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THE FORMATION OF PHOSPHOENZYME OF SARCOPLASMIC RETICULUM

REQUIREMENT FOR MEMBRANE-BOUND Ca^{2+}

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Summary

Membrane-bound Ca or Mg of sarcoplasmic reticulum fragments were removed by treating the membrane with EDTA or an acidic solution, and the changes in the enzymatic activities of sarcoplasmic reticulum fragments induced by these treatments were examined. With the decrease in the amount of membrane-bound Ca below $1—3 \cdot 10^{-8}$ mol/mg protein, it was demonstrated that the activity of $(Ca^{2+} + Mg^{2+})$-ATPase transiently increased and then diminished, that the Ca-uptake and phosphoenzyme formation declined gradually, and that the activity of Mg^{2+}-ATPase was affected to a less extent. Sodium dodecyl sulfate-gel electrophoretic patterns of peptides from the metal-deficient membranes were the same as those of the untreated material.

The level of the phosphoenzyme formation of the metal-deficient membrane was restored by increasing the amount of membrane-bound Ca, but not by increasing the amount of membrane-bound Mg.

Introduction

Ca^{2+}-uptake by sarcoplasmic reticulum fragments is known to be energized by ATP through $(Ca^{2+} + Mg^{2+})$-ATPase [1,2]. In this process, at least, two types of Ca binding to the membrane are involved; one of them takes place at sites probably located on the $(Ca^{2+} + Mg^{2+})$-ATPase [3,4], and the other at sites on Ca-binding proteins [5—7]. Moreover, it has been reported that the purified $(Ca^{2+} + Mg^{2+})$-ATPase itself contains three classes of Ca-binding sites [8]. As to the role of the membrane-bound Ca, it has been shown that when the bound Ca

Abbreviations: EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl-ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

was removed from sarcoplasmic reticulum fragments by EDTA [9] or by lowering the pH [10], the permeability of the membrane and the activity of (Ca^{2+} + Mg^{2+})-ATPase increased, though the Ca-uptake decreased. It is well known that the presence of free Ca^{2+} in a reaction medium is required for the phosphorylation of the ATPase induced by ATP and that the ratio of the phosphorylation of the ATPase to externally bound Ca^{2+} is close to 2 [3,11].

We attempted to determine how the endogenous membrane-bound Ca or Mg in sarcoplasmic reticulum fragments is involved in the Ca^{2+} translocation. In this paper we report that the activities of (Ca^{2+} + Mg^{2+})-ATPase, phosphoenzyme formation and Ca-uptake decrease by removing the membrane-bound metals, and that these activities can be restored by increasing the amount of membrane-bound Ca.

Materials and Methods

Preparation of sarcoplasmic reticulum fragments

Sarcoplasmic reticulum fragments were prepared by the method of Weber [12] with some modifications. Rabbits were killed by decapitation. Skeletal muscles (100 g) were homogenized by 4 volumes of 0.1 M KCl containing 5 mM histidine (pH 7.5) for 1.5 min. The homogenate was centrifuged at 1000 $\times g$ for 20 min, and the supernatant was centrifuged at 8000 $\times g$ for 30 min to remove mitochondria. This supernatant was filtered through No. 5A filter paper. The filtrate was centrifuged at 30000 $\times g$ for 50 min. The pellets were suspended in 30 volumes of 0.6 M KCl containing 5 mM Tris-maleate (pH 6.8) to remove contaminating myosin. This suspension was centrifuged at 35000 $\times g$ for 50 min. The pellets were then suspended in 0.12 M KCl, and centrifuged at 35000 $\times g$ for 50 min. They were again suspended in 0.12 M KCl containing 5 mM Tris-maleate (pH 6.8). This suspension was stored as sarcoplasmic reticulum fragments at 0°C (final concentration, 14—20 mg protein/ml). SDS-gel electrophoresis pattern of peptides of the preparation is presented in Fig. 1. The major band of the peptides, (Ca^{2+} + Mg^{2+})-ATPase, was approximately 90% of total peptides. Sarcoplasmic reticulum fragments used here showed low activity of (Ca^{2+} + Mg^{2+})-ATPase and relatively high activity of Mg^{2+}-ATPase (Table I). It was observed that the (Ca^{2+} + Mg^{2+})-ATPase converted from low activity form to high activity form during the storage in the preparation. Then, it is considered that low activity form of the (Ca^{2+} + Mg^{2+})-ATPase is that of the fresh preparation. In this paper, we used the freshly prepared sarcoplasmic reticulum fragments.

Removal of membrane-bound metals with EDTA

Sarcoplasmic reticulum fragments, the protein concentration of which was adjusted to 0.5—1.0 mg/ml, were incubated overnight at 4°C in a solution containing 20 mM Tris-maleate (pH 6.8) and 0—$1 \cdot 10^{-2}$ M EDTA. As the control, sarcoplasmic reticulum fragments were incubated with $1 \cdot 10^{-4}$ M $CaCl_2$ in the same buffer.

Removal of membrane-bound metals with acidic buffers

Sarcoplasmic reticulum fragment suspensions (8—10 mg protein/ml, 0.12 M

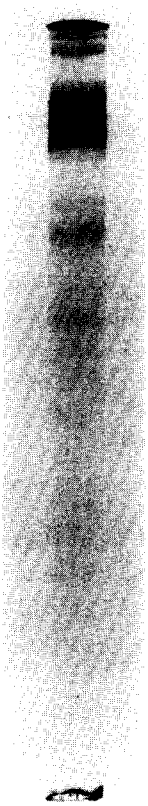

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sarcoplasmic reticulum fragments. Electrophoresis was carried out with 50 μg of protein on 7.5% polyacrylamide gel according to Weber and Osborn [16]. Sarcoplasmic reticulum fragments were dissolved in a solution of 4 M urea, 1% sodium dodecyl sulfate, 0.1 M phosphate buffer (pH 7.2) and 1% mercaptoethenol. Gel was stained with Comassie Brilliant Blue.

KCl, 20 mM Tris-maleate) were adjusted by 1 M HCl to a given pH from 4.2 to 6.8, and incubated for 1 h at 0° C.

In the experiments to restore sarcoplasmic reticulum fragment activities (ATPase, Ca-uptake, [^{32}P]phosphoenzyme formation), the pH of the sarcoplasmic reticulum fragment suspension was adjusted by dialysis against 20 mM K-acetate buffer (pH 5.2), or 40 mM Tris-maleate buffer (pH 6.0) to remove the membrane-bound metals, both containing 0.12 M KCl, $5 \cdot 10^{-4}$ M dithiothreitol, and was allowed to stand overnight at 4° C. Then the Visking tube was removed and dialyzed against 20 mM Tris · HCl buffer (pH 8.0) containing 0.12 M KCl, $5 \cdot 10^{-4}$ M dithiothreitol and also the materials indicated in Table IA, IB and IC, overnight at room temperature. The Visking tube used was boiled in 5% Na_2CO_3 containing 50 mM EDTA.

Measurement of membrane-bound Ca and Mg

After incubating sarcoplasmic reticulum fragments under the conditions mentioned above to remove the membrane-bound metals, they were washed twice with 20 mM Tris-maleate (pH 6.8) containing 50 mM KCl; sedimentation

by centrifugation and homogenization by Teflon homogenizer were repeated three times. Ca and Mg in the washed sarcoplasmic reticulum fragments were measured as membrane-bound metals. Ca and Mg were determined using a Hitachi Perkin-Elmer atomic absorption spectrophotometer. Standard Ca and Mg solutions contained 0.5% $LaCl_3$ and 10% trichloroacetic acid. Protein was removed with 10% trichloroacetic acid in the presence of 0.5% $LaCl_3$.

Assay of ATPase

The reaction mixture contained 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 20 mM Tris-maleate (pH 6.8), 0.04—0.07 mg protein/ml sarcoplasmic reticulum fragment and 1 mM EGTA or a given concentration of $CaCl_2$, in a total volume of 4 ml. After the preincubation for 5 min at 24°C, the reaction was initiated by adding ATP, and terminated by adding trichloroacetic acid. The amount of P_i liberated was measured according to the modified method of Allen [13].

Assay of Ca-uptake

The reaction mixture was the same as for the assay of ATPase, except it included $^{45}CaCl_2$. The reaction was terminated by filtrating the mixture with Millipore-filter (0.45 μm diameter). The amount of ATP-dependent Ca-uptake was calculated by the decrease of ^{45}Ca in the filtrate.

Assay of [^{32}P]phosphoenzyme

The reaction mixture contained 50 mM KCl, 1 mM $MgCl_2$, 10 μM [^{32}P]ATP, 5 mM $CaCl_2$, 20 mM Tris-maleate (pH 6.8) and 0.8—1.5 mg protein/ml sarcoplasmic reticulum fragments in a total volume of 2 ml. The reaction was initiated by adding 1 ml of sarcoplasmic reticulum fragment suspension. After incubating for 10 s at 0°C, the reaction was terminated by adding 4 ml of ice-cold 10% trichloroacetic acid containing 1 mM ATP, 10 mM P_i. Following centrifugation, the supernatant was decanted and the sediment was washed once with 7 ml of 5% trichloroacetic acid and three times with 7 ml of 2% trichloroacetic acid. The final sediment was solubilized in 2 ml of performic acid. Aliquots of 1 or 1.5 ml were assayed for radioactivity. Protein concentration was determined by the procedure of Lowry et al. [14] using bovine serum albumin as a standard.

Chemicals

Chemicals used were of analytical grade. ^{45}Ca, $^{32}P_i$ and [^{14}C]EDTA were purchased from the Japan Radioisotope Association. [γ-^{32}P]ATP was made by the procedure of Glynn and Chapell [15].

Results

Treatment of sarcoplasmic reticulum fragments with EDTA

In the presence of Mg^{2+}, ATP hydrolysis by sarcoplasmic reticulum fragments depends on the concentration of free Ca^{2+} in the reaction mixture, and the ATPase activity of sarcoplasmic reticulum fragments measured in the presence of Ca^{2+} and Mg^{2+} is called (Ca^{2+} + Mg^{2+})-ATPase. It has been shown that the Ca^{2+} dependency of the ATPase is biphasic and the optimum Ca^{2+} concen-

tration is $1 \cdot 10^{-5}$ M [1,2]. Such a biphasic Ca^{2+} response was also observed in this study with the EDTA-treated sarcoplasmic reticulum fragments (Fig. 2). However, in the case of sarcoplasmic reticulum fragments treated with $1 \cdot 10^{-2}$ M EDTA, the (Ca^{2+} + Mg^{2+})-ATPase activity at $1 \cdot 10^{-5}$ M Ca^{2+} was markedly depressed in comparison with that of untreated sarcoplasmic reticulum fragments (Fig. 2).

The relationship between the amount of remaining membrane-bound Ca and the activity of the (Ca^{2+} + Mg^{2+})-ATPase or the Ca-uptake of the EDTA-treated sarcoplasmic reticulum fragments are shown in Fig. 3. The decrease in the amount of the membrane-bound Ca from 2.5 mol/mg sarcoplasmic reticulum fragments protein to $0.9 \cdot 10^{-8}$ mol/mg sarcoplasmic reticulum fragments protein gave no effect either on the activities of the (Ca^{2+} + Mg^{2+})-ATPase or on the Ca-uptake. By further lowering the membrane-bound Ca, however, the activity of the Ca-uptake linearly diminished. On the other hand, the (Ca^{2+} + Mg^{2+})-ATPase was transiently activated with some increase in turbidity of the sarcoplasmic reticulum fragment suspension, which was observed after the removal of EDTA by washing, and consequently the activity declined. A similar relationship was observed between the amount of membrane-bound Mg and the activity of the (Ca^{2+} + Mg^{2+})-ATPase (Fig. 4). The critical concentrations for the transition of the ATPase activity were $0.9 \cdot 10^{-8}$ mol/mg protein for membrane-bound Ca and $0.05 \cdot 10^{-8}$ mol/mg protein for membrane-bound Mg, respectively. The value of the former varied in the range of $0.9–3.0 \cdot 10^{-8}$ mol Ca/mg protein in several sarcoplasmic reticulum fragment preparations. On the other hand, Mg^{2+}-ATPase activity showed little change by removing Ca (Fig. 5).

In these experiments with the EDTA-treated sarcoplasmic reticulum fragments, it was also demonstrated that the amount of remaining [^{14}C]EDTA in the treated sarcoplasmic reticulum fragments was negligible after washing twice with 20 mM Tris-maleate (pH 6.8) containing 50 mM KCl, and that the treated

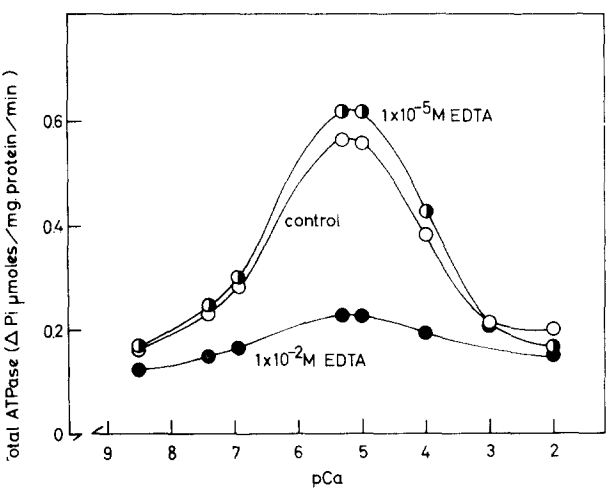

Fig. 2. Ca dependence of ATPase in sarcoplasmic reticulum fragments treated with EDTA. Sarcoplasmic reticulum fragments (1.0 mg protein/ml) were treated with 20 mM Tris-maleate (pH 6.8) (○———○), $1 \cdot 10^{-5}$ M EDTA (◑———◑) and $1 \cdot 10^{-2}$ M EDTA (●———●) overnight at 4°C. Ca^{2+} concentration in the assay medium was adjusted by use of EGTA; the association constant was $5 \cdot 10^{-5}$ M^{-1}.

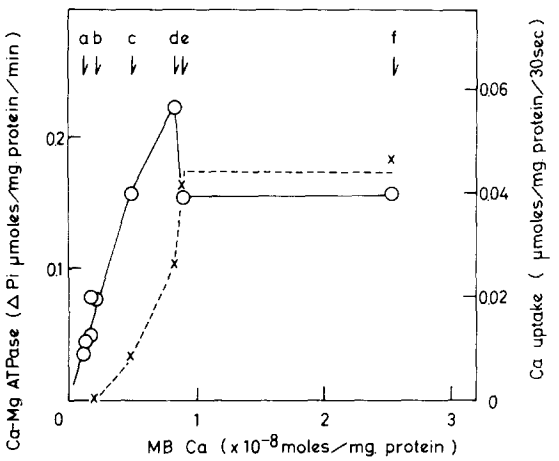

Fig. 3. The membrane-bound Ca (MB Ca), $(Ca^{2+} + Mg^{2+})$-ATPase and Ca-uptake of the EDTA-treated sarcoplasmic reticulum fragments. In Figs. 3, 4 and 5, the same sarcoplasmic reticulum fragment preparation was used. The membrane (0.5 mg protein/ml) was treated independently with $1 \cdot 10^{-4}$ M EDTA, $4 \cdot 10^{-5}$ M EDTA, $1 \cdot 10^{-5}$ M EDTA, $5 \cdot 10^{-6}$ M EDTA, only the buffer and $1 \cdot 10^{-4}$ M $CaCl_2$ at 4°C overnight, as represented respectively by a, b, c, d, e and f on arrows in the upper part of figure. Procedures of the assay for ATPase, Ca-uptake and the membrane-bound metals were described in Materials and Methods. ○———○, $(Ca^{2+} + Mg^{2+})$-ATPase; X———X, Ca-uptake.

sarcoplasmic reticulum fragments showed the same SDS-gel electrophoretic pattern of peptides [16] as the native sarcoplasmic reticulum fragments. The additional Ca-binding proteins in this preparation were not decreased even when the membrane-bound Ca was removed by EDTA at alkaline pH (8.5). Although we also attempted to restore the activities of sarcoplasmic reticulum fragments depressed by the EDTA treatment, it was not accomplished.

Treatment of sarcoplasmic reticulum fragments with acidic buffers

The membrane-bound metals were also removed by incubating sarcoplasmic reticulum fragments at acidic pH as described in Materials and Methods. This 'acidic pH method' has the advantage of reproducibility over the EDTA treat-

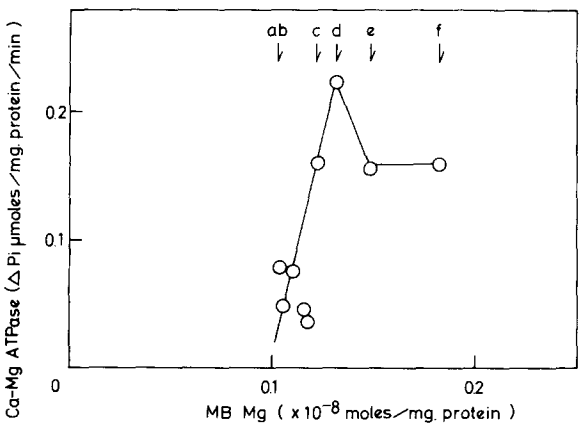

Fig. 4. The membrane-bound Mg (MB Mg) and $(Ca^{2+} + Mg^{2+})$-ATPase.

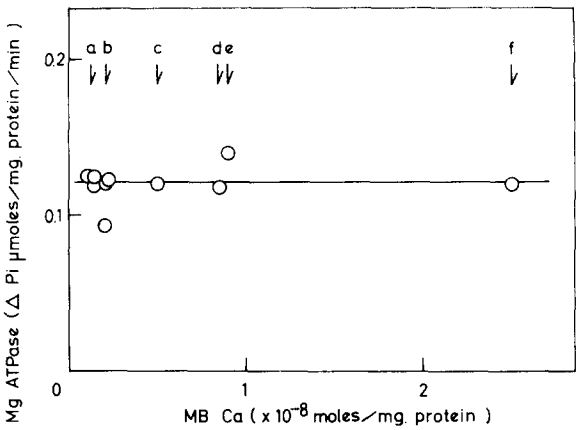

Fig. 5. The membrane-bound Ca (MB Ca) and Mg^{2+}-ATPase.

ment method. Furthermore, the 'washing' procedure required for the EDTA treatment can be omitted in this method. With this method, the removing of the membrane-bound metals from sarcoplasmic reticulum fragments of high concentrations was accomplished.

The relationships between the amounts of membrane-bound metals and the activities of (Ca^{2+} + Mg^{2+})-ATPase, Ca-uptake and phosphoenzyme formation obtained from sarcoplasmic reticulum fragments treated with acidic buffer were essentially the same as those of the EDTA-treated sarcoplasmic reticulum fragments (Fig. 6). However, it is difficult to understand the variation with the preparations in the amount of the membrane-bound Ca needed for full activity

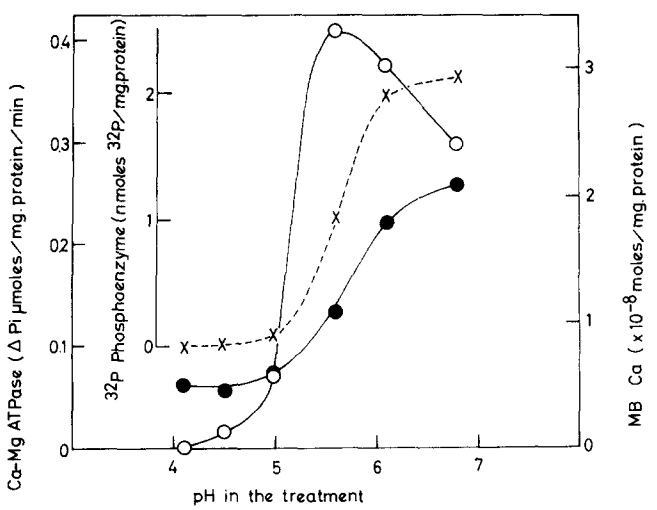

Fig. 6. The membrane-bound Ca (MB Ca), [^{32}P]phosphoenzyme formation and (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum fragments when treated at acidic pH. Each sarcoplasmic reticulum fragment preparation (8—10 mg protein/ml) was treated in a solution containing 20 mM Tris-maleate and 0.12 M KCl at the indicated pH in the abscissa at 0°C for 1 h, as described in Materials and Methods. Assay of [^{32}P]-phosphoenzyme and (Ca^{2+} + Mg^{2+})-ATPase were performed by the procedure described in Materials and Methods. ○———○, (Ca^{2+} + Mg^{2+})-ATPase; X- - - - -X, [^{32}P]phosphoenzyme; ●———●, membrane-bound Ca.

(Figs. 3 and 6). On the other hand, when the purified (Ca^{2+} + Mg^{2+})-ATPase was used, the amount of the membrane-bound Ca needed for full activity was approximately 1 mol/mol of ATPase. This result will be reported in detail in the following paper. The turbidity of sarcoplasmic reticulum fragment suspension also increased transiently by lowering the pH of the treating solution, while the Mg^{2+}-ATPase activity slightly decreased as the amount of membrane-bound Ca was lowered (Fig. 7). This result is different from the one obtained from the EDTA-treated sarcoplasmic reticulum fragments. Sarcoplasmic reticulum fragments treated with acidic buffer also showed the same patterns in SDS-gel electrophoresis [16] as the native sarcoplasmic reticulum fragments. We were unable to obtain the time course of the removal of the membrane-bound Ca, probably because it was difficult to terminate punctually the removal reaction with the repeated sedimentation and washing of sarcoplasmic reticulum fragments. The time course of the removal can be studied by using ^{45}Ca-labeled membrane.

In the cases of both EDTA treatment and the 'acidic pH' treatment, the activity of (Ca^{2+} + Mg^{2+})-ATPase, Ca-uptake and phosphoenzyme formation diminished as the amount of membrane-bound metals was lowered, except for the transient activation of (Ca^{2+} + Mg^{2+})-ATPase. Results of these experiments indicate that there is a close relationship between the activities of sarcoplasmic reticulum fragments and the amount of membrane-bound metals, and that bound metals of some types are responsible for the activities.

By decreasing the membrane-bound metals, it was observed that the transient activation of the (Ca^{2+} + Mg^{2+})-ATPase was accompanied by the decrease in Ca-uptake activity and the amount of phosphoenzyme. This phenomenon was observed both in the cases of acidic pH-treated sarcoplasmic reticulum fragments and EDTA-treated sarcoplasmic reticulum fragments. This observation may be explained as the result of the increase of the permeability of sarcoplasmic reticulum fragment membrane to Ca^{2+}. Duggan and Martonosi reported

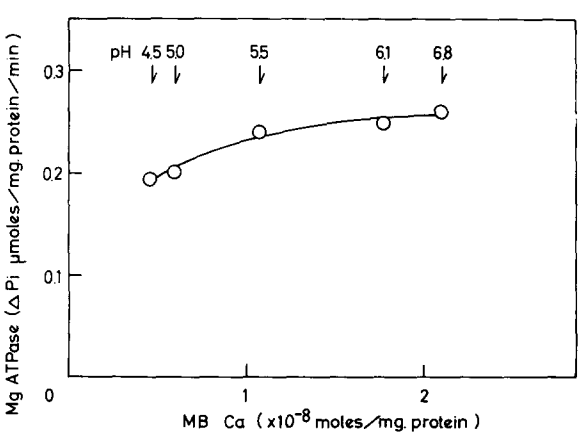

Fig. 7. The membrane-bound Ca (MB Ca) and Mg^{2+}-ATPase of sarcoplasmic reticulum fragments when treated at acidic pH. This sarcoplasmic reticulum fragment preparation was the same as in Fig. 6. The membrane was treated independently at pH 6.8, 6.1, 5.5, 4.5 and 4.1.

that the removal of the membrane-bound Ca gave rise to an increase in the permeability of the membrane to [^{14}C]inulin or [^{14}C]dextran, and an increase in the activity of (Ca^{2+} + Mg^{2+})-ATPase [8]. They also reported that C_1 and C_2 proteins, both Ca-binding proteins [7], were released from sarcoplasmic reticulum fragments by the EDTA treatment at alkaline pH. In our experiment, however, the EDTA treatment was performed at pH 6.8, and the protein profiles by SDS-gel electrophoresis of EDTA-treated and non-treated sarcoplasmic reticulum fragments were the same. These observations suggest that the increase of Ca^{2+} permeability of sarcoplasmic reticulum fragments by removing bound-Ca is not due to the detachment of some proteins from sarcoplasmic reticulum fragments.

Restoration of (Ca^{2+} + Mg^{2+})-ATPase activity, phosphoenzyme formation and Ca-uptake in the metal-deficient sarcoplasmic reticulum fragments

The (Ca^{2+} + Mg^{2+})-ATPase activity, the phosphoenzyme formation and the Ca-uptake of sarcoplasmic reticulum fragments were markedly suppressed with the decrease in the amount of the membrane-bound Ca and Mg by treating sarcoplasmic reticulum fragments with Tris-maleate buffer (pH 6.0) containing 0.12 M KCl, $5 \cdot 10^{-4}$ M dithiothreitol (Table IA).

When the treated sarcoplasmic reticulum fragments were dialyzed against the pH 8.0 Tris · HCl buffer containing various amounts of Ca^{2+}, the increase in the amount of the membrane-bound Ca and the decrease in the amount of the membrane-bound Mg were observed (Table IA). With the increase in the amount of the membrane-bound Ca from $0.4 \cdot 10^{-8}$ mol/mg protein to $2.8 \cdot 10^{-8}$ mol/mg protein, the amount of [^{32}P]phosphoenzyme increases from 0.2 to 0.65 nmol ^{32}P/mg protein. When the amount of the membrane-bound Ca was raised to $2.2 \cdot 10^{-8}$ mol/mg protein or to $2.8 \cdot 10^{-8}$ mol/mg protein, the phosphoenzyme formation of the 'acidic pH'-treated sarcoplasmic reticulum fragments was restored to 57 or 74% of the native sarcoplasmic reticulum fragments, respectively. On the other hand, recovery of the Ca-uptake was 38 and 76% of the native sarcoplasmic reticulum fragments in these cases. The extent of the recoveries of the phosphoenzyme formation and the Ca-uptake in sarcoplasmic reticulum fragments with $2.8 \cdot 10^{-8}$ mol Ca/mg protein remained the same, whereas sarcoplasmic reticulum fragments with $2.2 \cdot 10^{-8}$ mol Ca/mg protein showed higher recovery of the phosphoenzyme formation than that of the Ca-uptake. As to the discrepancy between these two activities, it can be considered that the membrane with $2.2 \cdot 10^{-8}$ mol Ca/mg protein was more leaky for Ca^{2+} than the membrane with $2.8 \cdot 10^{-8}$ mol/mg protein. This consideration was supported by the observation that the activity of (Ca^{2+} + Mg^{2+})-ATPase with $2.2 \cdot 10^{-8}$ mol Ca/mg protein was higher than that of (Ca^{2+} + Mg^{2+})-ATPase with $2.8 \cdot 10^{-8}$ mol Ca/mg protein. Dialysis of the treated sarcoplasmic reticulum fragments against Mg^{2+} resulted in a slight decrease in the amount of membrane-bound Ca and a marked increase in that of membrane-bound Mg (Table IA). On the other hand, the phosphoenzyme formation and Ca-uptake of the treated sarcoplasmic reticulum fragments were restored to a lesser extent than in the case of dialyzing against Ca^{2+}. Both activities were inhibited by raising the amount of the membrane-bound Mg. When the treated sarcoplasmic reticulum fragments were dialyzed against a solution containing

TABLE I

THE RESTORATION OF THE ACTIVITIES OF (Ca^{2+} + Mg^{2+})-ATPase, [^{32}P]PHOSPHOENZYME FORMATION AND Ca-UPTAKE OF Ca-DEFICIENT SARCOPLASMIC RETICULUM FRAGMENTS

The membranes were treated by dialysis against 40 mM Tris-Maleate (pH 6.0) (A and B) and or 20 mM K-acetate (pH 5.2) (C) containing 0.12 M KCl and $5 \cdot 10^{-4}$ M dithiothreitol at 4°C overnight. The membranes used in A, B and C were treated differntly and their protein concentrations were 8.9, 8.6 and 13.4 mg protein/ml, respectively. Then the Visking tubes were dialyzed against 20 mM Tris · HCl (pH 8.0) containing 0.12 M KCl, $5 \cdot 10^{-4}$ M dithiothreitol, the indicated concentrations of EDTA, Ca^{2+} and Mg^{2+} at room temperature, overnight.

	(Ca^{2+} + Mg^{2+})-ATPase (μmol/mg prot. per min)	Mg^{2+}-ATPase (μmol/mg prot. per min)	[^{32}P]Phosphoenzyme (nmol/mg prot.)	Membrane-bound Ca (10^{-8} mol/mg prot.)	Membrane-bound Mg (10^{-8} mol/mg prot.)	Ca-uptake (μmol/mg prot.)
A. Non-treated	0.252	0.264	0.88	4.1	0.59	0.084
Treated at pH 6.0	0.197	0.298	0.22	1.7	0.22	0.037
Dialysis against						
$1 \cdot 10^{-4}$ M EDTA	0.111	0.175	0.02	0.4	0.11	0.000
$1 \cdot 10^{-5}$ M Ca^{2+}	0.419	0.223	0.50	2.2	0.08	0.032
$1 \cdot 10^{-4}$ M Ca^{2+}	0.357	0.223	0.65	2.8	0.08	0.064
$1 \cdot 10^{-3}$ M Mg^{2+}	0.453	0.190	0.31	1.8	1.06	0.018
$1 \cdot 10^{-2}$ M Mg^{2+}	0.359	0.193	0.16	1.3	5.06	0.000
$1 \cdot 10^{-4}$ M Ca^{2+} + $1 \cdot 10^{-3}$ M Mg^{2+}	0.545	0.224	0.46	2.0	1.33	0.034
B. Non-treated	0.070	0.241	1.76	2.5	0.93	—
Treated at pH 6.0	0.000	0.265	0.00	1.2	0.18	—
Dialysis against						
$1 \cdot 10^{-4}$ M Ca^{2+}	0.247	0.124	0.55	2.1	0.15	—
$1 \cdot 10^{-3}$ M Mg^{2+}	0.130	0.105	0.11	1.0	0.57	—
C. Non-treated	0.188	0.299	0.85	1.5	0.34	—
Treated at pH 5.2	0.031	0.231	0.01	0.3	0.07	—
Dialysis against						
$1 \cdot 10^{-4}$ M EDTA	0.000	0.093	0.01	0.1	0.04	—
$1 \cdot 10^{-5}$ M Ca^{2+}	0.081	0.098	0.05	0.3	0.08	—
$1 \cdot 10^{-4}$ M Ca^{2+}	0.109	0.093	0.09	0.7	0.04	—
$1 \cdot 10^{-3}$ M Ca^{2+}	0.141	0.093	0.11	3.4	0.04	—

both Ca^{2+} and Mg^{2+}, the phosphoenzyme formation and Ca-uptake were restored more than in the case of the dialysis against Mg^{2+} and less than in the case of the dialysis against Ca^{2+}. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of the treated sarcoplasmic reticulum fragments was recovered by dialysis against Mg^{2+} to the same extent as the dialysis against Ca^{2+}. The activity was suppressed with further increase in the amount of the membrane-bound Mg. These observations suggest that the restored activities obtained by dialyzing the treated sarcoplasmic reticulum fragments against Mg^{2+} are due to the contaminant Ca^{2+} present in the dialyzing buffer.

As shown in Table IB, a preparation which formed almost no phosphoenzyme after the treatment at pH 6.0 restored the activity with increasing membrane-bound Ca by dialyzing against Ca^{2+} solution. The recovery was not achieved by dialyzing against Mg^{2+} solution. When the membrane-bound metals were removed at lower pH (5.2), the recovery of phosphoenzyme formation was very low, while the restoration of membrane-bound Ca was accomplished (Table IC). A tendency of increase in the amount of phosphoenzyme, however, was also observed with the increase in the amount of membrane-bound Ca in this preparation treated at lower pH. These observations were consistent with the results described in Table IA. We could not establish a correlation between the amount of Ca rebound and the various types of activities. Because rebound Ca binds not only to 'the endogenous Ca binding site', but also to the various types of Ca binding sites, we can not distinguish the amount of 'the rebound endogenous Ca' from the total amount of the rebound Ca. The Ca-uptake of the Ca-deficient sarcoplasmic reticulum fragments was easily restored with good reproducibility by incubating the membrane in the alkaline buffer containing Ca^{2+}, when the bound Ca was removed at pH 6.0. When most of the bound Ca was removed at pH 5.2, no restoration of the Ca-uptake of the Ca-deficient membrane was observed even in the solution containing a higher concentration of Ca^{2+}.

These observations indicate that a part of the membrane-bound Ca is necessary for the phosphoenzyme formation and that another part of the membrane-bound Ca is responsible for Ca-uptake of the membrane. The membrane-bound Mg, however, is not able to substitute for the Ca, but is inhibitive to both phosphoenzyme formation and Ca-uptake.

Discussion

The membrane-bound Ca and Mg of sarcoplasmic reticulum fragments were removed by treating the membrane with acidic buffer or with EDTA. The amount of membrane-bound metals of the untreated sarcoplasmic reticulum fragments were $2—4 \cdot 10^{-8}$ mol/mg protein for Ca and $0.2—1.0 \cdot 10^{-8}$ mol/mg protein for Mg, respectively. The amount of membrane-bound Ca was the same as observed by Makinose and Hasselbach, Duggann and Martonosi, and Yasukawa [17,9,10].

With the decrease in the amount of the membrane-bound Ca below $1—3 \cdot 10^{-8}$ mol/mg protein, it was observed that the transient activation of $(Ca^{2+} + Mg^{2+})$-ATPase of the treated sarcoplasmic reticulum fragments was accompanied with decrease in Ca-uptake as well as phosphoenzyme formation. This

result suggests that the transient activation of $(Ca^{2+} + Mg^{2+})$-ATPase is due to the increase in the permeability of the membrane to Ca^{2+}. The activation of $(Ca^{2+} + Mg^{2+})$-ATPase accompanying the inhibition of Ca-uptake has been reported by Duggan and Martonosi [9]. Yasukawa also reported that the Ca-uptake of Ca-deficient membrane was restored by increasing the amount of membrane-bound Ca [10].

By removing the membrane-bound metals, the turbidity of the membrane suspension increased and finally a sediment was formed. This might correspond to the aggregation of sarcoplasmic reticulum fragment vesicles and the shrinkage of the membrane surface as reported by Yasukawa [18].

The formation of the phosphoenzyme in the metal-deficient membrane was shown to be low, though enough Ca^{2+} was added to the assay medium. When the membrane-bound metals were removed by dialyzing sarcoplasmic reticulum fragments at pH 6.0, the activity of $(Ca^{2+} + Mg^{2+})$-ATPase and the phosphoenzyme formation of the metal-deficient membrane can be restored by dialyzing the membrane against alkaline buffer (pH 8.0) containing Ca^{2+}, at room temperature. In these procedures of the restoration of the activities, it is important to adjust the pH of the dialyzing solution at 8.0. The restoration was not accomplished by dialyzing at pH 6.8. Further systematical studies on the pH dependence of the Ca removal and rebinding are required. The amount of phosphoenzyme in the sarcoplasmic reticulum fragments treated at pH 6.0 was restored with the increase in the amount of membrane-bound Ca, and was suppressed with the increase in the amount of membrane-bound Mg. When phosphorylation of the metal-deficient membrane was assayed at pH 8.0, the amount of phosphoenzyme was the same as that at pH 6.8, the condition generally used in our study. This result indicates that free Ca^{2+} in the assay medium is not readily substituted for membrane-bound Ca.

It has been known that the phosphoenzyme of the untreated sarcoplasmic reticulum fragments could not be formed in the assay medium free from Ca^{2+}. On the other hand, Ca-deficient sarcoplasmic reticulum fragments could not be phosphorylated even in the presence of enough Ca^{2+} in the assay medium. SDS-gel electrophoretic patterns of the metal-deficient membranes were the same as those of the untreated membrane. These observations led us to the conclusion that there is an endogeous membrane-bound Ca necessary to the formation of the phosphoenzyme, and that Ca^{2+} in the assay medium is unable to substitute for the membrane-bound Ca in function. Yet it has been reported that Ca^{2+} in the external medium was exchangable for the membrane-bound Ca of the untreated membrane [17]. It is probable that the endogenous membrane-bound Ca is different from the Ca bound to the $(Ca^{2+} + Mg^{2+})$-ATPase, calsequestrin and other Ca-binding proteins which is readily and reversibly removed by EDTA or EGTA. It seems likely that Ca of 1—3 mol/10^5 g protein, located on $(Ca^{2+} + Mg^{2+})$-ATPase molecule, takes part in the phosphoenzyme formation. It is generally accepted that the ability of phosphoenzyme formation of $(Ca^{2+} + Mg^{2+})$-ATPase is not dependent on the changes in the permeability of the membrane. We are studying the affection of the permeability to the change of phosphoenzyme formation by using the purified ATPase. It is of interest to know whether the bound Ca translocates itself during the hydrolysis of ATP by $(Ca^{2+} + Mg^{2+})$-ATPase.

The activity of Ca-uptake of Ca-deficient membrane was not restored with the increase in the amount of the membrane-bound Mg, but with the increase in the amount of the membrane-bound Ca. This is probably due to the inability of the membrane-bound Mg in maintaining the permeability of the membrane to Ca^{2+}.

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