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CALCIUM UPTAKE, CALCIUM RELEASE AND ADENOSINETRIPHOSPHATASE ACTIVITY IN SARCOPLASMIC RETICULUM FRAGMENTS DEPOSITED ON MILLIPORE FILTERS

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Summary

1. A semi-rapid perfusion technique is used for the study of ATP-dependent Ca uptake, Ca release and ATPase activity in sarcoplasmic reticulum fragments. It involves the deposition of the biological material on millipore filters through which solutions containing the substrate, cofactors and/or inhibitors are perfused. The results show well-known properties of isolated sarcoplasmic reticulum vesicles, Ca uptake calls for ATP and Mg, and is greatly enhanced by oxalate and P_i.

2. Ca uptake and ATPase activity are independent of the perfusion flow rate.

3. The method allows the detection of Ca uptake in less than a second, using Ca concentrations within the sarcoplasmic range.

4. While no differences are detected between ATPase activity measured by our or other common techniques, ATP-dependent Ca uptake is systematically lower with the perfusion method.

5. The addition of EGTA causes a decrease of ATPase activity and a sudden release of Ca. Very fast Ca release is also observed upon Ca depletion from the perfusing solutions.

6. Very fast Ca release occurs upon stopping the Ca pumping mechanism, either by subtraction of ATP or Mg from the perfusing solution. The rate of Ca release is no so fast when Ca uptake proceeds in the presence of oxalate or inorganic phosphate. After Ca release, ATP elicits Ca uptake again.

7. ^{45}Ca taken up in the presence of ATP is rapidly exchanged with ^{40}Ca, at a

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Abbreviation: EGTA, ethyleneglycol tetraacetic acid; CDTA *trans*-1,2-diaminocyclohexane tetraacetic acid.

constant Ca concentration. Very fast ^{45}Ca release is also observed when Ca concentration is raised from 5 μM to 5 mM. It comprises all ^{45}Ca taken up in the presence of ATP. Lower amounts of ^{45}Ca are released when the perfusing Ca concentration is raised for 5 to 8 μM. Under these conditions, ^{45}Ca is probably exchanged with ^{40}Ca.

8. Microsomes loaded with Ca in their suspension medium, release Ca at a very low rate when deposited on millipore filters and perfused with an ATP-containing solution.

9. The rapid rate of Ca release reported in this article may serve as a model of the rapid Ca release from sarcoplasmic reticulum, in vivo, which triggers muscle contraction, since it occurs by effect of physiological agents.

Introduction

The contractile mechanisms of muscle fibers is activated by the entrance of Ca in the myofibrilar space. In mammalian skeletal muscle most of this Ca is released from the sarcoplasmic reticulum in response to the depolarization of the transverse tubules, by a not well established mechanism [1—3].

Vesicular fragments of sarcoplasmic reticulum take up Ca from their suspension medium, in the presence of ATP and Mg [4,5]. It is generally agreed that this experimental fact has its counterpart in vivo when intact sarcoplasmic reticulum triggers relaxation taking up Ca from the sarcoplasm [1,6]. This fact has prompted researchers to use the same preparation as a model for the study of Ca release from intact sarcoplasmic reticulum, which elicits muscle contraction.

Ca uptake by sarcoplasmic reticulum vesicles is usually measured by the disappearance of Ca from the suspension medium. Sarcoplasmic reticulum fragments incubated under specific conditions, usually becomes separated from the medium, where Ca concentration is measured. The separation of the microsomes has been achieved either by ultracentrifugation [5,7,8] or by filtration of the incubation mixture through a suitable filter [9]. With the last method, short reactions must be sharply cut. Alternatively, continuous records of Ca^{2+} concentration through the incubation period have been achieved, making use of the Ca^{2+} indicator murexide [10], or with the aid of a Ca^{2+}-sensitive electrode [11].

A frequent experimental design for the study of Ca release from sarcoplasmic reticulum fragments promoted ATP-dependent Ca uptake for further determination of the effect that several agents had on Ca release from the vesicles. Tests have included the Ca depletion of the extravesicular medium by addition of EDTA or EGTA [12,13], the study of the effect of ATP depletion after long incubation periods or repeated washes [5,14], the replacement of K^+ by Na^+ [15,16], the modification of the ionic strength of the media [14], the addition of ADP and P_i to reverse the Ca pump [17], the attempt to change the sarcoplasmic reticulum membrane potential with ions of different permeability [18], the application of electric pulses [19,20] and the addition of nonphysiological agents like lanthanum, detergents or Ca ionophores [21].

Most results seem to indicate that Ca taken up in the presence of ATP is not readily exchangeable.

No fast Ca release, as it should occur in vivo, has been obtained in vitro by the effect of agents likely to be physiologically significant.

This paper describes the determination of Ca uptake and ATPase activity of sarcoplasmic reticulum fragments with an original technique based upon the continuous flow of a suitable medium through preparations previously deposited on millipore filters. This method allowed us to detect fast Ca release from Ca loaded sarcoplasmic reticulum vesicles avoiding the use of nonphysiological agents.

Methods

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared by differential ultracentrifugation, essentially by the procedure described by several authors [4,8,9]. The vesicles were finally suspended in 5 mM histidine and 125 mM KCl. The hind leg muscles from Wistar rats provided the material.

All the experiments were performed less than 5 h after the isolation of the sarcoplasmic reticulum fragments.

Determination of Ca uptake and ATPase activity by continuous perfusion of sarcoplasmic reticulum fragments deposited on millipore filters

An aliquot of the sarcoplasmic reticulum suspension containing 0.5—2.0 mg of protein, was filtered through a millipore filter (0.22 μm average pore size and 13 mm diameter). The filter with sarcoplasmic reticulum fragments was placed inside a filter-holder connected to teflon tubing (2 m long and 0.47 mm internal diameter) through which test solutions perfused the preparation, being collected at the exit of the filter-holder [22].

Solutions passing through the preparation deposited on the filter were impelled at constant flow by a syringe pump. The composition of the test solutions is indicated under "results" for each case. ^{45}Ca was used as a Ca tracer.

Any difference in Ca concentration between the perfusing solution and the effluent was attributed to retention or release of Ca by the microsomal preparation deposited on the filter.

Usual experiments involve the successive perfusion of two different solutions. The solutions run along the teflon tubing separated by a small drop of metallic mercury (2—4 μl), to avoid mixing. Mercury does not pass through the filter. Ca retention or release by the sarcoplasmic reticulum vesicles may occur when a perfusing solution is replaced by the following one, and it is thus related to the different composition of the solutions.

In order to differentiate the time at which a given solution replaces a previous one, tritiated water is added to one of the solutions.

In most experiments the effluent solution directly dropped into counting vials containing a liquid scintillation cocktail. ^{45}Ca and ^{3}H double-labeled samples were simultaneously measured at different channel windows, in a Beckman LS 200 B liquid sintillation counter.

Samples for determining ATPase activity were collected from test solutions,

after passing through the sarcoplasmic reticulum vesicles held by the filter. Inorganic phosphate (P_i) concentration was measured on these samples, and taken as an index of ATP hydrolysis. P_i concentration was determined by the method of Baginski et al. [23]. Results are expressed in μmol $P_i \cdot mg^{-1}$ protein $\cdot$ h^{-1}. Final values may be arrived at knowing the perfusion flow rate and the amount of microsomal protein deposited on the filter.

Further details about the perfusing technique are given in a previous paper [22].

Determination of Ca uptake and ATPase activity in sarcoplasmic reticulum fragments by other methods

Ca uptake and ATPase activity of sarcoplasmic reticulum fragments were also determined by methods commonly used by other authors. The microsomal preparations were incubated at 37°C in different media. The reactions were stopped by filtration through a millipore filter (0.22 μm average pore) at given times after the addition of the microsomes. ^{45}Ca and P_i were measured in the ultrafiltrate as described above.

Results thus obtained are only shown for comparison with those obtained by the perfusion technique.

The sarcoplasmic reticulum preparation shows the general properties described by other authors, when determinations are performed under the same conditions.

Determination of protein concentration

Protein concentration in the microsomal suspensions were determined by the method of Lowry et al. [24].

Materials

^{45}Ca (chloride salt) was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. Nucleotides were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. All other reagents were analytical grade, and deionized water was used for all the experiments.

Results

ATP-dependent Ca uptake by sarcoplasmic reticulum fragments deposited on millipore filters

Fig. 1 shows the results of four experiments which demonstrate ATP-dependent Ca uptake by sarcoplasmic reticulum fragments deposited on millipore filters.

Two solutions (1 and 2) were successively perfused through the system, in four experiments. Solution 2 contained 5 μM $^{45}CaCl_2$ and ^3H_2O, in addition to the components of solution 1. Experiment A was run without microsomes deposited on the filter, while the other experiments were performed with microsomes on the filters, as described under Methods. The solutions either contained ATP (A and D), or ADP (C) or no nucleotides (B).

Samples were successively collected at the exit of the filter-holder, and ^{45}Ca and ^3H were determined and expressed as percentages of the radioactivity in

solution 2. Upon replacement of solution 1 by solution 2, radioactivity appears in the effluent. The rate of radioactivity increase, in the absence of sarcoplasmic reticulum fragments on the filter (Fig. 1A), was similar for both isotopes, and the relation between ^{45}Ca and ^{3}H percentage concentrations was constant during the experimental period. This implies that no compartments with different specific activities of the isotopes, nor different distribution spaces of calcium and water could be detected in the perfusion system. Ca retention by any structure of the system or Ca exchange with other ions, eventually adsorbed to the system, was not detected. The delay for reaching the concentration of the second solution is attributed to the flow pattern within the system, which determines the mixing rate of both solutions. Traces of contaminating Ca in the millipore filters [25] could not be detected under our experimental conditions (Fig. 1A).

Experiments with sarcoplasmic reticulum fragments on the filter, without nucleotides in the perfusing solutions (Fig. 2B), show lower relative ^{45}Ca than ^{3}H amounts in the effluent, upon replacement of solution 1 by solution 2

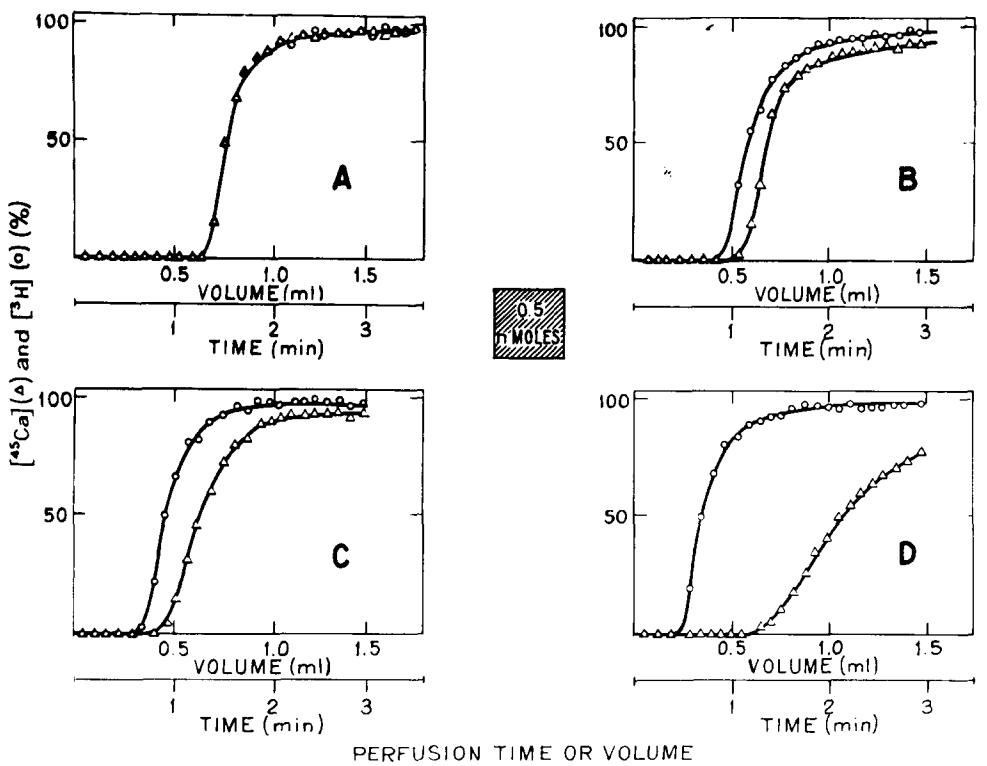


Fig. 1. Ca uptake by microsomes deposited on millipore filters. Successive perfusion of solutions 1 and 2, in four experiments (A, B, C and D), through sarcoplasmic reticulum fragments deposited on millipore filters as described under Methods. Solution 1 was Ca free and contained no radioisotopes. Solution 2 contained 5 μM $^{45}CaCl_2$ and $^{3}H_2O$. Both solutions 1 and 2 contained 5 mM $MgCl_2$, 100 mM KCl, 45 mM Tris · Cl (pH 7.4) and the following additions: A and D contained 4.2 mM Tris · ATP, C contained 4.2 mM Tris · ADP and B contained no nucleotides. A: No microsomes on the filter. B, C and D: 0.81 mg of microsomal protein were deposited on the filters. The perfusion flow was 0.5 ml/min, at 37°C. Samples were collected as detailed under Methods. The same microsomal preparation was used for all experiments.

implying that the perfusing Ca becomes bound to the microsomes.

The addition of ATP to the perfusing solutions leads to a great increase of Ca binding by the sarcoplasmic reticulum preparation (Fig. 2D). A steady state level was not reached within the experimental period. The difference between ^{3}H and ^{45}Ca percentages decreases with time, indicating a progressive saturation of the binding sites. The amount of Ca bound may be computed from the area limited by the ^{3}H and ^{45}Ca curves, comparing it with the area of the insert square.

The small amount of Ca binding induced by ADP (Fig. 2C) has been attributed to the presence of myokinase [5].

After a suitable microsomal perfusion time with a solution containing ^{45}Ca and no ATP, as in Fig. 2B, ^{45}Ca concentration in the effluent reaches a similar value as in the perfusing solution. A steady state is then achieved between membrane-bound Ca and that of the perfusion medium. Further introduction of an ATP containing solution allowed the detection of the ATP-dependent Ca uptake (Fig. 2).

The successive perfusion of two solutions (1 and 2) with similar ^{45}Ca specific activity and similar Ca concentration is shown in Fig. 2. Solution 2 contained ATP and $^{3}H_2O$, in addition to the components of solution 1. Ca uptake by the sarcoplasmic reticulum vesicles is observed as a downward deflection of the graph, upon the appearance of ATP. Its value can be quantified from the area limited by the experimental points and the horizontal line which represents Ca concentration in the perfusing solutions.

The effluent Ca is expressed by its molar concentration. It was calculated by

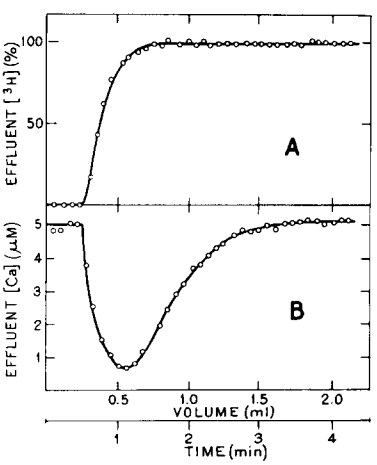


Fig. 2. ATP-dependent Ca uptake by micosomes deposited on millipore filters. Sarcoplasmic reticulum fragments deposited on a millipore filter, were successively perfused with two solutions: Solution 1: 100 mM KCl, 5 mM $MgCl_2$, 5 μM $^{45}CaCl_2$ and 45 mM Tris · Cl (pH 7.4). Solution 2: Same as 1 plus 4.9 mM Tris · ATP and $^{3}H_2O$. Perfusion was carried out at 0.5 ml/min and 37°C. 1.75 mg of protein were deposited on the filter. The collection of the samples and the determinations of radioactivity were performed as described under Methods. A: Time course of ^{3}H concentration. Values are expressed as percentages of ^{3}H concentration in solution 2. B: Time course of ^{45}Ca concentration. Values are expressed as molar Ca concentration, calculated from the ^{45}Ca concentration of the perfusing solutions. Both perfusing times and volumes, are indicated on the abscissa.

assuming similar ^{45}Ca specific activity in the effluent and in the perfusing solutions.

Furthermore, radioactivity determinations in aliquots of the solutions to be perfused enabled us to:

(1) Assure that the ^{45}Ca specific activity and Ca concentration in both solutions are equal.

(2) Calculate the volume of the samples, by comparing the ^{45}Ca activity in them with that of the samples obtained during the steady state period of perfusion with solution 1 (The first four points, in Fig. 2).

(3) Calculate the sampling time, from the volume of each sample and the flow rate, which was experimentally determined.

(4) Calculate ^{3}H activity in the effluent, which makes it necessary to know the ^{45}Ca channel ratio in ^{3}H-free samples.

In other experiments solution 1 contained ADP at similar concentration as ATP in solution 2, maintaining all the other conditions as in Fig. 2. Similar results to those shown in Fig. 2 were obtained (data no shown).

Several control experiments, were performed.

(a) Solutions 1 and 2 were successively perfused through filters without sarcoplasmic reticulum fragments.

(b) Solutions 1 and 2, independently, were continuously perfused through sarcoplasmic reticulum fragments separated in two fractions, with and without $^{3}H_2O$. The separation was achieved with a drop of metallic mercury, as in all other experiments. In both (a) and (b) the ^{45}Ca concentration in the effluent suffered no variation following the change of the perfusing solutions, in any of these cases (data not shown).

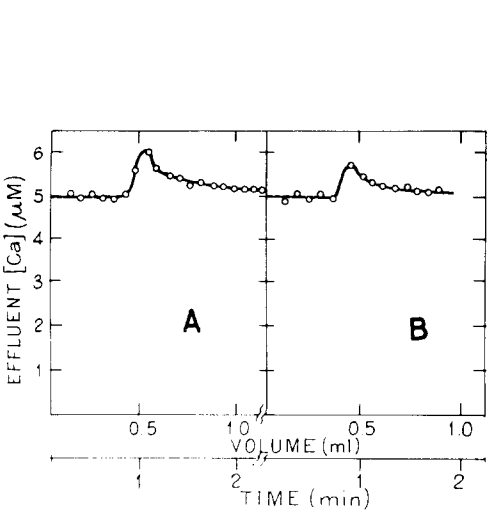
(c) The drop of metallic mercury was avoided. Either an air bubble was used to separate the solutions or no separation at all was attempted. In both cases, downward deflections of the graphs, limiting similar areas as in Fig. 2, were observed. The use of an air bubble determined variations in the sample volume at the time of changing solution 1 by solution 2, the points being scattered. When no separation between the solutions was attempted, smoother ^{3}H and ^{45}Ca deflections were observed (data not shown).

(d) Metallic mercury was also introduced in test solutions for determination of Ca uptake by sarcoplasmic reticulum fragments suspended in their incubation media. No effect of mercury on Ca uptake could be detected (data not shown).

Tritium concentration is not shown in later figures. It must be understood that ^{3}H curves are similar to those in Figs. 1 and 2.

Figs. 2 and 3A show similar experiments, except that Mg is absent from both perfusing solutions in Fig. 3A. ATP appearance does not elicit Ca uptake, as in Fig. 2. Instead, a small amount of Ca is released (Fig. 3A). This is adscribed to the formation of a Ca-ATP complex in solution 2, which lowers the concentration of free Ca in the perfusing medium. A new steady state level is thus attained between perfusing Ca and exchangeable Ca (or other cations) passively bound to the membrane.

Fig. 3B shows an experiment without ATP in both perfusing solutions. Mg is introduced in solution 2 after perfusion with a Mg-free solution. Again, we observed the release of a small amount of Ca which is now attributed to a Ca-



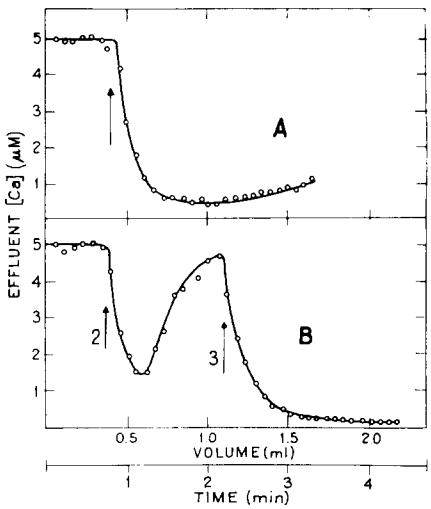


Fig. 3. Ca is not taken up in the absence of Mg (A) or ATP (B). Two solutions (1 and 2) were successively perfused through sarcoplasmic reticulum fragments deposited on millipore filters in two experiments (A and B). Solution 2 was labeled with ^3H_2O. Tritium activity is not shown in the figure; its appearance in the effluent is marked by the arrows, the curves being similar to those shown in Figs. 1 and 2. Solution 1 contained 100 mM KCl, 45 mM Tris · Cl (pH 7.4) and 5 μM ^{45}Ca Cl_2. Solution 2 contained, in addition 4.2 mM Tris · ATP (A) or 5 mM $MgCl_2$ (B). The same microsomal preparation was used in both experiments, with 0.66 mg of protein deposited on the filters. All the other conditions were as for Fig. 2.

Fig. 4. Effect of oxalate on Ca uptake by microsomes deposited on millipore filters. A: Two solutions (1 and 2) were successively perfused through sarcoplasmic reticulum fragments deposited on a millipore filter. Conditions are similar to those in Fig. 2 except for the addition of 5 mM potassium oxalate to both perfusing solutions. Microsomal protein deposited on the filter totaled 1.08 mg. The arrow shows the replacement of solution 1 by solution 2. B: Three solutions (1, 2 and 3) were successively perfused through 0.54 mg of microsomal protein deposited on a millipore filter. Solutions 1 and 2 were as in Fig. 2. The downward deflection detected upon introduction of solution 2 (arrow 2) represents Ca uptake under similar conditions as those shown in Fig. 2. Solution 3 (introduced at arrow 3) contained 5 mM potassium oxalate, in addition to the components of solution 2.

Mg exchange, at nonspecific Ca binding sites. Nonspecific Ca binding sites have been identified in sarcoplasmic reticulum, and differentiated from Ca binding specific sites [26,27].

When conditions are similar to those shown in Fig. 3B, except for the addition of ATP to both solutions, Ca uptake, is observed, as in Fig. 2, when the Mg-containing solution reaches the preparation (data not shown).

Oxalate and P_i greatly increase ATP-dependent Ca uptake by sarcoplasmic reticulum vesicles [1,6]. In Fig. 4A two solutions containing 5 mM potassium oxalate, successively perfused the microsomal preparation deposited on a filter. The other components, as well as the experimental conditions, were similar to those from Fig. 2. The experiment show increased Ca uptake, when compared with data obtained in the absence of oxalate.

In Fig. 4B sarcoplasmic reticulum fragments were successively perfused with three solutions. The first two solutions were as in Fig. 2, while the third solution contained 5 mM potassium oxalate, in addition to the components of solution 2. Ca uptake in the absence of oxalate was observed upon replacement of solution 1 by solution 2, and further uptake was immediately observed upon replacement of solution 2 by solution 3, when oxalate reaches the microsomes.

Similar results were obtained with 10 mM KH_2PO_4 (data not shown).

Ca uptake by sarcoplasmic reticulum vesicles deposited on millipore filters was determined at different flow rates, under the experimental conditions shown in Fig. 2. Within 0.3—1.2 ml/min flow rate limits, no difference was found in the amount of Ca uptake. A similar pattern was observed for the effluent ^{45}Ca concentration curves at different perfusing flow rates, when plotted against the perfused volumes (data not shown).

We experimented with a ten times higher flow rate (Fig. 5). The results are compared with those obtained in a parallel experiment at a lower flow rate. The effluent ^{3}H concentrations of the high flow rate experiment are also shown.

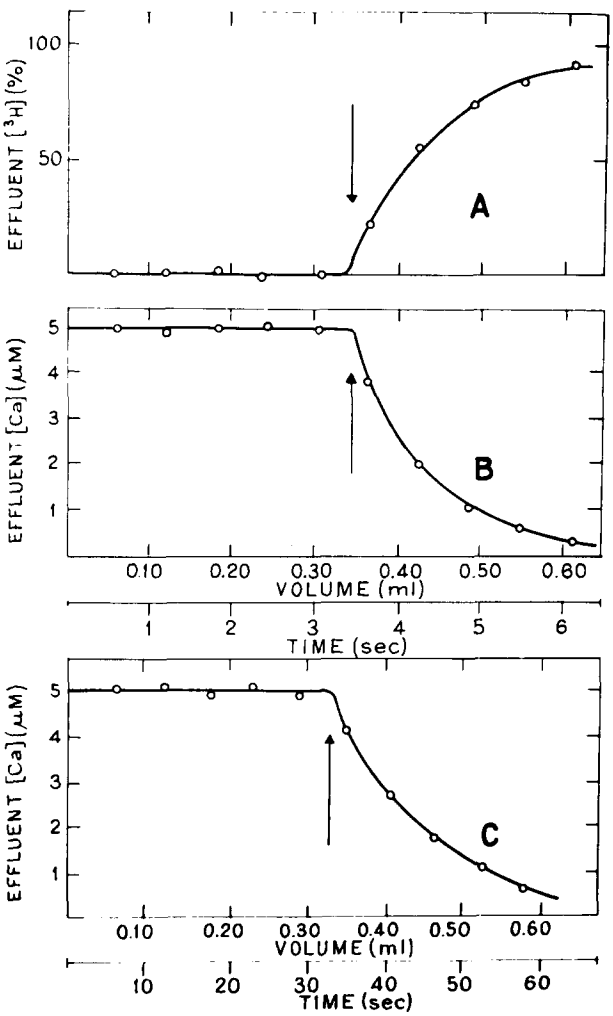


Fig. 5. Detection of ATP-dependent Ca uptake by rapid perfusion of test solutions through sarcoplasmic reticulum fragments deposited on millipore filters. Two solutions were successively perfused at 6.0 ml/min (A, B) and 0.6 ml/min (C). A: Effluent ^{3}H concentration at high perfusion rate. B and C: Effluent ^{45}Ca concentration at high and low perfusion rates, respectively. Microsomal protein on the filters totaled 0.93 mg. ATP concentration in the second solutions was 4.2 mM. All the other conditions were as for Fig. 2. The same microsomal preparation was used for both experiments.

Ca concentration curves (Figs. 5B and 5C) are similar, indicating that the uptake rate is fast enough to preclude the detection of differences between the downward slopes. The pattern of the curve, in the high flow rate experiment, is also determined by the mixing characteristics of the system.

Smooth curves passing through the ^{3}H and ^{45}Ca experimental points, in the high flow rate experiment, cut the horizontal lines within 0.1 s, indicating the high velocity at which Ca uptake begins, after introduction of ATP. Measurable Ca amounts are taken up in less than 1 s (Fig. 5B).

ATP-dependent Ca uptake is higher when sarcoplasmic reticulum fragments are incubated in their suspension media than when they are deposited on millipore filters. When the microsomes were suspended in media, similar to solution 2 from Fig. 2, 50% Ca was taken up in 1 min, which averaged 14 ± 2 nmol of Ca per mg of microsomal protein (n : 5), while Ca uptake determined by the perfusion technique was only 2.33 ± 0.26 nmol of Ca per mg of protein.

In Fig. 6 sarcoplasmic reticulum fragments were incubated in a suspension medium containing ATP and Mg. After 15 min, an aliquot of this microsomal suspension, solution 2, was pumped at constant flow through the perfusion system. It was preceeded by solution 1 and followed by solution 3, which were similar to the original suspension medium.

The effluent Ca concentration decreases upon introduction of solution 2, due to Ca uptake by the vesicles, which are retained by the filter. Solution 3 raises Ca concentration to the original level and goes slightly above it, indicating a slow Ca release from the sarcoplasmic reticulum fragments.

The vesicles deposited on the filter were unable to retain Ca taken up from their suspension medium, despite the fact that both the original suspension and the perfusing media, are similar. This results is coherent with the dissimilar ATP-dependent Ca uptake by sarcoplasmic reticulum fragments when deposited on millipore filters or incubated in their medium.

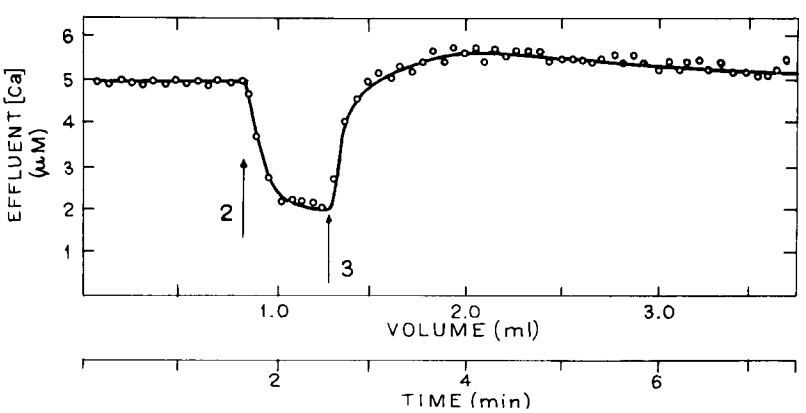


Fig. 6. Ca retention capacity of vesicles loaded in their suspension medium, when deposited on millipore filters. 0.15 mg of microsomal protein per ml were incubated at 37°C in a medium containing 45 mM Tris · Cl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 0.005 mM $^{45}CaCl_2$ and 3.8 mM Tris · ATP. A 0.5 ml aliquot (solution 2) was perfused at constant flow through a millipore filter after a 15 min incubation. Two solutions (1 and 3) similar to the initial incubation medium, preceeded and followed solution 2. Two other aliquots from the same incubation mixture, taken before and after the perfusion experiment, were tested for Ca uptake by filtration through a millipore filter. Radioactivity was analyzed in the ultrafiltrate. Uptake of total Ca by the suspended microsomes was 72% at 10 min and 92% at 25 min.

ATPase activity studied with the perfusion technique

ATPase activity was determined on sarcoplasmic reticulum fragments deposited on millipore filters, by analyzing P_i concentrations in the effluents. No significant differences were detected between determinations of ATPase activity by this or by conventional techniques (Table I). This indicates a similar exposure of the membranes to ATP with either technique.

In the ATPase test using the perfusion technique, the concentration of P_i in the effluent depends upon the flow rate (Fig. 7). As equilateral hyperbola can be fitted to the experimental points, an inverse relationship between flow rates and P_i concentrations becomes evident. This shows that in the perfusion technique, the flow pattern through the sarcoplasmic reticulum preparation is not modified by its rate within the experimental range.

The higher ATP hydrolysis at the lower flow rate is ascribed to the longer contact between the perfusing solution and the membranes deposited on the filters. Under these conditions, the high percentage of ATP hydrolysis observed, qualitatively indicates the relative absence of preferential channels through the system. As a consequence, we may speak of topographic homogeneity of all the lines of flow passing through the sarcoplasmic reticulum preparation as regards the potential hydrolysis of ATP transported by them.

Fig. 8 shows the successive perfusion of three solutions. The effluent samples were analyzed, alternatively, for ^{45}Ca (Fig. 8A) and P_i (Fig. 8B). The first two solutions were as in Fig. 2. The third solution contains, in addition to the components of the second, 0.1 mM EGTA.

Solution 2 elicits Ca uptake, and P_i appears in the effluent. P_i increased to a steady state level, reproducing the pattern of the ^3H curves (Figs. 1 and 2). This gradual increase is ascribed to the mixing process of the solutions inside the system rather than to a delayed reaction. Once the maximal concentration was reached, it maintained a constant level. In contrast, the net Ca uptake is a transient process.

TABLE I

ATPase ACTIVITY OF SARCOPLASMIC RETICULUM FRAGMENTS

Suspension: Sarcoplasmic reticulum fragments were incubated in a bath, at 37°C, for 5 min. Reactions were started by addition of the microsomal suspension to the media and stopped by filtration through a millipore filter (0.22 μm average pore). P_i was analyzed in the ultrafiltrate. Microsomal protein concentration was 0.18 ± 0.02 mg/ml.

Perfusion: Sarcoplasmic reticulum fragments were deposited on millipore filters and perfused with the test solutions. Samples were collected in the effluent for P_i analysis, after a suitable perfusion period with the ATP-containing solution, so that a steady state ATP concentration could be achieved. Perfusions were at 0.5 ml/min. Microsomal protein deposited on the filters was 0.90 ± 0.10 mg. Both incubation and perfusing media contained (mM): KCl 100, $MgCl_2$ 5, Tris · Cl (pH 7.4) 45, and Tris · ATP 4.5, plus $CaCl_2$ 0.005 or EGTA 0.1. Results are the average obtained from 5 microsomal preparations, with indication of the standard error of the mean.

	ATPase activity (μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$)	
	Suspension	Perfusion
With Ca	69.0 ± 4.3	61.0 ± 3.7
Without Ca	43.4 ± 4.5	31.6 ± 3.6

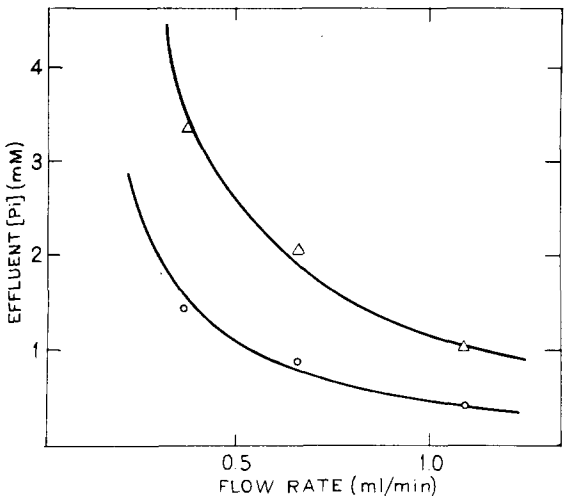


Fig. 7. ATPase activity determined with the perfusion technique at different flow rate. 1.06 mg of microsomal protein deposited on millipore filters were perfused at the flow rates indicated in the abscissa, with solutions containing 100 mM KCl, 45 mM Tris · Cl (pH 8.4), 5 mM $MgCl_2$ and 4.2 mM Tris · ATP, plus 5 μM $CaCl_2$ (△) or 0.1 mM EDTA (○). Effluent samples were collected after a suitable perfusion period for P_i analysis. Flow rates were experimentally determined.

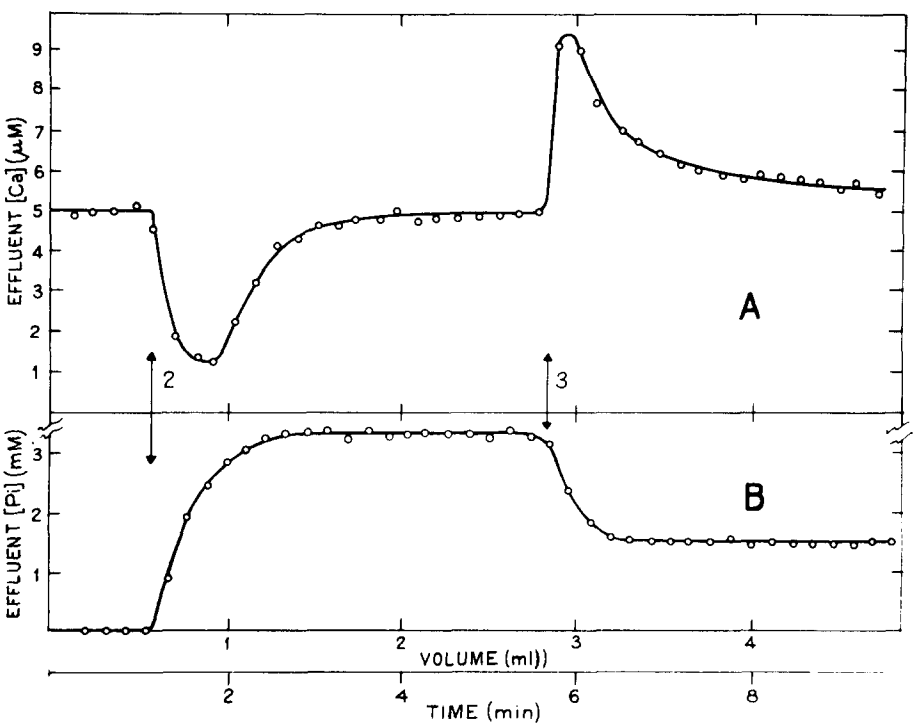


Fig. 8. Effect of EGTA on Ca uptake and ATPase activity in microsomes deposited on millipore filters. ^{45}Ca and P_i effluent concentrations of three solutions (1,2 and 3) passed through sarcoplasmic reticulum fragments deposited on millipore filters. The solutions contained 100 mM KCl, 5 mM $MgCl_2$, 5 μM $^{45}CaCl_2$ and 45 mM Tris · Cl (pH 7.4) plus the following additions 3.8 mM Tris-ATP in solutions 2 and 3, ^3H_2O in solution 2 and 0.1 mM EGTA in solution 3. The arrows indicate the appearance of solutions 2 and 3 in the effluent. The collected samples were alternatively assayed for ^{45}Ca and P_i. Microsomal protein on the filter amounted to 0.70 mg. All the other conditions were as for Fig. 2.

Further introduction of EGTA (solution 3) determines the decrease of P_i concentration in the effluent and a fast Ca release. The estimated amount released is of the same order of the ATP-dependent Ca uptake detected during the first phase of the experiment. The abrupt rise of the curve suggests that the detection of the velocity of Ca release is limited by the mixing time of the solutions inside the system.

Stopping the Ca pump causes Ca release

The effect of interrupting the catalytic reaction which energizes Ca uptake by sarcoplasmic reticulum, was studied by sudden depletion of ATP or Mg from the perfusing solutions. Fig. 9 shows the results of two experiments in which three solutions were successively perfused through sarcoplasmic reticulum fragments deposited on millipore filters. Solution 2 contains ATP in addi-

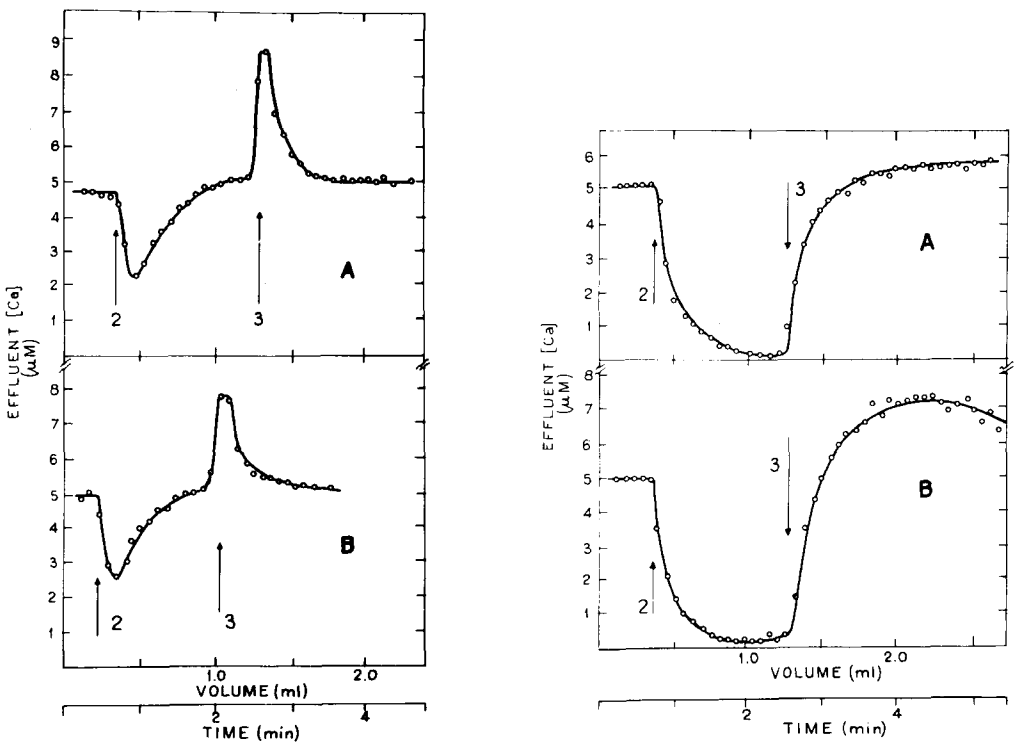


Fig. 9. Ca release from microsomes deposited on millipore filters upon ATP or Mg depletion in the perfusing media. Three solutions (1, 2 and 3) were successively perfused through sarcoplasmic reticulum fragments deposited on millipore filters, in two experiments (A and B). Solution 1 contained: 45 mM Tris · Cl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl and 0.005 mM $^{45}CaCl_2$. Solution 2: same as 1 plus 4.9 mM Tris · ATP. Solution 3: Same as 2 but without ATP (A) or without $MgCl_2$ (B). Solution 2 was labeled with ^3H_2O. Arrows indicate the arrival of solutions 2 and 3 at the filter. Microsomal protein deposited on the filters was 0.84 mg (A) and 0.42 mg (B)

Fig. 10. Ca release from sarcoplasmic reticulum vesicles loaded in the presence of oxalate or inorganic phosphate. Three solutions (1, 2 and 3) were successively perfused through sarcoplasmic reticulum fragments deposited on millipore filters, in two experiments (A and B). Solutions 1 and 3 contained: 45 mM Tris · Cl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 0.005 $^{45}CaCl_2$ and 5 mM potassium oxalate (A) or 10 mM K_2HPO_4 (B). Solution 2 also contained 3.7 mM Tris · ATP. 1.08 mg of microsomal protein were deposited on the filter in experiment A and 0.61 in experiment B.

tion to the components of solution 1. Solution 3 was similar to solution 2, either without ATP (Fig. 9A) or without Mg^{2+} (Fig. 9B).

Ca release is observed when solution 3 reaches the preparation. The amount of Ca released is similar to that previously taken up in the ATP-dependent reactions.

The rate of Ca release is quite high. The detection of this rate is limited by the mechanical characteristics of the system, as previously discussed for Ca uptake. When the preparation was perfused with a fourth, ATP-containing solution, Ca uptake was observed again, indicating that the functional state of the vesicles had not been affected by the previous uptake and release (data not shown).

Fig. 10 shows Ca release elicited by sudden depletion of ATP, either after Ca uptake in the presence of oxalate (Fig. 10A) or P_i (Fig. 10B). Three solutions successively perfused sarcoplasmic reticulum fragments deposited on the filters. Solution 2 contain ATP, while solutions 1 and 3 do not.

Ca uptake is elicited by solution 2. Upon perfusion with solution 3, uptake ceases immediately and Ca is released from the vesicles.

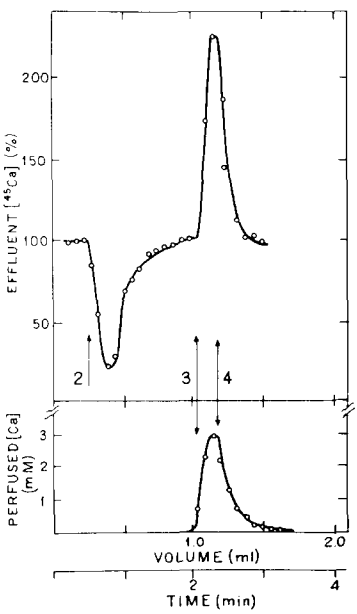
Under these conditions the rate of Ca release is lower than in Fig. 9. It is higher in the experiment with P_i (Fig. 10B) than in the presence of oxalate (Fig. 10A). This may be attributed to the different K_d values for calcium phosphate and calcium oxalate complexes, which would make the concentration of intravesicular free Ca different, in both cases.

Effect of modifications in the perfused Ca concentration on ^{45}Ca retention by sarcoplasmic reticulum fragments

Fig. 11 shows the effect of increasing the perfusing cold-Ca concentration. A highly concentrated solution of cold Ca was introduced briefly after an ATP-dependent Ca uptake. This caused a sudden decrease of the perfusing ^{45}Ca specific activity. The effluent ^{45}Ca concentration is shown in Fig. 11A, while the introduction of the brief pulse of unlabelled Ca in the perfusing solution is shown in Fig. 11B. ^{45}Ca was suddenly released from the microsomes. More ^{45}Ca was released than had previously been taken by effect of ATP, presumably because ATP-independent ^{45}Ca uptake was involved. The rate of ^{45}Ca release is very high.

It has been suggested [2] that Ca release from sarcoplasmic reticulum in vivo could be triggered, by means of a regenerative process, following a small increase in intracellular Ca concentration. The effect of a slight increase in the perfusing Ca was tested, as shown in Fig. 12. Non-radioactive-Ca introduced in the perfusing solution slightly lowered ^{45}Ca specific activity (Fig. 12B). ^{45}Ca concentration in the effluent is shown in Fig. 12A. The ^{45}Ca released is now less than the ATP-dependent ^{45}Ca previously uptaken.

Fig. 13 shows three experiments where Ca was eliminated from the perfusing solutions. In Fig. 13A no microsomes were deposited on the filter. A Ca- and ^{3}H-free solution (solution 2) was introduced after the concentration of the isotopes in the effluent equalled that of solution 1. Upon introduction of solution 2, the radioactivity decrease in the effluent is identical for both isotopes, indicating that tritium and Ca were not selectively accumulated by any structure of the system.



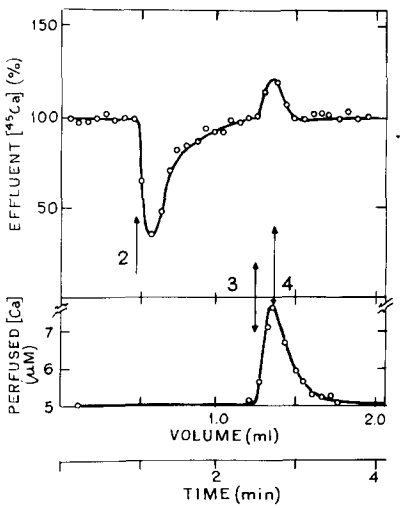


Fig. 11. ^{45}Ca release by effect of increasing the perfusing Ca concentration. The upper graph shows effluent ^{45}Ca activity after perfusion of 1.10 mg of microsomal protein deposited on a millipore filter with four solutions (1, 2, 3 and 4). Solution 1 contained 45 mM Tris · Cl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl and 0.005 mM $^{45}CaCl_2$. Solutions 2 and 4 also contained 4.1 mM Tris · ATP. Solution 3: same as 2 and 4, plus 5 mM $CaCl_2$ and ^3H_2O. Ca concentration reaching the microsomal preparation, as calculated by determination of ^3H activity, is shown below.

Fig. 12. ^{45}Ca release by effect of a very slight increase in the perfusing Ca concentration. Conditions are as in Fig. 11, except that solution 3 contained 0.010 mM $CaCl_2$. ^{45}Ca activity in solutions 1, 2, 3 and 4 was identical. 0.76 mg of microsomal protein were deposited on the filter. Tris · ATP in solutions 2, 3 and 4 was 3.7 mM.

When sarcoplasmic reticulum fragments are deposited on the filter and perfused with solutions without ATP (Fig. 13B), the percentual amount of Ca remains higher than the ^3H values after the introduction of the solution 2. This result reflects the release of ATP-independent Ca previously bound to the microsomes. This type of bound Ca is thus freely exchangeable with other cations from the perfusing solutions.

In Fig. 13C ATP-dependent Ca uptake had taken place previously to washing away ^3H and Ca. The disappearance of ^3H and Ca from the effluent is indicated by highly different curves. The area limited by the ^3H and Ca downward curves is similar to that representing ATP-dependent Ca uptake. This area reflects the release of Ca previously taken up by the microsomes.

Identical results to those in Fig. 13 were obtained when all solutions contained Ca at similar concentration (5 μM), the last one being ^{45}Ca-free. This experiment shows that the ATP-dependent Ca taken up is rapidly exchanged by perfusing Ca at constant concentration. A continuous Ca exchange takes place while the Ca pump remains fully activated during the steady state.

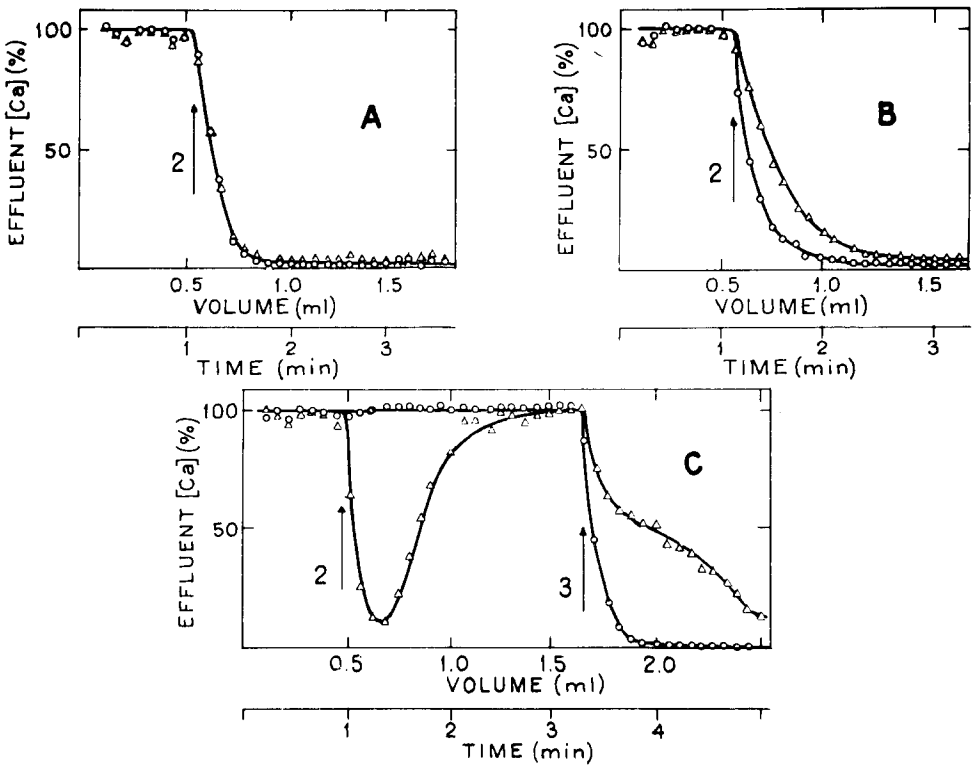


Fig. 13. ^{45}Ca release upon washing away total Ca or ^{45}Ca from the perfusing solution. Two solutions (1 and 2) were successively perfused through the system in experiments A and B. A: No microsomes were deposited on the filter. B: 0.72 mg of microsomal protein were deposited on the filter. Solutions 1 contained 45 mM Tris · Cl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 0.005 mM $^{45}CaCl_2$ and ^3H_2O. Solutions 2 were calcium and tritium free. In experiment C three solutions (1, 2 and 3) were perfused through similar amounts of the same microsomal preparation as in experiment B. Solution 1 was as in experiments A and B. Solution 2 also contained 3.8 mM Tris · ATP. Solution 3: Same as solution 2 but without ^3H_2O and $^{45}CaCl_2$. In a parallel experiment (not shown) all solutions contained similar amounts of $CaCl_2$ (0.005 mM). A tracer amount of $^{45}CaCl_2$ was added to solution 1 in experiments A and B and solutions 1 and 2 in experiment C. The results did not differ from those shown in the figure. (○), [^{3}H]; (△), [^{45}Ca].

Discussion

Isolated fragments of sarcoplasmic reticulum deposited on millipore filters take up Ca from perfusing solutions containing ATP and Mg. Simultaneously, P_i is released from ATP in a Ca-dependent reaction. The results must be analyzed in the light of some characteristics of the method which differentiates it in several aspects from others traditionally used for similar purposes:

(1) The method make the use of controls other than the previous state of the same microsomal population unnecessary for the study of a variable.

(2) It is possible to introduce or subtract any reactant without affecting the concentration of the others.

(3) The method replaces Ca-buffer or ATP-regenerating systems, as the concentrations of the reactants is constant in the perfusing solution. Determinations of Ca uptake from very low Ca concentration media are possible without

extravesicular Ca depletion. ADP and P_i resulting from ATP hydrolysis are also constant, in contrast with the depletion of ATP and the accumulation of ADP and P_i which progressively occurs when the microsomes are suspended in the incubation medium.

(4) Sarcoplasmic reticulum preparations contain Ca and Mg traces which might take part in the reactions [28]. The effect of contaminating cations is minimized, since free contaminating ions are washed away by the previous perfusion. Firmly bound cations are to be considered structural components of the membranes, not playing any role in the reactions under study [29]. Such contaminating cations, if involved in the reactions, would be washed away after a reaction cycle, disappearing from the medium.

(5) The method allows relatively fast sampling. Ca uptake has been followed during very short periods using the Ca^{2+} indicator murexide [10,30]. Although our time resolution is far from that of the murexide method, we could measure Ca uptake in less than 1 s (Fig. 5) using Ca concentrations within the sarcoplasmic range. On the other hand, high Ca concentrations (10^{-4} M) are required by murexide for detection [1,30].

ATPase activity and Ca-dependent/Ca-independent ATPase activity ratio are similar when tested by the perfusion technique or by methods commonly used by other authors (Table I). The perfusion rate does not affect ATPase activity (Fig. 7). It is concluded that the perfusion technique does not introduce any factor which affects ATPase activity, and sites with difficult access for the substrate cannot be detected.

Ca-dependent ATPase activity remains constant after the transient net Ca uptake proceeds (Fig. 8). This observation is at variance with results showing a burst of ATPase activity coincident with the net Ca uptake process. They were interpreted as a late inhibition of the enzyme by high intravesicular Ca concentration [21,31].

ATP-dependent Ca uptake can be demonstrated in sarcoplasmic reticulum fragments deposited on millipore filters. The results suggest that Ca uptake proceeds as found by other researchers with different techniques. Authors agree that oxalate and phosphate enhance Ca uptake by forming intravesicular complexes, which lower Ca^{2+} concentration, thus allowing the continuity of the transport process. As ATP-dependent Ca uptake detected with the perfusion technique is enhanced by oxalate or P_i, we also conclude that, under our experimental conditions, Ca is transported across the vesicular membrane rather than becoming bound to external sites of the vesicles. Other authors [13,30] arrived at a similar conclusion for the rapid initial phase of Ca uptake.

There is general agreement in that once bound Ca is firmly retained by the sarcoplasmic reticulum vesicles [1,5,12—21]. The results of our experiments dealing with Ca release showed striking differences with those previously reported. We observed fast Ca release upon ATP depletion from the perfusing solutions (Fig. 9A). Carvalho and Leo [14] showed that Ca is retained after ATP depletion from the medium. Ebashi and Lipman [5] found that the release of Ca by the vesicles was slow. We also observed fast Ca release when Mg was washed away from the medium.

In media containing oxalate or P_i, the rate of Ca release upon ATP depletion is lower (Fig. 10). Since the solubility product is lower for calcium oxalate than

for calcium phosphate, the observed rates of Ca release are proportional to the intravesicular free-Ca concentration.

The treatment of membranes with EGTA and CDTA [12.29] increases their permeability for Ca. It may account for the rapid Ca release observed upon addition of EGTA (Fig. 8). However, the sudden omission of Ca from the perfusing solutions has a similar effect (Fig. 13). Then, it is likely that the EGTA effect may be due to quelation of extravesicular Ca which originates the Ca gradient which promotes its release, since the quelator does not penetrate within the vesicles [32]. These results are at variance with those from other authors who found Ca retention upon addition of EGTA, at neutral pH [12].

For the experiments shown in Figs. 8—10, a ^{45}Ca-^{40}Ca exchange reaction must be ruled out, since the microsomes deposited on the filteres were successively perfused with solutions of similar ^{45}Ca specific activity and similar Ca concentration. Thus, in all cases in which the Ca pumping mechanism is abolished, a net loss of Ca from the sarcoplasmic reticulum fragments occurs. This inference is at variance with the lack of Ca release by effect of lanthanum [21], an agent which inhibits Ca-dependent ATPase activity.

The rapid exchangeability of Ca taken up in the presence of ATP is further appreciated in the experiments where ^{45}Ca specific activity of the perfusing solutions was changed (Figs. 11—13). When $^{45}CaCl_2$ is replaced by $^{40}CaCl_2$ at similar concentration (data similar to those in Fig. 13), the vesicles appear to exchange ^{45}Ca by ^{40}Ca while the Ca pumping activity and the Ca gradient across the membrane are held constant. The sudden reduction of ^{45}Ca specific activity by perfusion of a highly concentrated solution of non-radioactive Ca promotes a sudden and transient increase in the effluent ^{45}Ca (Fig. 11), despite the constancy of the perfusing ^{45}Ca concentration. Again, a ^{45}Ca-^{40}Ca exchange probably occurs. A moderate decrease in the perfusing ^{45}Ca specific activity causes the release of lower amounts of ^{45}Ca than those previously taken up in the presence of ATP (Fig. 12). This ^{45}Ca release tends to equilibrate the intravesicular ^{45}Ca specific activity with that in the surrounding medium. This result does not corroborate the suggestion [2] that a small increase in myoplasmic Ca^{2+}, in vivo, triggers Ca release in greater amounts by means of a regenerative process.

An increase in Ca permeability provides the simplest explanation for the decreased Ca uptake associated with fast Ca release and unmodified ATPase activity, observed for microsomes deposited on millipore filters. However, the excess Ca taken up by suspended microsomes is not readily released upon deposition of the Ca loaded vesicles on a filter (Fig. 6). It discards the possibility of a change of the membrane permeability as a consequence of the experimental manipulation. It follows that this fraction of bound Ca is released from a slowly exchangeable Ca pool (Fig. 6), in contrast to the fastly released Ca, which comes from a rapidly exchangeable source (Figs. 8—13). The observed differences in Ca uptake and release can be explained in terms of differences in the physical state of Ca take up by the microsomes, which is not well established: Several arguments favor Ca compartmentalization in suspended sarcoplasmic reticulum vesicles [33—36]. Intravesicular Ca has been postulated to be mostly in free form [6], or bound to anionic sites [1]. Ca-binding proteins have been postulated to be located inside the vesicles [27]. Mermier and Hasselbach [36], using a continuous dyalisis method, have shown evidences in favor of the occur-

rence of Ca uptake in two steps: an initial and fast Ca uptake followed by a slow phase of Ca uptake.

The different conditions in suspended and perfused vesicles might determine differences in the physical state of the retained Ca. Taking into account previous results, it would appear as if the perfusing technique could only detect the rapid phase of the uptake process, but not the slow one following it. The fast Ca release indicates a rapidly exchangeable form of Ca storage. It may be intravesicular free Ca. The retention of Ca as a free cation is also supported by the decreased rate of release observed in the presence of oxalate or P_i. Our results can be explained by assuming that the membrane permeability for Ca allows the rapid release or exchange of intravesicular free Ca, which concentration is maintained by the constant activity of the Ca pump.

Ca release from sarcoplasmic reticulum fragments must be very rapid to allow its correlation with the fast Ca release occurring in vivo. The results presented in this article, as an effect of stopping the Ca pump, fulfil this requirement. The suggestion [1] that Ca release in response to a single depolarization, in vivo, is an all-or-none phenomenon, provides support to introduce the hypothesis that Ca released to the myoplasm by a single depolarization pulse may be that intravesicularly free and rapidly exchangeable.

Acknowledgments

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