Journal of Bioenergetics and Biomembranes, Vol. 14, No. 2, 1982

Effect of ATP/ADP/Phosphate Potential on the Maximal Steady-State Uptake of Ca^{2+} by Skeletal Sarcoplasmic Reticulum

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Received June 30, 1981; revised August 19, 1981

Abstract

The ability of the Ca^{2+}-Mg^{2+} ATPase pump of skeletal SR to produce and maintain a Ca^{2+} gradient was studied as a function of the ATP/ADP/P_i ratio. The internal free Ca^{2+} concentration $[Ca^{2}]_i$ was monitored by changes in fluorescence of CTC. Increasing ADP concentrations in the medium reduce the maximal $[Ca^{2+}]_i$ concentration achieved. The inclusion or the omission of 4×10^{-4} M P_i or doubling the absolute ATP and ADP concentrations at a constant ATP/ADP ratio does not affect the level obtained. The level depends primarily on the ATP/ADP ratio. The $[Ca^{2+}]$ concentration shows a 1.5 power dependence on the ATP/ADP ratio. Further, $[Ca^{2+}]_i$ achieved at steady state does not depend on whether the pump had been working in the forward or the reverse direction prior to testing. Analysis shows that the levels of Ca^{2+} achieved are much lower than the levels predicted thermodynamically under the assumption of ideal coupling between Ca^{2+} transport and ATP hydrolysis with a stoichiometry of 2:1. Under this condition the "osmotic" energy of the $[Ca^{2+}]_i/[Ca^{2+}]_o$ ratio was shown to be 48% as large as the free energy of hydrolysis of ATP, giving an overall thermodynamic efficiency of 48%. Analysis shows that maximal steady-state uptake is determined by the balance between the rates of uptake by the pump and rates of leak processes (intrinsic or extrinsic to the pump). Comparison with other studies shows that the $[Ca^{2+}]_i$ achieved results in trans-inhibition of the pump by tying up the Ca^{2+} translocator in the inwardly oriented phosphorylated form. The absence of an effect of P_i can be taken as evidence that the dissociation of Ca^{2+} from the inwardly oriented translocator on the phosphoylated enzyme must precede the dephosphorylation of the enzyme.

Key Words: Ca^{2+} transport; sarcoplasmic reticulum; Ca^{2+}-Mg^{2+}-ATPase; ion transport; phosphate potential; bioenergetics; ion gradient; chlorotetracycline.

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0145-479X/82/0400-0087$03.00/0

Introduction

Calcium uptake by the sarcoplasmic reticulum (SR)[3] (Hasselbach and Makinose, 1963) is driven by a Ca^{2+}-dependent ATPase pump. The pump has been subjected to a large amount of biochemical and biophysical study (MacLennan and Holland, 1975). Early work showed that two Ca^{2+} are taken up per ATP split (Hasselbach and Makinose, 1963) and that the pump is able to reduce the external Ca^{2+} concentration to submicromolar concentrations (Weber *et al.*, 1966). The transport reaction has been shown to involve Ca^{2+} binding to high-affinity sites of the enzyme located on the outer surface, followed by phosphorylation of the enzyme, translocation of the occupied binding site, and expulsion of the bound Ca^{2+} into the vesicle lumen (Kanazawa *et al.*, 1971; Inesi, 1972; Froehlich and Taylor, 1975; Froehlich and Taylor, 1976; Hasselbach, 1978; Tada *et al.*, 1978; de Meis and Vianna, 1979). The Ca^{2+} transport and ATP hydrolysis are shown to be tightly coupled in experiments demonstrating that the rate of ATP splitting is reduced as the maximal level of accumulating calcium is reached (Kanazawa *et al.*, 1971). Tight coupling is also supported by the observation (Makinose and Hasselbach, 1971) that the pump can be reversed to synthesize ATP from ADP and phosphate when Ca^{2+} efflux is induced by EGTA addition to Ca^{2+}-loaded vesicles. The present study is concerned with quantitative testing of the tightness of coupling by the systematic study of the dependence of the Ca^{2+} gradient on the ATP to ADP and phosphate ratio under conditions of active uptake and pump reversal.

The early work of Hasselbach and Makinose (1963) in oxalate-supported Ca^{2+} uptake showed that the inside-to-outside Ca^{2+} ratio is 500 or greater, corresponding to a thermodynamic efficiency of about 45%. We have recently shown (Chiu and Haynes, 1980) that under optimal conditions (approximately 29 μM Ca^{2+} outside, 5 mM Mg-ATP, and 50 mM KCl) that a $[Ca^{2+}]_i/[Ca^{2+}]_o$ ratio of 2.8×10^3 can be achieved. In that publication we analyzed the Ca^{2+} uptake rates and maximal steady-state levels as a function of the K^+ and Mg^{2+} concentrations in the medium and showed that optimal rates and levels can be achieved when these cations are exposed to the luminal surface. The K^+ effect was analyzed and shown to be consistent with the K^+ counter-transport mechanism of the pump put forward by Kanazawa et al. (1971). The Mg^{2+} effect was analyzed and was shown to be explained most simply by its catalysis of the dephosphorylation of the pump in the inwardly oriented form (Kanazawa *et al.*, 1971; Froehlich and Taylor, 1975; Froehlich

[3]Abbreviations: chlorotetracycline, CTC; internal free Ca^{2+} concentration, $[Ca^{2+}]_i$; ATP and Ca^{2+} transport supported fluorescence increase observed in plateau phase, $\Delta F1$; value of $\Delta F1$ obtained for [ADP] = 0, $\Delta F1_{max}$; inorganic phosphate, P_i.

and Taylor, 1976). We presented evidence that both K^+ and possibly Mg^{2+} can compete for occupation of the inwardly oriented carrier, but that Mg^{2+} is not counter-transported to a significant extent (Chiu and Haynes, 1980).

In the present study, we report the dependence of the maximal internal free Ca^{2+} concentration achieved by the pump as a function of the ATP/ADP/P_i ratio. The experiments were carried out under the condition of maximal K^+ activation (100 mM KCl), but less than optimal Mg^{2+} concentration (0.1 mM). Under these conditions, the pump has been shown to be capable of transporting calcium from an external concentration of 29 μM to achieve an internal concentration of 14 mM, producing a Ca^{2+} concentration ratio of 482. The low Mg^{2+} concentration was chosen to reduce the influence of ATP-independent enzyme phosphorylation which has been observed with millimolar concentrations of Mg^{2+} and inorganic phosphate (Masuda and de Meis, 1973; de Meis and Masuda, 1974). Our studies are thus appropriate for characterizing the enzyme in the absence of appreciable amounts of this phosphoenzyme intermediate.

Materials and Methods

Active uptake was studied using the low density ATPase-rich fraction of rabbit skeletal muscle sarcoplasmic reticulum (Meissner, 1975). The procedure for isolation is as described previously (Chiu *et al.*, 1980). Ca^{2+} uptake was monitored using the increase in the fluorescence of chlorotetracycline (Caswell and Hutchison, 1971). The maximal value of fluorescence increase after ATP addition has been shown to be proportional to the free internal Ca^{2+} concentration in the sarcoplasmic reticulum (SR) lumen when the SR concentration in the suspension is kept low (Millman *et al.*, 1980). The latter condition is necessary in order that the concentration of free CTC in the aqueous medium be constant during the course of the active uptake reaction. An SR concentration of 0.05 mg/ml was used in all of the experiments reported here. The reaction medium contained 0.6 M sucrose, 100 mM KCl, 20 mM Hepes buffer, pH 7.0, 1×10^{-4} M Mg^{2+}, 1×10^{-5} M CTC, and 1×10^{-4} M Ca-EGTA. The reaction was initiated by the addition of ATP (sodium salt) to a final concentration of 4×10^{-4} M. The fluorescence was measured in a Hitachi Perkin-Elmer MPF-3L spectrofluorometer set at 398 nm (excitation) and 518 nm (emission).

Using the stability constants tabulated by Fabiato and Fabiato (1978) we calculate that the uptake occurred at a free Ca^{2+} concentration of 29 μM and a Mg-ATP concentration of 7.6×10^{-5} M. The free concentration of Mg^{2+} was 1×10^{-4} M inside and 2.4×10^{-5} M outside. When ADP was added, extra Mg^{2+} was added concomitantly at a ratio of 0.057 Mg/ADP in

order to assure constancy of the free Mg^{2+} and Mg-ATP concentrations. The ATP/ADP ratio was shown to be constant for the duration of the experiments by the following criteria: (1) calculations showed that less than 1% of the added ATP was hydrolyzed and (2) the fluorescence increases per milligram SR protein did not vary as a function of the SR concentration in the SR concentration range used.

Results

Figure 1 shows Ca^{2+} uptake monitored by CTC fluorescence. After the ATP addition the internal Ca^{2+} concentration increases, reaches a plateau, and then declines slowly. Previous work (Millman *et al.*, 1980) has shown that the level of fluorescence at steady state is proportional to the free internal Ca^{2+} concentration present at that time. However, the initial rate of Ca^{2+} uptake is not measured by the rate of fluorescence due to an approximately 30-sec half-time for CTC permeation. The figure shows that the maximal steady state level of $[Ca^{2+}]_i$ is sensitive to the concentration of ADP in the medium. The experiments below report the effect of ATP/ADP ratio on the maximal steady-state uptake.

Figure 2 shows that ADP addition after maximal uptake causes a partial release of Ca^{2+} in a graded manner. Addition of 1×10^{-3} M ADP brings an 81% decrease. A stable level of $[Ca^{2+}]_i$ is achieved. A subsequent addition of 1×10^{-3} M ADP reduced the internal Ca^{2+} concentration to its pre-active uptake level. This may be due to pump inhibition or possibly pump reversal of the type described previously (Makinose and Hasselbach, 1971). A subsequent addition of ADP brings about a further decrease. Comparison of the

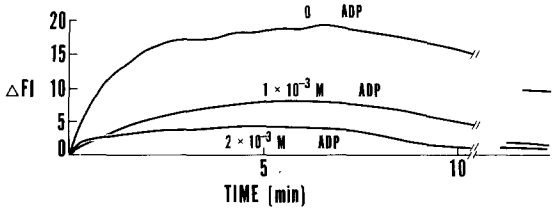

Fig. 1. Time course of CTC fluorescence increase resulting from active Ca^{2+} transport. Uptake initiated by the addition of 4×10^{-4} M ATP ($t = 0$). Sarcoplasmic reticulum was preincubated in a medium containing 0.6 M sucrose, 20 mM Hepes buffer, 100 mM KCl, 1×10^{-4} M Mg^{2+}, 1×10^{-4} M Ca EGTA, and 1×10^{-5} M CTC at a pH = 7.0. ADP (+0.057 extra Mg^{2+} per ADP) was present at the indicated concentrations.

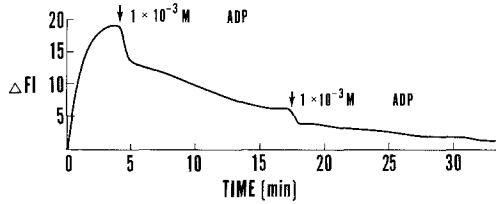

Fig. 2. Reversibility of active Ca^{2+} uptake. Active uptake was initiated ($t = 0$) by the addition of a saturating ATP concentration (4×10^{-4} M) to SR preincubated in the medium of Fig. 1. The uptake occurred in the presence of 4×10^{-4} M phosphate. The reaction was reversed by adding ADP (+0.057 extra Mg^{2+} per ADP) to the indicated concentration.

experiments of Figs. 1 and 2 shows that the level eventually reached does not depend upon whether the ATP and ADP were added simultaneously or serially. This is demonstrated in Fig. 3 in which the levels of fluorescence (ΔFl) are plotted as a function of the ATP/ADP ratio in logarithmic form. The dependence over most of the range is linear. A slope of 1.5 is observed for log ([Mg-ATP]/([ADP] · [P_i])) values below 3.4. For higher values (3.4 to infinity) only a small dependence was observed, corresponding to slopes at approximately zero.

Figure 4 compares the effect of ATP/ADP ratio on the maximal

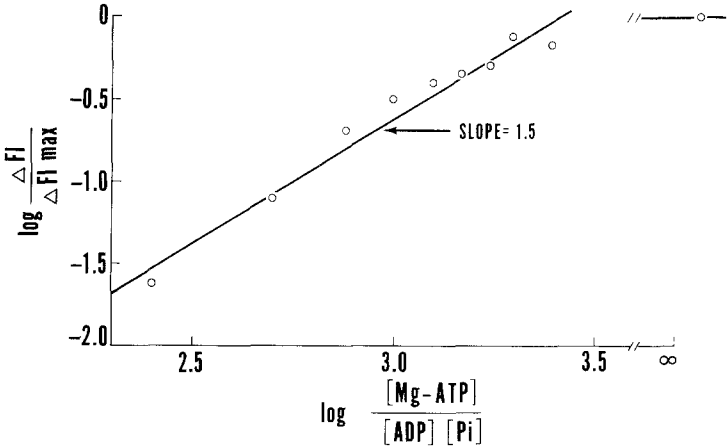

Fig. 3. Log of relative fluorescence increase ($\Delta Fl/\Delta Fl_{max}$) with ATP-driven transport vs. log of ATP/ADP/P_i ratio. ΔFl is maximal fluorescence observed at the experimentally adjusted ADP concentration. ΔFl_{max} is the value of ΔFl observed for [ADP] = 0. Maximal steady-state uptake was determined as a function of ADP concentration at 1×10^{-4} M phosphate. Experiments were carried out as in Fig. 1.

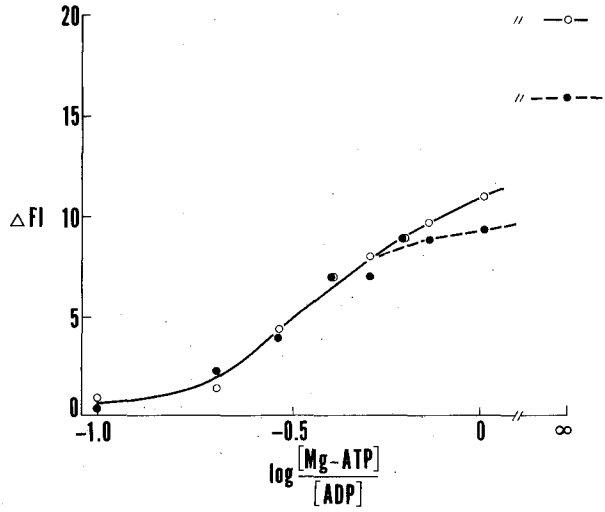

Fig. 4. Absence of an effect of P_i on maximal steady-state Ca^{2+} level. The experiments were carried out as in Fig. 1 (4×10^{-4} M ATP; variable ADP). The maximal steady-state fluorescence increase is plotted against the log of the ATP/ADP ratio. The experiments were carried out in the presence (●) and in the absence (○) of 4×10^{-4} M P_i.

steady-state uptake in the presence and absence of 10^{-4} M inorganic phosphate. Both the absolute values and the dependencies are not appreciably affected by the phosphate addition. This indicates that the contribution of inorganic phosphate to the ATP/ADP phosphate potential is not felt by the transport system and that inorganic phosphate does not give "product inhibition" at this level. The uptake experiments were repeated (data not shown) using twice the concentrations of ATP, and half-maximal uptake was observed at the same ATP/ADP ratio. This indicates that it is the ATP/ADP ratio, and not the absolute concentrations, which determines the maximal free internal Ca^{2+} concentration achieved by the pump.

Discussion

The present study has tested the ability of the Ca^{2+}-Mg^{2+}-ATPase pump to produce and maintain a gradient at saturating concentrations of external Ca^{2+} and Mg^{2+}-ATP. The concentrations of these two species are calculated to be 2.5×10^{-5} and 7.6×10^{-5}, respectively. These concentrations are at least one order of magnitude greater than the K_m values for transport or enzyme phosphorylation (Kanazawa *et al.*, 1971; Inesi, 1972; Froehlich and

Taylor, 1975; Froehlich and Taylor, 1976; Hasselbach, 1978; Tada *et al.*, 1978; de Meis and Vianna, 1979; Meissner, 1973). The Mg^{2+} concentration chosen was less than optimal. The total concentration of 10^{-4} was chosen to give an adequate concentration of Mg^{2+}-ATP but to be inadequate for the formation of Mg^{2+}-dependent, ATP-independent phosphoenzyme (de Meis and Masuda, 1974). The Mg^{2+} concentration chosen provides adequate ATP in the Mg^{2+} form with the bulk of the ADP in the free form. Since Mg-ATP is the substrate for the forward reaction and ADP is the substrate for the back reaction of the pump (Makinose and Boll, 1979; Yamada and Ikemoto, 1980), our conditions are optimal for the attainment of equilibrium between the Ca^{2+} gradient and ATP hydrolysis. We have shown that under the present condition, in the absence of ADP and P_i, the free internal Ca^{2+} concentration in the SR lumen is 14 ± 1 mM (Chiu and Haynes, 1980).

In agreement with Hasselbach and Makinose (1963) the thermodynamic efficiency of the pump is less than 100%. If the pump were ideally coupled the overall reaction would be

$$2Ca_o^{2+} + ATP\,(+4Cl_0^-) \rightarrow 2Ca_i^{2+} + ADP + P_i\,(+4Cl_i^-) \qquad (1)$$

In the absence of precipitating anions and in the presence of high KCl concentrations, the net reaction[4] will be coaccumulation of Ca^{2+} and Cl^-. When the Cl^- concentration is high we will have $[Cl^-]_i = 1.28[Cl^-]_o$ after transport of Ca^{2+} to internal concentration of 14 mM. The equilibrium constant of reaction (1) should then be close to that of ATP hydrolysis. The effects of membrane potential would also be minimal (Chiu and Haynes, 1980). Under the conditions of our experiments the free energy of hydrolysis of ATP is −9.4 kcal (Alberty, 1968; Shikama and Nakamura, 1973). The equilibrium constant for reaction (1) will therefore be given as $10^{(-\Delta G/2.303RT)} = 10^{6.71} = 5 \times 10^6$. The inside-to-outside Ca^{2+} ratio should therefore be given as

$$\frac{[Ca^{2+}]_i}{[Ca^{2+}]_o} = \left(5 \times 10^6 \frac{[ATP]}{[ADP][P_i]}\right)^{1/2} \qquad (2)$$

The equation makes predictions which are in disagreement with our experimental results. It predicts an infinite Ca^{2+} ratio at ADP or P_i equals zero (not observed), and it preducts that the Ca^{2+} ratio will vary as the one-half power of the ATP/ADP/P_i ratio (not observed). At low values of ATP/ADP/P_i ratio a 1.5 power dependence was actually observed. The lack of correspondence between simple expectations and observations indicates that the transport reaction in the sarcoplasmic reticulum is not ideally coupled.

The thermodynamic efficiency of the system can be calculated from the

[4]We have given evidence (Chiu and Haynes, 1980) that the actual mechanism is pump-mediated $Ca^{2+}/2K^+$ exchange followed by influx of K^+ and Cl^- via passive permeability mechanisms.

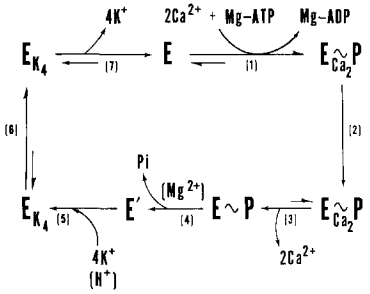

Fig. 5. Possible mechanism for Ca^{2+}-Mg^{2+}-ATPase action.

data of Fig. 3. Half maximal uptake (7 mM Ca^{2+} inside) is observed for log (ATP/ADP/P_i) equals 3.22. The free energy of ATP hydrolysis under this condition is given as $\Delta G = -9.4 - (1.40 \times 3.22) = -13.9$ kcal. The free energy of the Ca^{2+} gradient is given as $2 \times 1.40 \times \log (7.0 \text{ mM}/0.029 \text{ mM}) = 6.67$ kcal. The free energy of the calculated Cl^- gradient would be $4 \times 1.40 \times \log (107 \text{ mM}/100 \text{ mM}) = 0.16$ kcal. An efficiency value of 49% is calculated. This is close to the original estimate of Hasselbach and Makinose (1963).

The lack of ideal coupling and the low thermodynamic efficiency of the pump can be explained in two ways: Either (a) the pump is not ideally coupled due to shunt pathways in its cycle or (b) the pump functions ideally but works against a passive leak. Maximal steady-state uptake levels will be achieved when the rates of pumping and shunt or leak processes become equal. This will occur when the pump becomes inhibited by the buildup of high levels of internal Ca^{2+}. The process can be understood in terms of the simplified model of pump function given in Fig. 5. This model is based on mechanisms proposed in a number of studies (Kanazawa *et al.*, 1971; Inesi, 1972; Froehlich and Taylor, 1975; Froehlich and Taylor, 1976; Tada *et al.*, 1978; de Meis and Vianna, 1979; Makinose and Hasselbach, 1971; Chiu and Haynes, 1980). The initial velocity of the pump is determined by the rates of reactions 1–3 which are governed by the levels of enzyme phosphorylation. The inhibition of the enzyme at maximal steady-state uptake can be explained (but not uniquely) as the entrapment of the enzyme in the inwardly oriented $E_{Ca_2} \sim P$ state. The formation of this state and the release of Ca^{2+} into the lumen (reaction 3) precede the dephosphorylation of the enzyme. Thus the rate of Ca^{2+} pumping in the inhibited condition, in competition with the leak, would not be affected by 4×10^{-4} M phosphate in the medium. However, inhibition by phosphate might be expected at phosphate and Mg^{2+} concentrations sufficiently high to produce an ATP-independent phosphoenzyme. According to this reasoning, the ability of the pump to create and maintain a gradient against a shunt or passive leak would be proportional to

the degree of phosphorylation of the enzyme which in turn would be directly dependent on the ATP/ADP ratio. The equilibrium constants for this reaction have been evaluated by Meissner (1973) at 0°C and pH 7.5. He obtained an equilibrium constant of 1.5 for the process

$$E_{Ca_2} + ATP \rightleftharpoons E_{Ca_2} \sim P + ADP$$

The effect of ATP and ADP level on the initial rate of transport as measured by the ATPase reaction has been reported recently by Sakamoto and Tonomura (1980). They showed that ADP brings about a mixed inhibition of the initial rate at low Mg^{2+} concentrations (10 μM) and high Ca^{2+} concentrations (5 mM). They measured a K_i of 35 μM and showed that the value of the V_{max} was halved for 72 μM ADP. Their values predict that the initial rate observed with 10^{-4} M ATP and 10^{-3} M ADP (conditions giving half-maximal steady-state Ca^{2+} levels) would have given approximately 70% inhibition of our initial rates.

In conclusion, our study indicates that the less than ideal thermodynamic efficiency of the Ca^{2+}-Mg^{2+}-ATPase pump observed under our condition is the result of product (Ca^{2+} in) inhibition of the pump together with leakage through mechanisms intrinsic or extrinsic to the pump. The presence of these shunt or leak pathways may represent a flaw in the pump and membrane permeability system or may serve as a mechanism for limiting the maximal level of Ca^{2+} accumulated.

Acknowledgments

This work is supported by NIH grant GM 23990 and by a grant from the Florida Heart Association. Deborah Dixon and Adrian Corbett were supported by NIH Training Grant HL 07188.

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