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Effect of Temperature on the Reversal of the Calcium Ion Pump in Sarcoplasmic Reticulum

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Sarcoplasmic-reticulum vesicles were actively loaded with Ca^{2+} in the presence of phosphate, and the ADP-induced Ca^{2+} efflux and ATP synthesis were measured as a function of temperature. Arrhenius plots show break points for both processes at about 18 and 37°C. Between 18 and 37°C, Ca^{2+} efflux and ATP synthesis occur with an activation energy of 67.2–71.4 kJ/mol, whereas it is about 189–210 kJ/mol for temperatures below 18°C. Above 37°C, the rates of ADP-induced Ca^{2+} release and of ATP synthesis sharply decline until the temperature reaches about 42°C. Above this temperature, the Ca^{2+} efflux increases again even in absence of ADP, although the synthesis of ATP is inhibited, which reflects leakiness of the vesicles. The results show that the transition temperatures for ADP-induced Ca^{2+} efflux and for ATP synthesis resemble those for active Ca^{2+} uptake, which indicates that the same coupling mechanism is involved during the inward and outward Ca^{2+} translocations across the membrane.

It has been shown that, under certain conditions, the Ca^{2+} pump of isolated sarcoplasmic reticulum is reversed and ATP is synthesized coupled to Ca^{2+} release from the vesicles (Barlogie *et al.*, 1971; Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Deamer & Baskin, 1972; Yamada *et al.*, 1972; Yamada & Tonomura, 1973; Inesi *et al.*, 1973; De Meis, 1976*a*,*b*; Vale *et al.*, 1976). This phenomenon occurs either in vesicles previously loaded actively (Barlogie *et al.*, 1971; Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Deamer & Baskin, 1972; De Meis, 1976*a*,*b*) or passively with Ca^{2+} (Makinose, 1972; Yamada *et al.*, 1972; Yamada & Tonomura, 1973; Inesi *et al.*, 1973; Vale *et al.*, 1976), provided that ADP and P_i are present in the reaction medium. In both cases, the ratio of Ca^{2+} released to ATP synthesized is reported to be two (Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Deamer & Baskin, 1972; Yamada *et al.*, 1972; Vale *et al.*, 1976), which indicates that most of the Ca^{2+} is released from the vesicles through the pump system by an apparent reversal of the transport reactions.

By using a continuous recording system, we studied the effect of temperature on the ADP-induced Ca^{2+} efflux and on the ATP synthesis by sarcoplasmic-reticulum vesicles preloaded with Ca^{2+} in the presence of phosphate, which greatly increases the Ca^{2+} capacity of the vesicles (Hasselbach, 1964; Martonosi & Feretos, 1964; De Meis, 1967; De Meis *et al.*, 1974). Although the influence of temperature on reversal of the Ca^{2+} pump has been previously observed by several authors (Panet & Selinger, 1972; Inesi *et al.*, 1973; Masuda & De Meis, 1977), as far as we know, its detailed thermotropic characterization has not been reported. In the experiments reported in the present paper we determined the transition temperatures and we estimated the energies of activation of the ADP-induced Ca^{2+}-efflux and ATP-synthesis processes. The results show that the transition temperatures for the reversal of the Ca^{2+} pump in sarcoplasmic reticulum resemble those for active Ca^{2+} uptake.

Material and Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum was isolated from rabbit white skeletal muscle as described elsewhere (Vale & Carvalho, 1975).

Ca^{2+} uptake by sarcoplasmic-reticulum vesicles

The Ca^{2+} uptake by sarcoplasmic-reticulum vesicles was monitored spectrophotometrically as previously described (Fairhurst & Jenden, 1966). The incubation medium contained 20 mM-KCl, 4.0 mM-$MgCl_2$, 60 mM-KH_2PO_4, 0.14 mM-$CaCl_2$ and

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0.18mg of reticulum protein in a total volume of 3.5ml at pH7.0. The reaction was initiated by $MgATP^{2-}$ added to a final concentration of 1.4mM. The temperature was controlled by connecting the reaction vessel to a thermostatically controlled water bath maintained at 37°C.

In control experiments, acetyl phosphate (10mM), instead of ATP, was used as the energy source for Ca^{2+} transport by sarcoplasmic-reticulum vesicles.

Ca^{2+} efflux from sarcoplasmic-reticulum vesicles

After Ca^{2+} uptake, the reaction vessel was connected to one of several water baths equilibrated at the temperatures indicated in the legends. The Ca^{2+} release was initiated by adding 2.0mM-ADP and 5.0mM-EGTA.

The absorbance changes associated with Ca^{2+} uptake and Ca^{2+} release were followed at 380nm in a Spectronic 20 linked to a Vitatron recorder with the sensitivity adjusted so that full scale in the recorder was 0.1 absorbance units.

Ca^{2+} analysis

The Ca^{2+} retained by the membrane vesicles under several experimental conditions was measured by the Millipore-filtration technique (Martonosi & Feretos, 1964). The filters (HA 0.45μm) retaining the protein (0.05mg) were washed once by filtering 1.0ml of 0.25M-sucrose. Finally, they were immersed in 2.5ml of a solution containing 2% trichloroacetic acid and 0.5% La^{3+}, and after vigorous agitation, Ca^{2+} analysis was performed in this solution by atomic-absorption spectroscopy in a model 305 Perkin–Elmer spectrophotometer.

The protein was measured by the biuret method (Layne, 1957) with bovine serum albumin as standard.

Assay of ATP synthesis by sarcoplasmic-reticulum vesicles

The synthesis of ATP coupled with the Ca^{2+} efflux from sarcoplasmic-reticulum vesicles was determined by a continuous spectrophotometric assay described by Horgan (1978). After loading the vesicles with Ca^{2+} in the presence of acetyl phosphate, the reaction medium was incubated at each of several temperatures between 10 and 50°C, and the following substances were added: 10mg of fructose/ml, 0.5mg of $NADP^+$/ml, 1.4 units of hexokinase/ml, 1.7 units of glucose 6-phosphate dehydrogenase/ml, and 2.1 units of glucose phosphate isomerase/ml. The Ca^{2+} efflux was started by adding 2mM-ADP plus 5mM-EGTA, and the absorbance changes associated with formation of NADPH were measured at 340nm by using a Spectronic 20 instrument linked to a Vitatron recorder previously calibrated so that full scale in the recorder was 1.0 absorbance units.

The values for the synthesis of ATP were calculated by using a standard curve for glucose 6-phosphate. The ATP formed by reversal of the sarcoplasmic-reticulum Ca^{2+} pump was calculated after subtracting the ATP synthesized in the absence of Ca^{2+} efflux. This control was performed with unloaded vesicles in a medium with the same composition as described above.

Results

Effect of temperature on the ADP-induced Ca^{2+} efflux from sarcoplasmic-reticulum vesicles

Fig. 1 shows the Ca^{2+} uptake and Ca^{2+} release by sarcoplasmic reticulum determined by recording the absorbance changes associated with those processes. When Ca^{2+} uptake starts after addition of ATP, the absorbance of the reaction medium increases as calcium phosphate precipitates within the vesicles, and reaches a constant value when no more Ca^{2+} is taken up. On the other hand, the absorbance decreases when Ca^{2+} is released from the vesicles after adding ADP and EGTA.

The Ca^{2+} uptake was carried out at 37°C, whereas the Ca^{2+} release was studied at several temperatures in the range 8–50°C. At low temperatures (8°C), essentially no Ca^{2+} is released from the vesicles, but the Ca^{2+} efflux increases at the higher temperatures and reaches a maximum value at about 37°C. Above this temperature, the Ca^{2+} release decreases sharply, but increases again at temperatures of about 45–50°C. These variations are discussed below.

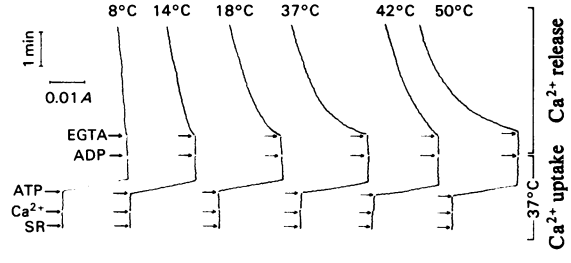

Fig. 1. *Absorbance changes during Ca^{2+} uptake at 37°C and Ca^{2+} release by sarcoplasmic reticulum at several temperatures*

The reactions were carried out in a medium containing 20mM-KCl, 4mM-$MgCl_2$, 60mM-KH_2PO_4, 0.14mM-$CaCl_2$ and 0.18mg of reticulum protein in a total volume of 3.5ml (pH 7.0). After equilibrating the medium at 37°C, Ca^{2+} uptake was initiated by adding 1.4mM-$MgATP^{2-}$. Ca^{2+} efflux was carried out at several temperatures between 8 and 50°C. Ca^{2+} release was started by adding 2mM-ADP and 5mM-EGTA. Abbreviation used: SR, sarcoplasmic reticulum. The arrows indicate additions.

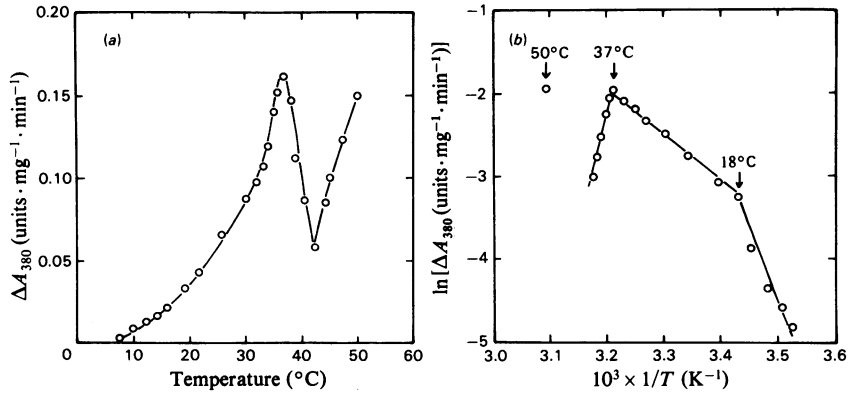

Fig. 2. *Effect of temperature on the ADP-induced Ca^{2+} efflux in sarcoplasmic-reticulum vesicles*

The experiments were performed as described in Fig. 1. The results represent the mean values for three individual experiments. (*a*) Initial rates of the absorbance changes associated with Ca^{2+} release from the vesicles as a function of temperature; (*b*) Arrhenius plot for the results depicted in (*a*).

The effect of temperature on the rate of Ca^{2+} efflux from sarcoplasmic reticulum is summarized in Fig. 2(*a*), in which the initial rates of absorbance changes per mg of protein were plotted against temperature. The rate of Ca^{2+} release increases steadily between about 8 and 37°C, but there is marked decrease (from 0.16 to 0.051*A* units · mg^{-1} · min^{-1}) on raising the temperature from 38 to 42°C. Above 45°C, the rate of absorbance change increases again, which indicates that, at these higher temperatures, the rate of Ca^{2+} efflux increases. This increased rate of Ca^{2+} release at the higher temperatures was shown to reflect loss of Ca^{2+} uncoupled to ATP synthesis.

Arrhenius plots of the rates of absorbance changes (Fig. 2*b*) show that an abrupt change in the apparent activation energy of the process occurs at about 18°C. Between 18 and 37°C the process has an energy of activation of about 67.2–71.4 kJ/mol, whereas below 18°C, the activation energy is about 189–210 kJ/mol. Above 37°C, the initial rate of the process sharply decreases. Therefore, two break points, at 18 and 37°C, can be defined in the Arrhenius plot. The point corresponding to the Ca^{2+} efflux observed at 50°C does not lie on the straight lines of the Arrhenius plot, which suggests that at this temperature another mechanism is operative in liberating Ca^{2+} from the vesicles.

The ADP-induced absorbance changes monitored by a continuous recording system can be correlated with the Ca^{2+} content of the vesicles determined by the Millipore-filtration technique. Fig. 3 shows that Ca^{2+} is indeed released from the vesicles after adding ADP plus EGTA to the reaction medium. The amount of Ca^{2+} released increases with the temperature and reaches a maximum value at 37°C. Between 37 and 42°C the ADP-induced Ca^{2+} efflux was decreased from 500 to 300 nmol/mg of protein, and it increased again at temperatures between 45 and 50°C, as was also observed by the simultaneous spectrophotometric analysis (Fig. 2*a*). The ratio $\Delta A/Ca^{2+}$ released remains constant (2×10^{-4}) over the range of temperatures studied, which indicates that good correlation indeed exists between absorbance changes and Ca^{2+} efflux.

Although the addition of ADP stimulated the Ca^{2+} release from sarcoplasmic reticulum, preliminary experiments showed that the mere addition of EGTA alone promotes some efflux of Ca^{2+}. This

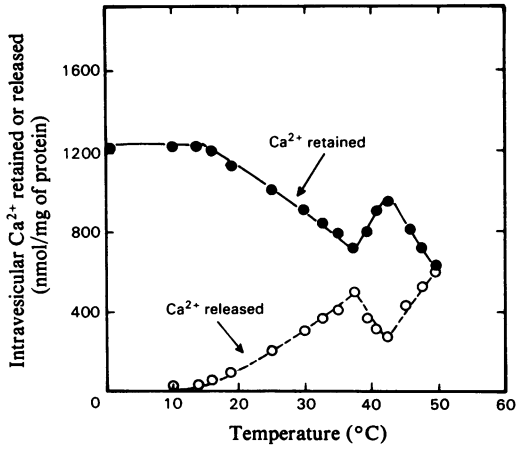

Fig. 3. *Ca^{2+} retained and Ca^{2+} released by sarcoplasmic-reticulum vesicles after addition of ADP at several temperatures*

The experimental conditions were those described for Fig. 1. The Ca^{2+} efflux was initiated by adding ADP (2 mM) plus EGTA (5 mM); 1 min later a 1.0 ml portion (0.05 mg of protein) was withdrawn and was filtered through Millipore filters.

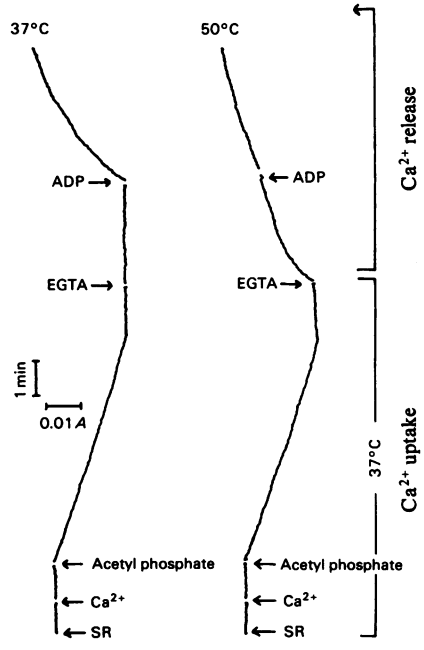

Fig. 4. *Effect of ADP on the Ca^{2+} efflux from sarcoplasmic-reticulum vesicles after Ca^{2+} uptake driven by acetyl phosphate*

The experimental conditions were similar to those described in Fig. 1, except that 10mM-acetyl phosphate, instead of ATP, was utilized for the measurement of Ca^{2+} uptake, and ADP was added after EGTA for the measurement of Ca^{2+} release. The experiments were performed at 37 and 50°C. Abbreviation used: SR, sarcoplasmic reticulum. The arrows indicate additions.

may be due to the presence of ADP resulting from the ATP hydrolysis during the Ca^{2+} uptake, or to leakiness of the vesicles. Control experiments were carried out in which the energy donor for Ca^{2+} uptake was acetyl phosphate instead of ATP, to prevent ADP accumulation in the reaction medium (Fig. 4). The acetyl phosphate can also support Ca^{2+} transport, but at a very much slower rate than that supported by ATP (Makinose, 1972; Panet & Selinger, 1972).

The results reported in Fig. 4 show that, at 37°C, EGTA alone does not release Ca^{2+} in a medium free of ADP, and that ADP is necessary for the Ca^{2+} efflux. In contrast, at 50°C, the Ca^{2+} efflux occurs even in the absence of ADP when EGTA is added, which indicates that at this temperature, the membranes are leaky and the Ca^{2+} bypasses the pump.

Measurements of the Ca^{2+} retained utilizing the Millipore technique showed that, at 37°C, EGTA alone in fact releases about 90nmol of Ca^{2+} per mg of protein. This is probably Ca^{2+} externally adsorbed on the surface of the vesicles (Kim *et al.*, 1976), since its liberation cannot be detected by the absorbance technique (Fig. 4).

Effect of temperature on the ATP synthesis by sarcoplasmic reticulum

The synthesis of ATP coupled to the Ca^{2+} efflux from actively loaded sarcoplasmic-reticulum vesicles was measured as a function of temperature. Fig. 5 shows the spectrophotometric records of the $NADP^+$ reduction associated with the formation of glucose 6-phosphate, which reflect the amount of ATP synthesized (Horgan, 1978). The first part of the curve depends on the amount of Ca^{2+} released from the sarcoplasmic-reticulum vesicles, whereas the second part occurs even in the absence of Ca^{2+} efflux, and probably reflects the synthesis of ATP catalysed by certain enzymes that normally contaminate the sarcoplasmic-reticulum preparations (Horgan, 1978). The adenylate kinase (Weber *et al.*, 1966) and the myokinase (Deamer & Baskin, 1972) are the enzymes present in sarcoplasmic reticulum responsible for the extra synthesis of ATP. The rate of ATP synthesis associated with Ca^{2+} efflux was easily calculated by subtracting the rate of the second component from the initial rate (Horgan, 1978). As shown in Fig. 5, the rate of ATP synthesis increased with temperature, reaching a maximum value at 37°C. Above this temperature, the rate of the second component of the reaction is still increasing, whereas the rate of the Ca^{2+} efflux-dependent ATP synthesis is significantly decreased.

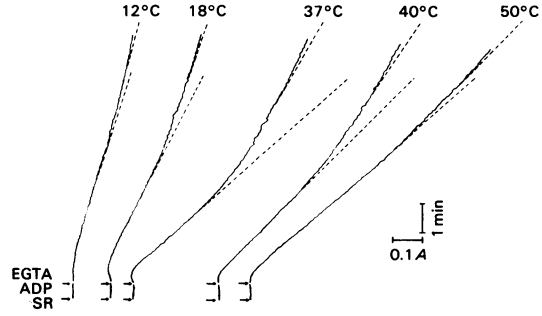

Fig. 5. *Spectrophotometric records of ATP synthesis by sarcoplasmic-reticulum vesicles*

Sarcoplasmic-reticulum vesicles were preloaded with Ca^{2+} during 15min in the presence of acetyl phosphate in a medium otherwise similar to that described in Fig. 1. Then, 10mg of fructose/ml, 0.5mg of $NADP^+$/ml, 1.4 units of hexokinase/ml, 1.7 units of glucose 6-phosphate dehydrogenase/ml and 2.1 units of glucose phosphate isomerase/ml were added to the reaction medium, which was incubated at several temperatures between 10 and 55°C. The Ca^{2+} efflux was started by adding 2mM-ADP plus 5mM-EGTA. Abbreviation used: SR, sarcoplasmic reticulum. The arrows indicate additions.

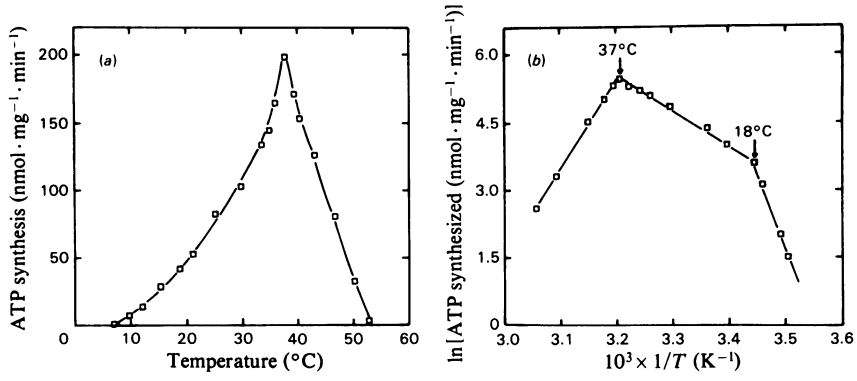

Fig. 6. *Effect of temperature on the ATP synthesis by sarcoplasmic-reticulum membranes*
The graph was constructed from the data obtained in the experiments depicted in Fig. 5. (*a*) shows the rate of ATP synthesis coupled to Ca^{2+} efflux from the reticulum vesicles as a function of temperature; (*b*) shows an Arrhenius plot for the results depicted in (*a*).

The results of these experiments are summarized in Fig. 6. Fig. 6(*a*) shows that the rate of ATP synthesis per mg of protein increases gradually with the temperature and reaches a maximum value of about 200nmol of ATP synthesized/mg of protein per min at 37°C. Above this temperature the synthesis of ATP is decreased, and it is zero at about 55°C. We observed similar effects of temperature on the ADP-induced Ca^{2+} efflux measured by spectrophotometric assay (Figs. 1 and 2*a*) or by analysis of the Ca^{2+} content of the vesicles (Fig. 3), except that, at the highest temperatures (>45°C), there is a rapid Ca^{2+} efflux, whereas the rate of ATP synthesis is decreased. This observation is in accord with the results reported in Fig. 4, which suggest that above 45°C, most of the Ca^{2+} bypasses the pump. Table 1 shows the values for the ratio of the Ca^{2+} released to ATP synthesized obtained at the various temperatures between 14 and 50°C. Below 45°C the value is about 2, as previously reported (Makinose & Hasselbach, 1971; Makinose, 1972; Panet & Selinger, 1972; Deamer & Baskin, 1972). At temperatures above 45°C, the ratio is greatly enhanced (values >5), which reflects uncoupling between both processes. The externally adsorbed Ca^{2+}, whose release is temperature-independent, was not included in these calculations.

Arrhenius plots of the rates of ATP synthesis (Fig. 6*b*) show break points at 18 and 37°C. Between these temperatures the process has an apparent activation energy of about 67.2kJ/mol, whereas at temperatures below 18°C the energy of activation is about 210kJ/mol. The similar thermotropic characteristics of the ADP-induced Ca^{2+} efflux (Fig. 2*b*) and of the ATP synthesis probably reflect a common mechanism for both processes.

To determine whether the effect of temperature is reversible at the higher temperature range, we submitted sarcoplasmic reticulum loaded at 37°C with Ca^{2+} in the presence of acetyl phosphate to temperatures up to 50°C. After equilibration at these temperatures for 3min, the loaded sarcoplasmic reticulum was utilized to synthesize ATP at 37°C in a medium containing ADP + EGTA. The results, summarized in Fig. 7, show that equilibration of the loaded vesicles at 38–45°C has only a small inhibitory effect on subsequent ATP synthesis at 37°C. For temperatures above 45°C the heat treatment has irreversible effects.

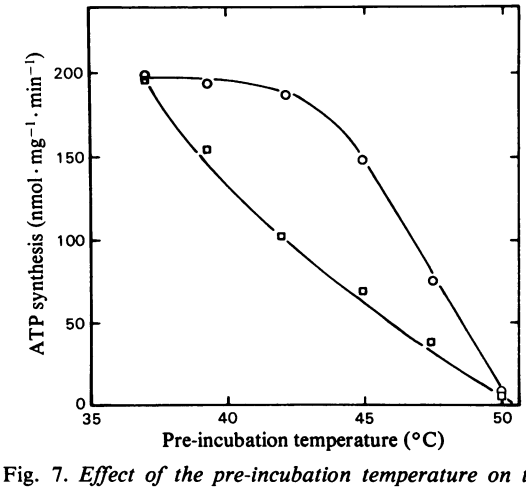

Fig. 7. *Effect of the pre-incubation temperature on the ATP synthesis by sarcoplasmic-reticulum membranes*
Sarcoplasmic reticulum loaded at 37°C with Ca^{2+} in the presence of acetyl phosphate was incubated at temperatures between 37 and 50°C for 3min. The loaded vesicles were utilized to synthesize ATP at 37°C as described in Fig. 5. Symbols: O, synthesis of ATP at 37°C after pre-incubation at various temperatures; □, synthesis of ATP at the various preincubation temperatures.

Table 1. *Coupling between Ca^{2+} release and ATP synthesis by sarcoplasmic-reticulum vesicles incubated at several temperatures*

Sarcoplasmic reticulum was actively loaded with Ca^{2+} in the presence of acetyl phosphate as described in the text. The synthesis of ATP coupled with the Ca^{2+} efflux from the vesicles was continuously followed spectrophotometrically, and the Ca^{2+} efflux was measured by the Millipore-filtration technique. The results represent the mean values obtained for 1 min of reaction in three experiments with different sarcoplasmic-reticulum preparations.

	Ca^{2+} release and ATP synthesis										
Temperature (°C) ...	14	16	18	25	30	35	37	39	40	42	45	50
Ca^{2+} released (nmol/mg of protein)	27	40	90	200	280	360	500	306	262	302	410	600
ATP synthesized (nmol/mg of protein)	14	25	43	85	115	147	210	166	153	130	70	32
Ratio of Ca^{2+} released/ATP synthesized	1.9	1.6	2.1	2.3	2.4	2.4	2.3	1.8	1.7	2.3	5.8	18.7

Discussion

The results reported in the present paper give new evidence about the reversal of the Ca^{2+} pump in sarcoplasmic reticulum. The results show that ADP-induced Ca^{2+} release and ATP synthesis have thermotropic characteristics similar to those observed in the active Ca^{2+} uptake. We found transition points at about 18 and 37°C, which are about the same temperatures as those obtained for the transitions of inward Ca^{2+} transport (Inesi *et al.*, 1973). Between 18 and 37°C, the activation energies for Ca^{2+} efflux and ATP synthesis are about 67.2–71.4 kJ/mol, which are similar to the values reported for Ca^{2+} uptake and ATP hydrolysis (Inesi *et al.*, 1973). Below 18°C, we calculate an activation energy of about 189–210 kJ/mol for the ADP-induced Ca^{2+} efflux and ATP synthesis. In preliminary experiments we obtained about 202 kJ/mol and 67.2 kJ/mol for the Ca^{2+} uptake by sarcoplasmic reticulum measured spectrophotometrically at temperatures below 18°C and between 18–37°C respectively. Therefore, we found close similarities between the thermotropic behaviour of Ca^{2+} uptake and of ATP synthesis in sarcoplasmic-reticulum membranes.

The transition temperatures described in the present paper are probably related to the phase transitions of the membrane molecular components occurring at these temperatures. In studies of Ca^{2+} uptake, Inesi *et al.* (1973) suggested that the membrane lipids are primarily involved in the transition at 18–20°C, whereas at 37–40°C, the transition may correspond primarily to the transition of the membrane protein. Recently, Anzai *et al.* (1978) reported that the break at 18°C is related to a change in the conformation of the ATPase molecule when in fluid lipids. These transitions appear to be reversible and only at the higher temperatures (> 45°C) is the process irreversible.

We found that at 50°C the ATP synthesis is greatly decreased and the Ca^{2+} efflux does not depend on the presence of ADP, which indicates that at this temperature, Ca^{2+} is passively released through the leaky membranes rather than through the pump system (Fig. 4). Indeed, it was found that the ATPase enzyme is inactivated at this temperature (Inesi *et al.*, 1973; Carvalho & Santos, 1976).

The study of the thermotropic behaviour of the reversal of the Ca^{2+} pump reported in the present paper indicates that coupling exists between Ca^{2+} efflux and ATP synthesis and that there is a common system for the inward and outward Ca^{2+} translocations across the sarcoplasmic-reticulum membranes.

Although reversal of the pump has been demonstrated by several investigators (Barlogie *et al.*, 1971; Makinose & Hasselbach, 1971; Makinose,

1972; Panet & Selinger, 1972; Deamer & Baskin, 1972; Yamada *et al.*, 1972; Yamada & Tonomura, 1973; Inesi *et al.*, 1973; De Meis, 1976*a*,*b*; Vale *et al.*, 1976), further information is necessary to understand the mechanism of coupling a Ca^{2+} gradient to the synthesis of ATP. Energy-rich phosphoprotein intermediates are formed during the functional activity of the reticulum membranes (Yamamoto & Tonomura, 1967; Makinose, 1969; Martonosi, 1969; Makinose, 1972; De Meis, 1976*a*,*b*; Yamada *et al.*, 1972; De Meis, 1972, 1974; Masuda & De Meis, 1973; De Meis & Carvalho, 1974; Osório e Castro *et al.*, 1976), but the relation of this phosphoprotein to the proton gradient generated during Ca^{2+} uptake (Madeira, 1978) is not clear. Further experiments will be necessary to clarify the mechanism of energy interconversion in sarcoplasmic reticulum.

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