Biochimica et Biophysica Acta, 465 (1977) 210—223

BBA 77626

EFFECTS OF ANIONS, pH AND MAGNESIUM ON CALCIUM ACCUMULATION AND RELEASE BY SARCOPLASMIC RETICULUM VESICLES

M.M. SORENSON * and L. DE MEIS

Instituto de Biofisica, Centro de Ciencias da Saude, Bloco G, Universidade Federal do Rio de Janeiro, Ilha do Fundao, Cidade Universitaria, ZC 32, Rio de Janeiro, RJ, 20.000 (Brasil)

(Received July 12th, 1976)

Summary

In the absence of oxalate, Ca^{2+} accumulation by isolated sarcoplasmic reticulum vesicles may show a transient behavior in which the vesicles accumulate during the first 2 min of incubation as much as twice the amount of Ca^{2+} which is retained after 5—7 min, when Ca^{2+} accumulation approaches a steady state. Before Ca^{2+} release begins, the Ca^{2+} accumulation can reach 200—250 nmol/mg protein. The spontaneous release of the "extra" Ca^{2+} initially accumulated appears to be triggered by the attainment of a sufficiently high concentration of free Ca^{2+} inside the vesicles.

The amplitude of the transient phase of Ca^{2+} accumulation reaches a high value near pH 6.0 and is increased by free Mg^{2+}. At optimal concentrations of H^+ and Mg^{2+}, the amount of Ca^{2+} accumulated during the transient is augmented by various anions, in the order maleate $\geqslant$ propionate $\geqslant$ succinate $>$ chloride $>$ sulfate $>$ acetylglycine. The divalent anions have their maximum effects at 20—40 mM and the monovalent anions, at 40—200 mM. At 200 mM, all of the carboxylic anions tested significantly reduce the amount of Ca^{2+} retained in the steady state.

Introduction

In a number of studies of Ca^{2+} accumulation by isolated sarcoplasmic reticulum vesicles in the absence of Ca^{2+}-precipitating agents, a spontaneous release of part of the Ca^{2+} initially accumulated by the vesicles has been described. Cal-

* To whom reprint requests should be addressed. Present address: Laboratory of Neurophysiology, Department of Neurology, College of Physicians and Surgeons of Columbia University, 630 West 168th St. New York City, N.Y., 10032, U.S.A.

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

cium accumulation by vesicles isolated from both cardiac muscle and white skeletal muscle reaches a maximum in less than a minute; subsequently a portion of the accumulated Ca^{2+} is released over a period of several minutes, and the vesicles approach a new steady state. Measurements of ATPase activity or the use of an ATP-regenerating system have excluded ATP depletion as a cause of the Ca^{2+} release [1—6].

The Ca^{2+} release described above seems to occur only under specific experimental conditions. Huxtable and Bressler [5] showed that 25% of the Ca^{2+} accumulated at pH 6.5 and 37°C in the first 30 s of incubation was released in 4 min. If the pH was raised to 7.2, the net accumulation of Ca^{2+} was nearly 50% lower than the maximum amount accumulated at pH 6.5, but the level attained at 30 s was maintained for several minutes. Other investigators have demonstrated that the Ca^{2+} release depends on temperature. At temperatures between 15 and 25°C, maximal Ca^{2+} uptake was similar to that observed at 35—37°C, but the spontaneous release of Ca^{2+} was greatly slowed or abolished [2,3]. The release was not observed in the presence of oxalate [2,3,5].

In this paper, we investigate the parameters affecting the transient accumulation and subsequent release of Ca^{2+}.

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle using KCl, EDTA, phosphate buffer, sucrose, and MgATP, as previously described [7]. In the last two washes at 70 000 × *g*, 0.1 M KCl was used. The vesicles were re-suspended in 0.1 M KCl or in 0.7 M enzyme-grade sucrose at 10—40 mg protein/ml, and stored at 0—4°C for no longer than 3 days. As others have noted (e.g. ref. 3) vesicle preparations from different rabbits vary considerably in their capacity for accumulating Ca^{2+}. Vesicles referred to in Results as having high or low capacities for Ca^{2+} accumulation were prepared identically, but were selected according to the amplitude of the overshoot obtained at optimal concentrations of H^+, maleate, and Mg^{2+} (details in legends to Figs. 6 and 7).

Ca^{2+} uptake was measured at 30°C in the absence of oxalate in media containing $Tris_2/Na_2ATP/Mg^{2+}/^{45}CaCl_2$ buffer, and other additions as described in the legends of the figures. The pH of each assay was adjusted to within 0.05 units of the stated value beforehand, and checked at the end of the incubation period using a Radiometer pH meter. Potassium salts other than KCl and K_2SO_4 were prepared by titration of the acid with KOH. For the experiments described in connection with Fig. 7, magnesium maleate was prepared by titration of MgO with maleic acid. In some experiments, and ATP-regenerating system was used, consisting of 4 or 5 mM phosphoenolpyruvate (monocyclohexylamine salt, neutralized with Tris base) and 0.1 mg/ml ATP : pyruvate phosphotransferase, preincubated with the assay medium at 30°C for 3—5 min before starting the reaction.

The reactions were started by the addition of vesicles to a final concentration of 0.2—0.4 mg protein/ml, and stopped at various intervals by filtration of 0.3—0.5-ml aliquots through Millipore or Gelman filters of average pore diameter of 0.45 μm. An aliquot of the filtrate was counted in a liquid scintil-

lation counter and compared with an aliquot of the unfiltered assay medium to determine the amount of Ca^{2+} accumulated by the vesicles. In some experiments, ATPase activity was measured by determining the concentration of P_i in the filtrate [8].

Materials. Enzymes, substrates, Tris, and enzyme-grade sucrose were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). $^{45}CaCl_2$ was purchased from the Radiochemical Centre (Amersham, U.K.). All other reagents were analytical grade.

For the calculation of free Mg^{2+} and $MgATP^{2-}$ complex in the experiments of Figs. 6 and 7, a computer program and association constants already described from this laboratory were used [9]. The complexes of H^+, Mg^{2+}, and Ca^{2+} with maleate were calculated using the pK_a values 1.92 and 6.22 and the association constant $10^{2.4}$ M^{-1} for the formation of calcium maleate [10]. The association constant for the formation of magnesium maleate was estimated at 63% of the value for the formation of calcium maleate, by analogy with other four-carbon dicarboxylic acids for which the association constants of the complexes with both metals have been measured [10].

Results

It will be shown that the transient accumulation and release of Ca^{2+} at 30°C depends on three factors: pH, the concentration and nature of the anion present in the medium, and the concentration of Mg^{2+}. As far as possible, the influence of each of these three parameters was studied while maintaining the other two at near-optimal concentrations.

Dependence on pH

The effect of pH is illustrated in Fig. 1A, which shows the time course of Ca^{2+} uptake at pH 6.1, 6.4 and 7.1 in a medium containing 40 mM maleate and 100 mM KCl. At the lowest pH, Ca^{2+} accumulation reaches its maximum rapidly as the vesicles take up 94% of the Ca^{2+} present in the medium in 40 s. Half of this Ca^{2+} is subsequently released, and the level of Ca^{2+} retained by the vesicles approaches a new steady state in 5—8 min. The initial accumulation of "extra" Ca^{2+} may be termed an "overshoot" or a "transient", since it is followed by Ca^{2+} release to a lower value which is then maintained. After a much smaller and slower overshoot at pH 6.4, a similar steady state is reached. At pH 7.1, no overshoot occurs and a steady state does not appear to have been completely established in 7 min. Experiments were not prolonged beyond 7 or 8 min in order to avoid building up in the medium enough inorganic phosphate from ATP hydrolysis to permit the accumulation of calcium phosphate by the vesicles [11]. Passive binding of Ca^{2+} (without ATP) determined in several preparations at pH 6.4—6.5 under the conditions of Fig. 1 was less than 10 nmol/mg protein.

The results of three experiments similar to that of Fig. 1A are plotted in Fig. 1B, showing the effect of pH on the maximum amount of Ca^{2+} accumulated during the overshoot and on the amount retained near the steady state at 8 min. Thus when maleate is the major anion present, the maximum overshoot occurs at pH 5.8—6.0 and above pH 6.8 no overshoot is observed. The steady

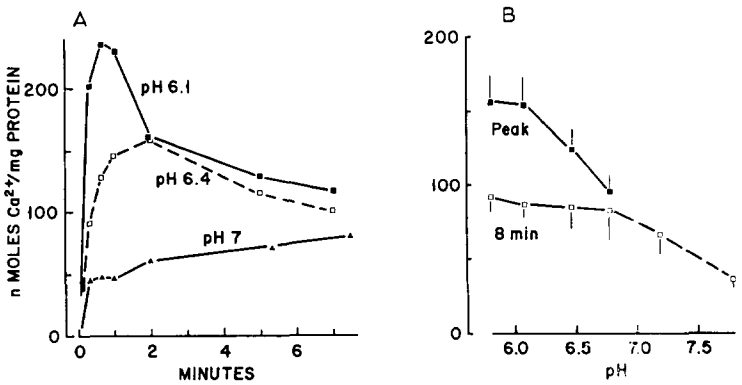

Fig. 1. (A) Time course of Ca^{2+} accumulation at different pH values. The assay media contained 3 mM ATP, 2 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 104 mM KCl, and 40 mM maleic acid adjusted to pH 6.1 (■), 6.4 (□), or 7.0 (▲) by the addition of Tris base, at 30°C. The reaction was started by the addition of vesicles to a final concentration of 0.4 mg protein/ml, and stopped at various intervals by filtration as described in Methods. Similar results were obtained in more than five other experiments. (B) Effect of pH on Ca^{2+} accumulation transient and steady state. The assay media were as described for (A), but contained 4 mM KCl. Aliquots filtered at 20, 40, 60 or 120 s were used for the determination of maximum accumulation (■), and aliquots filtered at 8 min (□) provided the steady-state levels of accumulation. Values represent means and standard errors of experiments on three different vesicle preparations.

state shows a broad plateau, from pH 5.8—6.8.

The amplitude of the overshoot in a given set of conditions varied from preparation to preparation. In 18 preparations tested under conditions similar to those of Fig. 1 at pH 6.0—6.2, the maximum amount of Ca^{2+} accumulated during the overshoot ranged from 126—270 nmol/mg protein. The steady-state accumulation ranged from 69—138 nmol/mg protein. The overshoot and spontaneous release were seen in all the preparations. The fraction of the maximal load accumulated during the overshoot which was spontaneously released after 5—8 min ranged from 27—58%.

ATP-regenerating system

Addition of an ATP-regenerating system to the assay medium had no effect, either on the amplitude of the overshoot or on the spontaneous release phase. In eight preparations tested under various conditions with an ATP-regenerating system present, the overshoot and the steady state were 98 ± 6% and 99 ± 7% (mean ± S.E.) of the controls without regenerating system, respectively. Persistence of the spontaneous release phase in the presence of an ATP-regenerating system (cf. also Figs. 6 and 7, Table I, and ref. 3) indicates that the spontaneous release of Ca^{2+} is not due to depletion of ATP or to accumulation of ADP in the medium.

Reversibility

Fig. 1A shows that the overshoot and the spontaneous Ca^{2+} release which occur at low pH are virtually complete within 2—3 min. If the vesicles are preincubated in the assay medium for this length of time before ATP is added, a large overshoot still occurs and the steady state of Ca^{2+} accumulation is unaffected (data not shown). Others have shown that the overshoot and release

TABLE I

HALF-TIMES OF Ca^{2+} RELEASE FROM DIFFERENT LOADS

Assay media contained (A) 1 mM or (B) 20 mM $MgCl_2$, 1 mM ATP, 0.1 mM $^{45}CaCl_2$, 100 mM KCl, 40 mM Tris maleate (pH 6.15), 4 mM phosphoenolpyruvate, 0.1 mg/ml ATP : pyruvate phosphotransferase, and 0.2 mg vesicle protein/ml, at 30°C. Aliquots were filtered at 20, 40, 60, 120 and 300 s and the half-time of release was taken as the interval between the peak of the overshoot and the loss of one-half of the difference between the peak and the steady-state loads at 300 s. The variation in amplitude of the overshoot due to different concentrations of $MgCl_2$ in A and B is described in the preceding section of the text. Values represent means and standard errors of n experiments.

	Ca^{2+} accumulated (nmol/mg protein)		Half-time (s)	n
	Overshoot	Steady state		
(A)	190 ± 7	103 ± 6	52 ± 6	7
(B)	242 ± 12	101 ± 11	48 ± 2	5

cycle can be repeated if a second aliquot of ATP is added to the medium after most of the ATP initially present has been hydrolyzed [1,6].

Effects of anions

The time course of Ca^{2+} accumulation is greatly modified by the anion present. In the absence of maleate, Ca^{2+} accumulation under the conditions of Fig. 1B shows no release whatsoever between pH 6.05 and 7.5. At pH 6 and in the presence of 20 mM $MgCl_2$, conditions which are optimal for the overshoot when maleate is present, little Ca^{2+} release is observed in the absence of maleate (Fig. 2). When either Tris maleate or potassium maleate is added in increasing

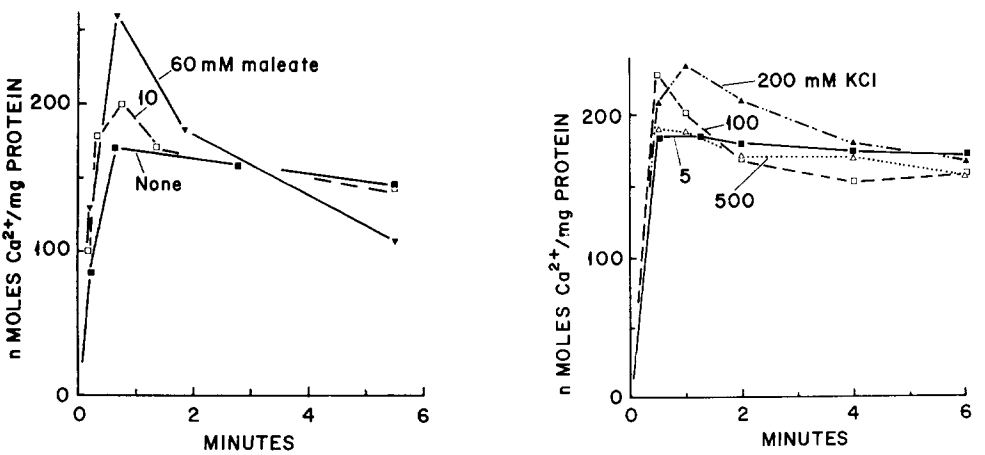

Fig. 2. Activation of Ca^{2+} accumulation transient by maleate at low pH. Assay media contained 4 mM ATP, 20 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 1.2 mM KCl, 0.2 or 0.25 mg sarcoplasmic reticulum vesicle protein/ml, and 0 (■), 10 (□), or 60 (▼) mM maleate adjusted with KOH or Tris base to pH 6.05, at 30°C. The curves are averages from two preparations.

Fig. 3. Effect of chloride on Ca^{2+} accumulation transient at low pH. In addition to the concentrations of KCl shown in the figure, the assay media contained 4 mM ATP, 20 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 0.6 mM KCl, and 0.2 mg vesicle protein/ml, at 30°C. The pH was adjusted to pH 6.05 with HCl or Tris base, and did not vary more than 0.1 unit during the experiments. Concentrations of KCl added were (in mM) 5 (■), 100 (□), 200 (▲), and 500 (△).

amounts, the overshoot becomes progressively larger (Fig. 2). Potassium maleate does not appear to be more effective than Tris maleate.

Enhancement of the overshoot is not unique to maleate. Concentrations of KCl op to 200 mM also augment the overshoot, although not as much as maleate (Fig. 3). Fig. 4 shows the results of experiments similar to those of Figs. 2 and 3, using the potassium salts of Cl^-, SO_4^{2-}, and various mono- and di-carboxylic anions. The divalent anions tested are maximally effective in the 20—40 mM range (Fig. 4, right), the monovalent anions require 40—200 mM for their maximal effects (Fig. 4, left). At the optimum concentration for each anion, the order of effectiveness in enhancing the overshoot is maleate $\geqslant$ propionate $\geqslant$ succinate $>$ Cl^- $>$ SO_4^{2-} $>$ acetylglycine. At these same concentrations, propionate and maleate slightly inhibit the steady-state accumulation of Ca^{2+}; the other anions have no significant effect (Fig. 4). At higher concentrations (100—300 mM), the organic anions can markedly depress the amount of Ca^{2+} retained by the vesicles in the steady state. Since in this higher concentration range there is a parallel inhibition of the overshoot, the total amount of Ca^{2+} released by the vesicles following the overshoot remains relatively constant (Fig. 5). In contrast to the other anions, SO_4^{2-} above 100 mM abolishes the overshoot.

The decrease in Ca^{2+} accumulation at the steady state in the concentration range of 100–300 mM may be related to the acid dissociation constants of the organic anions. At any given concentration between 100 and 300 mM, the inhibitory effectiveness of the different anions is in the same order as the concentration of undissociated carboxyl groups provided by each compound [34]. Thus Cl^- and SO_4^{2-} have no effect, acetylglycine (pK_a 3.7) and propionate (pK_a 4.9) are moderately inhibitory, and succinate (pK_1 5.6) and maleate (pK_1 6.2) are most inhibitory.

Mg^{2+} dependence

In preliminary tests for the effect of Mg^{2+} on the amplitude of the over-

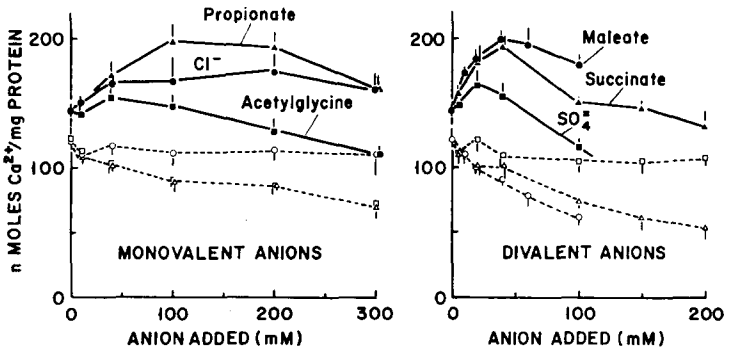

Fig. 4. Effects of anions on Ca^{2+} accumulation transient and steady state at low pH. Assay media contained 4 mM ATP, 20 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 2 mM KCl, and 0.2 mg protein/ml. at pH 6.0 and 30°C, in addition to the concentrations of potassium salts shown on the abscissae. Values are means and standard errors of five vesicle preparations. Solid lines, peak of transient at 20 or 40 s; dashed lines, steady-state at 5.5 min. Left, monovalent anions added were chloride (●,○), propionate (▲,△), and acetylglycine (■,□). Right, divalent anions added were maleate (●,○), succinate (▲,△), and sulfate (■,□).

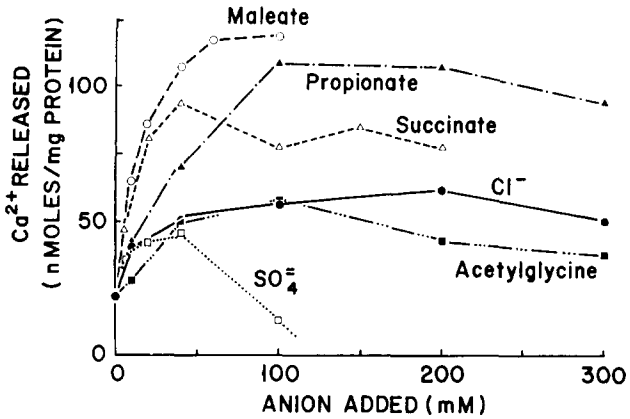

Fig. 5. Ca^{2+} release following the overshoot in the presence of different anions. Data are taken from Fig. 4, showing the difference between the amount of Ca^{2+} accumulated by the vesicles at the peak of the overshoot and the amount retained at the steady state, at each concentration of anion shown on the abscissa.

shoot, we observed differences in the responses of different preparations which appeared to depend on the degree of Ca^{2+} loading. Accordingly, the Mg^{2+} dependence was studied in detail on two groups of vesicles, one having a Ca^{2+} capacity about 50% greater than the other at the peak of the overshoot in 20—30 mM magnesium. In these experiments the pH and the concentration of maleate were maintained near their optimal values for the overshoot.

Increasing the concentration of magnesium at a fixed concentration of ATP increases both the rate and amount of Ca^{2+} accumulation during the overshoot (Figs. 6 and 7). In the higher-capacity vesicles (Fig. 6, left), the overshoot continues to increase as the concentration of magnesium is raised to 10 and 20 mM. The amount of Ca^{2+} retained in the steady state does not vary with magnesium. In the lower-capacity preparations (Fig. 7), the overshoot is maximal with 3 mM magnesium present; higher concentrations do not increase it further. In these vesicles, the steady-state accumulation also increases with magnesium, although the effect is less than on the overshoot.

The contrast between the data of Figs. 6 and 7, using vesicles with different maximal capacities for Ca^{2+}, may indicate that the concentration of Ca^{2+} inside the vesicles influences the magnesium requirement for activation of the overshoot.

In order to determine whether the activation shown in Fig. 6 is due to free Mg^{2+} or to $MgATP^{2-}$ complex, the Ca^{2+} loads at peak and steady state were measured in the presence of 3 mM and 0.01—0.03 mM ATP, with 3 mM magnesium present in the medium. An ATP-regenerating system was used to maintain constant the concentrations of ATP. The free Mg^{2+} concentrations were similar in the two media (pMg^{2+} 3.5 and 3.1, respectively), but there was a 100-fold difference in MgATP concentrations. In the three preparations tested, the average amount of Ca^{2+} accumulated at the peak of the overshoot in the two conditions was the same (224 and 215 nmol/mg protein). The steady-state loads were also unaffected by the difference in MgATP concentrations. A more complete test was performed using low-capacity vesicles, as shown in Fig. 7.

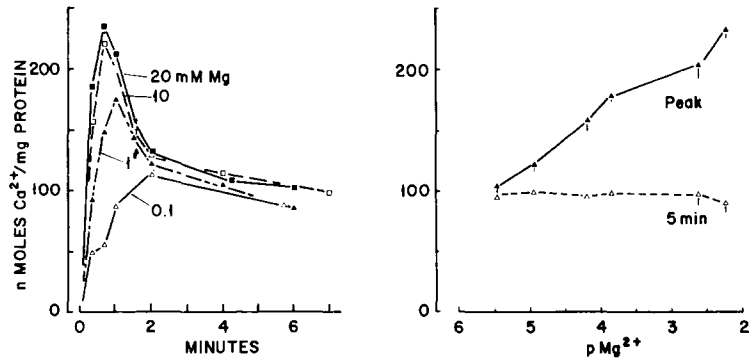

Fig. 6. Effect of Mg^{2+} on Ca^{2+} accumulation by high-capacity vesicles. Vesicles which accumulated at the maximum 219—244 nmol Ca^{2+}/mg protein were used. Left, assay media contained 1 mM ATP, 0.1 mM $^{45}CaCl_2$, 100 mM KCl, 4 mM phosphoenolpyruvate, 0.1 mg/ml ATP : pyruvic phosphotransferase, 40 mM Tris maleate (pH 6.2), and 0.1 (△), 1.0 (▲), 10 (□), or 20 (■) mM $MgCl_2$ at 30°C. The reactions were started by the addition of vesicles to a final concentration of 0.2 mg protein/ml. Right, averages of three experiments similar to those at left, but varying $MgCl_2$ from 0.03—20 mM. Solid lines, maximum accumulation at 20, 40, 60 or 120 s; dashed lines, steady-state accumulation at 5 min. Free Mg^{2+} concentrations were calculated as described in Methods.

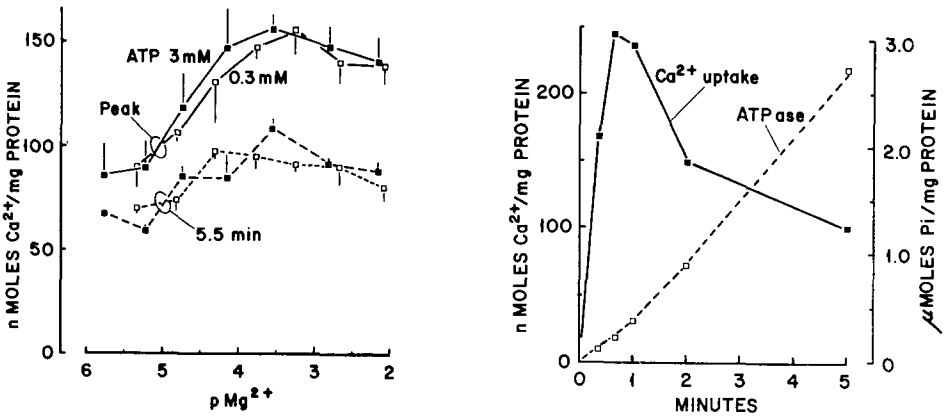

Fig. 7. Effect of Mg^{2+} on Ca^{2+} accumulation by low-capacity vesicles. Vesicles which accumulated at the maximum 149—168 nmol Ca^{2+}/mg protein were used. Assay media contained 3.0 (■) or 0.3 (□) mM ATP, 0.1 mM $^{45}CaCl_2$, 10 mM His · HCl, 0.1 mg/ml ATP : pyruvate phosphotransferase, 4 mM phosphoenolpyruvate, potassium maleate as required to keep the total concentration of maleate at 60 mM, 0.2 mg vesicle protein/ml and 0.03—30 mM magnesium maleate at pH 6.0 and 30°C. Free Mg^{2+} concentrations were calculated as described in Methods. Solid lines, maximum accumulation at 20, 40, 60 or 120 s. Dashed lines, steady state at 5.5 or 6.5 min. Values are the means and standard errors of experiments on three preparations. In five similar experiments in which the Cl^- concentration was maintained at 100 mM as $MgCl_2$ was added in the presence of 3 mM ATP, both the transient and the steady state also showed optima at pMg^{2+} 3.5.

Fig. 8. Ca^{2+}-activated ATP hydrolysis during the overshoot. Assay medium contained 1 mM ATP, 20 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 0.03 mM EGTA, 100 mM KCl, 40 mM maleic acid adjusted to pH 6.15 with Tris base, and 0.2 mg vesicle protein/ml, at 30°C. The assay was started by the addition of vesicles and stopped by filtration at the times indicated. Liberation of inorganic phosphate (□) and ^{45}Ca uptake (■) were measured as described in Methods, using, respectively, 0.2 and 0.1 ml of each filtrate. A Mg^{2+}-dependent ATPase activity of 0.097 μmol/mg protein per min, measured in the same medium but with $CaCl_2$ omitted, was subtracted from the total activity to obtain the Ca^{2+}-dependent activity shown.

For this experiment the magnesium concentration was increased over four orders of magnitude at two different ATP concentrations, and the data plotted as a function of free Mg^{2+}. The curves obtained with different ATP concentrations virtually superimpose when plotted in this way. These data indicate that the overshoot is activated by free Mg^{2+}.

ATP hydrolysis

In a number of experiments, ATP hydrolysis and Ca^{2+} accumulation were measured simultaneously in order to determine whether the Ca^{2+} release following the overshoot might be due to an abrupt inhibition of the Ca^{2+} pump (Fig. 8). The release of Ca^{2+} following the overshoot is not accompanied by any decrease in the rate of Ca^{2+}-dependent hydrolysis of ATP. A similar finding was reported by Huxtable and Bressler [5].

Effect of Ca^{2+} load

The next experiments were designed to explore the role of the Ca^{2+} concentrations inside and outside the vesicles. The time course of Ca^{2+} accumulation from media containing a fixed concentration of $^{45}CaCl_2$ and four different concentrations of vesicular protein is shown in Fig. 9 (left). The increase in protein concentration leads to removal of proportionately increasing amounts of Ca^{2+} from the medium: with 0.4 mg protein/ml, at the peak of the overshoot only 7% (7 μM) of the Ca^{2+} initially added to the medium still remains. In these experiments larger protein concentrations were avoided in order to ensure that at the peak of the overshoot, the Ca^{2+} concentration remaining in the assay medium was sufficient to maximally activate the transport ATPase [12]. In Fig. 9 (right), the data are re-plotted to show the amount of Ca^{2+} accumulated per mg of protein at the peak of the overshoot and in the steady state, as a function of the concentration of Ca^{2+} remaining in the medium at those two incubation times. This experiment shows that the overshoot is independent of

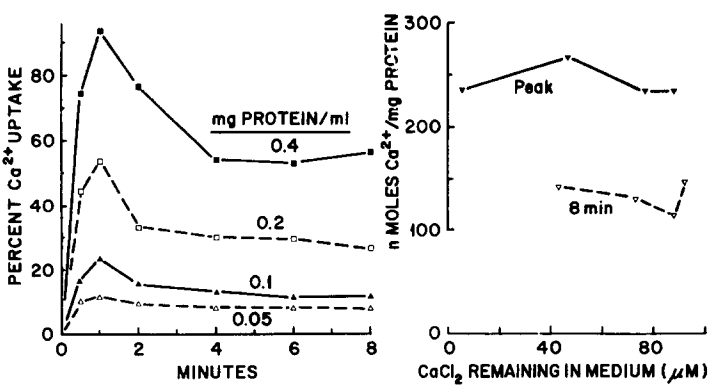

Fig. 9. Effect of external Ca^{2+} concentration on overshoot and steady state of Ca^{2+} accumulation. Assay media contained 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 20 mM KCl, 40 mM maleic acid adjusted to pH 6.4 with Tris base, and the concentrations of vesicle protein shown, at 30°C. Left, time course of Ca^{2+} accumulation at vesicle protein concentration of 0.05 (△), 0.1 (▲), 0.2 (□), or 0.4 (■) mg/ml. Right, data at left re-plotted to show Ca^{2+} accumulation at peak of overshoot (▼) and at steady state (▽) as a function of the concentration of calcium remaining in the medium at each of those incubation times.

external $CaCl_2$ over the range of 7—88 μM. Since the calcium load at the peak of the overshoot is the same even when the external calcium concentration is reduced 10-fold, these data also indicate that the overshoot can withstand a 5—10-fold increase in the transmembrane Ca^{2+} concentration gradient without alteration.

At the steady state the Ca^{2+} load in the experiment of Fig. 9 was independent of external $CaCl_2$ between 46 and 92 μM. In another experiment, the peak and steady-state loads were measured at a constant protein concentration (0.2 mg/ml) but in media containing different $CaCl_2$ concentrations. The peak and the steady-state Ca^{2+} loads were the same whether the medium initially contained 30 or 100 μM $CaCl_2$. Final concentrations at the steady state were 20 and 90 μM, respectively. This experiment confirms that of Fig. 9 and extends down to 20 μM the range in which the steady-state load is independent of external calcium.

It is somewhat more difficult experimentally to vary the internal Ca^{2+} concentration. In designing the next experiments we have assumed that Ca^{2+} is accumulated during the overshoot by being actively transported into the vesicles, where some of it remains free. It may be in equilibrium with another fraction that is bound to components of the membrane [13,14]. The results of adding oxalate to the medium (Fig. 10) indicate a role for internal Ca^{2+} in the spontaneous release of Ca^{2+} that follows the overshoot. Vesicles incubated with ATP, Ca^{2+}, Mg^{2+} and oxalate accumulate calcium actively and oxalate passively until the limit of solubility is reached inside the vesicles, and calcium oxalate precipitates [14—17]. As calcium accumulation continues, the free Ca^{2+} concentration inside the vesicles remains constant. This concentration is lower the higher the oxalate concentration of the assay medium [14—16]. Fig. 10 shows that the presence of small amounts of oxalate (up to 0.5 mM) in the assay medium abolishes the release phase following the overshoot, without greatly affecting the rate of uptake. In three similar experiments, the Ca^{2+} release phase was

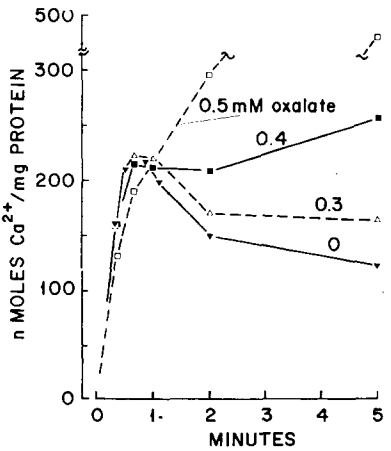

Fig.10. Loss of spontaneous Ca^{2+} release in the presence of oxalate. Assay media contained 1 mM ATP, 5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 100 mM KCl, 4 mM phosphoenolpyruvate, 0.1 mg/ml ATP : pyruvate phosphotransferase, 40 mM Tris maleate (pH 6.2), and 0 (▼), 0.3 (△), 0.4 (■), or 0.5 (□) mM potassium oxalate, at 30°C. The reactions were started by the addition of vesicles to a final concentration of 0.2 mg protein/ml.

attenuated or abolished at oxalate concentrations between 0.2 and 0.3 mM. At oxalate concentrations above 0.5—1 mM, Ca^{2+} uptake becomes very rapid and the steady-state capacity of the vesicles is greatly increased (data not shown).

If the Ca^{2+} release depends on internal Ca^{2+}, its rate should increase as the internal Ca^{2+} concentration increases. In the experiment of Table I, the time course of the spontaneous Ca^{2+} release was studied in vesicles which had been loaded to two different levels at the peak of the overshoot. This was accomplished by taking advantage of the Mg^{2+} effect described in a preceding section. High-capacity vesicles were allowed to accumulate Ca^{2+} under the conditions described for Fig. 6 in the presence of 1 or 20 mM $MgCl_2$. Identical half-times are observed for the spontaneous Ca^{2+} release from the two different degrees of filling thus obtained. We assume that the different Ca^{2+} loads in A and B are reflected by differences in the internal Ca^{2+} concentrations. The data of Table I would thus be consistent with Ca^{2+} outflow at a rate which is proportional to the internal Ca^{2+} concentration.

A second point can be made from the data in Table I. If the lower overshoot obtained in the presence of the lower concentration of $MgCl_2$ were simply due to a greater leakiness of the membrane, one would expect a smaller half-time and a lower steady-state capacity for this experimental condition. As this was not the case, it is unlikely that different amplitudes of the overshoots reflect differences in the membrane's passive permeability to Ca^{2+}.

Discussion

The data presented in this paper document ionic requirements for a transient accumulation and spontaneous release of Ca^{2+} by isolated sarcoplasmic reticulum vesicles in the absence of Ca^{2+}-precipitating agents. The data of Fig. 9 and Table I establish that the overshoot and the steady state represent two distinctly different capacities for Ca^{2+} retention. Optimal concentrations of anions, H^+ and Mg^{2+} all increase the maximum capacity during the overshoot, with lesser effects on the lower, steady-state capacity. The spontaneous release of Ca^{2+} which follows the overshoot is a reversible process and represents a transition between the high- and low-capacity states.

Although new information is provided about the conditions which are optimal for observing the early, high-capacity state which characterizes the overshoot, it is still not possible to describe completely the mechanism by which it is induced. The overshoot is accompanied by ATP hydrolysis (Fig. 8) and is too large to be accounted for by the small amount of passive binding found in the absence of ATP. Our data do not exclude the possibility that the "extra" Ca^{2+} accumulated during the overshoot represents a bound fraction whose binding depends on ATP [18] and is affected by pH, Mg^{2+} and anions. Nevertheless, the graded effects of low concentrations of oxalate which abolish the Ca^{2+} release (Fig. 10) indicate that the free Ca^{2+} concentration inside the vesicles is an important factor in controlling the overshoot. We suggest that the amplitude of the overshoot is limited by attainment of a critical Ca^{2+} concentration inside the vesicles. Low concentrations of oxalate suffice to prevent this critical internal Ca^{2+} concentration from being reached. In the absence of oxalate, the critical internal Ca^{2+} concentration triggers readjustment to a low-capacity state,

and Ca^{2+} is then released by passive diffusion (Table I) until the new steady state is attained.

The different amplitudes of the overshoot which are observed when the ionic conditions are varied in the absence of oxalate may correspond to alterations in the threshold concentration of Ca^{2+} inside the vesicles which is required to trigger the release phenomenon. An alternative which is made less likely by the data of Table I is that a higher overshoot simply reflects a lower passive permeability to Ca^{2+} already accumulated. Since the release occurs while the pump is still operating normally, as indicated by the ATPase activity (Fig. 8 and ref. 5), it is not a result of the pump suddenly being arrested. Nor does it appear to be the consequence of reversal of the Ca^{2+} pump [19], since it is not blocked by the use of a regenerating system that removes ADP from the medium (Figs. 6 and 7 and ref. 3).

Anions. Activation by anions of Ca^{2+} accumulation by isolated vesicles has not previously been reported. Using intact vesicles and concentrations of Mg^{2+} and H^+ which are optimal for activating the overshoot, we have been unable to detect any activation of the ATPase activity by 40 mM maleate which could provide the increased Ca^{2+} influx necessary to produce an overshoot. A study of the effects of anions on the kinetics of the Ca^{2+}-dependent ATPase activity is in progress, using vesicles purposely made leaky in order to permit control of the Ca^{2+} concentration at the inner surface of the vesicle membrane.

Earlier studies of anion effects have been confined to pH 7, where an overshoot does not occur, and have been focussed on chaotropic anions. Various degrees of inhibition of the steady-state capacity were observed at anion concentrations between 50 and 300 mM [20—24]. Both increased leakiness of the vesicles and direct inhibition of the Ca^{2+} transport ATPase contributed to the decrease in Ca^{2+} capacity [23,24]. We have not attempted to estimate membrane permeability in the presence of anion concentrations which inhibit the steady-state capacity (Fig. 4). It may be that some permeability increase occurs at these higher concentrations.

Comparison with skinned fibers. This study includes anions which have been shown to promote uptake or release of Ca^{2+} by elements of the sarcoplasmic reticulum which persist in isolated "skinned" fibers, from which the sarcolemma has been removed [25—31]. In these preparations, the sarcoplasmic reticulum accumulates Ca^{2+} from bathing media containing Mg^{2+}, Ca^{2+}, ATP and one of a number of potassium salts. Ca^{2+} accumulation is greater in the presence of "more permeant" anions such as Cl^- than in the presence of propionate or SO_4^{2-} [28]. In the isolated vesicles, these anions differ greatly in their ability to induce an overshoot at their optimal concentrations (Fig. 4). Nevertheless their effects are not in the same order as their reported actions on skinned fibers. Propionate, for example, enhanced the overshoot more than Cl^-, but SO_4^{2-} depressed it.

pH and Mg^{2+}. Our data confirm previous reports of a transient accumulation and release of Ca^{2+} which showed that it occurs at pH 6.5 but not at pH 7.2 [5]. However, we observed a maximum at pH 6 or below (Fig. 1B), whereas for Huxtable and Bressler [5], pH 6.5 was optimal. A comparison of Figs. 1—3, and also Fig. 6, suggests that differences may be expected on the basis of the different anions (110 mM Cl^- vs. 40 mM maleate), and possibly the different

Mg^{2+} concentrations used. Using maleate and a large excess of Mg^{2+}, Nakamaru and Schwartz [32] also found an increase in the amplitude of the overshoot as the pH was reduced below 6.5.

The pH profile which we observe for the overshoot is in striking contrast to that which we find for the lower levels of Ca^{2+} accumulation that are associated with the steady state (Fig. 1B). Several authors who have studied pH effects on the steady state in the presence of oxalate have reported that the pH optimum shifts to more acidic values when either Ca^{2+} accumulation or ATPase activity is measured in the absence of oxalate [3,5,33]. This shift has been attributed to the higher internal free Ca^{2+} concentrations which prevail in the absence of oxalate [33]. It may be that the lower pH optimum observed for the overshoot, in contrast to the steady state (Fig. 1), is due to the higher internal Ca^{2+} concentrations present at the peak of the overshoot.

The Mg^{2+} dependence at pH 6 has not been studied before. The Mg^{2+} profile for low-capacity vesicles at pH 6 (Fig. 7) is similar to that reported for vesicles having a Ca^{2+} capacity in this same range or lower at pH 6.6—6.7, where no overshoot would occur [13,21]. In the high-capacity vesicles, the Mg^{2+} becomes more effective in inducing the overshoot (Fig. 6).

Acknowledgements

This investigation was supported in part by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Brasil; by the Conselho de Ensino para Graduados do U.F.R.J.; and by FINEP-FNDCT-314/CT. M.S. was the recipient of a fellowship from the CNPq. The excellent technical assistance of Mr. Antonio Carlos Miranda da Silva is acknowledged.

References

1 Ohnishi, T. and Ebashi, S. (1963) J. Biochem. Tokyo 54, 506—511
2 Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 781—794
3 Sreter, F.A. (1969) Arch. Biochem. Biophys. 134, 25—33
4 Schwartz, A. (1971) in Calcium and the Heart (Harris, P. and Opie, L., eds.), pp. 66—92, Academic Press, London
5 Huxtable, R. and Bressler, R. (1974) J. Membrane Biol. 17, 189—197
6 Besch, Jr., H.R. and Watanabe, A.M. (1975) in Basic Functions of Cations in Myocardial Activity (Fleckenstein, A. and Dhalla, N.S., Eds.), pp. 143—149, University Park Press, Baltimore
7 de Meis, L. and Hasselbach, W. (1971) J. Biol. Chem. 246, 4759—4763
8 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
9 Vianna, A.L. (1975) Biochim Biophys. Acta 410, 389—406
10 O'Sullivan, W.J. (1969) in Data for Biochemical Research (Dawson, R.M.C., ed.), 2d edn., pp. 423—434, Oxford University Press, Oxford
11 Weber, A., Herz, R. and Reiss, I. (1964) Fed. Proc. 23, 896—900
12 Weber, A. (1971) J. Gen. Physiol. 57, 50—63
13 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329—369
14 Hasselbach, W. (1974) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. 10, pp. 431—467, Academic Press, New York
15 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94—111
16 Makinose, M. and Hasselbach, W. (1965) Biochem. Z. 343, 360—382
17 de Meis, L., Hasselbach, W. and Machado, R.D. (1974) J. Cell Biol. 62, 505—509
18 Ebashi, S. and Endo, M. (1968) Prog. Biophys. 18, 123—183
19 Barlogie, B., Hasselbach, W. and Makinose, M. (1971) FEBS Lett. 12, 267—268
20 Ebashi, S. (1964) in Conference on the Biochemistry of Muscle Contraction (1962) (Gergely, J., ed.), pp. 197—206, Little, Brown and Co., Boston

21 Carvalho, A.P. and Leo, B. (1967) J. Gen. Physiol. 50, 1327—1352
22 Chimoskey, J.E. and Gergely, J. (1968) Arch. Biochem. Biophys. 128, 601—605
23 Vianna, A.L., Bhatnagar, G.M. and Gergely, J. (1971) Physiol. Chem. Phys. 3, 536—548
24 The, R. and Hasselbach, W. (1975) Eur. J. Biochem. 53, 105—113
25 Natori, R. (1954) Jikeikai Med. J. 1, 119
26 Costantin, L.L. and Podolsky, R.J. (1967) J. Gen. Physiol. 50, 1101—1124
27 Reuben, J.P., Brandt, P.W. and Grundfest, H. (1967) J. Gen. Physiol. 50, 2501
28 Nakajima, Y. and Endo, M. (1973) Nat. New Biol. 246, 216—218
29 Orentlicher, M., Reuben, J.P., Grundfest, H. and Brandt, P.W. (1974) J. Gen. Physiol. 63, 168—186
30 Reuben, J.P., Wood, D.S., Zollman, J.R. and Brandt, P.W. (1975) in Concepts of Membranes in Regulation and Excitation (Rocha e Silva, M. and Suarez-Kurtz, G., eds.), pp. 21—39, Raven Press, New York
31 Thorens, S. and Endo, M. (1975) Proc. Jap. Acad. 51, 473—478
32 Nakamaru, Y. and Schwartz, A. (1970) Biochem. Biophys. Res. Commun, 41, 830—836
33 Verjovski-Almeida, S. and de Meis, L. (1975) An. Acad. Brasil. Cienc. 47, 351—356
34 Robinson, R.A. and Stokes, R.H. (1969) Electrolyte Solutions, 2nd edn., Butterworths, London