Correlation between Ca^{2+} uptake, Ca^{2+} efflux and phosphoenzyme level in sarcoplasmic-reticulum vesicles

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Previously [Inesi & de Meis (1989) *J. Biol. Chem.* **264**, 5929–5936] it was shown that dimethyl sulphoxide (Me_2SO) increases the amount of Ca^{2+} accumulated by sarcoplasmic-reticulum vesicles. This effect was related to a decrease in the enzyme affinity for ADP from $< 20 \mu\text{M}$ to 1 mM. In the present work the apparent affinity of the ADP-sensitive phosphoenzyme for ADP was determined by measuring the rate of ATP synthesis in vesicles previously loaded with Ca^{2+} , at different pH values and in the presence and absence of Me_2SO (20%, v/v) and KCl. In all conditions tested, addition of Me_2SO never promoted an increase of the apparent K_m for ADP to a value higher than $25 \mu\text{M}$. ADP inhibits the phosphorylation of the enzyme by P_i . Two components, with K_i values of $80 \mu\text{M}$ and 8 mM, were detected when vesicles previously loaded with Ca^{2+} were used. The high-affinity component (K_i $80 \mu\text{M}$) was abolished after addition of Me_2SO to the medium. Empty vesicles, on the other hand, exhibited only the low-affinity component (K_i 8 mM). During ATP synthesis in a totally aqueous medium, there was a decrease in the phosphoenzyme formed by P_i , after addition of $80\text{--}100 \mu\text{M}$ -ADP to the medium. In the presence of Me_2SO this decrease was smaller. The sum of the fractions of phosphoenzyme formed by ATP and by P_i during Ca^{2+} uptake was higher in the presence of Me_2SO than in a totally aqueous medium. Me_2SO decreased the passive efflux of Ca^{2+} measured in the presence of 0.1 mM- P_i and 0.1 mM- MgCl_2 . In a totally aqueous medium the same decrease was observed when P_i and MgCl_2 concentrations were raised to 4 mM. These data suggest that ADP binds to two different enzyme conformations. The binding to one of these conformations (*E) inhibits the phosphorylation of the enzyme by P_i , increases the efflux of Ca^{2+} and decreases the amount of Ca^{2+} accumulated by the vesicles.

INTRODUCTION

Sarcoplasmic-reticulum vesicles retain a membrane-bound ATPase which catalyses both the hydrolysis and the synthesis of ATP [1–4]. There are two different functional states of the enzyme, E and *E (Fig. 1). The E form binds Ca^{2+} with high affinity at the outer surface of the vesicles (K_d $1.0 \mu\text{M}$ at pH 7.0), and can be phosphorylated by ATP, ITP and acetyl phosphate, but not by P_i . In the *E form the Ca^{2+} -binding site faces the inner surface of the vesicles and binds Ca^{2+} with low affinity (K_d 1 mM at pH 7.0). This form is phosphorylated by P_i , but not by nucleoside triphosphates. Three different forms of phosphoenzyme appear in the sequence, but only 2Ca:E-P is capable of transferring its phosphate to ADP, leading to the synthesis of ATP. Recently [5,6] it has been shown that, when ATP is used as substrate, there is an increase in Ca^{2+} uptake when either an ATP-regenerating system or dimethyl sulphoxide (Me_2SO) is included in the assay medium. According to these data, small concentrations of ADP (less than $20 \mu\text{M}$) inhibited Ca^{2+} accumulation, whereas Me_2SO abolished the effect of ADP by decreasing the affinity of the enzyme for ADP to less than $20 \mu\text{M}$ to 1.0 mM. At present, it is not known which step of the catalytic cycle is altered by ADP interactions decreasing Ca^{2+} uptake. In the present work, the effect of ADP on the Ca^{2+} pump was studied in water and in 20% (v/v) Me_2SO .

MATERIALS AND METHODS

Sarcoplasmic-reticulum vesicles were prepared from rabbit skeletal muscle as described by Eletr & Inesi [7].

Ca^{2+} uptake and Ca^{2+} efflux were measured by the filtration method by using Millipore filters (average pore diameter

$0.45 \mu\text{m}$), as described by Chiesi & Inesi [8]. [^{32}P]P_i was obtained from the Brazilian Institute of Atomic Energy and purified as previously described [9].

The vesicles were loaded at 35 °C with Ca^{2+} in a medium containing 50 mM-Mops/Tris buffer (pH 7.0), 20 mM- MgCl_2 , 0.3 mM- CaCl_2 , 80 mM-KCl and either 10 mM-acetyl phosphate or 20 mM- P_i and 2 mM-ATP. After 40 min incubation at 35 °C, the vesicles were sedimented by centrifugation at 40000 g for 15 min at 5 °C; the supernatant was blotted to minimize the remaining volume of loading medium. The vesicles were kept in ice and resuspended in a small volume of ice-cold water. The suspension of loaded vesicles was diluted 7.5-fold in a reaction medium. Protein concentration was determined as described by Lowry *et al.* [10], with bovine serum albumin as standard. Synthesis of ATP was assayed by measuring the formation of [$\gamma\text{-}^{32}\text{P}$]ATP from ADP and [^{32}P]P_i [9].

Phosphorylation of the ATPase by [^{32}P]phosphate or by [$\gamma\text{-}^{32}\text{P}$]ATP was determined with Millipore filters as previously described and corrected for non-specific binding [9]. The reactions were quenched with 1 vol. of a mixture of 20% (w/v) trichloroacetic acid and 4 mM- P_i . The filters were washed with $15 \times 5 \text{ ml}$ of an ice-cold solution containing 0.1 M-HCl and 2 mM- P_i and counted for radioactivity in a liquid-scintillation counter.

[$\gamma\text{-}^{32}\text{P}$]ATP was prepared as described by Glynn & Chappell [11].

All experiments were performed at 35 °C.

RESULTS

Effect of ADP on Ca^{2+} uptake and ATP synthesis

The experiment of Fig. 2 confirmed previous observations [5]

Abbreviation used: Me_2SO , dimethyl sulphoxide.

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Fig. 1. Reaction sequence

Fig. 2. Effect of Me_2SO and ATP-regenerating system on Ca^{2+} uptake

(a) The assay medium composition was 50 mM-Mops/Tris (pH 7.0), 80 mM-KCl, 10 mM- MgCl_2 , 0.1 mM- $^{45}\text{CaCl}_2$, 1 mM- P_i , 20 μg of vesicle protein/ml and 1 mM-ATP. ○, Without additions; ▲, 20% (v/v) Me_2SO ; △, 20% Me_2SO plus 2 mM-phosphoenolpyruvate and 20 μg of pyruvate kinase/ml; ●, 2 mM-phosphoenolpyruvate and 20 μg of pyruvate kinase/ml. (b) The assay medium was as in (a), except for the pH of the buffer, which was either 6.5 (□, ■) or 7.5 (▽, ▼). □, ▽, Without organic solvent; ■, ▼, in the presence of 20% Me_2SO .

that, in the presence of 1 mM- P_i , addition to the medium of either Me_2SO or an ATP-regenerating system promotes an increase in the amount of Ca^{2+} accumulated by the vesicles. The effects of the regenerating system and of Me_2SO were not additive. The amount of Ca^{2+} accumulated by vesicles incubated in media containing only an ATP-regenerating system and in a medium that, besides the regenerating system, also contained 20% Me_2SO was essentially the same (Fig. 2). Activation of Ca^{2+} uptake by Me_2SO was observed at pH 6.5, 7.0 and 7.5 (Fig. 2). A possible mechanism by which ADP inhibits the Ca^{2+} uptake is by reversal of reaction 3 in Fig. 1. In this case, Me_2SO would greatly decrease the affinity of the enzyme form $2\text{Ca}:\text{E}\cdot\text{P}$ for ADP, and the small amount of nucleoside diphosphate formed during hydrolysis of ATP would no longer be sufficient to promote the reversal of reaction 3, and thus enhance the forward rate of the cycle. This hypothesis was excluded by the data in Table 1. The affinity of the enzyme form $2\text{Ca}:\text{E}\cdot\text{P}$ for ADP can be determined by measuring the rate of ATP synthesis in vesicles previously loaded with Ca^{2+} [12]. In all conditions tested, Me_2SO promoted a small increase in the apparent K_m for ADP. The increase was more pronounced at pH 7.5 than at pH 6.0. This was not sufficient to account for the increase in the K_i for ADP from $< 20 \mu\text{M}$ to 1.0 mM [5] promoted by Me_2SO in the Ca^{2+} -uptake measurements. In the absence of KCl, both the K_m for ADP and the V_{max} of ATP synthesis decreased as the pH of the medium was raised from 6.0 to 7.5 (Table 1). This decrease was observed

both in the absence and in the presence of 20% Me_2SO . In a previous report [13], it was shown that KCl modified the synthesis of ATP in different ways, depending on the pH of the medium

Table 1. Synthesis of ATP: K_m for ADP

The assay-medium composition was 50 mM-Mops/Tris adjusted to the pH values shown in the Table, 10 mM- MgCl_2 , 1 mM- $^{32}\text{P}_i$, 5 mM-EGTA and ADP concentrations ranging from 1 to 100 μM . The reaction was quenched after 15 s incubation. The values shown are averages of two to six different experiments.

Additions	Totally aqueous medium		Me_2SO (20%, v/v)	
	K_m (μM)	V_{max} ($\mu\text{mol/min per g}$)	K_m (μM)	V_{max} ($\mu\text{mol/min per g}$)
Without KCl				
pH 6.0	10.5	146.3	25.0	61.4
pH 6.5	6.0	128.9	14.5	49.0
pH 7.5	1.9	17.9	17.2	26.5
With 80 mM-KCl				
pH 6.0	13.9	119.0	25.0	50.0
pH 6.5	8.0	98.1	13.7	159.8
pH 7.5	2.6	20.4	10.5	111.3

Fig. 3. Effect of ADP and Me_2SO on the enzyme phosphorylation by P_i (a) in the absence or (b) in the presence of a Ca^{2+} gradient

The assay medium composition was 50 mM-Mops/Tris buffer (pH 6.0), 5 mM-EGTA, 10 mM- MgCl_2 , 1 mM- P_i , 0.16 mg of vesicle protein/ml and the ADP concentrations indicated in the Figure in the absence (○) or in the presence (●) of 20% (v/v) Me_2SO . The reaction time was 15 s. In (a) empty vesicles were used; 100% phosphoenzyme in a totally aqueous medium was equivalent to 1.3 ± 0.1 (S.E.M.) $\mu\text{mol/g}$ of protein, and in the presence of 20% Me_2SO it was 2.4 ± 0.5 (S.E.M.) $\mu\text{mol/g}$. In (b) vesicles loaded with $0.8\text{--}1.0$ μmol of Ca^{2+} /mg of protein were used; 100% of phosphoenzyme (E-P) was equivalent to 1.98 ± 0.1 (S.E.M.) $\mu\text{mol/g}$ of protein in totally aqueous medium and 2.90 ± 0.1 (S.E.M.) $\mu\text{mol/g}$ in 20% Me_2SO . The values in the Figure are averages of three experiments.

Fig. 4. Inhibition by ADP in totally aqueous medium

The reaction-medium composition was as in Fig. 3 with (○) 1 mM- or (●) 2 mM- P_i . In (a) empty vesicles and in (b) vesicles loaded with Ca^{2+} were used.

and on whether or not Me_2SO was included in the assay medium. This observation was confirmed by the results in Table 1, which in addition show that the effect of KCl was associated with a change in V_{max} , having practically no effect on the K_m for ADP.

Phosphorylation by P_i

In previous reports [6,14] it was shown that ADP inhibited the phosphorylation of the enzyme by P_i in a competitive manner (reactions 6 and 7 in Fig. 1). We now show that, in a totally aqueous medium, the effect of ADP on the phosphoenzyme level varies depending on whether empty vesicles or vesicles previously loaded with Ca^{2+} are used. In empty vesicles (Fig. 3), a significant decrease in phosphoenzyme was only observed when the ADP concentration was raised above 1 mM. In a Dixon plot the K_i

Fig. 5. The two components of ADP inhibition observed in totally aqueous medium

The reaction-medium composition was as in Fig. 3, with vesicles loaded with Ca^{2+} . ○, Totally aqueous medium; ●, 20% Me_2SO . The inset shows values obtained at higher ADP concentrations.

found was 8 mM (Fig. 4a). With the use of vesicles previously loaded with Ca^{2+} , two components were observed (Figs. 3b and 5). A first fall in the phosphoenzyme level appeared with the use of micromolar concentrations of ADP, with a K_i of 80 μM (Figs. 3b, 4b and 5). A second decrease was observed when the ADP concentration was raised to the millimolar range. The K_i of this second component was the same as that measured in empty vesicles, i.e. 8 mM-ADP (Figs. 3, 4a and 5). In vesicles previously loaded with Ca^{2+} , the high-affinity component was abolished when Me_2SO was included in the medium, and a decrease in the level of phosphoenzyme was only observed with millimolar concentrations of ADP. Fig. 3(a) shows that in empty vesicles the inhibitory activity of ADP was essentially the same with or without Me_2SO .

ATP synthesis

The two components observed in Figs. 3(b) and 5 suggested that ADP may be involved in more than one step of the catalytic

Fig. 6. Effect of ADP on phosphoenzyme level and on the rate of ATP synthesis

The reaction mixture composition was 50 mM-Mops/Tris (pH 6.0), 5 mM-EGTA, 10 mM-MgCl₂, 1 mM-P_i, 0.13 mg of vesicles loaded with 1.1 μmol of Ca²⁺/mg of vesicle protein, (○) without or (●) with 20% Me₂SO. The reaction was started by the addition of vesicles and stopped after 15 s. In (a), 100% phosphoenzyme (E-P) was equivalent to 1.90 μmol/g of protein in totally aqueous medium and 2.95 μmol/g of protein in 20% Me₂SO. In (b), 100% of ATP synthesis was equivalent to 184 and 56 nmol/min per mg in totally aqueous medium and 20% Me₂SO respectively.

Table 2. Effect of ADP and Me₂SO on the enzyme phosphorylated by P_i and by ATP

The assay-medium composition was 50 mM-Mops/Tris buffer (pH 7.0), 80 mM-KCl, 10 mM-MgCl₂, 0.2 mM-CaCl₂, 0.1 mg of vesicle protein/ml, 1 mM-ATP and 1 mM-P_i. When added, the concentration of ADP was 1 mM. The values are means ± S.E.M. of *n* experiments. The differences between the values marked with * and † were statistically significant (*P* < 0.05; Student's *t* test). Differences among the other values in the Table were not statistically significant. The reaction time was 1 min.

Additions	Radioactive phosphoenzyme (μmol/g of protein)	
	Totally aqueous medium	Me ₂ SO (20%, v/v)
No added ADP		
A. [³² P]P _i , ATP	0.05 ± 0.01 (<i>n</i> = 6)	0.52 ± 0.03* (<i>n</i> = 12)
B. [γ- ³² P]ATP, P _i	3.95 ± 0.15† (<i>n</i> = 6)	3.75 ± 0.11 (<i>n</i> = 9)
A + B	4.00	4.27
With 1 mM-ADP		
A. [³² P]P _i , ATP	0	0.30 ± 0.06* (<i>n</i> = 6)
B. [γ- ³² P]ATP, P _i	2.71 ± 0.16† (<i>n</i> = 6)	3.42 ± 0.16 (<i>n</i> = 6)
A + B	2.71	3.72

cycle of the ATPase. In order to ascertain which of the two components was related to the transfer of phosphate from the phosphoenzyme to ADP (reaction 3 in Fig. 1), the effects of ADP on the rate of ATP synthesis and on the level of phosphoenzyme were simultaneously measured by using vesicles previously loaded with Ca²⁺ both in the absence and in the presence of Me₂SO (Fig. 6). In a totally aqueous medium, the synthesis of ATP was accompanied by a decrease in the phosphoenzyme level. In the presence of Me₂SO this was not observed, the increment in ATP synthesis being accompanied by only a small decrease in the

phosphoenzyme level. In a totally aqueous medium the concentration of ADP that promoted a half-maximal decrease in the first phosphoenzyme component was higher (80–100 μM) than that which promoted a half-maximal increase in the rate of ATP synthesis. This discrepancy is probably related to the availability of different forms of phosphoenzyme during steady state [1–4]. The radioactive phosphoenzyme level measured represents the sum of three different species, *E-P, 2Ca:*E-P and 2Ca:E-P. The synthesis of ATP depends on the rate of phosphate transfer from only one form of phosphoenzyme (reaction 3 in Fig. 1), whereas the steady-state level of the phosphoenzyme depends also on the rates of *E-P formation (reactions 6 and 7) and on interconversion of the different forms of phosphoenzyme.

Phosphorylation of the ATPase by P_i and by ATP during Ca²⁺ uptake

During Ca²⁺ uptake the different enzyme forms are continuously interconverted, including those which are phosphorylated by ATP and by P_i [15]. The fractions of each of these two enzyme forms available during Ca²⁺ uptake varies depending on the experimental conditions used. The amount of phosphoenzyme formed by ATP during Ca²⁺ uptake was the same in totally aqueous medium and in the presence of Me₂SO (Table 2). However, a significant phosphorylation by P_i was detected only when Me₂SO was included in the assay medium. In totally aqueous medium, the addition of 1 mM-ADP to the medium promoted a significant decrease in the level of phosphoenzyme formed by both ATP and P_i (Table 2). This decrease was less pronounced in the presence of 20% Me₂SO. Thus the total fraction of enzyme phosphorylated by both ATP and P_i during Ca²⁺ uptake becomes significantly smaller in totally aqueous medium rather than in the presence of organic solvent. Finally, in totally aqueous medium the fractions of enzyme phosphorylated by either ATP or P_i did not vary when an ATP-regenerating system was included in the assay medium (results not shown).

Enzyme phosphorylation by P_i and passive Ca²⁺ efflux

Recently [16], evidence has been presented showing that phosphorylation of the enzyme by P_i inhibited the passive efflux of Ca²⁺ measured in vesicles actively loaded with Ca²⁺ and

Fig. 7. Effect of Me_2SO and Mg-P_i on passive Ca^{2+} efflux

The assay-medium composition was 50 mM-Mops/Tris buffer (pH 6.8), 5 mM-EGTA and (a) 0.1 mM- P_i and 0.1 mM- MgCl_2 or (b) 4.0 mM- P_i and 4.0 mM- MgCl_2 . The reaction was started by the addition of loaded vesicles to a final concentration of 0.03 mg/ml, and the Ca^{2+} remaining in the vesicles after different incubation intervals is shown in the Figure. In (a) and (b): $\circ$, totally aqueous medium; $\bullet$, 20% Me_2SO .

Fig. 8. Correlation between rate of Ca^{2+} efflux and level of enzyme phosphorylation by P_i

The assay medium composition was 50 mM-Mops/Tris buffer (pH 7.2), 5 mM-EGTA, 4 mM- P_i , 0.13 mg of vesicles/ml loaded with 4.0 μmol of Ca^{2+} /mg of protein. For Ca^{2+} efflux ($\circ$) the vesicles were loaded with ^{45}Ca , and the P_i added to the efflux medium was non-radioactive. For the phosphorylation of the enzyme by P_i ($\bullet$), the vesicles were loaded with non-radioactive Ca^{2+} and $[^{32}\text{P}]\text{P}_i$ was used in the efflux medium.

incubated in a medium containing EGTA. The experiments of Fig. 7 show that Me_2SO decreased the passive efflux of Ca^{2+} and that this effect could be associated with the phosphorylation of the enzyme by P_i .

In media containing 0.1 mM- P_i and 0.1 mM- MgCl_2 , the efflux of Ca^{2+} measured in totally aqueous media was faster than that measured in the presence of 20% Me_2SO (Fig. 7a). In this experiment the phosphoenzyme level measured in totally aqueous media was less than 0.1 $\mu\text{mol/g}$ of protein, and ranged between 1.4 and 2.2 $\mu\text{mol/g}$ of protein in the presence of Me_2SO . The rates of Ca^{2+} efflux were practically the same in the presence and in the absence of Me_2SO when the P_i and MgCl_2 concentrations were raised to 4 mM (Fig. 7b). In these conditions, the levels of phosphoenzyme in totally aqueous media and in the presence of Me_2SO were practically the same, and among four vesicle

preparations tested ranged between 2.5 and 3.2 $\mu\text{mol/g}$ of protein.

In totally aqueous media there was a decrease in the rate of Ca^{2+} efflux and a concomitant increase in the phosphoenzyme level as the MgCl_2 concentrations in the medium were progressively raised from zero (2 mM-EDTA) to 4 mM (Fig. 8).

DISCUSSION

Data on Ca^{2+} efflux obtained in different laboratories indicate that Ca^{2+} can be released from the vesicles by the ATPase in a process not necessarily linked to the synthesis of ATP [17–21]. This leakage seems to be mediated by the interconversion of the enzyme form $2\text{Ca}^+ \text{E}$ into E [18] as shown in Fig. 1, reactions 9, 10 and 11. Thus Ca^{2+} may leave the vesicles through two different routes. One is coupled to the synthesis of ATP and is mediated by the reversal of reaction 3. The second route is not coupled with ATP synthesis, and is perhaps mediated by the interconversion of the enzyme forms $2\text{Ca}^+ \text{E}$ into $2\text{Ca}^+ \text{E}$. Thus three different conditions can be identified during Ca^{2+} uptake.

(a) Totally aqueous medium and non ATP-regenerating system; in this condition, Ca^{2+} is released through the two routes, coupled (reaction 3) and uncoupled (reactions 9–11).

(b) Totally aqueous medium with an ATP-regenerating system; Ca^{2+} is released only through the uncoupled route, since there is no ADP available to activate the reversal of reaction 3.

(c) In the presence of Me_2SO ; the rate of Ca^{2+} release through the two routes is smaller than that measured in totally aqueous media. This is related to a decrease of the fraction of dephosphoenzyme available during Ca^{2+} uptake (Table 2).

The amount of Ca^{2+} accumulated by the vesicles after each incubation interval in Fig. 2 depends on the rate at which Ca^{2+} is pumped inside the vesicle minus the rate at which Ca^{2+} leaves the vesicles through the two routes, coupled and uncoupled. During the Ca^{2+} uptake measured with Me_2SO (Fig. 2), only a small fraction of the enzyme is involved in the reversal of the Ca^{2+} pump. This was deduced from the finding showing that the addition of an ATP-regenerating system in media containing Me_2SO has no effect on the amount of Ca^{2+} accumulated by the vesicles. On the other hand, in a totally aqueous medium the major contribution of Ca^{2+} efflux during Ca^{2+} uptake is probably derived from the release of Ca^{2+} through the uncoupled route, because a large fraction of the enzyme is not phosphorylated by either ATP or P_i (Table 2), and can be engaged in reactions 9–11.

High concentrations of ADP would compete with P_i for the reversal of reactions 6 and 7 and thus increase the rate of Ca^{2+} efflux through the uncoupled route, both in totally aqueous medium and in the presence of solvent. Therefore the second K_m of lower affinity for ADP detected in Figs. 3 and 5 is related to the binding of ADP to the enzyme form E which is phosphorylated by P_i .

The experiments of Fig. 3 suggests that Me_2SO , in addition to increasing the apparent affinity of the enzyme for P_i , also decreases the rate of interconversion of the ADP-insensitive form into the ADP-sensitive phosphoenzyme (reaction 4), i.e. stabilizes the phosphoenzyme forms ADP-insensitive $2\text{Ca}^+ \text{E-P}$ and E-P . During reversal of the Ca^{2+} pump, the steady-state level of phosphoenzyme depends on the rates of formation of the ADP-insensitive phosphoenzyme (reactions 7–4 backwards) and on the rate of transfer of phosphate from the phosphoenzyme to ADP (reversal of reaction 3). Figs. 3(b) and 6 show that in totally aqueous medium the synthesis of ATP is accompanied by a significant decrease in the phosphoenzyme level in totally aqueous medium, but not in Me_2SO . These experiments were performed at pH 6.0, a condition in which K^+ has little or no effect on the V_{max} of ATP synthesis. At this pH the V_{max} of ATP synthesis was

about twice as fast in water as in Me_2SO . These data can be readily accounted for by assuming that Me_2SO decreases the rate of reaction 4 backwards. Thus, after the addition of ADP in Fig. 6, the higher level of phosphoenzyme found in the presence of Me_2SO would be accounted for by a larger fraction of ADP-insensitive phosphoenzyme.

Finally, we still do not know why in the presence of Me_2SO the effect of K^+ on the V_{max} of ATP synthesis varies depending on the pH of the medium (Table 1).

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