm in the reaction mixture.

TABLE I. **Effects of heat treatment of scallop SR[a] in the presence of Ca^{2+}, EGTA, nucleotides or vanadate on the catalytic activity.**

Conditions	Activities (μmol/mg SR·min)	
	ATP hydrolysis	Ca^{2+} transport
Control (no treatment)	0.48	0.91
	0.32	0.59
	0.24	0.53
5 μM ATP, 1 mM EGTA	0.20	0.035
5 μM ATP, 0.1 mM $CaCl_2$	0.12	0.030
0.4 mM ATP, 1 mM EGTA	0.27	0.045
0.4 mM ATP, 0.1 mM $CaCl_2$	0.15	0.022
9.2 μM AMPPNP	0.07	0.017
0.23 mM	0.45	—
1.84 mM	0.46	0.036
5 μM vanadate, 1 mM EGTA	0.27	0.59
	—	0.50
	—	0.41
5 μM vanadate, 0.1 mM $CaCl_2$	*0.0*	0.07
	—	0.002
No addition, 0.1 mM $CaCl_2$	0.036	0.012
	0.004	0.0
	0.0	0.02

[a]Scallop SR vesicles were incubated for 2–3 min at 40°C under the conditions indicated. SR vesicles were washed 3 times by centrifugation in a solution containing 10% glycerol, 0.1 M KCl, and 10 mM Tris-maleate (pH 7.0). The ATP hydrolysis and Ca^{2+} transport activities were measured under the conditions given in Fig. 1.

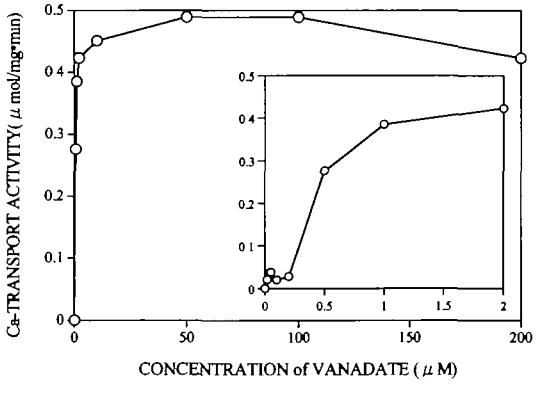


Fig. 2. **Dependence of heat stabilization of scallop SR on the concentration of vanadate.** Scallop SR was incubated at 38°C for 2 min with various concentrations of vanadate in the presence of 2 mM EGTA and 5 mM $MgCl_2$. The resulting SR was washed by centrifugation and resuspended at 0.02–0.05 mg/ml in the standard solution. Ca^{2+} transport activity was measured as in Fig. 1. Inset: Heat treatment of scallop SR was carried out in a low concentration range of vanadate.

of the solution from syringe B, which contained 50 μM ATP. Immediately after mixing, the Ca^{2+} concentration in the reaction medium, as determined as the absorption of APIII, decreased on the movement of Ca^{2+} into the SR lumen and reached a low steady level in 10 s. No significant time lag was observed in the initial phase of the reaction. Similar time courses were observed with rabbit SR vesicles.

Figure 4 shows the time courses of Ca^{2+} release and binding to SR measured in the same way as in Fig. 3, except that the experiments were carried out at 0°C in the presence of 2 μM A23187, and the concentration of ATP was varied from 3 to 9 μM. On mixing of the solutions from syringes A and B, the Ca^{2+} level in the medium increased to the maximum level in several seconds, and then slowly decreased to the initial low level.

The rising phase of the Ca^{2+} time course could be related to the rapid dissociation of Ca^{2+} from the enzyme and the subsequent slow decay phase of the Ca^{2+} time course due to the reassociation of Ca^{2+} with the enzyme. Under the conditions used, Ca^{2+} binding sites were previously saturated. Thus, during the ATP hydrolysis cycle, nearly the same amount of Ca^{2+} as its binding sites would be dissociated during the conversion from E1P to E2P. The amount of released Ca^{2+} appeared to increase from 3.2 to 4.4 nmol/mg protein for the rabbit SR and 1 to 3.7 nmol/mg protein for the scallop SR as the ATP level increased from 3 to 9 μM. The acceleration of Ca^{2+} release by ATP may be due to the binding of ATP to the regulatory site that enhances the formation and hydrolysis of E2P (*28*). The slow decay phase of the Ca^{2+} time course could be related to refilling of the empty site of the enzyme with the released Ca^{2+}. In general, the rate of E2P decomposition into P_i and E2, as well as that from E2 to E1, is considered to be very low compared with that of conversion of E1P into E2P. This may be why the rate of Ca^{2+} rebinding to the enzyme is much lower than that of the release. This possibility was examined by means of the experiment shown in Fig. 5.

Rabbit SR was preincubated for several minutes with 5 μM ATP in the presence of the regeneration system. On this incubation, all of the Ca^{2+} bound to the Ca-ATPase was expected to be removed from the binding sites. When Ca^{2+} uptake was initiated by the addition of $CaCl_2$, the initial level of Ca^{2+} in the medium decreased to the baseline with time (Fig. 5, trace b). When the same experiment was carried out in the presence of 2 μM A23187, a Ca^{2+} transient curve such as that previously seen, see Fig. 4, was no longer observed (Fig. 5, trace a). The pronounced difference between the kinetics of Ca^{2+} release and binding, as illus-

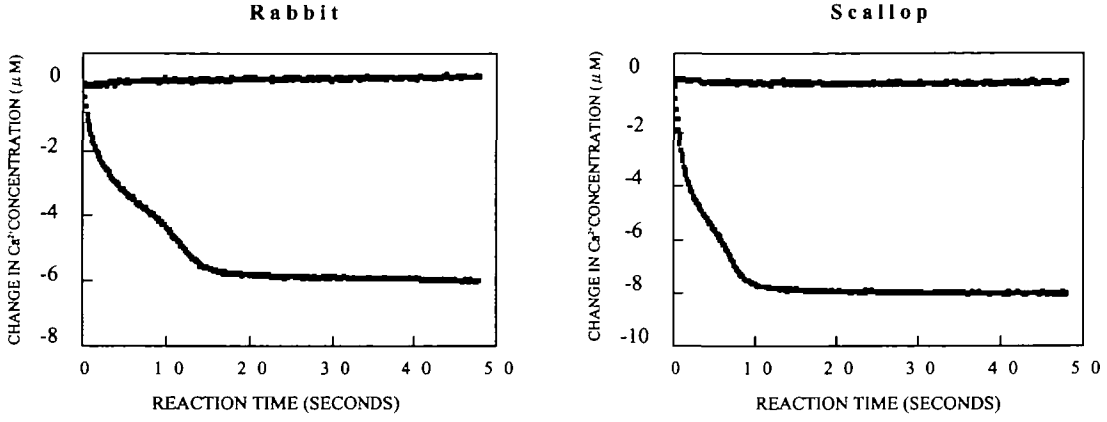


Fig. 3. **Comparison of the Ca^{2+} transport kinetics in rabbit and scallop SR measured using a stopped flow spectrophotometer.** In syringe A, 0.1 mg/ml rabbit (left panel) or scallop (right panel) SR was incubated with 5 μM $CaCl_2$ in the standard solution containing 0.2 mM APIII, 5 mM oxalate, 0.5 mM $MgCl_2$, 0.1 M KCl, 10% glycerol, and 20 mM Tris-maleate (pH 7.0). The reaction was initiated at 27°C by mixing an equal volume of the solution from syringe B, which contained 50 μM (lower traces) or 0 μM (upper traces) ATP in the standard solution. Changes in the Ca^{2+} concentration were followed by monitoring the absorption of APIII at 700 nm.

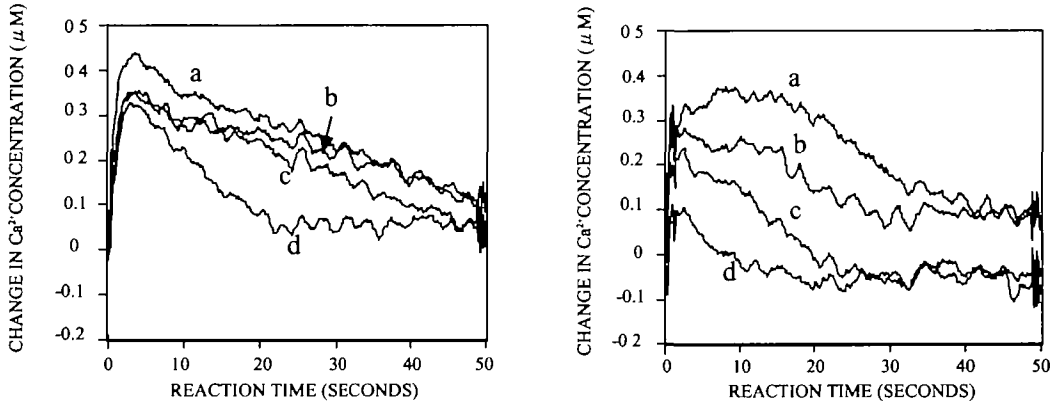


Fig. 4. **Dependence of the level of Ca^{2+} release on the concentration of ATP.** In syringe A, rabbit SR (left panel) or scallop SR (right panel) was incubated at 0.1 mg/ml with 5 μM $CaCl_2$ and 2 μM A23187 in the standard solution, and then an equal volume of solution from syringe B, which contained 9 (a), 7 (b), 5 (c), or 3 (d) μM ATP in the standard solution, was added to start the reaction.

trated in Figs. 4 and 5 (trace a), can be attributed to whether the respective Ca^{2+} binding sites of the enzyme were occupied with Ca^{2+} or were empty prior to initiation of the reaction. These results and considerations indicated that dissociation of Ca^{2+} from and association of it with the enzyme are tightly coupled with the elementary steps in the ATP hydrolysis cycle, and that the basic aspects of the coupling mechanism appear to be similar for rabbit and scallop SR.

Effect of Heat Treatment of SR in the Presence of EGTA on Ca^{2+} Binding and Release from the Enzyme—A major question arising during these experiments is which step of the catalytic cycle is the most sensitive to thermal denaturation of scallop SR in the presence of EGTA. We examined the possibility that the Ca^{2+} bound to the enzyme would be dissociated at the cytoplasmically surfacing site during the reaction step of conversion of E1P to E2P (Scheme 1, Step 3). In the experiment in Fig. 6, after several minutes incubation of scallop SR vesicles with 5 μM $CaCl_2$ in the absence of A-23187, the Ca^{2+} transport reaction was initiated by the addition of 5 μM ATP.

The intact SR showed a typical time course of Ca^{2+} transport, as measured as the Ca^{2+} level in the external medium (Fig. 6, curve c). In contrast, in the case of the scallop SR vesicles, which had been incubated with EGTA at high temperature, the release of Ca^{2+} into the cytosolic medium was found (Fig. 6, curve a). The amount of Ca^{2+} released was estimated to be 2–3 nmol/mg protein, which seems to correspond to that bound to the transport sites of the enzyme. These results suggest that the heat treatment of scallop SR with EGTA induced a change of the enzyme structure with loss of the vectorial movement of Ca^{2+}. Support for this came from the experimental results presented in Fig. 7.

The time courses of Ca^{2+} release and binding to scallop SR were measured in the presence of A23187 under similar conditions to in the experiment shown in Fig. 6 (curve a). For the intact scallop SR (Fig. 7, left panel), upon the addition of ATP, the Ca^{2+} level in the medium transiently increased to the maximum level in several seconds, and then slowly decreased to the initial low level. A fundamentally similar transient curve was obtained with the uncoupled

SR (Fig. 7, right panel), although the amount of Ca^{2+} released from SR (about 2 nmol Ca^{2+}/mg protein) was slightly lower than that in the control (about 3 nmol Ca^{2+}/mg protein). These results support the hypothesis that elementary steps of Ca^{2+} release and rebinding proceed in the uncoupled SR essentially in the same manner as in the intact one. Each of these time courses showed a good fit with the theoretical one when the transient curve is assumed to be a combination of the initial increasing curve, which corresponds to Ca^{2+} deocclusion, and the following slow one, which corresponds to Ca^{2+} rebinding to the enzyme, and the ratio of their rate constants is 1.56:1 for the intact SR and 1.87:1 for the uncoupled SR. These findings suggested that the uncoupling of the ATP hydrolytic reaction from Ca^{2+} transport in scallop SR occurs in the reaction step of conversion of E1P to E2P, where Ca^{2+} dissociates from the enzyme at the cytoplasmic surface but does not dissociates onto the lumenal side of the SR vesicles.

DISCUSSION

Heat treatment of scallop SR with EGTA may directly alter the conformation of the scallop Ca^{2+} pump ATPase such that ATP hydrolysis becomes uncoupled from Ca^{2+} transport. This is based on the following evidence. Heat treatment did not increase the permeability of the SR membrane for Ca^{2+} (*22*). Heat inactivation was also observed with proteoliposomes reconstituted from the scallop Ca^{2+} ATPase and soybean phospholipid (*23*). The concentration of Ca^{2+} required for the heat inactivation was about 0.5 μM (*22*). This value is very close to that giving the half maxi-

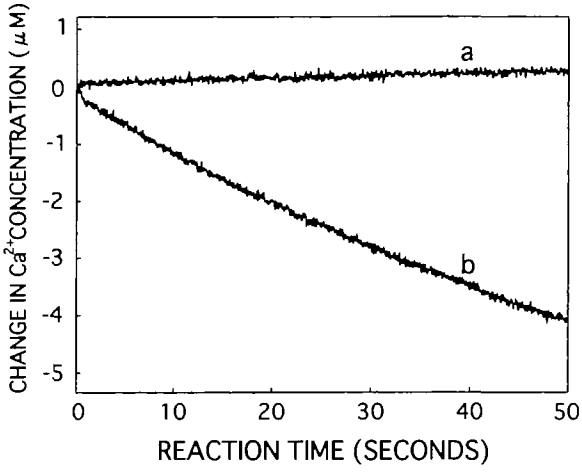


Fig. 5. **Disappearance of the transient Ca^{2+} rise after initiation of the reaction by the addition of Ca^{2+}.** In syringe A, rabbit SR (0.1 mg/ml) was incubated for longer than 5 min with 10 μM ATP in the presence of the ATP regeneration system. The change in the Ca^{2+} concentration of the reaction medium was measured after initiation of the reaction by the addition of an equal volume of the solution in syringe B, which contained 10 μM $CaCl_2$ with (trace a) or without (trace b) 2 μM A23187.

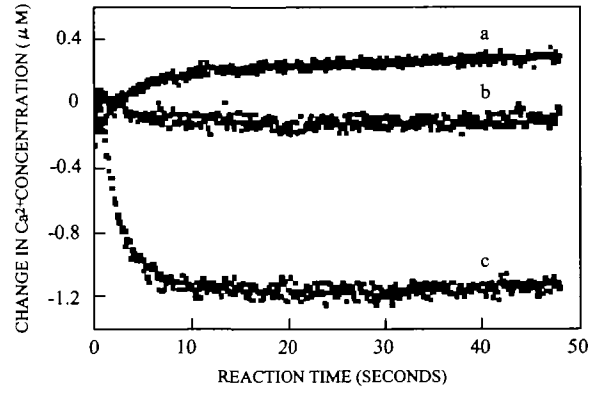


Fig. 6. **Deocclusion of Ca^{2+} at the cytoplasmic surface of the uncoupled scallop SR during the ATP hydrolysis cycle.** In syringe A, intact scallop SR (lower trace) or heat treated-scallop SR (upper and middle traces) was incubated at 0.1 mg/ml with 5 μM $CaCl_2$ without A23187 in the standard solution, and then an equal volume of solution from syringe B containing 9 μM (upper trace) or 0 μM ATP in the standard solution was added to start the reaction at 0°C.

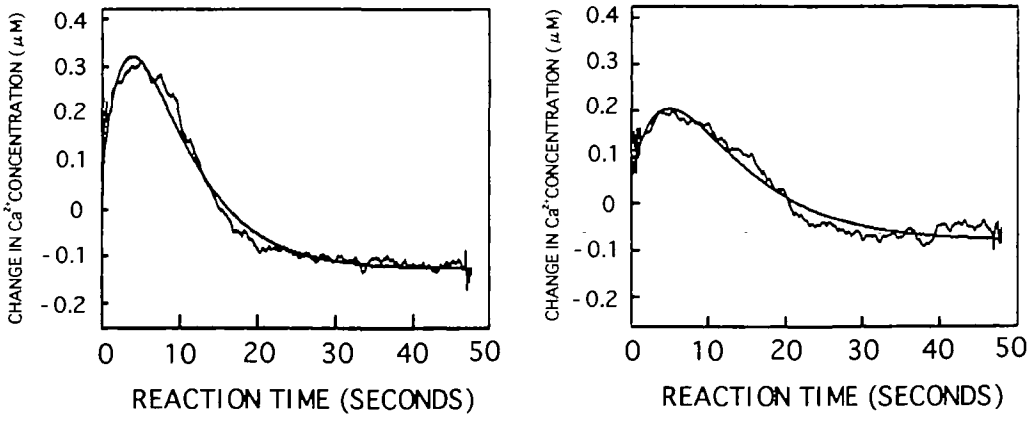


Fig. 7. **Time courses of deocclusion of Ca^{2+} from and reassociation of it with intact and heat-treated scallop SR in the presence of A23187.** Intact scallop (left panel) or uncoupled scallop SR (right panel) was preincubated in syringe A with 5 μM $CaCl_2$ in the presence of A23187, and then the reaction was started at 0°C by the addition of an equal volume of the ATP solution from syringe B. Solid smooth lines show simulation curves that were obtained by assuming that the ratio of the rate constant (k_1) in the rising phase to that (k_2) in the slow decay phase was $k_1/k_2 = 1.56$ for the intact SR and $k_1/k_2 = 1.88$ for the uncoupled SR (see text), and that the maximum level of Ca^{2+} release was the same for the intact and heat-treated SR.

mum activation of the Ca^{2+} pump activity. As shown in Table I, vanadate was found to be able to prevent the heat inactivation of Ca^{2+} transport of scallop SR in the concentration range of 0.2 to 0.5 μM. This indicates that direct binding of vanadate to the scallop ATPase changed its heat stability. The uncoupled SR has also been obtained with rabbit SR by incubation at 37°C in the presence of EGTA (*29–31*) or incubation at 25°C for longer than 10 min with an extremely low Ca^{2+} concentration (*18*). The uncoupling of rabbit SR can be partially restored by the addition of Ca^{2+} (*31*). This contradicts our finding that Ca^{2+} ions enhanced thermal denaturation of the scallop Ca-ATPase. The difference in the Ca^{2+} effect between scallop and rabbit SR may be related to partial differences in their molecular structures, as indicated by our previous works (*32, 33*).

In addition to Ca^{2+} binding to the transport site, this cation has been shown to be necessary for full activation of the Ca-ATPase of SR (*34*) or plasma membrane Ca-ATPase (*35*). Recently, Berman (*14*) demonstrated that thapsigargin can release Ca^{2+} from SR vesicles at a concentration much higher than that for half inhibition of the Ca^{2+} pump activity, suggesting that since both high and low Ca^{2+} binding sites are vacant under these conditions, the Ca^{2+} ions must be released from the site that is separated from the transport site. Therefore, the possibility can not be eliminated that Ca^{2+} binding to sites other than the transport sites may be involved in the thermostability of the Ca-ATPase in scallop SR.

The Ca-ATPase of SR uses the energy of ATP hydrolysis to transport Ca^{2+} into the SR lumen through a series of conformational changes that are involved in the vectorial movement of the Ca^{2+} ions. A major question is, in terms of the vectorial movement of Ca^{2+} coupled to ATP hydrolysis, which step of the catalytic cycle is a heat-labile one. Comparison between the time courses of changes in the Ca^{2+} level of the medium after initiation of the reaction by adding ATP (Fig. 4) and $CaCl_2$ (Fig. 5, trace a) revealed that the rapid dissociation of Ca^{2+} from the enzyme occurs in the step of conversion of E1P to E2P (Step 3), while subsequent slow reassociation of Ca^{2+} occurs during reaction Steps 4 to 6 in Scheme 1, and that the basic aspects of the coupling mechanism appear to be similar for scallop and rabbit SR.

The most surprising finding in this study is that when the uncoupled scallop SR vesicles were incubated with Ca^{2+} in the absence of A23187 and then the reaction was initiated by the addition of ATP, they released Ca^{2+} into the external medium (Fig. 6, trace a), while the intact SR vesicles incorporated Ca^{2+} into the SR lumen (Fig. 6, trace c). Under these conditions, the uncoupled scallop Ca-ATPase is fully activated without the accumulation of Ca^{2+} inside the vesicles. On the contrary, both the uncoupled or intact scallop SR vesicles showed a transient increase in the Ca^{2+} concentration of the medium when the reaction was initiated by the addition of ATP in the presence of A23187 (Fig. 7). These observations lead us to conclude that the conformational change accompanied by the conversion of the enzyme state from E1P to E2P plays an important role in controlling the direction of Ca^{2+} movement through the channel of the enzyme protein and that the heat treatment of scallop SR in EGTA prevents the vectorial transport of Ca^{2+} by attacking the translocation site of the enzyme to change its structure. These conclusions are in good agreement with the finding by McIntosh and Woolley (*11, 36*) that K492 and R678 in the rabbit Ca^{2+}-ATPase cross-linked with glutaraldehyde lead to blocking of the E1P to E2P transition, and that the Ca^{2+} at the transport site is forced to leave the channel from the side it entered on hydrolysis of the phosphoenzyme.

In Fig. 4, the decrease of Ca^{2+} due to the reassociation of Ca^{2+} with the enzyme was observed in the presence of A23187, whereas no decrease of Ca^{2+} was observed in Fig. 6. This discrepancy is considered to be caused by the difference in the passage through which the deoccluded Ca^{2+} ions come out from SR vesicles. In the presence of A23187, the Ca^{2+} ions can be readily released into the outer medium through the ionophore. While in the absence of the ionophore, the Ca^{2+} ions will be passed through the Ca^{2+} pump protein to the cytosolic surface of the SR membrane. In the latter case, it is necessary to determine the translocation site that might be altered by the heat treatment. Comparative studies on the molecular structure of the Ca^{2+} pump ATPase in the intact and heat-treated scallop SR are presently underway to gain further insight into the uncoupling mechanism of the Ca^{2+} pump.

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