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THE ADP- AND Mg^{2+}-REACTIVE CALCIUM COMPLEX OF THE PHOSPHOENZYME IN SKELETAL SARCOPLASMIC RETICULUM Ca^{2+}-ATPase *

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The effects of ATP on Ca^{2+} binding in the absence of added Mg^{2+} to the purified sarcoplasmic reticulum Ca^{2+}-ATPase were studied at pH 7.0 and 0°C. ATP increased the number of Ca^{2+}-binding sites of the enzyme from 2 to 3 mol per mol of phosphorylatable enzyme. The association constant for the ATP-induced Ca^{2+} binding was $4 \cdot 10^5$ M^{-1}, which was not significantly different from that obtained in the absence of ATP. Ado*P*[CH_2]*PP* had little effect on the Ca^{2+}-binding process. The amount of phosphoenzyme formed was equivalent to the level of ATP-induced Ca^{2+} binding. ADP decreased the level of ATP-induced Ca^{2+} binding and phosphoenzyme by the same amount. These results suggest that ATP-induced Ca^{2+} binding exists in the form of an ADP-reactive phosphoenzyme·Ca complex. In addition, the Ca^{2+} bound to the enzyme in the presence of ATP was released on the addition of 1 mM $MgCl_2$; after the release of Ca^{2+}, the phosphoenzyme decayed. These observations suggest that Mg^{2+}, added after the ATP-induced Ca^{2+}-binding process, may replace the Ca^{2+} on the phosphoenzyme and initiate phosphoenzyme decomposition.

Introduction

It is generally accepted that the sarcoplasmic reticulum produces muscle relaxation by removing Ca^{2+} from the contractile apparatus. Calcium transport in isolated sarcoplasmic reticulum membrane vesicles is an active process, dependent upon a membrane-bound Ca^{2+}-ATPase [1–4]. The ATPase protein has high-affinity Ca^{2+}-binding sites [5–8]. It has been reported that in the absence of ATP, the affinity of the enzyme for Ca^{2+} is about 30000 higher than that for Mg^{2+}, whereas, in the presence of ATP the affinity ratio for Ca^{2+} compared to Mg^{2+} is only 2.5 [9]. It has been suggested that Ca^{2+}-binding sites required for the phosphorylated enzyme formation are distinct from Mg^{2+}-binding site(s) required for the phosphoenzyme decomposition [10–12]. In fact, Yamada and Ikemoto [13] have suggested that the substrate acceptor site for Ca^{2+} derived from Ca·ATP is separate from the Ca^{2+}-transport sites.

Calcium binding to the sarcoplasmic reticulum Ca^{2+}-ATPase has been studied mostly in the presence of Mg^{2+} at millimolar concentration [5,7,8,14]. Very little information is available regarding Ca^{2+} binding in the absence of added Mg^{2+} [14–16]. It was reported, e.g., that in the absence of added Mg^{2+}, the substrate ATP did not affect Ca^{2+} binding to phospholipase A-digested microsomal sarcoplasmic reticulum vesicles

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Ado*P*[CH_2]*PP*, adenosine 5′-[α,β-methylene]triphosphate.

[14]. By the use of ^{45}Ca, however, we observed that in the absence of added Mg^{2+}, ATP induced an increase in the amount of $^{45}Ca^{2+}$ which was bound to a purified Ca^{2+}-ATPase and the $^{45}Ca^{2+}$ was released on the addition of 1 mM $MgCl_2$ [15]. Recently, Dupont [16] also observed ATP-induced $^{45}Ca^{2+}$ incorporation into microsomal sarcoplasmic reticulum and reported that the incorporated $^{45}Ca^{2+}$ was released by EDTA, which brought about a change in the phosphoenzyme reactivity toward ADP. Since the sarcoplasmic reticulum may contain intrinsic Ca^{2+} which is inaccessible to Ca^{2+} chelators [17–19], it is uncertain whether the increased $^{45}Ca^{2+}$ in our and Dupont's reports is due to an actual increase in Ca^{2+} binding or is a result of substitution or exchange of $^{45}Ca^{2+}$ for the intrinsic Ca^{2+}. To answer this question, we studied ATP-induced Ca^{2+} binding to a purified Ca^{2+}-ATPase in the absence of added Mg^{2+} by $^{45}Ca^{2+}$ and by atomic abosrption spectrophotometry. We report that either in the presence or in the absence of ATP, the enzyme binds Ca^{2+}, but the substitution or exchange of external Ca^{2+} for intrinsic Ca^{2+} is negligible. Also, in the absence of Mg^{2+}, ATP induces an increase in the number of Ca^{2+}-binding sites on the enzyme and the bound Ca^{2+} is in the form of an ADP-reactive phosphoenzyme · Ca complex and is replaceable by Mg^{2+}.

Materials and Methods

Chemicals

Chemicals used in this work were of analytical grade. Creatine kinase and Ado*P*[CH_2]*PP* were obtained from Sigma Chemical Co. $^{45}CaCl_2$, [^{14}C]ATP, and [^{32}P]ATP were purchased from the Radiochemical Centre, Amersham. Deoxycholate was purified by the method of McLennan [20].

Purification of Ca^{2+}-ATPase

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle by the method of Weber et al. [21] with some modification [17]. Ca^{2+}-ATPase of the sarcoplasmic reticulum was purified by the following two procedures.

Deoxycholate-solubilized Ca^{2+}-ATPase. Sarcoplasmic reticulum was solubilized with sodium deoxycholate (deoxycholate/protein = 1 : 3, w/w) in a 20 mM Tris-HCl buffer (pH 8.0) solution containing 0.5 M KCl, 0.3 M sucrose, 10 μM $CaCl_2$ and 0.1% β-mercaptoethanol at 0°C for 10 min. Insoluble materials were removed by centrifugation at 165 000 × *g* for 60 min. The supernatant was placed on a Sephadex G-100 column equilibrated with a solution containing 0.5 M KCl, 10 μM $CaCl_2$ and 0.1% β-mercaptoethanol. The fractions eluted immediately at the void volume were pooled and then sedimented at 53 000 × *g* for 60 min. The pellet was suspended in a buffer solution containing 0.5 M KCl, 0.3 M sucrose and 10 μM $CaCl_2$, and was sedimented again at 53 000 × *g* for 60 min. The resultant pellet was resuspended in a 20 mM Tris-maleate buffer (pH 7.0) containing 0.5 M KCl and 0.3 M sucrose. The suspension was used as the deoxycholate-solubilized Ca^{2+}-ATPase.

Deoxycholate-extracted Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles were partially solubilized by sodium deoxycholate (deoxycholate/protein = 1 : 4, w/w) in the same solubilization buffer as described above. The suspension was centrifuged at 40 000 × *g* for 60 min. The pellet was washed to remove detergent, resuspended in a 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M KCl, 0.3 M sucrose and 10 μM $CaCl_2$, and centrifuged at 40 000 × *g* for 60 min. After one additional centrifugation in fresh buffer, the resultant pellet was resuspended in a 20 mM Tris-maleate buffer (pH 7.0) solution containing 0.5 M KCl and 0.3 M sucrose. The suspension was used as deoxycholate-extracted Ca^{2+}-ATPase.

The deoxycholate-solubilized or deoxycholate-extracted Ca^{2+}-ATPase was precipitable by centrifugation and does not filter through a Millipore filter of 0.45 μm pore size, indicating the possibility that these enzymes are still membrane bound. The two preparations did not accumulate Ca^{2+} in the presence of potassium oxalate (Table I), and their SDS gel electrophoretic patterns were identical (Fig. 1). The 115 kDa Ca^{2+}-ATPase [22] was 93% and the 55 kDa high-affinity, Ca^{2+}-binding protein [23] 3.4% of the total protein. Calsequestrin (44 kDa) [24,25] was not detected. The purified Ca^{2+}-ATPases (10–25 mg protein/ml) were stored at 0°C and used within 2 days after purification. These preparations were 'contaminated' by 0.5–2.0 nmol Mg^{2+}/mg protein. In the procedures for purification, washing

TABLE I

ATP-INITIATED Ca^{2+} UPTAKE BY SARCOPLASMIC RETICULUM, DEOXYCHOLATE-EXTRACTED AND DEOXYCHOLATE-SOLUBILIZED Ca^{2+}-ATPase

Ca^{2+} uptake was assayed at 24°C in a 20 mM Tris-maleate buffer solution (pH 6.8) containing 0.12 M KCl, 25–40 μM $CaCl_2$, 1 mM $MgCl_2$, 5 mM potassium oxalate, 1 mM ATP and 0.01–1.03 mg protein/ml of sarcoplasmic reticulum, deoxycholate-extracted and deoxycholate-solubilized Ca^{2+}-ATPase. The reaction was terminated by filtration through a Millipore filter in the case of sarcoplasmic reticulum and through a glass filter in the case of deoxycholate-extracted or deoxycholate-solubilized Ca^{2+}-ATPase. The amount of Ca^{2+} uptake was calculated from the total radioactivity in the whole mixture minus the radioactivity in the filtrate. The ATP-initiated Ca^{2+} uptake is presented as the difference between the amount of Ca^{2+} uptake in the presence and absence of ATP. Results are expressed as nmol/mg per min.

	Sarcoplasmic reticulum	Deoxycholate-extracted Ca^{2+}-ATPase	Deoxycholate-solubilized Ca^{2+}-ATPase
In complete system (reaction time 3 min)	829.0	2.9	1.0
−oxalate (reaction time 1 min)	78.0	−1.5	−1.4

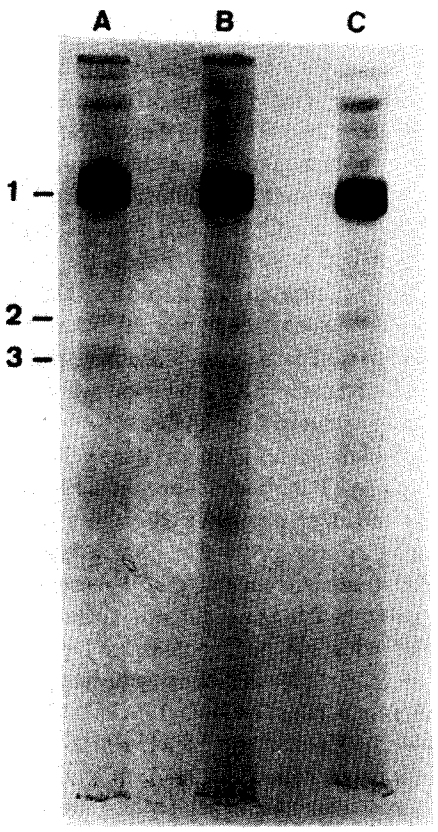

Fig. 1. SDS-polyacrylamide gel electrophoretic profiles of sarcoplasmic reticulum (A), deoxycholate-solubilized Ca^{2+}-ATPase (B) and deoxycholate-extracted Ca^{2+}-ATPase (C) showing: Ca^{2+}-ATPase (1), high-affinity Ca^{2+}-binding protein (2) and calsequestrin (3).

the enzyme with $CaCl_2$ at higher than 10 μM decreased the Mg^{2+} contamination to below 0.5 nmol/mg protein, but the enzyme aggregated when this high $CaCl_2$ was present.

Assays

Ca^{2+} binding. Unless otherwise indicated the Ca^{2+}-binding reaction was carried out at 0°C in 1 or 2 ml of 20 mM Tris-maleate buffer solution (pH 7.0) containing 0.12 M KCl, 1 mM creatine phosphate, 0.3 mg/ml creatine kinase, 1.02–2.50 mg/ml Ca^{2+}-ATPase and various concentrations of $^{45}CaCl_2$ and EGTA with or without ATP. The reaction mixture in the absence of added Mg^{2+} was contaminated with 10–15 μM Mg^{2+}, as measured by atomic absorption spectrometry. Most of the contaminating Mg^{2+} originated in creatine kinase used in the ATP-regenerating system. The Mg^{2+} at concentrations less than 100 μM does not affect the Ca^{2+} binding to the enzyme in the presence of ATP (see Fig. 6). The Ca^{2+}-binding reaction was initiated by adding ATP. The Ca^{2+}-bound proteins of the Ca^{2+}-ATPase or phosphoenzymes were separated by a glass filter, Millipore filter, or the centrifugation procedure described below.

Glass filter procedure. The membrane-bound proteins were removed by filtration with two layers of a Whatmann glass filter (0.7 μm pore size, GF/F). The radioactivity of ^{45}Ca in both the original reaction mixture and the filtrate was mea-

sured; the difference determined the amount of $^{45}Ca^{2+}$ which was bound to the enzyme.

Millipore filter procedure. A Millipore filter (0.45 μm pore size, HA) was used. The filter was washed once with 2 ml of 20 mM Tris-maleate (pH 7.0) containing 0.12 M of KCl and various concentrations of $^{45}CaCl_2$ before it was used for filtering 0.2 ml of the reaction mixture. The Ca^{2+}-bound proteins on the filter were washed a few times within 1 min to remove ATP, creatine kinase and creatine phosphate. For each washing 2 ml of the washing solution were used. After the washing, the enzyme retained on the filter was washed with 2 ml of the washing solution containing ADP or Mg^{2+}. The amount of Ca^{2+} binding was calculated from the radioactivity of the enzyme-retaining filter subtracted from the radioactivity of the enzyme-free filter.

Centrifugation. The Ca^{2+}-binding reaction was carried out in a centrifuge tube for 15 min. After the reaction, the membrane-bound proteins were separated from 2 to 4 ml of reaction medium by centrifugation at 0–5°C and 109000 × g for 60 min. The Ca^{2+} concentrations in the original mixture and the supernatant were both measured by an atomic absorption spectroscopy method as described previously [17] and by a $^{45}Ca^{2+}$ method; the difference determined the amount of Ca^{2+} which was bound to the enzyme.

In the above three procedures, the centrifugation procedure gave the lowest blank values and the most precise Ca^{2+}-binding values. The Millipore filtration procedure gave results with large variations. The glass filtration procedure gave high blank values.

Residual ATP. ATP which remained on the enzyme was assayed by measuring [^{14}C]ATP which remained on the enzyme using the Millipore filtration method as described for the Ca^{2+}-binding assay. The amount of remaining ATP was calculated from the radioactivity of the enzyme-retaining filter subtracted from the radioactivity of the enzyme-free filter which was prewashed with blank solution containing [^{14}C]ATP.

Phosphoenzyme. The phosphorylation reaction was carried out in the same solution as that for the Ca^{2+} binding, and the assay of ^{32}P-labeled phosphoenzyme was carried out by the Millipore filtration method. Creatine phosphate and creatine kinase were omitted. The concentration of the contaminated Mg^{2+} in the reaction mixture was 0.5–2.0 μM. The reaction was terminated by washing the filter with 2 ml of ice-cold 5% trichloroacetic acid. The filter was washed four times with 2 ml of 5% trichloroacetic acid containing 0.1 mM ATP and 0.1 mM P_i. The amount of ^{32}P-labeled phosphoenzyme was calculated from the radioactivity of the phosphoenzyme-retaining filter, and that of [^{32}P]ATP was calculated from the radioactivity of the filtrate which contained [^{32}P]ATP extracted by the method of Martin and Doty [26]. The total amount of phosphorylatable enzyme was the maximum phosphoenzyme obtained in 0.1 mM $CaCl_2$, 0.1 mM ATP and 5 mM $MgCl_2$.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide gel according to the method of Weber and Osborn [27]. Gels were stained with Coomassie blue. Protein concentration was determined by the procedures of Lowry et al. [28] using bovine serum albumin as the standard.

Results

The amounts of Ca^{2+} binding to Ca^{2+}-ATPase determined by atomic absorption spectroscopy and by $^{45}Ca^{2+}$ methods are summarized in Table II. Both methods gave similar results. Calcium binding was the highest when the reaction was carried out in the presence of ATP and absence of Mg^{2+}. In the presence of Mg^{2+} or the absence of ATP with or without Mg^{2+}, Ca^{2+} binding was reduced 30%.

The time course of Ca^{2+} binding to the enzyme is shown in Fig. 2. ATP increased the amount of Ca^{2+} binding from 13.2 to 18.1 nmol/mg protein in 10 s, and most of the bound Ca^{2+} increased by ATP was released by 1 mM $MgCl_2$ within 10 s. When ATP was not regenerated during the reaction, the amount of ATP-induced Ca^{2+} binding was diminished within 30–60 s after the addition of ATP.

The association constants of Ca^{2+}-ATPase for Ca^{2+} in both the presence and absence of ATP without added Mg^{2+} were not significantly different as determined by Scatchard analysis (Fig. 3); ATP increased the number of Ca^{2+}-binding sites from 10.2 to 15.6 nmol/mg protein.

TABLE II

THE Ca^{2+} BINDING ASSAYED BY ATOMIC ABSORPTION SPECTROSCOPY AND ^{45}Ca METHODS

Reaction conditions: Ca^{2+}-ATPase (deoxycholate-solubilized), 2.5 mg/ml; $CaCl_2$, 46 μM; ATP, 100 μM. The centrifugation procedure was used for these experiments. The bound Ca^{2+} was measured from the total concentration of Ca^{2+} in the original reaction mixture minus that in the supernatant.

Method	Bound Ca^{2+} (nmol/mg protein)			
	−ATP		+ATP	
	−Mg	+Mg	−Mg	+Mg
Atomic absorption, mean ± S.E. ($n = 4$)	10.8 ± 0.8	10.8 ± 2.0	15.2 ± 0.8	11.2 ± 0.6
^{45}Ca	9.7 [a]	9.4 [a]	13.7 [a]	9.8 [a]
	9.2 [b]	8.9 [b]	14.1 [b]	8.7 [b]
	9.0 [b]	9.3 [b]	13.5 [b]	8.5 [b]

[a] Assayed under the same condition as that in atomic absorption.
[b] Assayed by using 1.3 mg/ml Ca^{2+}-ATPase, 37 μM $CaCl_2$ and 100 μM ATP.

Ado*P*[CH_2]*PP* at 10 μM did not increase the amount of Ca^{2+} binding; whereas, ATP did increase the binding from 12.2 to a maximum level of 17.5 nmol/mg protein (Fig. 4). In some of the Ca^{2+}-ATPase preparations, ATP-induced Ca^{2+} binding was not observed at concentrations of ATP higher than 1 mM. The steady-state level of Ca^{2+} binding dependent upon ATP was 5.3 nmol/mg protein. During the course of the reaction, acid-precipitable phosphoenzyme is formed.

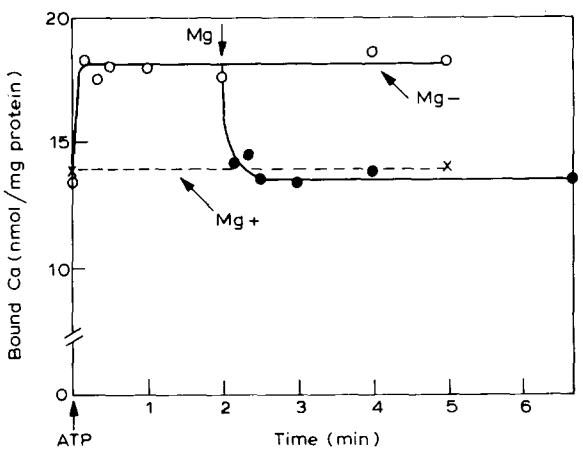

Fig. 2. The time courses of ATP-initiated Ca^{2+} binding in the presence (×- - -×) and absence (○——○) of 1 mM Mg^{2+}. Mg↓ indicates the addition of 1 mM Mg^{2+} to the Ca^{2+}-binding reaction mixture which is originally without added Mg^{2+}. Following the addition of Mg^{2+}, the Ca^{2+} binding decreases (●——●). Other conditions were: Ca^{2+}-ATPase (deoxycholate-extracted), 1.02 mg/ml; ATP, 50 μM; $CaCl_2$, 20 μM. The glass filtration procedure was used for these experiments.

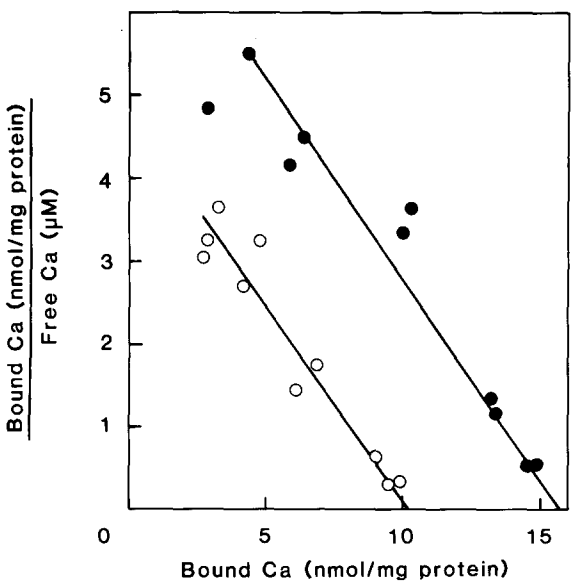

Fig. 3. Scatchard plots of Ca^{2+} binding in the absence of added Mg^{2+}. (●——●) In the presence of 50 μM ATP, (○——○) in the absence of ATP. The various Ca^{2+} concentrations were adjusted by mixing 44 μM Ca^{2+} with 0–100 μM EGTA, the association constant for EGTA-Ca being $1.3 \cdot 10^{-6}$ M^{-1}. The concentration of Ca^{2+}-ATPase (deoxycholate-extracted) was 1.06 mg/ml. The centrifugation procedure was used for these experiments. Ca^{2+}-ATPase used here had a maximum phosphoenzyme level of 4.6 nmol/mg protein.

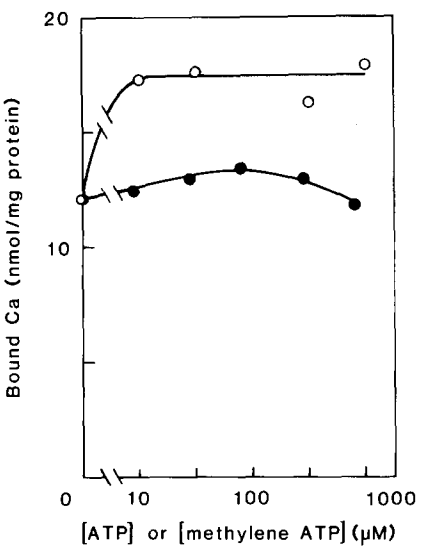

Fig. 4. Effects of ATP (○———○) and Ado*P*[CH_2]*PP* (methylene ATP) (●———●) on the Ca^{2+} binding in the absence of added Mg^{2+}. Reaction conditions: Ca^{2+}-ATPase (deoxycholate-extracted), 1.04 mg/ml; $CaCl_2$, 38 μM. The reaction time was 4 min. The glass filtration procedure was used for these experiments.

After exhaustive washing (up to eight times) of the preparation to remove excess ATP which remained on the enzyme, the phosphoenzyme was measured at a constant level of 3.9 ± 0.1, and the Ca^{2+} apparently bound to the phosphoenzyme remains at 5.0 ± 0.6 nmol/mg protein (Fig. 5).

The effects of ADP on ATP-induced Ca^{2+} binding and phosphoenzyme level are summarized in Table III. ADP lowered both the amount of ATP-induced Ca^{2+} binding and the amount of phosphoenzyme formed, in contrast to Ca^{2+} binding that occurred in the absence of ATP which was only slightly affected by ADP. The amount of ATP-induced Ca^{2+} binding was decreased from 4.4 to 0.5 nmol/mg protein by increasing the concentration of ADP from 0 to 100 μM. The amount of phosphoenzyme was also decreased from 3.5 to 0.2 nmol/mg protein by increasing the concentration of ADP from 0 to 100 μM. About 97% of ^{32}P in the ^{32}P-labeled phosphoenzyme was recovered as [^{32}P]ATP. The ratio of the amount of the ATP-induced Ca^{2+} binding to that of phosphoenzyme that was decreased by ADP was 0.8–1.2.

The Ca^{2+} that was bound to the enzyme could be increased by ATP only at lower than 100 μM Mg^{2+}. However, Mg^{2+} at concentrations higher than 100 μM reduced the ATP-induced Ca^{2+} binding, and no ATP-induced Ca^{2+} binding was observed when Mg^{2+} concentration was higher than 1 mM (Fig. 6). Furthermore, the bound Ca^{2+} which was increased by ATP could be released by 1 mM (Table IV and Fig. 2). However, the amount of phosphoenzyme that was decreased by Mg^{2+} was relatively small, only from 3.3 to 2.9 nmol/mg

TABLE III

THE EFFECTS OF ADP ON ATP-INDUCED Ca^{2+} BINDING AND PHOSPHOENZYME LEVEL

Reaction conditions: Ca^{2+}-ATPase (deoxycholate-extracted), 1.0 mg/ml; $CaCl_2$, 12 μM; ATP, 50 μM. The Millipore filter procedure was used for these experiments. After the fourth washing of the enzyme retained on the filter with washing solution containing 14 μM $CaCl_2$, ATP which remained on the enzyme was 0.03 nmol/mg protein. Then, the enzyme was chased three times with the washing solution containing 14 μM $CaCl_2$ and the indicated concentrations of ADP. The triple chasings were done within 20 s. The amount of ATP-induced Ca^{2+} binding is presented as the difference between that of Ca^{2+} binding with ATP and that without ATP. ΔCa and Δphosphoenzyme are the amounts of ATP-induced Ca^{2+} binding and phosphoenzyme which are reduced by ADP.

[ADP] (μM)	Ca^{2+} binding without ATP (nmol/mg protein)	ATP-induced Ca^{2+} binding (nmol/mg protein)	Phosphoenzyme (nmol/mg protein)	ΔCa/ Δphosphoenzyme
0	11.1	4.0	3.1	
1	9.8	1.9	0.5	0.8
5	9.3	1.9	0.5	0.8
10	10.2	1.2	0.3	1.0
50	9.5	0.9	0.2	1.1
100	8.4	0.5	0.2	1.2

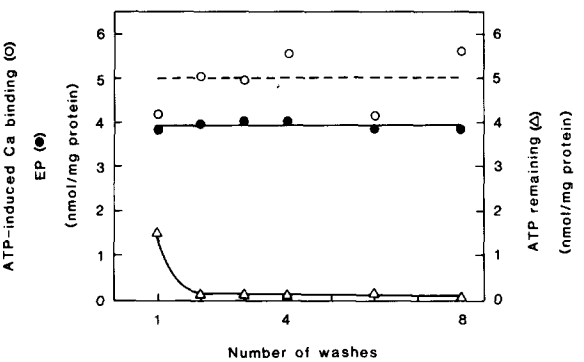

Fig. 5. Levels of ATP-induced Ca^{2+} binding (O------O), phosphoenzyme (EP) (●———●) and the remaining ATP (Δ———Δ) in the absence of added Mg^{2+} with respect to washing. Reaction conditions: Ca^{2+}-ATPase (deoxycholate-extracted), 1.3 mg/ml; $CaCl_2$, 19.2 μM; ATP, 50 μM. The reaction time was 5 min. The Millipore filtration procedure was used for these experiments. Washings were done within 1 min with washing solution containing 24 μM $CaCl_2$.

TABLE IV

THE EFFECT OF Mg^{2+} ON ATP-INDUCED Ca^{2+} BINDING AND PHOSPHOENZYME LEVEL

Reaction conditions: Ca^{2+}-ATPase (deoxycholate-extracted), 1.12 mg/ml; $CaCl_2$, 28 μM; ATP, 50 μM. The Millipore filtration procedure was used for these experiments. The enzyme retained on the filter was washed once with a washing solution containing 22 μM $CaCl_2$ to reduce the concentration of the remaining ATP. The washed enzyme was chased one to three times with a solution containing 1 mM $MgCl_2$ and 22 μM $CaCl_2$. Each chasing was carried out for 5–8 s. Results are expressed as nmol/mg protein.

	Times of chase with Mg^{2+}			
	0	1	2	3
ATP-induced Ca^{2+} binding	3.6	0.4	−1.0	−1.4
Phosphoenzyme	3.3	2.9	2.5	2.5
ATP remaining	0.43	0.07	0.06	0.03

protein (Table IV); most of the phosphoenzyme was decomposed by Mg^{2+} only when the reaction time was increased. Mg^{2+} at a concentration of 1 mM did not affect the Ca^{2+} that was bound to the enzyme in the absence of ATP (Table II and Fig. 6), and, even at 10 mM Mg^{2+}, Ca^{2+} binding was decreased only 5–20% (data not shown). The Mg^{2+} sensitivity of the ATP-induced Ca^{2+} binding

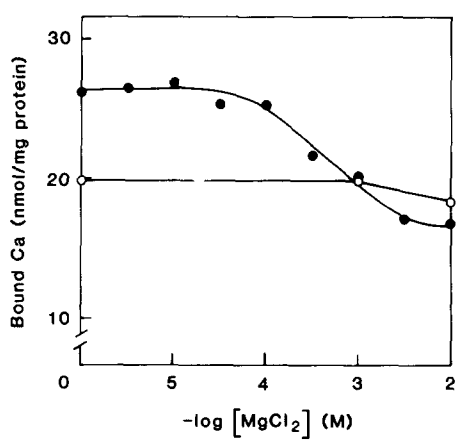

Fig. 6. The Mg^{2+} dependency of Ca^{2+} binding in the presence (●———●) and absence (O———O) of ATP. Reaction conditions: Ca^{2+}-ATPase (deoxycholate-solubilized), 1.5 mg/ml; $CaCl_2$, 28 μM; ATP, 20 μM. The reaction time was 4 min. The glass filtration procedure was used for these experiments.

declined with the time of storage of the enzyme at −80°C (data not shown).

Discussion

In the present study, the difference between the Ca^{2+} bound to the enzyme in the presence and absence of ATP without addition of Mg^{2+} determined by the ^{45}Ca method (13.9–9.3 = 4.6 nmol/mg protein; average of three values listed in Table II) is not significantly different from that determined by the atomic absorption method (15.2–10.8 = 4.4 nmol/mg protein), suggesting that the exchange process, even if it takes place, does not contribute significantly to the net ATP-increased Ca^{2+} binding to the enzyme. Calcium binding is, therefore, largely due to external Ca^{2+}.

We have also clearly shown that ATP-induced Ca^{2+} binding to the Ca^{2+}-ATPase in the absence of added Mg^{2+} is associated with the formation of phosphorylated enzyme which contributes to the increase in the number of Ca^{2+}-binding sites to 5.4 nmol/mg protein, but not to the affinities of the enzyme for the Ca^{2+}. Based on a molecular mass of 115 kDa for the Ca^{2+}-ATPase [22] and its maximum phosphoenzyme level of 4.6 nmol/mg protein, the results indicate that ATP increased the Ca^{2+}-binding sites from 2 to 3 mol Ca^{2+} per mol

phosphorylable enzyme. The formation of phosphoenzyme which increased Ca^{2+} binding is in accord with the observations of Takisawa and Makinose [29], who attributed the increase in Ca^{2+} binding in a soluble enzyme preparation to the formation of phosphoenzyme · Ca_2 complex from enzyme · Ca complex. Vale and Carvalho [30] also observed an ATP-induced Ca^{2+} binding to microsomal sarcoplasmic reticulum vesicles in the presence of 5 mM Mg^{2+}. But in this case, because the sarcoplasmic reticulum prepared under these conditions may contain other Ca^{2+}-binding proteins [23–25] in addition to the Ca^{2+}-ATPase, and because the amount of Ca^{2+} binding (30 nmol/mg protein) was nearly 6-times as great as the present finding for the purified Ca^{2+}-ATPase in the absence of Mg^{2+}, the ATP-induced Ca^{2+} binding to the contaminating proteins in the presence of Mg^{2+} might be present.

The phosphoenzyme formation resulting in the increase in Ca^{2+} binding is further supported by the fact that Ado*P*[CH_2]*PP*, despite its higher affinities for the enzyme [31] and Ca^{2+} [32], did not increase the amount of Ca^{2+} binding. Also, on addition of ADP, both the ATP-induced Ca^{2+} binding and phosphoenzyme decreased with a ratio of about 1.

The bound Ca^{2+} induced by ATP could also be released by 1 mM $MgCl_2$ prior to phosphoenzyme decomposition by Mg^{2+}. This may be a result of an exchange of Mg^{2+} for the Ca^{2+} on the ADP-reactive phosphoenzyme. This Mg^{2+}-enhanced Ca^{2+} release does not appear to be due to the release from ADP-unreactive phosphoenzyme (which would have a lower affinity for Ca^{2+} [33]), because the transition of ADP-reactive phosphoenzyme to ADP-unreactive phosphoenzyme is not enhanced by Mg^{2+} [33]. Also, it appears that the Ca^{2+} that can be replaced by Mg^{2+} may be bound to the substrate acceptor site because the Ca^{2+} on this site is not specific for Ca^{2+} [13]. On the other hand, the Ca^{2+} on the transport site is Ca^{2+} specific [7,13] and cannot be replaced by Mg^{2+}. The existence of such a substrate acceptor site has been shown for the divalent cations of the metal-ATP complexes [12,13]. Recently, Nakamura and Tonomura [34] also observed the ATP-induced Ca^{2+} binding attributable to ADP-reactive phosphoenzyme. The Ca^{2+} binding, however, was not decreased by Mg^{2+} in contrast to our results. Because of the fact that the enzyme, stored for more than 1 month at −80°C without prefreezing by liquid N_2, lost the Mg^{2+} sensitivity of the Ca^{2+} binding (data not shown), it is probably that their enzyme, stored at −80°C after freezing by liquid N_2, also lost the Mg^{2+} sensitivity.

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