Biochimica et Biophysica Acta, 773 (1984) 99–105
Elsevier

BBA 72113

THE RATE OF Ca^{2+} TRANSLOCATION BY SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase MEASURED WITH INTRAVESICULAR ARSENAZO III

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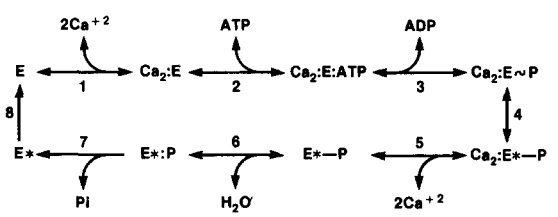
(Received December 21st, 1983)

Key words: (Ca^{2+} + Mg^{2+})-ATPase; Ca^{2+} transport; Arsenazo III; Membrane vesicle; Sarcoplasmic reticulum; (Rabbit skeletal muscle)

Release of Ca^{2+} from the (Ca^{2+} + Mg^{2+})-ATPase into the interior of intact sarcoplasmic reticulum vesicles was measured using arsenazo III, a metallochromic indicator of Ca^{2+}. Arsenazo III was placed inside the sarcoplasmic reticulum vesicles by making the vesicles transiently leaky with an osmotic gradient in the presence of arsenazo III. External arsenazo III was then removed by centrifugation. Addition of ATP to the (Ca^{2+} + Mg^{2+})-ATPase in the presence of Ca^{2+} causes the rapid phosphorylation of the enzyme at which time the bound Ca^{2+} becomes inaccessible to external EGTA. The release of Ca^{2+} from the (Ca^{2+} + Mg^{2+})-ATPase to the interior of the vesicle measured with intravesicular arsenazo III was much slower indicating that there is an occluded form of the Ca^{2+}-binding site which precedes the release of Ca^{2+} into the vesicle. The rate of Ca^{2+} accumulation by sarcoplasmic reticulum vesicles is increased by K^+ (5–100 mM) and ATP (50–1000 μM) but the initial rate of Ca^{2+} translocation measured after the simultaneous addition of ATP and EGTA to vesicles that were preincubated in Ca^{2+} was not influenced by these concentrations of K^+ and ATP.

Introduction

Ca^{2+} transport by sarcoplasmic reticulum vesicles is mediated by the (Ca^{2+} + Mg^{2+})-ATPase. The generally accepted mechanism for (Ca^{2+} + Mg^{2+})-ATPase is shown in Scheme I [1–4].



Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Following the binding of external Ca^{2+} to the high affinity Ca^{2+}-binding sites on the (Ca^{2+} + Mg^{2+})-ATPase (Step 1), the enzyme is rapidly phosphorylated by ATP (Step 3). The phosphorylation presumably causes a conformational change of the ATPase which alters both the affinity and orientation of the Ca^{2+} binding sites (Step 4). Ca^{2+} is then released into the lumen of the sarcoplasmic reticulum (Step 5) and the phosphoenzyme bond is hydrolyzed (step 6).

Upon phosphorylation, the Ca^{2+} bound to the (Ca^{2+} + Mg^{2+})-ATPase rapidly becomes inaccessible to external EGTA [5–8]. Addition of ADP along with EGTA causes the rapid release of Ca^{2+} due to the reversal of Step 3 [8–10]. Dupont demonstrated that the release of Ca^{2+} by EGTA + ADP was much faster than the relase of Ca^{2+}

from the phosphoenzyme by EGTA + X537A (a Ca^{2+} ionophore) [8]. The former condition causes the reversal of Step 3 while the latter condition depends on the rate at which Ca^{2+} is released from the (Ca^{2+} + Mg^{2+})-ATPase into the interior of the sarcoplasmic reticulum vesicle and translocated out of the vesicle by X537A. It is also possible that X537A directly removes Ca^{2+} bound to the protein at a site not exposed to the aqueous phase [11,12].

We have developed a more direct method to measure the rate of Ca^{2+} release from the (Ca^{2+} + Mg^{2+})-ATPase into intact vesicles using the Ca^{2+} indicator arsenazo III inside the vesicle. Our results indicate that the release of Ca^{2+} into the interior of the vesicle is relatively slow while the removal of bound Ca^{2+} from the external medium is quite rapid. Therefore the Ca^{2+} must reside in an occluded form during much of the transport cycle.

Methods and Materials

$^{45}CaCl_2$ and [γ-^{32}P]ATP were purchased from New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

Methods

Preparation of arsenazo III-loaded sarcoplasmic reticulum vesicles. The microsomal fraction from rabbit skeletal muscle was prepared as previously described [13]. The microsome fraction contains mostly fragmented sarcoplasmic reticulum vesicles. This fraction was then placed on a 30–45% sucrose gradient and centrifuged for 15 h at 4°C at 100 000 × *g*. The low-density sarcoplasmic reticulum vesicle fraction (32–34%) was removed from the gradient and diluted 1 : 7 into 20 mM arsenazo III/25 mM $MgSO_4$ (pH 6.8). The osmotic imbalance across the membrane of the vesicles causes them to swell [14] allowing the influx of arsenazo III. The free arsenazo III was then removed by collecting the sarcoplasmic reticulum by centrifugation (100 000 × *g* for 30 min.) and resuspending them in arsenazo III-free medium (0.15 M potassium glutamate/10 mM histidine/5 mM $MgSO_4$/0.5 mM EGTA/0.45 mM $CaCl_2$). The centrifugation was repeated four times. The final pellet was resuspended to a protein concentration of 16 mg/ml, frozen in liquid nitrogen, and stored at −70°C.

Phosphorylation of the (Ca^{2+} + Mg^{2+})-ATPase by ATP. Ca^{2+} transport was initiated in medium containing 0.1 mM [γ-^{32}P]ATP (1 μCi/ml). At various times the reaction was stopped by the addition of 5% trichloroacetic acid, 0.1 M KH_2PO_4, 5% polyphosphate. The samples were centrifuged at 1000 × *g* for 20 min. The supernatant was removed and the amount of $^{32}PO_4$ determined by the method described by De Meis and Carvalho [15]. The pellets were washed three times by centrifugation using the trichloroacetic acid solution. The final pellet was resuspended in Lowry C reagent [16] and the amount of ^{32}P in the pellet was determined by measuring the Cerenkov radiation in a scintillation counter. The protein content in each sample was then assayed by the method of Lowry et al. [16].

Results

Our procedure for preparing arsenazo III-loaded sarcoplasmic reticulum vesicles did not differ from the standard way of isolating sarcoplasmic reticulum vesicles except that the vesicles obtained from the sucrose gradient following centrifugation were diluted into an arsenazo III-containing medium. The transient swelling of the sucrose-equilibrated vesicles following dilution in a hypotonic solution made the membrane leaky [14] allowing arsenazo III to enter the vesicles. Following equilibration of the vesicles, the extravesicular arsenazo III was then removed by centrifugation. Neither the

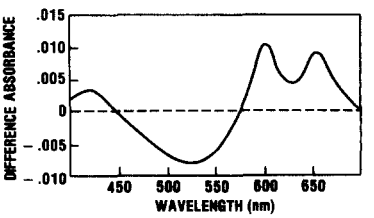


Fig. 1. Difference spectrum of arsenazo III-loaded sarcoplasmic reticulum vesicles following Ca^{2+} uptake. The sample and reference cuvettes contained 0.15 M potassium glutamate, 10 mM histidine, 5 mM $MgSO_4$, 50 μM $CaCl_2$ and arsenazo III-loaded sarcoplasmic reticulum (0.4 mg/ml protein). The spectrum was taken 1 min after the addition of 0.1 mM ATP to the sample cuvette. Temperature = 1°C.

Ca^{2+}-loading capacity (120–150 nmol Ca^{2+}/mg) nor the Ca^{2+}-dependent ATPase activity (0.5–0.8 μmol/mg min) at 25 °C was significantly altered by the arsenazo III loading procedure.

Fig. 1 shows the difference spectrum of arsenazo III-loaded vesicles following the addition of ATP in the presence of Ca^{2+}. Under these conditions the vesicles accumulate Ca^{2+}. The spectrum is identical to the difference spectrum obtained when 2.5 mM Ca^{2+} is added to the sarcoplasmic reticulum vesicles in the presence of the Ca^{2+} ionophore, A23187. In the absence of free Ca^{2+}, ATP did not alter the arsenazo III spectrum. Following Ca^{2+} uptake by the sarcoplasmic reticulum vesicles, removal of the free extravesicular Ca^{2+} by EGTA did not have an immediate effect on the arsenazo III spectrum. These experiments demonstrate that arsenazo III was responding only to intravesicular Ca^{2+}.

The use of intravesicular arsenazo III the measure the rate of Ca^{2+} influx is demonstrated in Fig. 2. Ca^{2+} influx at 1 °C was initiated by the additionof 2.5 mM Ca^{2+} or the addition of ATP to activate Ca^{2+} transport. The rate of passive Ca^{2+} influx was dependent on the external Ca^{2+} concentration and was increased by the addition of A23187. At 2.5 mM Ca^{2+}, the absorbance change of the intravesicular arsenazo III was not linear. About 17% of the Ca^{2+}-induced absorbance change occurred relatively fast (first order rate constant = 0.23 s^{-1}). This initial phase was

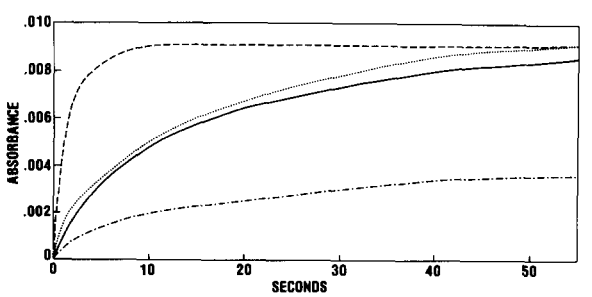


Fig. 2. Absorbance change of arsenazo III-loaded sarcoplasmic reticulum during Ca^{2+} influx. The sample contained 0.1 M potassium glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, 50 μM $CaCl_2$ and arsenazo III-loaded vesicles (0.4 mg/ml protein). Ca^{2+} influx was initiated by the addition of 2.5 mM $CaCl_2$ (– · –), 2.5 mM $CaCl_2$ and 2.5 μM A23187 (· · · · · ·), 2.5 mM $CaCl_2$ and 10 μM A23187 (– – –), or 0.1 mM ATP (———). The absorbance change of arsenazo III was monitored at 660 nm using 685 nm as a reference wavelength.

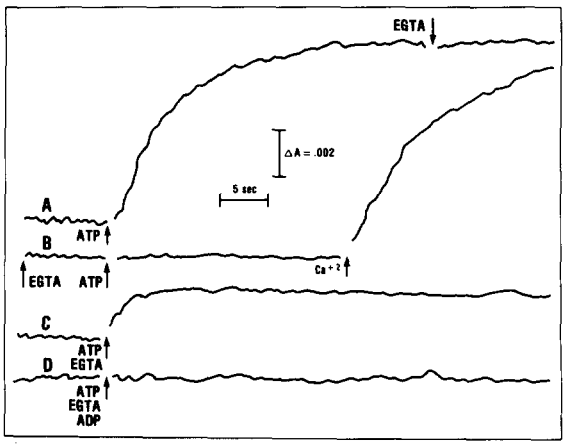


Fig. 3. Effect of EGTA on the absorbance change of arsenazo III-loaded sarcoplasmic reticulum vesicles during Ca^{2+} uptake. The samples contain 0.1 M potassium glutamate, 10 mM histidine, 5 mM $MgSO_4$, 50 μM $CaCl_2$ and arsenazo III-loaded sarcoplasmic reticulum vesicles (0.4 mg/ml protein) at 1 °C. At the indicated time, ATP (0.1 mM), EGTA (1 mM), $CaCl_2$ (0.9 mM) or ADP (1 mM) was added. The absorbance change of arsenazo III at 660 nm was monitored using 685 nm as a reference wavelength.

followed by a much slower one in which the absorbance change was less than $3 \cdot 10^{-5} \Delta A$/min. The addition of 10 μM A23187 increased the rate of Ca^{2+} influx 300-fold. The rate of Ca^{2+} influx in the presence of 2.5 mM $CaCl_2$ and 2.5 μM A23187 was similar to the rate measured during active Ca^{2+} transport at an external Ca^{2+} concentration of 50 μM.

As stated before, the removal of external Ca^{2+} by EGTA after Ca^{2+} loading does not cause an immediate change in the arsenazo III absorbance (Fig. 3, trace A). But the addition of EGTA to the vesicles before ATP prevents Ca^{2+} uptake and the corresponding absorbance change of arsenazo III (Fig. 3, trace B).

When ATP and EGTA are added at the same time, only the Ca^{2+} initially bound to the (Ca^{2+} + Mg^{2+})-ATPase can be transported (Fig. 3, trace C) [5,7,22–24]. This is demonstrated by the experiment shown in Fig. 4. Ca^{2+} uptake was measured using $^{45}Ca^{2+}$ as a tracer. When the arsenazo III-loaded vesicles were equilibrated with the $^{45}Ca^{2+}$ containing solution, addition of ATP caused a rapid accumulation of about 4 nmol Ca^{2+}/mg protein which was followed by a much slower rate of Ca^{2+} uptake (0.4 nmol/s per mg). The steady-

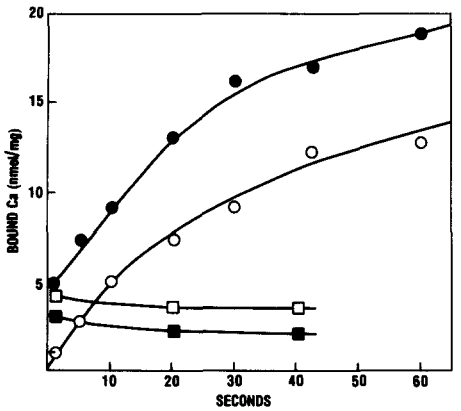


Fig. 4. Ca^{2+} uptake by sarcoplasmic reticulum. The medium contained 0.1 M potassium glutamate, 10 mM histidine (pH 6.8), 5.0 mM $MgSO_4$ 50 μM^{45} $CaCl_2$ (1 μCi/ml), and either arsenazo III-loaded sarcoplasmic reticulum vesicles (0.4 mg/ml protein) (●,□,■) or 0.1 mM ATP (○) at 1°C. Ca^{2+} uptake was initiated by the addition of either 0.1 mM ATP (●,□), 0.1 mM ATP + 1 mM EGTA (■), or sarcoplasmic reticulum vesicles (0.4 mg/ml protein) (○) preincubated in potassium glutamate medium without ^{45}Ca. To one of the samples (□), 1 mM EGTA was added 1 s after the ATP addition. At various times, the samples were diluted 1 : 10 with 0.1 M potassium glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$ and 1 mM EGTA and passed through Millipore HA filters. The filters were immediately washed with 2 ml of EGTA wash solution. After drying the filters, the amount of $^{45}Ca^{2+}$ bound to the filters was measured in a scintillation counter using a nonaqueous counting solution.

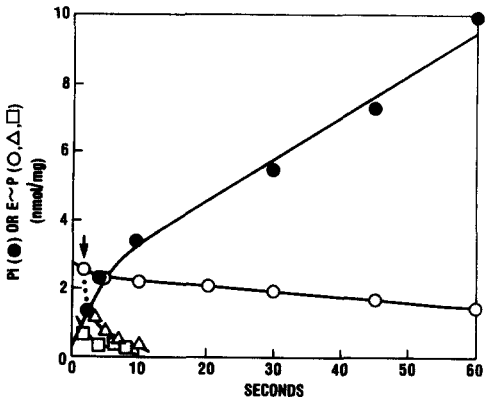


Fig. 5. Formation of the phosphorylated enzyme intermediate and the release of inorganic phosphate by the $(Ca^{2+} + Mg^{2+})$-ATPase during Ca^{2+} transport. The medium contained 0.1 M potassium glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, 0.1 mM [γ-^{32}P]ATP (1 μCi/ml), and either 50 μM $CaCl_2$ (△,●,○) or 1.0 mM EGTA (□). Ca^{2+} transport was initiated by the addition of sarcoplasmic reticulum vesicles (0.4 mg/ml) which were equilibrated in potassium glutamate solution containing 50 μM $CaCl_2$. To one sample, 1 mM EGTA was added to the medium 2 s after the reaction was initiated (△). All values represent the Ca^{2+}-dependent E ~ P formation or Ca^{2+}-dependent release since the background obtained from experiments in which the vesicles and media contained 1 mM EGTA was subtacted from the data obtained when the medium and/or the vesicles contained Ca^{2+}.

state level of the phosphorylated intermediate of these vesicles was about 2.5 nmol E-P/mg protein (Fig. 5). Even when EGTA was added together with ATP there was a rapid uptake of about 3 nmol Ca^{2+}/mg but no subsequent accumulation since the free Ca^{2+} was chelated by EGTA. When Ca^{2+} transport was initiated by the simultaneous addition of ATP and $^{45}Ca^{2+}$ to vesicles equilibrated in the absence of ^{45}Ca, there was no burst of $^{45}Ca^{2+}$ uptake since only the unlabeled bound Ca^{2+} was carried by the first turnover. The appearance of the bound Ca^{2+} in the interior of sarcoplasmic reticulum vesicle following ATP addition as detected by intravesicular arsenazo III was much slower (first order rate = 0.45 s^{-1}) (Fig. 3, trace C) than the rate at which it becomes inaccessible to external EGTA. The rate of Ca^{2+} release into the interior of the vesicle was comparable to the rate at which the phosphoenzyme intermediate decomposes (Fig. 5).

When 1 mM ADP was added along with ATP and EGTA, neither Ca^{2+} translocation (Fig. 3, trace D) nor $^{45}Ca^{2+}$ uptake (data not shown) [9,10] was observed. Since ADP accelerates the reversal of Step 3 of Scheme I it is likely that in the presence of ADP the rate of Ca^{2+} release by the reversal of steps 1–4 or steps 1–3 is much faster than Ca^{2+} translocation (steps 3–5).

The $(Ca^{2+} + Mg^{2+})$-ATPase is activated by K^+ [25–31] and high concentrations of ATP [32–35]. The initial rate of absorbance change of arsenazo III-loaded vesicles during Ca^{2+} uptake at 1°C increased from $5.1 \cdot 10^{-4}$ $\Delta A/s$ in the presence of 2 mM K^+ to $11 \cdot 10^{-4}$ $\Delta A/s$ in the presence of 100 mM K^+ (Fig. 6). Increasing the ATP concentration from 50 μM to 1.0 mM increased the initial rate of absorbance change from $9.5 \cdot 10^{-4}$ $\Delta A/s$ to $21 \cdot 10^{-4}$ $\Delta A/s$ (Fig. 7A). Since the K_m of the Ca^{2+}-ATPase for ATP is about 3 μM [9],

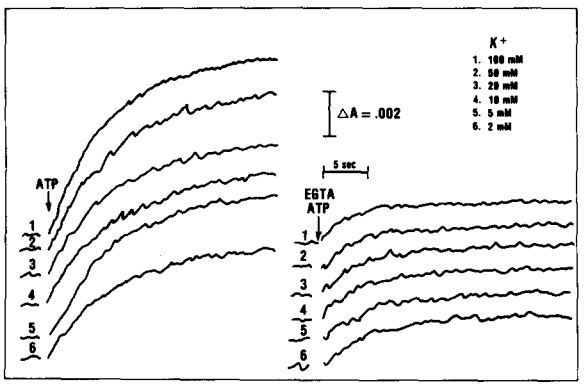


Fig. 6. Effect of K^+ on the rate of Ca^{2+} translocation by sarcoplasmic reticulum. The medium contained 10 mM histidine (pH 6.8), 5.0 mM $MgSO_4$, 50 μM $CaCl_2$, arsenazo III-loaded sarcoplasmic reticulum vesicles (0.4 mg/ml) and varying ratios of 0.2 M glycine and 0.1 M potassium glutamate. Ca^{2+} uptake was initiated by the addition of 0.1 mM ATP (left) or 0.1 mM ATP+1 mM EGTA (right). The absorbance change at 660 nm was monitored in an Aminco DW-2 spectrophotometer in the dual wavelength mode using 685 nm as the reference wavelength.

most of this increase in the uptake rate is attributed to an activation of an intermediate step in the reaction cycle. When Ca^{2+} transport was initiated by the simultaneous addition of ATP and EGTA there was no effect of high concentrations of ATP (0.1–1.0 mM) (Fig. 7B) or K^+ (Fig. 6) on the appearance of Ca^{2+} inside the vesicles. This data suggests that K^+ and ATP activate an intermediate reaction which follows the release of Ca^{2+} into the vesicle.

It was previously demonstrated that Ca^{2+} transport is activated by inside negative membrane potentials generated by K^+ gradients in the presence of the K^+ ionophore valinomycin [36–38]. In order to measure the effect of K^+ gradients on the rate of Ca^{2+} transport at 1°C, arsenazo III-loaded vesicles equilibrated in potassium glutamate were diluted 50-fold into either glycine or potassium glutamate medium containing valinomycin. When Ca^{2+} transport was initiated at the time of the dilution, the rate of Ca^{2+} uptake in the glycine and potassium glutamate solutions monitored by both the absorbance change of the intravesicular

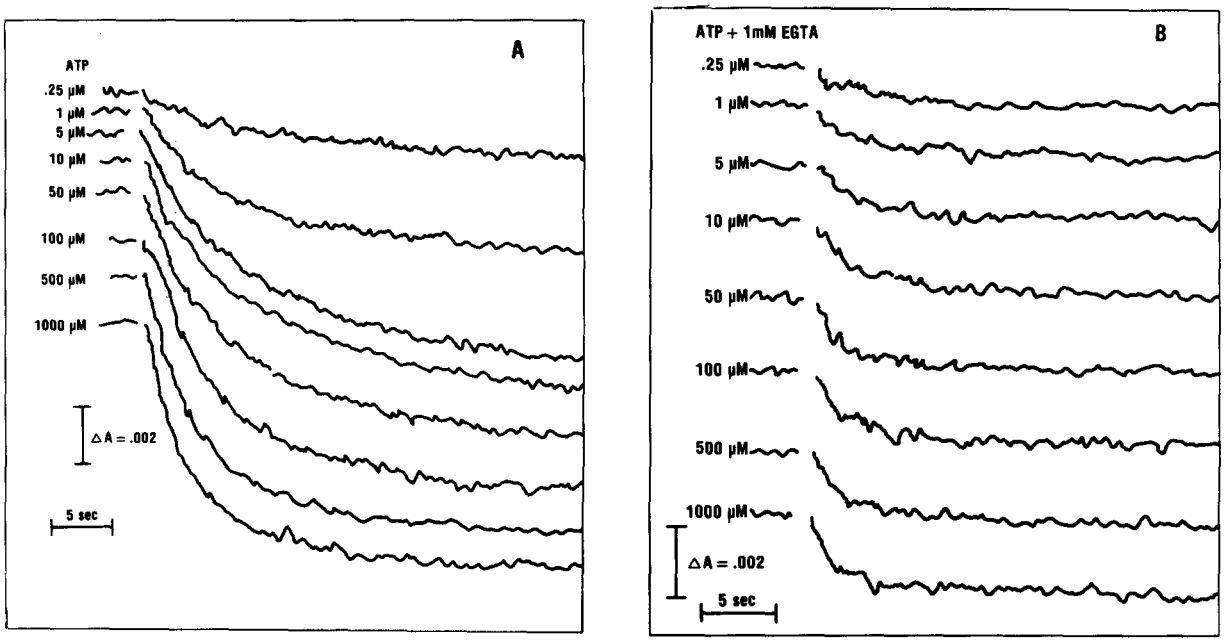


Fig. 7. Effect of ATP on the initial rate of Ca^{2+} translocation into sarcoplasmic reticulum vesicles. The medium contained 0.1 M potassium glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, 50 μM $CaCl_2$, and arsenazo III-loaded sarcoplasmic vesicles (0.4 mg/ml). Ca^{2+} transport was initiated by the addition of varying amounts of ATP with (Panel B) or without (Panel A) 1 mM EGTA. The absorbance change of arsenazo III at 660 nm was monitored in an Aminco DW-2 spectrophotometer in the dual wavelength mode using 685 nm as a reference wavelength. An increase in the asorbance at 660 nm is indicated by downward trace.

arsenazo III (as in Fig. 6) or by the accumulation of $^{45}Ca^{2+}$ (as in Fig. 5) was essentially the same. Allowing the vesicles to equilibrate in the dilution medium before the initiation of Ca^{2+} transport gives the same results as shown in Fig. 6. Under these conditions (1°C) the activation of Ca^{2+} transport by K^+ is the same whether the K^+ is on both sides of the membrane or just on the internal side. At 15°C, the rate of Ca^{2+} accumulation of K^+-equilibrated vesicles was much greater in glycine (+valinomycin) than in potassium glutamate (+valinomycin) medium [37].

The rate of Ca^{2+} translocation measured by the absorbance change of arsenazo III-loaded sarcoplasmic reticulum vesicles equilibrated in potassium glutamate medium following a 50-fold dilution into glycine medium containing ATP, EGTA and valinomycin did not significantly differ from that observed after diluting into potassium glutamate medium containing ATP and EGTA. These experiments along with the one in Fig. 6 indicate that the rate of the initial Ca^{2+} translocation by vesicle equilibrated in K^+ medium is the same in glycine medium as that in potassium glutamate even when a K^+ gradient (and therefore a membrane potential [37–39]) is present.

Discussion

A technique was developed to prepare sarcoplasmic reticulum vesicles loaded with arsenazo III. Other optical probes such as chlortetracycline [40,41] and 8-anilino-1-naphthalenesulfonate [12,42–44] can measure internal Ca^{2+} but they lack specificity and have slow response times. The response time of arsenazo III is quite rapid (Ca^{2+} on an off time = 2–5 ms) [45,46] and the change in the absorbance spectrum of arsenazo III due to Ca^{2+} is very different than that caused by changes in the Mg^{2+} or H^+ concentration. Because the affinity of arsenazo III for Ca^{2+} is so high and the amount of dye that is trapped by the vesicles ($\cong$ 3 nmol/mg protein) was well below the capacity of the vesicles to accumulate Ca^{2+}, the intravesicular arsenazo III rapidly became saturated with Ca^{2+} after initiation of Ca^{2+} transport. This makes quantitation of the amount of Ca^{2+} translocated difficult. A Ca^{2+} indication of much lower affinity would be more useful in measuring the intravesicular Ca^{2+} concentration during Ca^{2+} accumulation. Using the same loading technique we were unable to prepare vesicles with the Ca^{2+} indicators, murexide or antipyrylazo III, due to problems with solubility of the probes and damage to the vesicles.

The appearance of Ca^{2+} within the vesicle upon the initiation of Ca^{2+} transport was much slower than its removal from the external medium. This provides direct evidence for an occluded state during Ca^{2+} uptake [8,24,47]. Ikemoto [48] measured the rate of Ca^{2+} release from isolated (Ca^{2+} + Mg^{2+})-ATPase following phosphorylation with 5 μM ATP at 22°C. This preparation did not accumulate Ca^{2+} due to the leakiness of the membrane following Triton X-100 treatment. Ca^{2+} release followed E-P formation with a lag time of 15 ms. At this low ATP concentration, the maximum phosphorylation level (0.1 mol E-P/mol ATPase) was reached within 150 ms after ATP addition while the maximum level of Ca^{2+} release (0.24 mol Ca^{2+}/mol ATPase) was reached after 250 ms. The work presented here confirms that with intact vesicles, there is a lag time between phosphorylation and Ca^{2+} release from the (Ca^{2+} + Mg^{2+})-ATPase.

Although the rate of Ca^{2+} transport is increased by K^+ (10–100 mM) and by ATP (50–1000 μM), the initial rate of Ca^{2+} translocation was not influenced by K^+ or high ATP concentrations. The most likely explanation is that K^+ and ATP activates an intermediate reaction which follows the release of Ca^{2+} from the (Ca^{2+} + Mg^{2+})-ATPase into the interior of the vesicle.

Acknowledgements

This work was supported by research grants from the National Institutes of Health (GM 29300), from the Office of Naval Research and by the Uniformed Services University of the Health Sciences.

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