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The Influence of Calcium Pump Coupling on the Arrhenius Behavior of Sarcoplasmic Reticulum Ca^{2+}-ATPase

Martin D. King,[1,2] Peter J. Quinn,[1,3] Felix M. Munkonge,[1,4] and Thomas D. Madden[1,5]

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Abstract

Experiments were performed in which two batches of sarcoplasmic reticulum were isolated from rabbit hind leg muscle, one in the presence of dithiothreitol, the other in the absence of reducing agent. A comparative study was made of some of the properties of the two preparations, in particular, the Arrhenius behavior of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase isolated in the absence of dithiothreitol is thermally unstable with the result that a triphasic Arrhenius plot was obtained. This triphasic behavior is largely the consequence of an uncoupling of the hydrolytic machinery from the calcium pump. In contrast, the sarcoplasmic reticulum preparation obtained in the presence of dithiothreitol is thermally stable and yields a linear Arrhenius plot. The difference in the Arrhenius behavior shown by the two preparations was abolished when the measurements of Ca^{2+}-ATPase activity were made in the presence of the calcium ionophore, A23187.

Key Words: Calcium-ATPase; Arrhenius behavior; sarcoplasmic reticulum; ATPase.

Introduction

Sarcoplasmic reticulum in skeletal muscle regulates the contractibility of the tissue. As a result of an exciting potential, a portion of the accumulated

[1]Department of Biochemistry, King's College London, Campden Hill, London W8 7AH, U.K.
[2]Present address: Abteilung Spektroskopie, Max Planck Institut fur Biophysikalische Chemie, Göttingen, Federal Republic of Germany.
[3]To whom correspondence should be addressed.
[4]Present address: Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.
[5]Present address: Department of Biochemistry, The University of British Columbia, Vancouver, B.C. V6T 1W5, Canada.

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calcium ions is released from the sarcoplasmic reticulum space resulting in initiation of the formation of an actomyosin complex and tension development. Calcium is subsequently accumulated inside the sarcoplasmic reticulum network by an ATP-dependent Ca-pump thereby inducing muscle relaxation (Hasselbach and Makinose, 1961). Sarcoplasmic reticulum (SR) fragments prepared by differential centrifugation methods form closed vesicles in which Ca^{2+}-pump molecules are oriented in such a way that ATP-dependent Ca^{2+} accumulation into the sealed vesicles takes place (Inesi and Asai, 1968). Thus, the Ca^{2+}-pump consists of Ca^{2+}-dependent ATPase (E.C. 3.6.1.38) which couples ATP hydrolysis to the accumulation of Ca^{2+} ions. The system is controlled by calcium ions: it is activated by calcium ($\geqslant 100$ nm) outside the vesicles and inhibited by calcium ($\geqslant 50\,\mu$M) inside the vesicles (Weber, 1971). Ca^{2+} accumulation inside the vesicles causes inhibition of the Ca-pump, and outflux of Ca^{2+} from SR activates Ca-pump operation.

The Ca^{2+}-ATPase has been shown to exhibit nonlinear Arrhenius behavior, a phenomenon that was originally attributed to a perturbation caused by a phase transition in the membrane lipids (Inesi *et al.*, 1973). Since then a variety of alternative explanations have been suggested to account for the nonlinear behavior, including the formation of lipid clusters (Lee *et al.*, 1974), a reduction in motional anisotropy of parts of the lipid molecules (Davis *et al.*, 1976), and some interaction between the ATPase and a tightly bound lipid annulus (Hesketh *et al.*, 1976).

In a previous study of the temperature dependence of Ca^{2+}-ATPase activity of sarcoplasmic reticulum, a triphasic Arrhenius plot was reported (Madden and Quinn, 1979) which contrasted with other studies in which biphasic Arrhenius behavior is reported. The origin of this triphasic behavior appears to be the result of an uncoupling of the hydrolytic machinery from the calcium pumping process. A spontaneous uncoupling of the Ca^{2+}-ATPase has previously been reported by Berman's group (Berman *et al.*, 1977; McIntosh and Berman, 1978) where it was demonstrated that uncoupling was not caused by an enhanced permeability of the membrane to calcium, but rather by a change in the Ca^{2+}-ATPase itself such that the hydrolysis of ATP is no longer accompanied by the movement of calcium across the membrane. Other workers have shown that the sarcoplasmic reticulum calcium pump is more stable in vesicles isolated in the presence of the reducing agent dithiothreitol (DTT) (Van der Kloot, 1969; Johannsson *et al.*, 1981). We have therefore made a comparative study of the Arrhenius behavior of two types of sarcoplasmic reticulum preparation, one obtained in the presence and one in the absence of DTT.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit hind leg muscle by the method of Warren *et al.* (1974). In each experiment two batches of material were isolated, one of which was processed in buffers containing 1 mM DTT and the other was isolated in the absence of reducing agent. The protease inhibitor phenylmethylsulfonylfluoride was also present (5 μM) in the preparation containing DTT but in the absence of DTT it was found to have no protective effect by itself.

Calcium-dependent ATPase activities were measured in the presence of an ATP generating enzyme system (Madden *et al.*, 1979). Sarcoplasmic reticulum vesicles were added to a protein concentration of 1–20 μg/ml dependent upon the assay conditions. The reaction was initiated by the addition of calcium, and the Ca^{2+}-ATPase activity was measured by following the rate of NADH oxidation using a Pye Unicam SP1800 spectrophotometer. Where employed, A23187 was added to a final concentration of 6.4 μM.

Two different procedures were used to study the Arrhenius behavior of the hydrolytic reaction. Method 1: ATPase activity was measured at fixed temperatures in the range 3–43°C. This is referred to as the discrete temperature method. Method 2: ATPase activity was continuously recorded as the temperature of the reaction mixture was increased at a rate of 1°C/min over the temperature range 6–33°C or 25–43°C, as indicated. A least-squares polynomial was fitted to the digitized data, the first derivative of which was used to calculate the reaction rate at intervals of temperature. Assay temperatures were monitored with a thermocouple inserted into the cuvette.

Calcium uptake measurements were made using a Philips calcium-sensitive electrode. The output voltage was recorded on a pen recorder at a sensitivity of 100 mV full-scale deflection. The reaction mixtures all contain 20 mM PIPES, pH 7.4, buffer, 10 mM $MgCl^{2+}$, and 50 mM KCl. The sarcoplasmic reticulum vesicles (250 μg/ml protein) were preincubated for 10 min at 32°C before the addition of calcium or ATP as indicated.

Results and Discussion

It has been reported that the sarcoplasmic reticulum calcium ATPase, isolated in the absence of DTT, is thermally unstable (Van der Kloot, 1969; Johannson *et al.*, 1981), and that at temperatures greater than about 20°C, it loses the capacity to accumulate calcium. This is confirmed by the calcium uptake traces given in Fig. 1, which show that the Ca^{2+}-ATPase of preparations isolated in the absence of DTT loses its ability to accumulate calcium during a 10-min incubation at 32°C (Figs. 1c and 1d) while the Ca^{2+}-ATPase

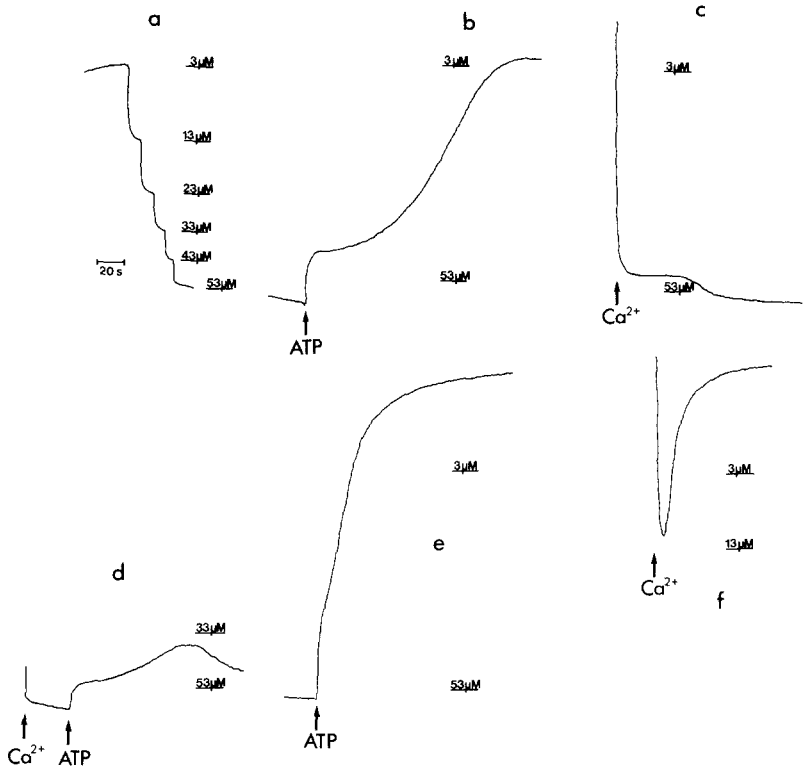

Fig. 1. Calcium uptake into sarcoplasmic reticulum vesicles. (a) Calibration trace. The calcium concentration was incremented in 10 μM steps, starting at a concentration of 3 μM. A 20-sec calibration is shown, time increasing from left to right. (b–d) Sarcoplasmic reticulum vesicles prepared in the absence of dithiothreitol. (e, f) Sarcoplasmic reticulum vesicles prepared in the presence of dithiothreitol. The reaction mixtures all contained 20 mM PIPES, pH 7.4, buffer, 10 mM $MgCl_2$, and 50 mM KCl. The vesicles were preincubated at 32°C for 10 min before adding calcium or ATP as indicated. In (c) and (f), ATP was present in the preincubation mixture at a concentration of 3 mM. In (b) and (e) the preincubation mixture contained calcium (60 μM), and ATP was added at the time indicated together with a small quantity of calcium sufficient to counteract the voltage response to the added ATP.

of preparations isolated in the presence of reducing agent retains its capacity for calcium uptake (Figs. 1e and 1f). The loss of calcium pumping activity in the – DDT Ca^{2+}-ATPase was not observed, however, if the 32°C incubation was carried out in the presence of calcium (Fig. 1b).

As might be expected from their differing thermal stabilities, the Arrhenius properties of the two types of Ca^{2+}-ATPase preparations are dissimilar. Arrhenius activity data were obtained using two different assay procedures. The first of these involved pre-equilibrating separate samples at each assay

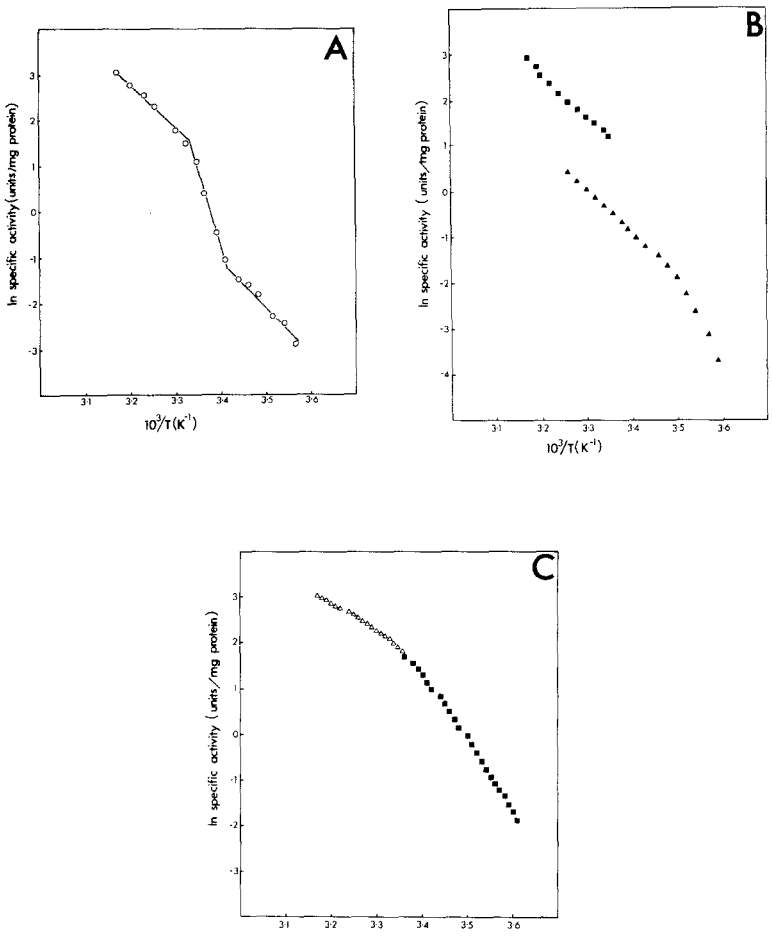

Fig. 2. Arrhenius plots of the Ca^{2+}-ATPase activity displayed by a sarcoplasmic reticulum preparation isolated in the absence of dithiothreitol. Enzyme assays were performed at intervals of temperature in the range 7–42°C (A), or using the continuous heating procedure (see Methods) (B, C). In (A) and (B), the enzyme activity was measured in the absence of an uncoupler, while in (C), 6.4 μM A23187 was present. All data are corrected for the calcium-independent rate.

temperature for 10 min before the reaction was initiated by the addition of calcium. The Arrhenius plot thus obtained for the $-$DDT Ca^{2+}-ATPase is shown in Fig. 2A and is triphasic. In the second procedure, ATPase hydrolytic activity was monitored continuously while the sample was slowly heated over a defined temperature range. Under these conditions the $-$DDT Ca^{2+}-ATPase did not give rise to a triphasic Arrhenius plot. Instead, a continuous curve was obtained, the vertical position of which depended on the pre-incubation

temperature (Fig. 2B). A detailed examination of Figs. 2A and 2B revealed that in the temperature range 25–43°C, the discrete temperature method yielded activity data similar to that obtained for samples preincubated at 25°C and measured using the continuous heating method. On the other hand, in the temperature range 6–18°C, the discrete temperature method yielded activities that were of the same magnitude as those obtained with the continuous heating procedure and with samples preincubated at 3°C. The 3°C-preincubated, continuously heated samples did not, however, give rise to an upward Arrhenius inflection at 18°C. These observations are consistent with the hypothesis that the Ca^{2+}-ATPase can adopt two activity states, the lower of which can undergo a spontaneous transformation into the higher activity state. It must be assumed that at 20°C, the transformation occurs at a rate sufficient to produce a significant amount of the higher activity form during 10-min preincubation and that the transformation is prevented in the absence of Ca^{2+}. These observations are also consistent with the results obtained from measurements of ATPase activity made at 32°C. In this experiment, samples were either preincubated at 32°C before the addition of calcium, or calcium was added at 0°C, after which the sample was rapidly heated to 32°C. – DDT Ca^{2+}-ATPase samples preincubated at 32°C in the absence of calcium (and absence of A23187) exhibited a significantly higher level of ATPase activity compared with those samples rapidly heated to 32°C in the presence of Ca^{2+}.

One mechanism by which an increase in ATPase activity may be mediated is via an uncoupling of the hydrolytic machinery from the calcium pump. In the coupled state, a high intravesicular calcium concentration is rapidly generated by the pump. After an initial burst of activity, the rate of hydrolysis decreases to a lower level as a result of the inhibitory effect of the accumulated calcium (Weber, 1971). In order to test the hypothesis that in the low activity state, an elevation of intravesicular calcium is the cause of the reduced level of hydrolytic activity, and that the heat-induced activation is caused by an uncoupling of the pump, measurements of ATPase activity were made in the presence of the calcium ionophore, A23187. This ionophore will dissipate the transmembrane gradient generated by the pump and should thus allow expression of the full hydrolytic activity. In the presence of the ionophore, the expected stimulation of ATPase activity was observed (Table I). The large difference in the activity exhibited by samples preincubated at 32°C in a calcium-free medium and those in which the calcium solution was added at 0°C was very substantially reduced (Table I).

The Arrhenius plot derived from measurements made in the presence of A23187 and using the continuous heating method is shown in Fig. 2C. The ionophore caused little stimulation of activity over the temperature range 25–43°C in samples preincubated at 25°C (compare Figs. 2A and C), while in samples preincubated at 3°C and then monitored over the range 5–28°C,

Table I. Effect of 32°C Preincubation in a Calcium-Free Medium on ATPase Activity[a]

Preparation	Temperature of calcium addition (°C)	Ca^{2+}-ATPase activity (units/mg protein)	
		−A23187	+A23187
−DTT	0	0.96 ± 0.03	10.5 ± 0.3
	32	4.6 ± 0.5	11.7 ± 0.3
+DTT	0	2.9 ± 0.2	15.8 ± 0.7
	32	2.8 ± 0.2	16.5 ± 0.5

[a] −DTT, sarcoplasmic reticulum vesicles isolated in the absence of dithothreitol and phenylmethylsulfonylfluoride; +DTT, sarcoplasmic reticulum isolated in the presence of dithiothreitol and phenylmethylsulfonylfluoride. Calcium was added at either 0 or 32°C. Following the addition of calcium at 0°C, the samples were rapidly heated to 32°C before measuring the ATPase activity. The other samples were preincubated at 32°C for 10 min prior to the addition of calcium at 32°C.

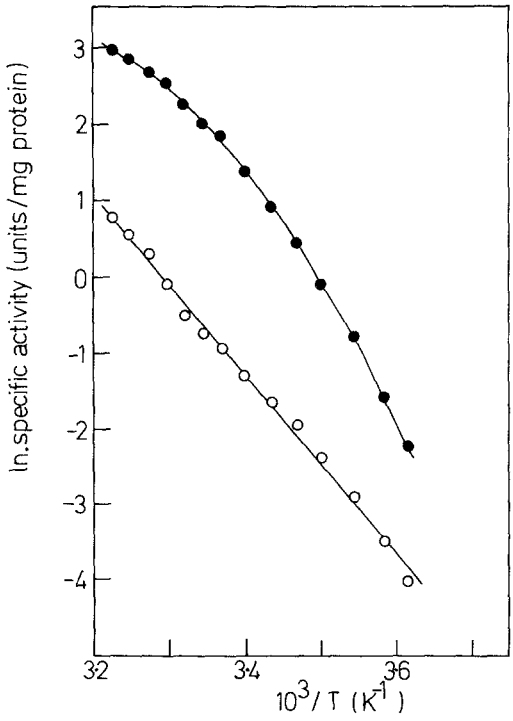

Fig. 3. An Arrhenius plot of the Ca^{2+}-ATPase activity displayed by a sarcoplasmic reticulum preparation isolted in the presence of dithiothreitol and measured in the absence of (○) and in the presence of (●) A23187 using the discrete temperature method.

the hydrolytic activity was considerably enhanced by the ionophore. These results are consistent with the hypothesis that the thermally induced increase in activity observed in the presence of A23187 is caused by an uncoupling of the calcium pump from the hydrolytic machinery and that the process does not occur in the presence of calcium.

Arrhenius plots of Ca^{2+}-ATPase of vesicles prepared with DTT obtained using the discrete temperature method in either the presence or absence of A23187 are shown in Fig. 3. In the absence of ionophore, the plot does not deviate significantly from a straight line, indicating that this material is thermally stable, in agreement with the Ca^{2+}-uptake data (Fig. 1). In the presence of A23187, however, the plot is nonlinear and is similar to that shown in Fig. 2C for the $-$DTT Ca^{2+}-ATPase.

The above results suggest that sarcoplasmic reticulum vesicles isolated in the absence of DTT are thermally unstable and that uncoupling of the pumping and hydrolytic processes is the major cause of its nonlinear Arrhenius behavior. When the full hydrolytic activity is unmasked by the addition of the calcium ionophore A23187, a less dramatic departure from classical Arrhenius behavior is observed. A similar gradual departure from linearity is seen in the Arrhenius plot derived from the A23187-uncoupled, thermally stable sarcoplasmic reticulum preparation obtained in the presence of DTT. The cause of this underlying nonlinearity is the subject of further studies.

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