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Osmotic Changes of Sarcoplasmic Reticulum Vesicles during Ca^{2+} Uptake

Troy Beeler
Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Md. 20814

Summary. The ATP-dependent accumulation of Ca^{2+} by sarcoplasmic reticulum vesicles at 37° C reaches a peak after approximately 100 sec. The Ca^{2+}-loading level then declines until a steady-state level is reached which is 20% less than the peak value. This spontaneous release of Ca^{2+} is enhanced by inclusion of maleate in the Ca^{2+} uptake medium. Increasing the extravesicular osmolarity by the addition of sucrose to the Ca^{2+} uptake medium prevents spontaneous Ca^{2+} release and increases the steady-state Ca^{2+}-loading capacity of sarcoplasmic reticulum vesicles. Swelling of sarcoplasmic reticulum vesicles during Ca^{2+} uptake in medium containing sucrose is indicated by changes in the light-scattering intensity. These experiments indicate that the capacity of sarcoplasmic reticulum vesicles to accumulate Ca^{2+} is limited by the osmotic gradient generated by the increase in intravesicular Ca^{2+}. Swelling of sarcoplasmic reticulum vesicles during Ca^{2+} uptake causes spontaneous Ca^{2+} release.

Key Words sarcoplasmic reticulum · Ca^{2+}-ATPase · Ca^{2+} transport · Ca^{2+} release · osmotic swelling

Introduction

Sarcoplasmic reticulum vesicles from skeletal muscle contain a Ca^{2+}-dependent ATPase which mediates the active transport of Ca^{2+} into the vesicles. The concentration of the Ca^{2+}-ATPase in the membrane is so high that only one turnover of the Ca^{2+} pumps would generate millimolar amounts of Ca^{2+} inside the sarcoplasmic reticulum vesicles [1]. Because this amount of internal Ca^{2+} is sufficient to inhibit the Ca^{2+}-ATPase, a linear rate of Ca^{2+} uptake is not expected [11, 23–25]. Since passive efflux of Ca^{2+} from sarcoplasmic reticulum vesicles was found to be linearly related to the Ca^{2+} gradient across the membrane [6], one might expect the rate of Ca^{2+} accumulation by sarcoplasmic reticulum vesicles to steadily decline until the rate of active transport matches the passive rate of Ca^{2+} efflux. At this point, a steady-state Ca^{2+}-loading level would be reached. But under various conditions, Ca^{2+} uptake by sarcoplasmic reticulum vesicles shows a transient behavior in which vesicles accumulate Ca^{2+} for 0.5 to 2 min, followed by a release phase in which up to 50% of the Ca^{2+} is lost [5, 8, 10–13, 17, 19, 20]. This spontaneous release of Ca^{2+} was shown not to be caused by depletion of ATP or a decrease in the Ca^{2+}-ATPase activity, but is probably due to an increased rate of Ca^{2+} efflux [12, 19]. It is reported here that spontaneous Ca^{2+} release can be initiated by osmotic swelling of the sarcoplasmic reticulum vesicles caused by the increase in the intravesicular Ca^{2+} concentration during Ca^{2+} uptake.

Materials and Methods

Arsenazo III (Aldrich, Milwaukee, Wis.) was further purified by the method of Kendrick [14]. All dicarboxylic acids were purchased from Aldrich. Adenosine-5′-triphosphate and all other chemicals were obtained from Sigma (St. Louis, Mo.).

Preparation of Sarcoplasmic Reticulum Vesicle

Sarcoplasmic reticulum vesicles were prepared from the back and hindleg skeletal muscles of rabbit as previously described [4]. The final pellet was resuspended in 25 mM K_2 maleate, 50 mM Mg maleate, 25 mM Tris maleate and 20 mM histidine (maleate medium) or 0.15 M KCl, 20 mM histidine, 5 mM $MgCl_2$ (KCl medium), frozen in liquid nitrogen and stored at −70° C until used.

Measurement of Ca^{2+} Accumulation by Sarcoplasmic Reticulum

Ca^{2+} uptake was measured using arsenazo III as an indicator of free Ca^{2+} [3]. The decrease in the absorbance of the Ca^{2+}-arsenazo III complex during Ca^{2+} uptake was monitored at 660 nm in an Aminco DW-2 spectrophotometer in the dual wavelength mode using 685 nm as the reference wavelength. In most experiments, Ca^{2+} transport was initiated by the addition of ATP. The baseline absorbance was measured after releasing the sequestered Ca^{2+} with A23187 at the end of each

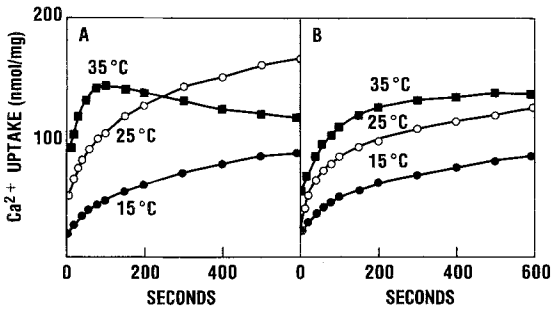

Fig. 1. Effect of temperature on Ca^{2+} uptake by sarcoplasmic reticulum vesicles. *A*. Ca^{2+} uptake was measured in medium containing 0.15 M KCl, 20 mM histidine (pH 6.8), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM arsenazo III and sarcoplasmic reticulum vesicles (0.2 mg protein/ml) at the indicated temperature. Ca^{2+} accumulation was monitored after the addition of 4 mM ATP by measuring the difference absorbance (660 to 685 nm) of the Ca^{2+}-arsenazo III complex. The amount of Ca^{2+} accumulated was determined using a calibration curve obtained by measuring the absorbance change of the Ca^{2+} uptake medium as the Ca^{2+} concentration was varied from 80 to 100 μM at the various temperatures. The baseline absorbance was established after releasing all the sequestered Ca^{2+} with A23187 (1 μg/ml). *B*. Same as *A*, except 80 mM sucrose was added to the Ca^{2+} uptake medium 2 min before the ATP addition

measurement. The optical response of the arsenazo III to Ca^{2+} was calibrated by the repeated additions of 4 μM $CaCl_2$ to the Ca^{2+} uptake medium.

Measurement of ATPase Activity

The rate of ATP hydrolysis was followed by measuring the release of inorganic phosphate from ATP. ATP hydrolysis was initiated by the addition of ATP (0.8 mM) to the medium containing sarcoplasmic reticulum vesicles (0.18 mg protein/ml). After incubations of 10, 20, 30, 60, 100, 150 and 200 sec, 0.6-ml aliquots were removed and mixed with 0.1 ml of 10% TCA at 1 °C. After removing the protein by centrifugation, the supernatant was analyzed for inorganic phosphate according to Fiske and Subbarow [7].

Light-Scattering Measurements

Changes in the volume of the sarcoplasmic reticulum vesicles were monitored by measuring the light-scattering intensity at 450 nm as described by Kometani and Kasai [15]. A Perkin-Elmer model 650 fluorescence spectrophotometer was used with both the excitation and emission monochrometers set at 450 nm.

Results

Spontaneous Ca^{2+} Release

Figure 1 shows the effect of temperature on the rate of Ca^{2+} uptake in KCl medium at pH 6.8. At 15 and 25 °C, the rate of Ca^{2+} accumulation steadily declined until a steady state was reached after 10 min in which the rate of Ca^{2+} efflux matched the rate of Ca^{2+} transport. On the other hand, the accumulation of Ca^{2+} at 35 °C reached a maximum after 100 sec, followed by a decline in the capacity of the sarcoplasmic reticulum vesicles to accumulate the Ca^{2+}. Similar observations have been reported by others [8, 20]. The Ca^{2+}-loading level goes from a peak of 143 nmol/mg protein after 1.5 min to 116 nmol/mg protein at 6 min. The rate of Ca^{2+}-dependent ATP hydrolysis at 35 °C is linear between 1 and 10 min, indicating that the decline in the Ca^{2+}-loading level is not due to an inactivation of the Ca^{2+}-ATPase.

Under conditions of low ATP or high protein concentrations, an apparent release of Ca^{2+} following the initial burst of Ca^{2+} uptake can be observed due to an accumulation of ADP and a depletion of ATP [18]. But under the experimental conditions used in this study, the spontaneous release of Ca^{2+} cannot be explained by ATP depletion or the build-up of ADP. Varying the MgATP concentration from 1.0 to 10.0 mM had no significant effect on the time course of Ca^{2+} uptake. Addition of an ATP-regenerating system (10 mM creatine phosphate, 1 mg/ml creatine phosphokinase) to prohibit the accumulation of ADP or the depletion of ATP had no influence on spontaneous Ca^{2+} release.

To test the effect of the extravesicular osmolarity on spontaneous Ca^{2+} release, 80 mM sucrose was added to the Ca^{2+} uptake medium (Fig. 1 *B*). The addition of sucrose inhibited the spontaneous release of Ca^{2+} at 37 °C and increased the steady-state Ca^{2+}-loading level. The initial rate of Ca^{2+} uptake was reduced by 80 mM sucrose, presumably due to a decrease in the volume of the sarcoplasmic reticulum vesicles caused by the efflux of water driven by the osmotic gradient. As the volume of the sarcoplasmic reticulum vesicles decreases, the amount of transported Ca^{2+} required to raise the internal Ca^{2+} concentration to inhibitory levels is also decreased. The rate of Ca^{2+}-dependent ATP hydrolysis by sarcoplasmic reticulum vesicles at 35 °C is not influenced by sucrose (0.02 to 0.25 M) in the presence of the Ca^{2+} ionophore, A23187, indicating that sucrose does not directly affect the Ca^{2+}-ATPase.

While the initial rate of Ca^{2+} accumulation was reduced when sucrose was included in the Ca^{2+} uptake medium, the addition of sucrose to the medium during spontaneous Ca^{2+} release increased the rate of Ca^{2+} accumulation by the sarcoplasmic reticulum vesicles. This is probably due to a decrease rate of osmotic-induced Ca^{2+} release.

In the presence of 50 mM phosphate, sucrose (20 to 100 mM) had no effect on the initial rate of Ca^{2+} uptake. Under these conditions, a de-

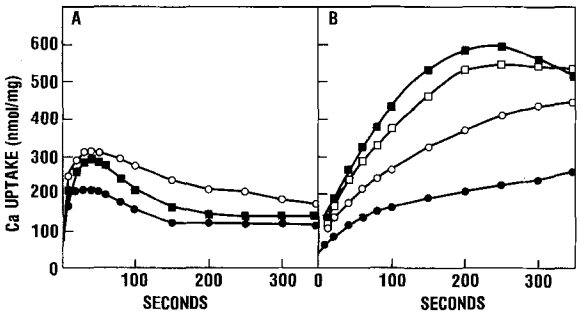

Fig. 2. Effect of maleate on Ca^{2+} uptake by sarcoplasmic reticulum vesicles. *A*. Ca^{2+} uptake at 37 °C was measured in medium containing 0.3 M KCl (●), 0.2 M KCl and 0.05 M K_2 maleate (○), 0.1 M KCl and 0.1 M K_2 maleate (□), or 0.15 M K_2 maleate (■) along with N-[2-acetamido]-iminodiacetic acid (pH 6.23), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM aresenazo III, 4 mM ATP and sarcoplasmic reticulum vesicles (0.2 mg/ml) as described in Fig. 1. *B*. Same as *A*, except 0.2 M sucrose was included in the Ca^{2+} uptake medium

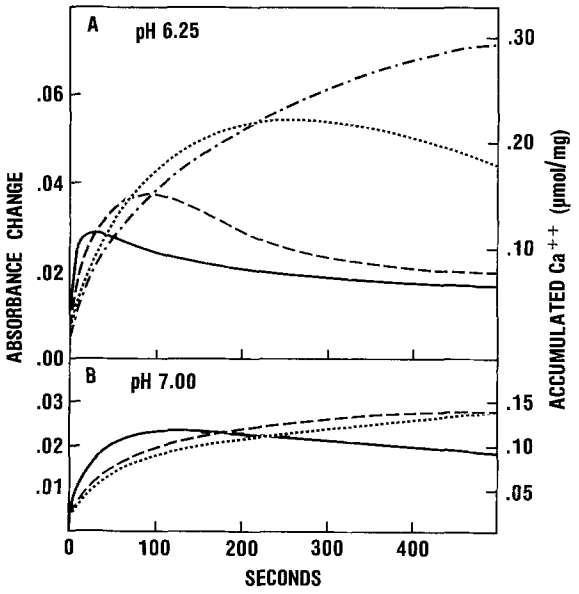

Fig. 3. Effect of sucrose concentration on Ca^{2+} uptake by sarcoplasmic reticulum vesicles in the presence of maleate. Ca^{2+} uptake at 37 °C was measured in medium containing sarcoplasmic reticulum vesicles (0.18 mg protein/ml), 25 mM K_2 maleate, 50 mM Mg maleate, 25 mM Tris maleate, 20 mM histidine, 0.1 mM Ca maleate and 0.1 mM arsenazo III adjusted to pH 6.25 (panel *A*) or 7.00 (panel *B*). The difference absorbance of the arsenazo III-Ca complex at 685 to 660 nm was monitored in an Aminco DW-2 spectrophotometer in the dual wavelength mode. Sucrose was added to some of the samples at a final concentration of 50 mM (---) 100 mM (· · ·) or 250 mM (— · —) 5 min before ATP was added to initiate Ca^{2+} transport. The baseline absorbance was established after releasing all the sequestered Ca^{2+} from the sarcoplasmic reticulum vesicles with 1 µg/ml A23187

crease in the volume of the sarcoplasmic reticulum vesicles would not influence the rate of Ca^{2+} transport since intravesicular Ca^{2+} is trapped by phosphate precipitation. This experiment further demonstrates that sucrose does not directly affect the rate of Ca^{2+} transport by the Ca^{2+}-ATPase.

Spontaneous Ca^{2+} release was also observed in the presence of 50 mM phosphate as reported by Katz and coworkers [12, 13]. This spontaneous Ca^{2+} release was also inhibited by increasing the external osmolarity with 100 mM sucrose.

Effect of Maleate on Ca^{2+} Uptake

Sorenson and DeMeis [19] have demonstrated that the rate and magnitude of spontaneous calcium release is increased by including maleate in the calcium uptake medium. In the presence of 0.2 M sucrose, maleate (50 to 150 mM) increased both the rate of Ca^{2+} accumulation and the level of Ca^{2+} loading by sarcoplasmic reticulum at pH 6.23 (Fig. 2*B*). Maleate probably activates Ca^{2+} uptake by binding the intravesicular Ca^{2+}, but unlike phosphate or oxalate, the Ca^{2+}-maleate complex does not precipitate and therefore remains osmotically active. Several other dicarboxylic acids (malonate, succinate, phthalate) gave results similar to maleate. In the absence of sucrose, increasing the maleate from 50 to 150 mM actually decreased the capacity of the sarcoplasmic reticulum vesicles to accumulate Ca^{2+} (Fig. 2*A*). This is apparently caused by an increased rate of spontaneous Ca^{2+} release at higher maleate concentrations.

The influence of the internal osmolarity in initiating spontaneous Ca^{2+} release was investigated further in media containing 100 mM maleate. The relationship between the sucrose concentration of the medium and Ca^{2+} uptake is shown in Fig. 3. The capacity of the vesicles to accumulate Ca^{2+} increased as the sucrose concentration was raised. The Ca^{2+}-loading capacity of the sarcoplasmic reticulum vesicles at pH 6.25 increased from 0.12 µmol Ca^{2+} per mg protein in the absence of sucrose to 0.23 µmol/mg in the presence of 100 mM sucrose. The time between the initiation of Ca^{2+} transport and spontaneous Ca^{2+} release also increased as the sucrose concentration was raised. Ca^{2+} release occurred 30 sec after ATP addition in the absence of sucrose and 260 sec in the presence of 100 mM sucrose. As in the absence of maleate, the rate of Ca^{2+} uptake decreased as the sucrose concentration was raised, presumably due to the reduction of the vesicle size as the external osmolarity is increased. Once again, the rate of ATP hydrolysis was not influenced by sucrose up to a concentration of 100 mM in the presence of A23187.

Raising the pH of the Ca^{2+} uptake medium from 6.2 to 7.0 reduced the effect of maleate on Ca^{2+} accumulation. This is probably due to the increased inhibition of the Ca^{2+}-ATPase by internal Ca^{2+} [22] and a decrease rate of maleate influx at the higher pH.

In the absence of maleate, increasing the sucrose concentration did delay the time of spontaneous Ca^{2+} release, but did not increase the loading capacity to the extent that was observed in the presence of 100 mM maleate. Spontaneous Ca^{2+} release began at the same loading level (160 to 200 nmol/mg) as is required to inhibit the Ca^{2+}-ATPase. So, even though spontaneous Ca^{2+} release is inhibited by increasing the external osmolarity, Ca^{2+} uptake does not continue due to the decrease rate of Ca^{2+} transport at high intravesicular Ca^{2+} levels. Because maleate can bind Ca^{2+} to lower the intravesicular free Ca^{2+} concentration, the loading capacity is greatly increased by adding maleate to the Ca^{2+} uptake media.

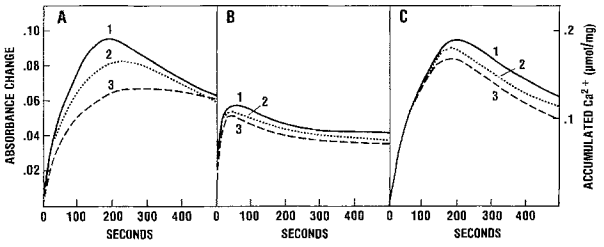

Fig. 4. Effect of the incubation time in sucrose on Ca^{2+} uptake by sarcoplasmic reticulum vesicles. *A*. Sarcoplasmic reticulum vesicles were equilibrated in 25 mM K_2 maleate, 25 mM Tris maleate, 50 mM Mg maleate, 20 mM histidine (pH 6.5) and 0.1 mM Ca maleate. Sucrose was added to the medium from a 1.0 M stock solution to give a final concentration of 0.1 M. After 5 (1), 200 (2) and 400 (3) min at 25 °C, aliquots were removed and assayed fro Ca^{2+} uptake at 37 °C using 0.1 mM arsenazo III, as described in Fig. 1. *B*. Same as *A*, except sucrose was not added to the medium. *C*. Same as *B*, except sucrose was added 2 min before the Ca^{2+} uptake assay was performed

For optimal inhibition of spontaneous Ca^{2+} release, sucrose is required only in the extravesicular medium (Fig. 4). The effect of 0.1 M sucrose on Ca^{2+} uptake was diminished after incubating the vesicles at 25 °C with the sucrose for 200 or 400 min to allow penetration of some of the sucrose into the vesicles before initiating Ca^{2+} uptake with ATP. In control samples in which sucrose was not added or added after the 200 or 400 min incubation, there was only a slight loss in the ability of sarcoplasmic reticulum to accumulate Ca^{2+}.

Spontaneous Ca^{2+} release was dependent on the level of Ca^{2+} loading (Fig. 5*A*). Sarcoplasmic reticulum vesicles were incubated in medium containing less than 1 μM Ca^{2+}. After ATP was added, successive additions of 4 μM Ca^{2+} (20 nmol Ca^{2+}/mg protein) were made and Ca^{2+} uptake monitored. Up to 60 nmol Ca^{2+}/mg protein were accumulated before any Ca^{2+} release was observed. When 80 nmol Ca^{2+}/mg protein were added to the medium a slow release of Ca^{2+} into the extravesicular medium was observed. Increasing the Ca^{2+} to 100 nmol/mg caused a further increase in the rate of Ca^{2+} release. Completely saturating the system with 160 nmol/mg caused an even greater rate of Ca^{2+} release. Under the right conditions,

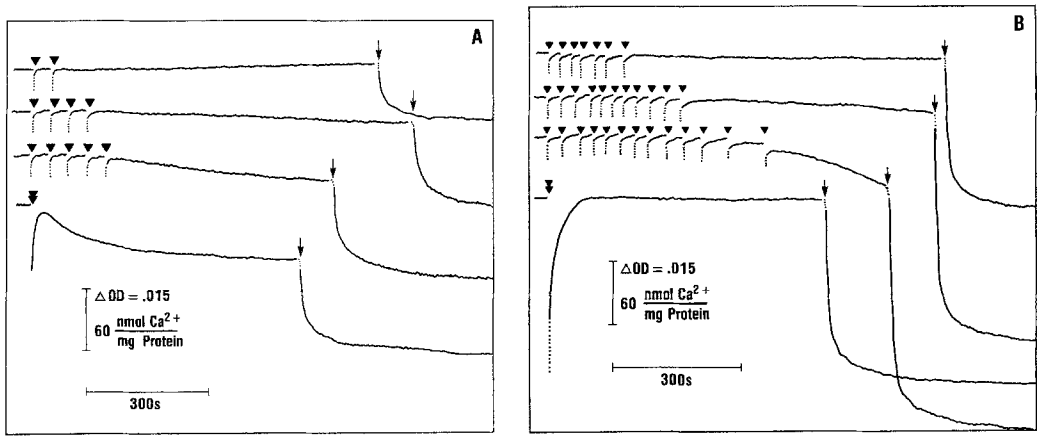

Fig. 5. Effect of the Ca^{2+} loading level on Ca^{2+} release from sarcoplasmic reticulum vesicles. *A*. Sarcoplasmic reticulum vesicles were equilibrated in 50 mM K_2 maleate, 20 mM $MgSO_4$, 25 mM Tris maleate, 20 mM histidine (pH 6.5) and 30 μM arsenazo III at a final protein concentration of 0.2 mg/ml. ATP (4 mM) was added and the difference absorbance of the Ca^{2+}-arsenazo III complex at 685 to 660 nm was measured. The temperature was kept at 37 °C. At the indicated times 4 μM (▼) or 32 μM (▼) $CaCl_2$ was added. A23187 (1 μg/ml) was added at the end of the trace (↓) to release the sequestered Ca^{2+}. *B*. Same as *A*, except 0.1 M sucrose was added to the medium 5 min before the ATP addition

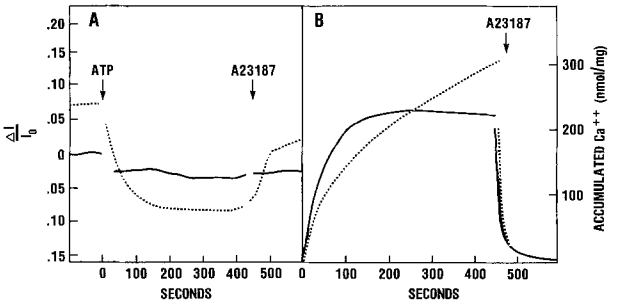

Fig. 6. Reswelling of shrunken sarcoplasmic reticulum vesicles during Ca^{2+} uptake. *A*. Effect of Ca^{2+} uptake on the light-scattering intensity. Sarcoplasmic reticulum vesicles (0.2 mg protein/ml) were equilibrated in 25 mM K_2 maleate, 50 mM Mg maleate, 25 mM Tris maleate, 20 mM histidine (pH 6.5) and 0.2 mM Ca maleate. To shrink the sarcoplasmic reticulum vesicles, 0.1 M sucrose was added to some of the samples (· · · ·); a control contained no sucrose (–). The changes in light-scattering intensity (ΔI) at right angles to the incident beam were measured in a fluorescence spectrophotometer with both the excitation and emission wavelength set at 450 nm. Ca^{2+} uptake was initiated by the addition of ATP (0.2 mM) and Ca^{2+} was released 7.5 min later by the addition of A23187 (1 μg/ml), temperature = 25 °C. *B*. Calcium uptake. All the additions were made as in *A*, except 0.1 mM arsenazo III was included in the medium and the rate of Ca^{2+} accumulation was monitored by measuring the difference absorbance at 685 to 660 nm as described in Fig. 1

the addition of Ca^{2+} to media containing saturated sarcoplasmic reticulum vesicles cause the release of a greater amount of Ca^{2+} from the vesicles. In the presence of 0.1 M sucrose, Ca^{2+} release was not observed until more than 200 nmol Ca^{2+} per mg protein were added (Fig. 5*B*).

In the experiments shown in Figs. 2 through 5, the amount of Ca^{2+} released never exceeded 50% of the initial, maximum loading level. To see if the vesicles which release their Ca^{2+} during spontaneous Ca^{2+} release were irreversibly damaged, sucrose was added after the initiation of Ca^{2+} uptake with ATP. Even after Ca^{2+} release had occurred, sucrose addition was still effective in restoring the maximum Ca^{2+}-uptake ability of the sarcoplasmic reticulum vesicles. It is therefore likely that osmotic-induced swelling of the vesicles during Ca^{2+} uptake induces the released Ca^{2+} but does not irreversibly damage the membrane. Ca^{2+} uptake and release can occur continuously, giving a steady-state loading level that is 50% of the initial peak level.

Kometani and Kasai [15, 16] have used light-scattering measurements to monitor the decrease in the volume of sarcoplasmic reticulum vesicles caused by the outflow of water driven by osmotic pressure differences. Increasing the extravesicular osmolarity led to an increase in the light-scattering intensity due to vesicle shrinkage. Decreasing the extravesicular osmolarity had little influence on the light-scattering intensity presumably because the vesicles are spherical to begin with, and the shape and size of the vesicles is not significantly altered by water influx [15]. Addition of 0.1 M sucrose to a suspension of sarcoplasmic reticulum vesicles caused a 7% increase in the light-scattering intensity (Fig. 6). Initiation of Ca^{2+} uptake with ATP reversed the change in light-scattering intensity caused by 0.1 M sucrose (Fig. 6). Releasing the accumulated Ca^{2+} with the Ca^{2+} ionophore, A23187, caused the light-scattering intensity to return to a higher value. In the absence of sucrose, a small decrease in light-scattering intensity followed the addition of ATP and no change was observed upon the addition of A23187. The small decrease in light scattering caused by ATP was also observed in the presence of A23187, indicating that it was caused by the interaction of ATP with the vesicles and not to Ca^{2+} uptake. The increased refractive index of the medium containing sucrose may be the reason why the light-scattering intensity of the vesicles in 0.1 M sucrose went below that of the vesicles in medium lacking sucrose during Ca^{2+} uptake [21].

Table. Effect of Ca^{2+} loading on the volume of sarcoplasmic reticulum vesicles

Medium	^3H_2O space (μl/mg protein)	Inulin-^{14}C space (μl/mg protein)	Inulin exclusion space (μl/mg protein)
Control	8.9	6.0	2.9
Control +0.17 M sucrose	6.9	6.4	0.5
Control +0.17 M sucrose +5 mM ATP	7.4	6.3	1.1

[a] Sarcoplasmic reticulum vesicles (1 mg protein/ml) were suspended in medium containing 0.05 M KCl, 0.05 M K_2 maleate, 10 mM histidine (pH 6.3), 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, ^3H_2O (0.1 μC_i/ml), and 25 μg/ml inulin-^{14}C (0.025 μC_i/ml). After the addition of 0.17 M sucrose and 5 mM ATP to the indicated samples, they were incubated at 25 °C for 5 min. The sarcoplasmic reticulum vesicles were then sedimented by centrifugation in a swinging bucket rotor for 5 min at 150,000 × *g*. The centrifuge temperature was set at 1 °C. The supernatants were removed and the pellet resuspended in 0.4 ml of 1% sodium dodecyl sulfate. Aliquots (100 μl) of the resuspended pellet were removed for protein determination and for the determination of ^3H_2O and inulin-^{14}C. The inulin exclusion space, calculated by subtracting the inulin-^{14}C space from the ^3H_2O space, was assumed to be the internal space of the sarcoplasmic reticulum vesicles since they are impermeable to inulin. The values are averages of 3 trials which did not vary more than 20%.

The inulin exclusion space caused by sarcoplasmic reticulm vesicles can also be used to measure changes in the vesicle volume [4] (Table). In maleate medium, sarcoplasmic reticulum vesicles had an inulin exclusion space of 3 μl/mg protein. Addition of 0.17 M sucrose to the medium reduced the inulin exclusion space to 0.5 μl/mg, while vesicles actively loaded with Ca^{2+} in the presence of 0.17 M sucrose had an inulin exclusion volume of 1.1 μl/mg. In the absence of sucrose, Ca^{2+} loading had no effect on the inulin exclusion space of sarcoplasmic reticulum vesicles. Since these measurements required the sedimentation of the sarcoplasmic reticulum vesicles, the measurement of the inulin exclusion space under optimal contitions for Ca^{2+} uptake was not possible.

Discussion

The rate of Ca^{2+} accumulation by sarcoplasmic reticulum vesicles is dependent on the difference between the rate of Ca^{2+} transport by the Ca^{2+}-ATPase and the rate of Ca^{2+} efflux from the vesicles. The passive efflux of Ca^{2+} from sarcoplasmic reticulum vesicles has been shown to be proportional to the free intravesicular Ca^{2+} [6]. The rate of Ca^{2+} transport by the Ca^{2+}-ATPase decreases as the internal Ca^{2+} concentration increases [9, 23, 24]. In the absence of an osmotic gradient, the Ca^{2+}-loading capacity (150 to 200 nmol/mg) is reached when the rate of Ca^{2+} efflux becomes equal to the rate of Ca^{2+} transport. But the Ca^{2+} gradient established by the Ca^{2+}-ATPase introduces an osmotic gradient which initiates Ca^{2+} release. Following the release of Ca^{2+}, a vesicle is able to once again reaccumulate Ca^{2+} until osmotic swelling reoccurs. Therefore, the steady-state Ca^{2+}-loading level is lowered since a certain fraction at the sarcoplasmic reticulum vesicles is always in the process of releasing or reaccumulating Ca^{2+}.

The intravesicular Ca^{2+} concentration required to inhibit the Ca^{2+}-ATPase (120 to 150 nmol/mg) is close to the level which causes osmotic-induced Ca^{2+} release. Therefore, in the absence of maleate, increasing the extravesicular osmolarity only increased the capacity of the vesicle to accumulate Ca^{2+} by 20 percent. But in the presence of maleate, the inhibition of the Ca^{2+}-ATPase by intravesicular Ca^{2+} is reduced due to the Ca^{2+} binding ability of maleate. Unlike Ca^{2+}-phosphate or Ca^{2+}-oxalate, the Ca^{2+}-maleate complex does not precipitate in the sarcoplasmic reticulum vesicles and is therefore osmotically active. The main factor which limits the accumulation of Ca^{2+} by sarcoplasmic reticulum in the presence of 0.1 M maleate is the osmotic gradient generated by the transport of Ca^{2+} and the passive influx of maleate. Addition of 0.2 M sucrose to the extravesicular medium increases the capacity of sarcoplasmic reticulum vesicles to accumulate Ca^{2+} by 400 percent.

An extensive study by Chu et al. [2] on the effects of anions on calcium transport by sarcoplasmic reticulum vesicles has recently been reported. Although their observations on the effect of anions on Ca^{2+} release are consistent with the proposal that permeant anions which buffer the internal Ca^{2+} can increase Ca^{2+} accumulation enough to cause osmotic swelling of the sarcoplasmic reticulum vesicles, they rejected any osmotic effect based on the inability of 16 mM mannitol to significantly alter spontaneous calcium release. Under their conditions, 16 mM mannitol would have altered the osmolarity by only 7%. Much greater amounts of sucrose are required to inhibit spontaneous Ca^{2+} release (Fig. 3).

In conclusion, the osmotic gradient produced by Ca^{2+} transport limits the capacity of sarcoplasmic reticulum vesicles to accumulate Ca^{2+}. Loading vesicles with Ca^{2+} above 110 nmol/mg is sufficient to induce the release of Ca^{2+}. To obtain optimal loading levels, the osmolarity of the extravesicular medium must be increased to compensate for the increase in the internal Ca^{2+} concentration.

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