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Characterization of the phosphoenzyme that is involved in the Ca^{2+}-Ca^{2+} exchange catalyzed by the Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles

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The rate of Ca^{2+} efflux was determined with $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum vesicles (mainly with the light fraction of vesicles) at pH 6.5 and 0°C. The efflux depended on external Ca^{2+}, Mg^{2+}, ATP and ADP, but it was not activated by AMP. The results indicate that the efflux is derived from Ca^{2+}-Ca^{2+} exchange mediated by the phosphoenzyme (EP) of membrane-bound Ca^{2+}-ATPase. EP was formed with Ca^{2+}-loaded vesicles (light fraction) under similar conditions without added ADP. The subsequent addition of EGTA and ADP induced triphasic EP dephosphorylation. Three species of EP (EP_1, EP_2, and EP_3) were distinguished on the basis of this dephosphorylation kinetics, EP_1, EP_2 and EP_3, corresponding to the first, second, and third phases of the dephosphorylation. Dephosphorylation of EP_1 and EP_2 resulted in stoichiometric ATP formation, while dephosphorylation of EP_3 led to stoichiometric P_i liberation. The rate of Ca^{2+} efflux was compatible with that of EP_2 dephosphorylation, whereas it was much lower than the rate of EP_1 dephosphorylation and much higher than the rate of EP_3 dephosphorylation. The intravesicular Ca^{2+} concentration dependence of the rate of EP_2 dephosphorylation agreed with that of the rate of Ca^{2+} efflux. The results suggest that isomerization between EP_1 and EP_2 is the rate-limiting process in the Ca^{2+}-Ca^{2+} exchange and that EP_3 is not involved in this exchange.

Introduction

The membrane-bound Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles catalyzes Ca^{2+} uptake that is coupled to ATP hydrolysis [1,2]. During the transport process, phosphoenzyme (EP) is formed from the enzyme and ATP in the presence of external Ca^{2+} [3–5].

Makinose [6] showed previously that the ADP-activated Ca^{2+}-Ca^{2+} exchange across the vesicular membrane occurred in the early stage of the transport cycle before the step of EP hydrolysis. Further investigations in other laboratories [7–9] as well as in ours [10–12] have indicated that the Ca^{2+}-Ca^{2+} exchange is mediated by EP. On the other hand, from the kinetic analysis of the Ca^{2+}-ATPase it has been suggested that there are more than two species of EP in the reaction sequence of the enzyme [13–17]. In this situation, detailed information on the relationship between the different species of EP and the Ca^{2+}-Ca^{2+} exchange could bring about a better understanding as to the mechanism of EP-mediated Ca^{2+} transport across the membrane.

In the present study, in order to characterize the species of EP that is involved in the Ca^{2+}-Ca^{2+} exchange, we have determined both the Ca^{2+} ef-

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Abbreviations: EP, phosphoenzyme; [^{32}P]EP, ^{32}P-labeled phosphoenzyme; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

flux in the Ca^{2+}-Ca^{2+} exchange and the EP dephosphorylation following addition of EGTA and ADP under similar conditions. The results demonstrate that three species of EP can be distinguished on the basis of the observed triphasic pattern of EP dephosphorylation, and further suggest that isomerization between two of these EP species is the rate-limiting process in the Ca^{2+}-Ca^{2+} exchange and that the third species of EP is not involved in this exchange.

Materials and Methods

Preparation of the light fraction of sarcoplasmic reticulum vesicles (light vesicles). Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described previously [18]. In order to obtain the light fraction, the vesicles were fractionated by sucrose density gradient centrifugation according to Morii and Tonomura [19]. The light vesicles were suspended in 0.1 M KCl/0.1 M sucrose/20 mM Tris-maleate (pH 6.5) and stored at −80°C.

Active loading of sarcoplasmic reticulum vesicles with $^{45}Ca^{2+}$. Sarcoplasmic reticulum vesicles without further fractionation were actively loaded with $^{45}Ca^{2+}$ as described previously [11]. K^+, Mg^{2+} and $^{45}Ca^{2+}$ in the external medium were removed by passing 0.5 ml of the suspension of the vesicles at 6°C successively through two columns equilibrated with 20 mM Tris-maleate (pH 6.5), the first being a column (1 × 11 cm) of Sephadex G-50 and the second being a column (1 × 5 cm) of Dowex chelating resin. The turbid fractions were collected.

Passive loading of light vesicles with $^{45}Ca^{2+}$. Light vesicles (1.5 mg) were incubated at 0°C overnight in 1 ml of a medium consisting of 50 mM KCl/5 mM $MgCl_2$/50 mM sucrose/20 mM Tris-maleate (pH 6.5), with $^{45}CaCl_2$ at various concentrations. In order to remove K^+ and $^{45}Ca^{2+}$ in the external medium, 0.5 ml of the incubation mixture was passed at 6°C successively through the above two columns equilibrated with 0.1 M LiCl/5 mM $MgCl_2$/50 mM sucrose/20 mM Tris-maleate (pH 6.5). The turbid fractions were collected.

$^{45}Ca^{2+}$ efflux. The reaction was started by adding 1 vol. of the suspension of $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum vesicles (or $^{45}Ca^{2+}$-loaded light vesicles) to 99 (or 19) vol. of a medium containing, unless otherwise stated, ATP, ADP, $MgCl_2$, $CaCl_2$ and EGTA. After incubation for different times, a 0.5 ml portion of the reaction mixture was filtered within 2 s through a Millipore filter (pore size 0.45 μm). The radioactivity of the filtrate was measured in a liquid scintillation mixture.

Passive loading of light vesicles with Ca^{2+}. In order to remove K^+ from the medium, the prepared light vesicles were diluted with 2–4 volumes of a medium comprising 0.1 M LiCl/5 mM $MgCl_2$/50 mM sucrose/20 mM Tris-maleate (pH 6.5) and the mixture centrifuged at 130000 × *g* for 45 min at 4°C. The pellet was suspended to give 2–8 mg light vesicles/ml in 0.1 M LiCl/5 mM $MgCl_2$/50 mM sucrose/20 mM Tris-maleate (pH 6.5), with $CaCl_2$ at various concentrations. The suspension was incubated overnight at 0°C.

Phosphorylation of light vesicles with [γ-^{32}P]ATP. The reaction was started by adding 1 vol. of the suspension of light vesicles to 4 vol. of a medium containing [γ-^{32}P]ATP, unless otherwise stated. In most of the measurements, [^{32}P]EP formation was terminated by adding excess EGTA or by adding a mixture of excess EGTA, ATP and ADP. The reaction was quenched with perchloric acid containing carrier ATP, PP_i, P_i and bovine serum albumin. The amounts of [^{32}P]EP [11], [γ-^{32}P]ATP [20] and [^{32}P]P_i [20] in the sample were determined essentially in the same way as described previously.

ATP-ADP exchange. The ATP-ADP exchange reaction was started by adding 0.02 ml of the suspension of the Ca^{2+}-loaded light vesicles to 0.18 ml of a medium containing [^{14}C]ADP. The reaction was stopped by adding 0.1 ml of 15% (w/v) trichloroacetic acid containing 15 mM ATP, 15 mM ADP and 15 mM AMP as carriers. The denatured sample was centrifuged at 2260 × *g* for 10 min at 0°C, and 40 μl of the resulting supernatant was placed on a poly(ethylene imine)-cellulose thin layer prepared by the method of Randerath and Randerath [21]. Thin-layer chromatography was performed according to Inoue and Tonomura [22]. The region of the cellulose layer containing the spot of ATP was scraped and

the radioactivity was measured in the liquid scintillation mixture.

All of the reactions in the present experiment were performed at 0°C and pH 6.5

Other procedures. $^{45}CaCl_2$ and [^{14}C]ADP were purchased from New England Nuclear. Na_2ATP was obtained from Boehringer-Mannheim. Na_2-ADP, free acid AMP, EGTA, poly(ethylene imine), and cellulose powder for thin-layer chromatography were from Sigma. Other reagents were of reagent grade. Na_2ATP and Na_2ADP were converted into Tris salts by passage through a Dowex 50W (Tris form) column. [γ-^{32}P]ATP was prepared according to Post and Sen [23]. Protein concentrations were determined by the method of Lowry et al. [24] with bovine serum albumin as a standard. Tris-maleate (pH 6.5) was prepared by adding maleic acid to Tris without NaOH. K^+ and Na^+ contaminating the reaction mixture were determined by flame photometry and were found to be less than 10 μM and 30 μM, respectively. Free Ca^{2+} concentrations in the external medium were adjusted with EGTA as described previously [11]. THe time-course of dephosphorylation was fitted by using a least-squares method.

Results

Ca^{2+} efflux in the Ca^{2+}-Ca^{2+} exchange catalyzed by Ca^{2+}-ATPase

In order to determine the rate of the Ca^{2+} efflux in the Ca^{2+}-Ca^{2+} exchange catalyzed by the Ca^{2+}-ATPase, $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum vesicles (or light vesicles) was followed in the presence of 10 μM ATP, 50 μM ADP, 5 mM $MgCl_2$ and 13 μM external Ca^{2+}. The efflux was almost linear with time for at least 2 min. Almost all of the $^{45}Ca^{2+}$ in the vesicles flowed out in about 30 min. In the following measurements, the initial rate of the $^{45}Ca^{2+}$ efflux was determined from the initial linear phase.

Table I and Fig. 1 demonstrate the requirements for the $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded vesicles, which were prepared without fractionation into light and heavy fractions. The results indicate that the efflux substantially depended on external Ca^{2+}, Mg^{2+}, ATP and ADP. Similar findings were obtained when light vesicles were used. The rate of the efflux in the absence of both ATP and ADP was essentially equivalent to that of the efflux in the absence of external Ca^{2+} (Table I). It is, therefore, most probable that the efflux without ATP and ADP represents passive Ca^{2+} leak from the vesicles. The efflux in the absence of ATP and presence of ADP was considerably faster than that in the absence of both ATP and ADP. This is possibly due to the presence of contaminant ATP in added ADP. The rate of the efflux increased with increasing concentration of added ADP and reached a maximum at about 10 μM ADP (Fig. 1). The free ADP (ADP^{3-}) concentration giving a half-maximum activation was extremely low, being about 0.2 μM.

The requirements for the Ca^{2+} efflux shown in Table I and Fig. 1 are in full accord with activating factors for the forward and backward reactions in the Ca^{2+}-ATPase. The results agree well with the previous observations [6,7,9–11], being consistent with the view that the (ATP, ADP)-dependent part of the Ca^{2+} efflux can be attributed to the Ca^{2+}-Ca^{2+} exchange catalyzed by the Ca^{2+}-ATPase as proposed earlier [6–12,25]. Table I also shows that the efflux was not activated by 10 mM AMP. This finding indicates that a possi-

TABLE I

REQUIREMENTS FOR $^{45}Ca^{2+}$ EFFLUX FROM $^{45}Ca^{2+}$-LOADED VESICLES

The initial rate of $^{45}Ca^{2+}$ efflux was measured with $^{45}Ca^{2+}$-loaded vesicles (not fractionated into light and heavy fractions). The reaction mixture (the complete system) had a final composition of 5.9–6.5 μg $^{45}Ca^{2+}$-loaded vesicles/ml, 10 μM ATP, 50 μM ADP, 5 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.5 mM EGTA, and 20 mM Tris-maleate (pH 6.5). Free Ca^{2+} in the external medium was 13 μM.

Reaction mixture	Initial rate of $^{45}Ca^{2+}$ efflux (nmol/min per mg)	
	high [a] intravesicular $^{45}Ca^{2+}$	low [b] intravesicular $^{45}Ca^{2+}$
Complete	7.26	1.39
− $CaCl_2$	2.16	0.38
− $MgCl_2$	1.91	0.62
− ATP	3.77	0.69
− ATP, − ADP	1.50	0.28
− ATP, − ADP, + 10 mM AMP	2.55	0.41

[a] 44.3 nmol $^{45}Ca^{2+}$/mg protein.

[b] 9.1 nmol $^{45}Ca^{2+}$/mg protein.

ble adenine nucleotide-dependent Ca^{2+}-induced Ca^{2+} release (previously suggested with heavy vesicles [19,26]) was not significantly involved in the Ca^{2+} efflux under the present conditions, even when the vesicles without fractionation were used.

Dependence of Ca^{2+} efflux on intravesicular Ca^{2+} concentration

The dependencies of the Ca^{2+} efflux on intravesicular Ca^{2+} concentration were determined (Fig. 2). Light vesicles passively loaded with Ca^{2+} or $^{45}Ca^{2+}$ were used in all of the following measurements.

The rates of the Ca^{2+} efflux increased with increasing concentrations of intravesicular Ca^{2+} in the presence and absence of ATP and ADP. The (ATP, ADP)-dependent part of the Ca^{2+} efflux was saturated with 80 nmol intravesicular Ca^{2+}/mg protein. In contrast, the efflux in the absence of ATP and ADP showed no tendency to be saturated with intravesicular Ca^{2+} up to 100 nmol/mg protein, again indicating that this efflux represents passive Ca^{2+} leak. The concentration of intravesicular Ca^{2+} giving a half-maximum activation was 30 nmol/mg protein, being equivalent to 6 mM on the assumption that an average vesicular water space was 5 μl/mg protein [27,28].

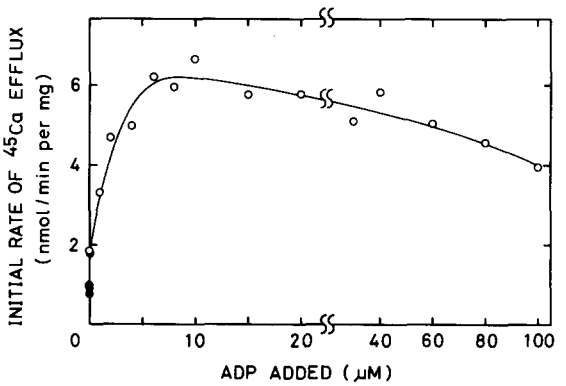

Fig. 1. Dependence of the initial rate of $^{45}Ca^{2+}$ efflux on ADP concentration. The initial rate of $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum vesicles was determined at different ADP concentrations (○), otherwise as described for Table I except for vesicles (7.1–7.4 μg/ml) loaded with $^{45}Ca^{2+}$ (30.7–36.4 nmol $^{45}Ca^{2+}$/mg protein) and 20 μM ATP. For the control (●), ATP and ADP were omitted.

Triphasic dephosphorylation of EP after addition of EGTA and ADP

Ca^{2+}-loaded light vesicles were phosphorylated with 10 μM [γ-^{32}P]ATP in the absence of KCl and ADP and in the presence of 5 mM $MgCl_2$ and 13 μM external Ca^{2+} (Fig. 3). When [^{32}P]EP reached a steady level, phosphorylation was terminated with a large volume of a medium containing 5 mM EGTA, 10 μM ATP and 50 μM ADP. The subsequent dephosphorylation of [^{32}P]-EP gave a triphasic pattern (the inset of Fig. 3A and Fig. 3B). [^{32}P]EP corresponding to the first phase (EP_1) showed very rapid dephosphorylation (Fig. 3B). [^{32}P]EP corresponding to the second phase (EP_2) showed dephosphorylation with an intermediate rate, its apparent first-order rate constant being 0.19 s^{-1} (Fig. 3B). [^{32}P]EP corresponding to the third phase (EP_3) showed much slower dephosphorylation, its apparent first-order rate constant being 0.043 s^{-1} (the inset of Fig. 3A). At the zero time in Fig. 3, the contents of EP_1, EP_2 and EP_3 were about 33%, 35% and 32%

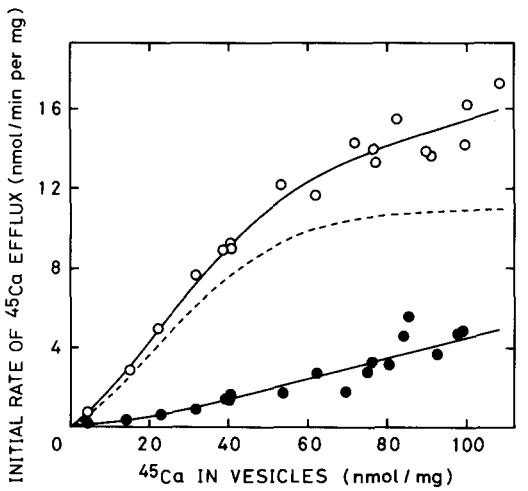

Fig. 2. Dependence of the initial rate of $^{45}Ca^{2+}$ efflux on intravesicular $^{45}Ca^{2+}$ concentration. The initial rate of $^{45}Ca^{2+}$ efflux (3.6–8.7 μg light vesicles/ml) was determined at different intravesicular $^{45}Ca^{2+}$ concentrations in the presence of 10 μM ATP, 50 μM ADP, 0.1 M LiCl, 5 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.5 mM EGTA, 50 mM sucrose, and 20 mM Tris-maleate (pH 6.5) (○). For the control (●), ATP and ADP were omitted. The broken line shows the initial rate of (ATP, ADP)-dependent $^{45}Ca^{2+}$ efflux, which was obtained by subtracting the initial rate of the efflux in the control from that of the efflux in the presence of ATP and ADP.

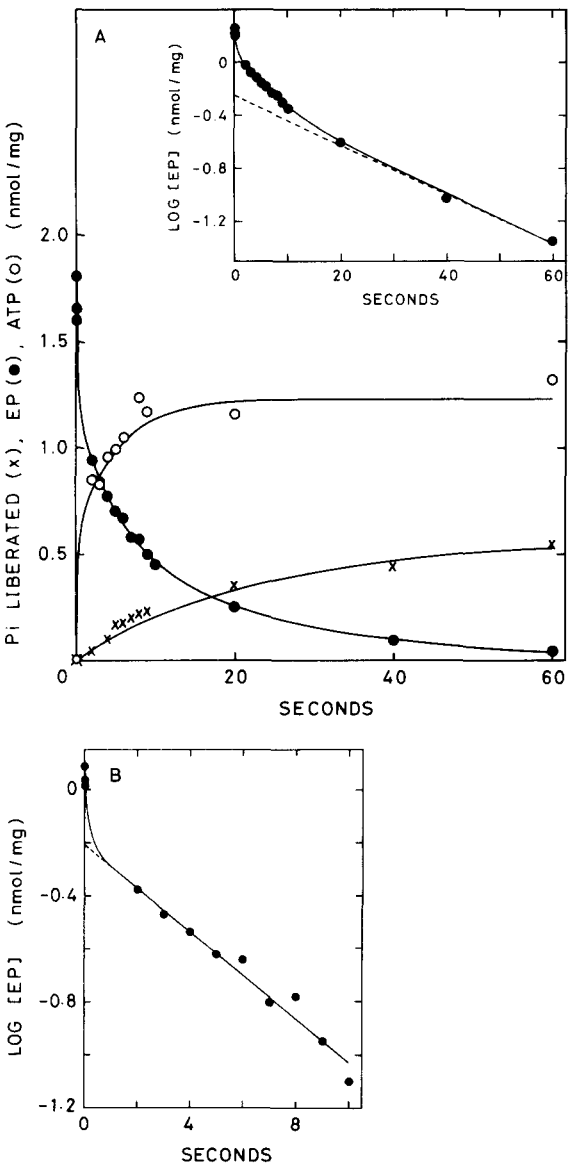

Fig. 3. [^{32}P]EP dephosphorylation, [γ-^{32}P]ATP formation, and [^{32}P]P_i liberation after addition of EGTA and ADP. (A) Phosphorylation of light vesicles (1.63 mg/ml) loaded with Ca^{2+} (30 nmol Ca^{2+}/mg protein) was started in 0.1 ml of a medium comprising 10 μM [γ-^{32}P]ATP/0.1 M LiCl/5 mM $MgCl_2$/0.7 mM $CaCl_2$/1.2 mM EGTA/50 mM sucrose/50 mM Tris-maleate (pH 6.5). Free Ca^{2+} in the external medium was 13 μM. After 15 s (at the zero time on the abscissa), 5 ml of a medium comprising 5 mM EGTA/10 μM ATP/50 μM ADP/0.1 M LiCl/5 mM $MgCl_2$/50 mM sucrose/50 mM Tris-maleate (pH 6.5) was added. For the control, the reaction was started in the presence of 20 mM EGTA, otherwise as described above. The amount of ^{32}P incorporated into protein in this control was less than 0.6 pmol/mg protein. The inset shows a semilogarithmic plot of the amount of [^{32}P]EP against time. The dotted line indicates the component (EP_3) showing the slowest dephosphorylation (corresponding to the third phase of dephosphorylation). (B) The amount of EP_3 was subtracted from the amount of [^{32}P]EP. The amount thus obtained was replotted in a semilogarithmic way against time. The dotted line indicates the component (EP_2) showing dephosphorylation with an intermediate rate (corresponding to the second phase of dephosphorylation). Subtraction of the amounts of EP_2 and EP_3 from the amount of [^{32}P]EP at the zero time gives the amount (at the zero time) of the component (EP_1) showing the fastest dephosphorylation (corresponding to the first phase of dephosphorylation).

of the total amount of [^{32}P]EP respectively.

The dephosphorylation of [^{32}P]EP brought about [γ-^{32}P]ATP formation and [^{32}P]P_i liberation. The time-course and the extent of [γ-^{32}P]ATP formation corresponded well to the time-course and the extent of dephosphorylation of EP_1 and EP_2. This finding indicates that the addition of EGTA and ADP caused almost complete conversion of EP_1 and EP_2 to [γ-^{32}P]ATP. On the other hand, the time-course and the extent of [^{32}P]P_i liberation corresponded to the time-course and the extent of EP_3 dephosphorylation. This shows that EP_3 did not react with added ADP and was entirely hydrolyzed to liberate [^{32}P]P_i.

Effect of intravesicular Ca^{2+} on EP_1, EP_2 and EP_3

The apparent first-order rate constant of EP_2 dephosphorylation (k_2) increased with increasing concentration of intravesicular Ca^{2+} (Fig. 4). It was saturated with 80 nmol intravesicular Ca^{2+}/mg protein. The concentration of intravesicular Ca^{2+} giving a half-maximum activation was 25 nmol/mg protein, being equivalent to 5 mM. In contrast, the apparent first-order rate constant of EP_3 dephosphorylation (k_3) decreased slightly as the intravesicular Ca^{2+} concentration was increased.

When the intravesicular Ca^{2+} concentration was elevated, EP_1 markedly increased and inversely EP_3 decreased (Fig. 5). The total amount of [^{32}P]EP remained constant over the whole range of intravesicular Ca^{2+} concentration tested.

Comparison between the rate of Ca^{2+} efflux and the rate of EP_2 dephosphorylation

Phosphorylation was performed under the same conditions as those for the Ca^{2+} efflux, and then

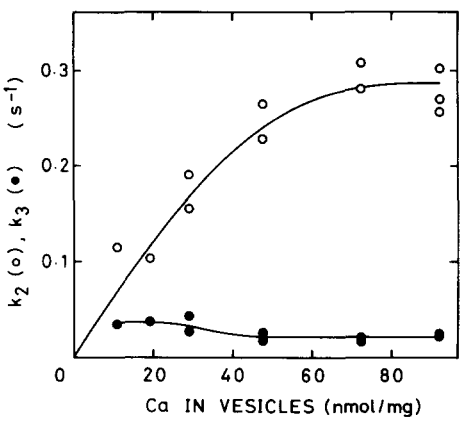

Fig. 4. Dependences of the apparent first-order rate constants of dephosphorylation of EP_2 and EP_3 on intravesicular Ca^{2+} concentration. Phosphorylation and dephosphorylation of the Ca^{2+}-loaded light vesicles (0.43–1.63 mg/ml) were performed at different intravesicular Ca^{2+} concentrations, otherwise as described for Fig. 3. The apparent first-order rate constants of dephosphorylation of EP_2 (k_2) and EP_3 (k_3) were determined by semilogarithmic plotting made in the same way as in Fig. 3.

dephosphorylation following addition of EGTA was determined (Table II). The rate of EP_2 dephosphorylation (v_2) increased when the intravesicular Ca^{2+} concentration was elevated. It was about two-thirds of the rate of the Ca^{2+} efflux at two different intravesicular Ca^{2+} concentrations used.

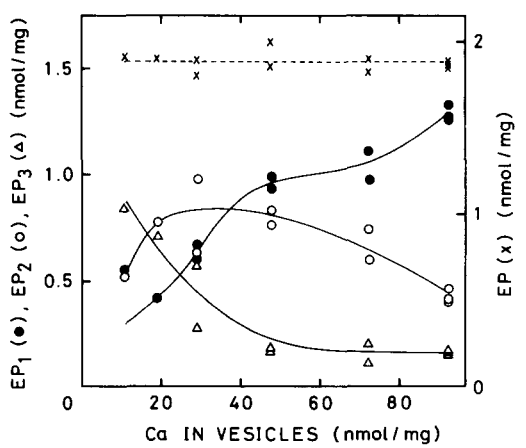

Fig. 5. Dependences of the amounts of EP_1, EP_2 and EP_3 on intravesicular Ca^{2+} concentration. The amounts of EP_1, EP_2 and EP_3 at the time of the addition of EGTA and ADP were determined from the measurements given in Fig. 4 in the same way as in Fig. 3. The total amount of [^{32}P]EP (the sum of the amounts of EP_1, EP_2, and EP_3) was also plotted (×).

ATP-ADP exchange

In order to estimate the lower limit of the rate of ATP dissociation from calcium-enzyme-ATP complex, the rate of the ATP-ADP exchange catalyzed by the Ca^{2+}-ATPase was measured under conditions similar to those for the Ca^{2+} efflux given in Fig. 2 except that light vesicles (9.9 μg/ml) had been loaded with unlabeled Ca^{2+} (30 nmol Ca^{2+}/mg protein) and that 50 μM [^{14}C]ADP was present. [^{14}C]ATP formation in this exchange was linear with time for at least 35 s. The rate of the ATP-ADP exchange was 106 nmol/min per mg. No significant [^{14}C]ATP formation was found in the presence of 10 mM EGTA without added $CaCl_2$.

Ca^{2+} dissociation from calcium-enzyme-ATP complex

The following experiment was performed with a handmade rapid mixing apparatus [29] in order to assess the rate of Ca^{2+} dissociation from calcium-enzyme-[γ-^{32}P]ATP complex. For the control measurement, the reaction was started by adding 0.046 ml of [γ-^{32}P]ATP to 0.184 ml of a mixture to give 40.2 μg light vesicles/ml, 100 μM [γ-^{32}P]ATP, 0.1 M LiCl, 5 mM $MgCl_2$ and 13 μM external Ca^{2+}. [^{32}P]EP reached a steady level (2.1 nmol/mg protein) in about 2 s. In the other measurement, the reaction was started as above, and at 0.1 s 0.301 ml of a medium comprising 153 mM EGTA/0.1 M LiCl/5 mM $MgCl_2$ was added. A small amount of [^{32}P]EP (0.25 nmol/mg protein) was formed before this addition of EGTA

TABLE II

COMPARISON BETWEEN THE RATE OF Ca^{2+} EFFLUX AND THE RATE OF EP_2 DEPHOSPHORYLATION

Phosphorylation and dephosphorylation of light vesicles (0.19 mg/ml) loaded with Ca^{2+} (30 or 50 nmol Ca^{2+}/mg protein) were performed as described for Fig. 3 except that 50 μM ADP was present at the start of phosphorylation. k_2 and EP_2 were determined in the same way as in Fig. 3.

Intravesicular Ca^{2+} (nmol/mg)	k_2 (s^{-1})	EP_2 (nmol/mg)		v_2 [a]	Ca^{2+} efflux [b] (nmol/min per mg)
30	0.18	0.30		3.2	5.4
50	0.28	0.34		5.7	8.7

[a] $v_2 = k_2 \cdot [EP_2]$.

[b] Data taken from Fig. 2.

and phosphorylation ceased completely in about 0.5 s after the EGTA addition. The amount of [^{32}P]EP formed after the addition of EGTA was 0.46 nmol/mg protein, being only a small fraction of [^{32}P]EP in the steady state in the control. Since saturating concentrations of [γ-^{32}P]ATP and external Ca^{2+} were used in this measurement, almost all of the enzyme should have been in the form of calcium-enzyme-[γ-^{32}P]ATP complex immediately after the start of the reaction. Therefore, the above finding indicates that Ca^{2+} dissociated rapidly (within 0.5 s) from the calcium-enzyme-[γ-^{32}P]ATP complex after the addition of EGTA.

K^+-induced acceleration of EP_3 dephosphorylation

In order to see the effect of K^+ on EP_3 dephosphorylation, phosphorylation was started in the absence of K^+ as described in Fig. 3 and then it was terminated by adding a large volume of a medium comprising 0.1 M KCl/5 mM EGTA/10 μM ATP/50 μM ADP (Fig. 6). The time-course of the dephosphorylation was biphasic (Fig. 6, inset). The amount of [^{32}P]EP in the slower phase of the dephosphorylation was 35% of the total amount of [^{32}P]EP at the zero time, the apparent first-order rate constant of this dephosphorylation being 0.41 s^{-1}. This rate was approximately twice the rate of EP_2 dephosphorylation without KCl addition (see Fig. 3B). Thus, the component of [^{32}P]EP showing very slow dephosphorylation was not found in this measurement. This indicates that EP_3 dephosphorylation was greatly accelerated by added K^+.

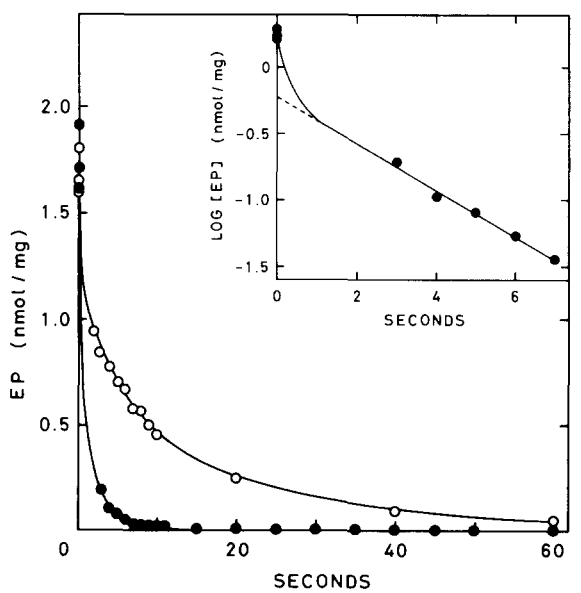

Fig. 6. K^+-induced acceleration of EP_3 dephosphorylation. Phosphorylation and dephosphorylation of light vesicles (0.78 mg/ml) loaded with Ca^{2+} (30 nmol Ca^{2+}/mg protein) were performed as described for Fig. 3 except that the medium containing 0.1 M KCl instead of LiCl was added at 15 s (at the zero time on the abscissa) after the start of phosphorylation (●). The inset indicates a semilogarithmic plot of the initial part of these data against time. Open circles show [^{32}P]EP dephosphorylation in the absence of KCl and presence of 0.1 M LiCl (the data were taken from Fig. 3A).

Discussion

The implications of the present results may be discussed conveniently in terms of the minimum scheme (Fig. 7) tentatively proposed for the Ca^{2+}-ATPase. This scheme is based on present and earlier findings [5,15,30,31].

The results (Fig. 1 and Table I) are consistent with the view that the (ATP, ADP)-dependent part of the Ca^{2+} efflux in the present experiment can be attributed to the EP-mediated Ca^{2+}-Ca^{2+} exchange (the dynamic reversal of steps 1–4 in the scheme) demonstrated previously [6–12,25]. The apparent high affinity for ADP in the Ca^{2+} efflux

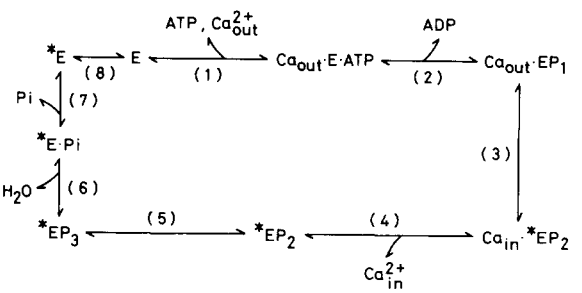

Fig. 7. Tentative scheme for the catalytic and transport cycle of the Ca^{2+}-ATPase in sarcoplasmic reticulum vesicles. Ca^{2+}_{out} and Ca^{2+}_{in} designate free Ca^{2+} in the external and internal media of vesicles. E and EP_1 denote the state of the enzyme which has transport sites with a high affinity for Ca^{2+}. In this state, the transport sites face on the external medium. *E, *EP_2 and *EP_3 indicate the state of the enzyme which has transport sites with a low affinity for Ca^{2+}. In this state, the transport sites face on the internal medium. Step 3 includes the processes of occlusion, translocation, and exposure of Ca^{2+} transport sites in EP. The stoichiometry of Ca^{2+} transport is omitted for simplicity.

(Fig. 1) is compatible with the high affinity of EP for ADP in transphosphorylation from EP to ADP (reversal of step 2) [30]. This finding also gives support to the above conclusion.

Three species of EP (EP_1, EP_2 and EP_3) can be distinguished on the basis of the triphasic pattern of EP dephosphorylation induced by addition of EGTA and ADP (Fig. 3). Under the conditions in which 50 μM ADP is present, the rate of EP_1 dephosphorylation is much higher than that of the Ca^{2+} efflux (Figs. 2 and 3B). Furthermore, EP_1 dephosphorylation is accompanied by stoichiometric ATP formation (Fig. 3A). It is, therefore, likely that this EP_1 corresponds to $Ca_{out} \cdot EP_1$ in the scheme. In contrast, the rate of EP_3 dephosphorylation is much lower than that of the Ca^{2+} efflux (Fig. 2 and the inset of Fig. 3A). In addition, EP_3 dephosphorylation results in stoichiometric P_i liberation (Fig. 3A). Accordingly, it is evident that EP_3 is not involved in the Ca^{2+} efflux.

On the other hand, EP_2 dephosphorylation results in stoichiometric ATP formation (Fig. 3A), and the rate of dephosphorylation agrees with that of the Ca^{2+} efflux with a stoichiometric ratio (Ca^{2+}/EP_2) of 1.5 to 1.7 (Table II). This stoichiometry is somewhat higher than that (Ca^{2+}/ATP ratio of 1.0) obtained previously at low temperature [10,11,32–34]. However, the results from some laboratories [35,36] show that the stoichiometry ranges from 1.4 to 2.0, even when the reaction temperature is low. The intravesicular Ca^{2+} concentration dependence of the rate of EP_2 dephosphorylation essentially agrees with that of the rate of EP-mediated Ca^{2+} efflux, and both the rates are saturated with considerably high concentrations of intravesicular Ca^{2+} (Figs. 2 and 4). All of these findings can be reasonably explained by the following assumptions; (a) EP_2 corresponds to $Ca_{in} \cdot {}^*EP_2$ and ${}^*EP_2$ in the scheme; (b) EP_2 dephosphorylation is derived from the reversal of steps 1–4; (c) steps 1 and 4 are both in rapid equilibrium; (d) step 3 is the rate-limiting process in the Ca^{2+} efflux, if ADP concentration is higher than 10 μM so that the reverse reaction of step 2 does not limit the rate of the Ca^{2+} efflux (see Fig. 1). In these assumptions, the rapid equilibrium in step 4 implies that the Ca^{2+} transport sites in EP_2 are in a state exposed to the internal medium.

Step 3 includes the process of occlusion of Ca^{2+} transport sites in EP. There is growing evidence [17,20,35,37,38] that dissociation of Ca^{2+} from occluded Ca^{2+} transport sites is rather slow. It is, therefore, highly probable that the exposure of occluded Ca^{2+} transport sites to the internal medium is the rate-limiting process in the EP-mediated Ca^{2+}-Ca^{2+} exchange.

Recently, Jencks and co-workers [39,40] showed that addition of ADP to EP causes biphasic EP dephosphorylation, resulting in stoichiometric ATP formation. They suggested that the fast phase of the dephosphorylation represents rapid formation of calcium-enzyme-ATP complex from calcium · EP and ADP and that the slow phase represents rate-limiting dissociation of ATP from the calcium-enzyme-ATP complex thus formed. In contrast with their suggestion, the rate of the ATP-ADP exchange (dynamic reversal of steps 1 and 2) in our experiment is much higher than that of EP_2 dephosphorylation (Fig. 3B and Table II). Therefore, it is unlikely that in the present experiment the phase of EP_2 dephosphorylation can be ascribed to dissociation of ATP from $Ca_{out} \cdot E \cdot ATP$ (step 1). This conclusion is consistent with the view obtained by Froehlich and Heller [17] from the transient kinetic study on EP dephosphorylation with leaky vesicles. The observed rapid ATP-ADP exchange is also compatible with the findings reported previously [6–8,41].

Chiesi and Inesi [42] and Ikemoto et al. [34] showed that, when EGTA and ATP are added simultaneously to calcium-enzyme complex at the beginning of the reaction, significant EP formation and Ca^{2+} transport occur before effective quenching takes place. This suggests that Ca^{2+} dissociation from calcium-enzyme complex is slower than EP formation and Ca^{2+} transport. Their conclusion appears to be in harmony with our present finding that EP formation is not promptly interrupted when EGTA is added at the early stage of the reaction. However, our results show that Ca^{2+} dissociation from $Ca_{out} \cdot E \cdot ATP$ is much more rapid than the Ca^{2+} efflux. This indicates that Ca^{2+} dissociation in step 1 is not the rate-limiting process in the Ca^{2+} efflux. It seems possible that the bound ATP in $Ca_{out} \cdot E \cdot ATP$ has a stimulatory effect on this Ca^{2+} dissociation.

EP_3 decreases with increasing concentration of intravesicular Ca^{2+} (Fig. 5). This indicates that

most of EP_3 is derived from Ca^{2+}-ATPase of tightly sealed vesicles but not from Ca^{2+}-ATPase of contaminating leaky vesicles. The effect of the intravesicular Ca^{2+} on EP_3 corresponds well to the intravesicular Ca^{2+} concentration dependences of Ca^{2+} efflux (Fig. 2) and EP_2 dephosphorylation (Fig. 4). This finding suggests that EP_3 has a low affinity for internal Ca^{2+} and is closely related to EP_2. EP_3 is insensitive to ADP (Fig. 3A) and sensitive to K^+ (Fig. 6). This is characteristic of the last species of EP in the reaction sequence of the Ca^{2+}-ATPase [43]. Therefore, it seems reasonable that EP_3 is tentatively placed in the late step of the reaction sequence ($*EP_3$ in the scheme). If this assumption is correct, the reverse reaction of step 5 should be much slower than hydrolysis of $*EP_3$, since EP_3 dephosphorylation leads to stoichiometric P_i liberation (Fig. 3A). However, the possibility cannot be excluded that EP_3 could be derived from a possible heterogeneity within light vesicles suggested by Watras and Katz [44].

Froehlich and Heller [17] showed that addition of 1.66 mM ADP to EP formed with leaky vesicles in the presence of 100 mM KCl at 21°C gave a triphasic pattern of dephosphorylation. This contrasts with our results that biphasic dephosphorylation was induced by addition of ADP and KCl to EP (Fig. 6). The discrepancy may be possibly due to difference in temperature, tightness of vesicles, or ADP concentration used.

It appears that the amount of heavy vesicles [45] contaminating light vesicles used in the present experiment is negligibly small. This is because the adenine nucleotide-dependent Ca^{2+}-induced Ca^{2+} release [19,26] did not occur to a significant extent with the light vesicles under the conditions (1 mM $MgCl_2$, pH 7.0, 0°C) in which the Ca^{2+} release occurred pronouncedly when the isolated heavy vesicles were used (Inao, S. and Kanazawa, T., unpublished data). It is, therefore, unlikely that the observed triphasic pattern of dephosphorylation was caused by heavy vesicles.

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