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UTILIZATION OF X-537A TO DISTINGUISH BETWEEN INTRAVESICULAR AND MEMBRANE-BOUND CALCIUM IONS IN SARCOPLASMIC RETICULUM

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SUMMARY

The Ca^{2+} ionophore X-537A is employed as a tool to distinguish between intravesicular Ca^{2+} and surface membrane-bound Ca^{2+} in sarcoplasmic reticulum isolated from rabbit skeletal muscle. When sarcoplasmic reticulum is incubated in 20 mM Ca^{2+} in the absence of ATP, 10–12 h are necessary for measurable amounts of Ca^{2+} to penetrate into the vesicular space, as determined by the fact that X-537A releases Ca^{2+} from 'loaded' vesicles only after this period of incubation. A fraction of Ca^{2+} of 50–60 nmol/mg protein, rapidly taken up by sarcoplasmic reticulum, exchanges with Mg^{2+} and K^+ in the medium and is readily released by ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid, but it is not released by X-537A. The slow-penetrating fraction of Ca^{2+} (30–40 nmol/mg protein) is rapidly released by X-537A. The results indicate that most of the Ca^{2+} retained by sarcoplasmic reticulum under conditions of passive uptake is bound to the external side of the membrane. The fraction of Ca^{2+} that slowly penetrates the vesicles remains essentially free inside the vesicles and only a small part is bound to the internal side of the membrane.

INTRODUCTION

Sarcoplasmic reticulum isolated from rabbit skeletal muscle transports Ca^{2+} activily in the presence of ATP [1–5] and during Ca^{2+} efflux the ATPase system may be reversed so that ATP synthesis takes place [6–11]. It has also been reported that ATP synthesis coupled to Ca^{2+} efflux occurs after sarcoplasmic reticulum is passively loaded with Ca^{2+} [12–13], and it was implied that Ca^{2+} liberated from passively loaded sarcoplasmic reticulum is largely intravesicular.

In view of the evidence that the sarcoplasmic reticulum membranes have a high density of charge [14–16] and, therefore, a high Ca^{2+}-binding capacity, and that a Ca^{2+} gradient is not essential for phosphoprotein formation [17, 18] in sarco-

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

plasmic reticulum, it appeared to be of interest to differentiate between the amount of Ca^{2+} retained passively in sarcoplasmic reticulum which is bound externally and the amount which is retained intravesicularly. Differentiation between these two fractions of Ca^{2+} was possible in the experiments reported here since the Ca^{2+} ionophore X-537A can be utilized to release intravesicular Ca^{2+} [19], whereas ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA) rapidly releases externally bound Ca^{2+}, and releases the intravesicular Ca^{2+} only very slowly.

The results show that a large fraction of the Ca^{2+} retained by sarcoplasmic reticulum under conditions of passive uptake is bound to the external side of the membrane. The Ca^{2+} which slowly reaches the intravesicular region remains essentially free inside the vesicles and only a small part is bound to the internal side of the membrane. In a subsequent paper, we will report the relationship between ATP synthesis by sarcoplasmic reticulum and the release of intravesicular and membrane-bound Ca^{2+}.

METHODS

Isolation of sarcoplasmic reticulum. The sarcoplasmic reticulum vesicles were obtained by homogenization and differential centrifugation of rabbit white skeletal muscle in a medium containing 0.1 M KCl and 10 mM Tris/maleate at a pH value of 7.0, according to a method previously described [20]. To remove the contaminating actomyosin, the sarcoplasmic reticulum at a concentration of about 1 mg/ml was washed once with a solution containing 0.6 M KCl and 10 mM Tris/maleate at a pH value of 7.0. Finally, the sarcoplasmic reticulum was resuspended in the isolation medium (0.1 M KCl and 10 mM Tris/maleate at a pH value of 7.0 and stored at O °C).

Passive uptake and binding of Ca^{2+} by isolated sarcoplasmic reticulum. The incubations were carried out as described in legends of the figures. The sarcoplasmic reticulum vesicles were removed from the suspending medium by the Millipore filtration technique [21].

Ca^{2+} efflux promoted by the ionophore X-537A. At various incubation times, as described in legends of the figures, 20 μM of the ionophore X-537A was added and the protein was removed by the filtration method at several time intervals.

In all cases, after the filtration of aliquots containing 0.5 mg of protein, the filters (Millipore HA 0.45 μm) were washed twice by filtering each time 1.0 ml of 0.25 M sucrose. The filters were finally immersed in 2.5 ml of a solution containing 2 % trichloroacetic acid and 0.5 % La^{3+}, and after vigorous agitation, Ca^{2+} and Mg^{2+} analyses were performed in this solution by atomic absorption spectroscopy in a Perkin-Elmer spectrophotometer, Model 305. The protein was measured by the biuret method using bovine serum albumin as a standard.

Reagents. All reagents were of analytical grade. The ionophore X-537A was a generous gift of Dr Julius Berger of Hoffman-La Roche, Nutley, New Jersey 07110, U.S.A.

RESULTS

Effect of the ionophore X-537A on the passive retention of Ca^{2+} by sarcoplasmic reticulum

Under the experimental conditions reported here, it is shown that Ca^{2+} can exist free inside the vesicles as well as bound to the internal and external sides of the membranes. Fig. 1 represents the kinetics of the Ca^{2+} uptake by sarcoplasmic reticulum when it is incubated in media containing 20 mM $CaCl_2$, 100 mM KCl and 2 mM $MgCl_2$ buffered at pH 6.9 with 20 mM Tris/maleate. After various periods of incubation, the reaction mixture was diluted 20-fold with the reaction medium free of Ca^{2+}, so that the final Ca^{2+} concentration was 1 mM.

After a rapid initial uptake, there is a gradual increase of the Ca^{2+} taken up by the vesicles, which reaches a plateau at approx. 14 h of incubation. The uptake of Ca^{2+} can be subdivided into two distinct phases. The first phase, representing the rapid uptake (50 nmol Ca^{2+}/mg protein), probably corresponds to external surface binding of Ca^{2+}. Preliminary experiments showed that the amount of Ca^{2+} rapidly taken up is higher (100 nmol/mg protein) if the incubation medium is diluted with 0.25 M sucrose instead with the Ca^{2+}-free reaction medium. This difference probably reflects displacement of Ca^{2+} by the cations (Mg^{2+} and K^+) of the medium. None of this Ca^{2+} fraction is released by X-537A (Fig. 1). After the first hour of incubation, a second phase of Ca^{2+} uptake begins which is complete at about 12–14 h. During this

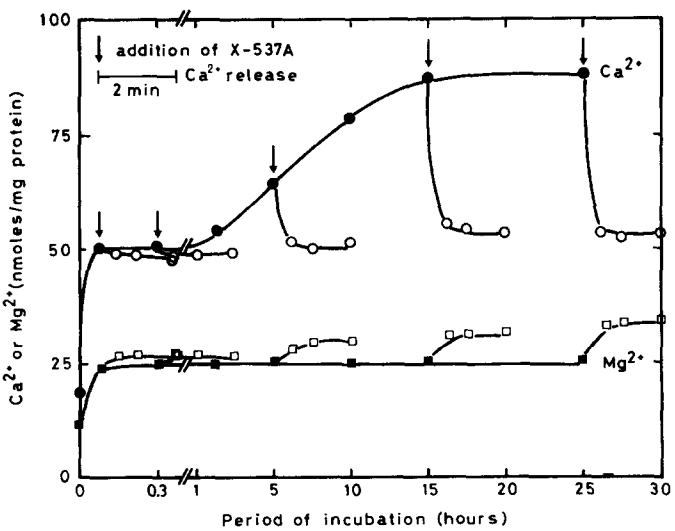

Fig. 1. Passive Ca^{2+} uptake by sarcoplasmic reticulum vesicles. The reaction medium contained 20 mM Tris maleate (pH 6.9), 100 mM KCl, 2 mM $MgCl_2$, 20 mM $CaCl_2$ and 60 mg sarcoplasmic reticulum in a total volume of 6.0 ml. After various periods of incubation at 3 °C, aliquots of 0.5 ml were diluted 20-fold with Ca^{2+}-free reaction medium at room temperature (25 °C), and 0.5 mg of protein was removed by Millipore filtration at 30 s after dilution. To the remaining medium was added X-537A to a final concentration of 20 μM and 0.5 mg of protein were removed at 0.5, 1 and 2 min. Ca^{2+} (●) and Mg^{2+} (■) retained by sarcoplasmic reticulum after dilution with Ca^{2+}-free reaction medium. Ca^{2+} (○) and Mg^{2+} (□) retained by sarcoplasmic reticulum after addition of X-537A.

second phase of Ca^{2+} uptake, about 30–40 nmol/mg protein are taken up and this Ca^{2+} can all be released by X-537A, which suggests that this fraction of Ca^{2+} diffused out across the membrane.

Since the intrinsic Ca^{2+} of sarcoplasmic reticulum is about 20 nmol/mg protein, we conclude that an additional 30 nmol Ca^{2+}/mg protein remain bound to the external side of the membranes when they were washed with standard medium and that of the 100 nmol Ca^{2+}/mg protein retained when the vesicles are washed with 0.25 M sucrose, 50 nmol are lost during the wash with standard medium in exchange for Mg^{2+} and K^{+}.

The difference between the amount of Ca^{2+} retained during the first and second phases represents the amount of Ca^{2+} that enters the vesicles gradually (30–40 nmol Ca^{2+}/mg protein), and is not easily accessible from the outside unless X-537A is present. These results are in accord with the observation that the sarcoplasmic reticulum membrane is relatively impermeable to Ca^{2+} [4, 22]. Furthermore, we observed that the loaded vesicles have a very slow passive Ca^{2+} efflux when they are transferred to a low-Ca^{2+} medium.

The Mg^{2+} curve of Fig. 1 shows that the Mg^{2} content of the vesicles (about 25 nmol/mg protein) is increased when Ca^{2+} is released by the ionophore X-537A. A cation exchange occurring simultaneously with the Ca^{2+} efflux promoted by the ionophore is illustrated in Fig. 2, in which Ca^{2+} loading of sarcoplasmic reticulum was carried out in absence and in the presence of KCl in the reaction medium. In the

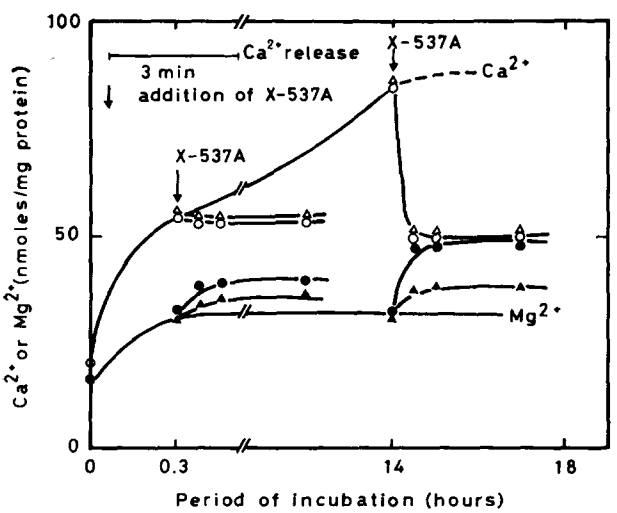

Fig. 2. Release of Ca^{2+} induced by X-537A from passively loaded sarcoplasmic reticulum vesicles in the presence and in the absence of 100 mM KCl. Reaction medium contained 20 mM Tris/maleate (pH 6.9), 100 mM KCl (if present), 2 mM $MgCl_2$, 20 mM $CaCl_2$ and 30 mg protein in a total volume of 3.0 ml. After 20 min or 14 h of incubation at 3 °C, the reaction medium was diluted 20-fold with the Ca^{2+}-free reaction medium at room temperature (25 °C), and an aliquot containing 0.5 mg protein was removed by Millipore filtration at 30 s after dilution. To the remaining medium was added X-537A to a final concentration of 20 μM, and 0.5 mg protein were removed at 0.5, 1 and 3 min. Ca^{2+} (△) and Mg^{2+} (▲) retained by sarcoplasmic reticulum vesicles in the presence of KCl in the reaction medium. Ca^{2+} (○) and Mg^{2+} (●) retained by sarcoplasmic reticulum vesicles in the absence of KCl.

absence of KCl, the penetration of Mg^{2+} into the vesicles, occurring concurrently with the Ca^{2+} efflux promoted by X-537A, is higher than in presence of KCl (about 20 nmol Mg^{2+}/mg protein against 10 nmol Mg^{2+}/mg protein, respectively). Thus, these results indicate that both Mg^{2+} and K^+ can exchange with Ca^{2+}.

Distinction between Ca^{2+} binding to the external and internal sides of the sarcoplasmic reticulum membrane

The ionophore X-537A increases the sarcoplasmic reticulum membrane permeability to Ca^{2+} so that the intravesicular Ca^{2+} concentration equilibrates rapidly with the extravesicular Ca^{2+} concentration after addition of X-537A. In Fig. 3, the sarcoplasmic reticulum vesicles were previously loaded passively with 20 mM Ca^{2+} for 14 h, after which the medium was diluted 20-fold with Ca^{2+}-free medium. Subsequent addition of X-537A released the Ca^{2+} trapped inside the vesicles, which corresponds to about 30 nmol Ca^{2+}/mg protein. The Ca^{2+} retained by the sarcoplasmic reticulum in the presence of X-537A represents essentially Ca^{2+} bound to the external and to the internal Ca^{2+}-binding sites of the membrane in equilibrium with 1.0 mM external Ca^{2+}. This bound Ca^{2+} can be released by EGTA and represents about 50–60 nmol Ca^{2+}/mg protein (Fig. 3).

The results depicted in Fig. 3 also allow some conclusion regarding the Ca^{2+} binding to the external side of the membrane, if we assume that all Ca^{2+} released by EGTA in the absence of X-537A (control, Fig. 3) is externally bound Ca^{2+}. The

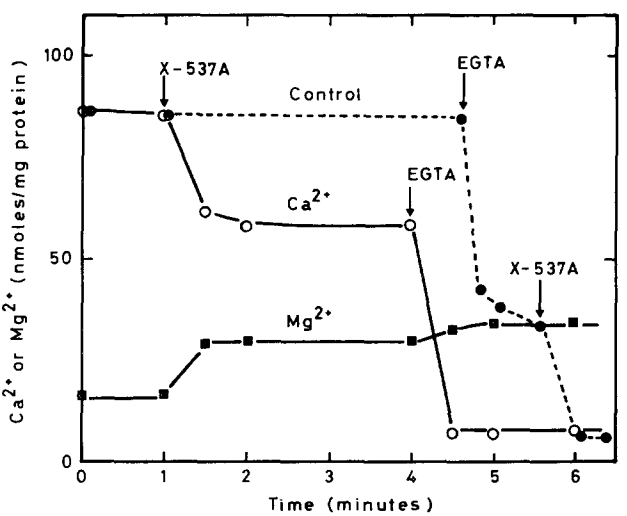

Fig. 3. Release of specific Ca^{2+} fractions from sarcoplasmic reticulum by X-537A and EGTA. Sarcoplasmic reticulum (10 mg/ml) was incubated 14 h at 3 °C in a medium containing 20 mM Tris/maleate (pH 6.9), 100 mM KCl, 2 mM $MgCl_2$ and 20 mM $CaCl_2$. After the incubation period, the mixture was diluted 20-fold with the Ca^{2+}-free reaction medium at room temperature (25 °C) and additions of X-537A (20 μM) followed by EGTA (5 mM) or vice-versa, were made. Before and after addition of these substances, aliquots containing 0.5 mg protein were taken and were filtered through Millipore filters. Ca^{2+} (○) and Mg^{2+} (■) retained by sarcoplasmic reticulum before and after addition of first X-537A and then EGTA. Ca^{2+} (●) retained by sarcoplasmic reticulum after the addition of first EGTA and then X-537A.

value for this fraction of Ca^{2+} is shown in Fig. 3 to be about 50 nmol Ca^{2+}/mg protein. Addition of X-537A after the external Ca^{2+} was liberated by EGTA caused further release of 30 nmol Ca^{2+} from the intravesicular region. This fraction of Ca^{2+} probably includes both Ca^{2+} that was free inside the vesicles and some intravesicular bound Ca^{2+} which exists in equilibrium with the free intravesicular Ca^{2+}.

Therefore it is concluded that, under the conditions of these experiments, the external bound Ca^{2+} is about twice as high as the total intravesicular Ca^{2+}. This conclusion is further supported by Fig. 4, which depicts the results of experiments where sarcoplasmic reticulum (5 mg) was incubated either 10 min or 14 h in the usual medium containing 20 mM $CaCl_2$ in the absence or in the presence of 200 μM X-537A in a total volume of 0.5 ml. After incubation, the medium was diluted 20-fold with 0.25 M sucrose at a pH value of 7.0. Dilution with sucrose assures that the bound cations are not lost during the dilution procedure and permits us to obtain a more accurate distribution of the binding sites.

It is shown in Fig. 4A that the amount of Ca^{2+} retained by the membranes is higher (110 nmol/mg protein) in the presence of X-537A than in its absence (95 nmol/mg protein) for short periods of incubation (10 min). The amount of Ca^{2+} retained in the presence of X-537A in excess of the amount retained in its absence after short incubation periods (about 15 nmol Ca^{2+}/mg protein) represents the intravesicular Ca^{2+} fraction, which probably is bound, since free intravesicular Ca^{2+} diffused out during the dilution. After longer periods of incubation (Fig. 4B), Ca^{2+}

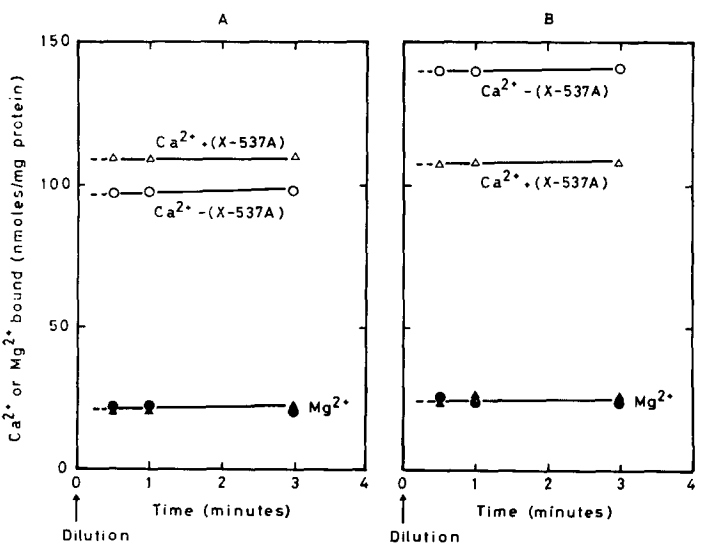

Fig. 4. Passive uptake of Ca^{2+} by sarcoplasmic reticulum in the presence and in the absence of X-537A. Reaction medium contained 20 mM Tris/maleate (pH 6.9), 100 mM KCl, 2 mM $MgCl_2$, 20 mM $CaCl_2$, and 5 mg protein in a total volume of 0.5 ml, either in the presence or in the absence of 200 μM X-537A. After 10 min (A) or 14 h (B) of incubation at 3 °C, the medium was diluted 20-fold with 0.25 M sucrose (pH 7.0) at room temperature (25 °C) and the protein (0.5 mg) was removed by Millipore filtration at 0.5, 1 and 3 min. Ca^{2+} (△) and Mg^{2+} (▲) retained by sarcoplasmic reticulum vesicles in presence of X-537A. Ca^{2+} (○) and Mg^{2+} (●) retained by sarcoplasmic reticulum in absence of X-537A.

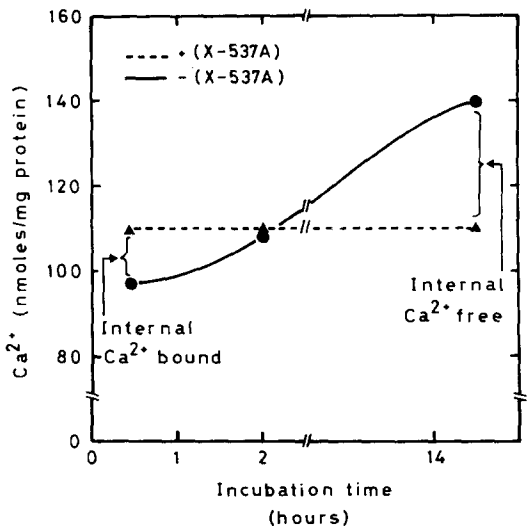

Fig. 5. Evidence for the free and bound fractions of intravesicular Ca^{2+} in passively loaded sarcoplasmic reticulum. This graph is constracted with the data obtained in experiments similar to those described in legend of Fig. 4. Ca^{2+} (●) retained by sarcoplasmic reticulum in absence of X-537A. Ca^{2+} (▲) retained by sarcoplasmic reticulum in the presence of X-537A.

will have penetrated the vesicles and remains there after dilution in the absence of X-537A. In this case the amount of Ca^{2+} retained, which is higher than the values obtained in the presence of the ionophore, represents the fraction of free Ca^{2+} inside the vesicles.

This distinction between the internal Ca^{2+} bound (15 nmol/mg protein) and the internal Ca^{2+} free (25–30 nmol/mg protein) is evident in Fig. 5. The total Mg^{2+} retained in both conditions was about 20 nmol Mg^{2+}/mg protein. The Ca^{2+} concentration in the reaction medium was much higher (20 mM) than the Mg^{2+} concentration (2mM), which probably accounts for the low Mg^{2+} binding.

Passive uptake and binding of Ca^{2+} by sarcoplasmic reticulum vesicles at low external Ca^{2+} concentration in presence of phosphate

In this series, experiments were performed with sarcoplasmic reticulum under conditions which have been utilized by some workers to load sarcoplasmic reticulum vesicles passively with Ca^{2+} before measuring ATP synthesis during Ca^{2+} efflux [12]. The medium utilized for this purpose by Makinose [12] contained 4 mM $CaCl_2$ as well as 5 mM sodium phosphate, which was absent in our previous group of experiments carried out with 20 mM $CaCl_2$.

Fig. 6 shows that, after several incubation periods, when the vesicles were removed by filtration without previous dilution, the amount of Ca^{2+} retained by the sarcoplasmic reticulum increases only slightly with the incubation time, (from 95 nmol Ca^{2+}/mg protein at the beginning of incubation to 110 nmol Ca^{2+}/mg protein at the end of an incubation period of 24 h). Furthermore, the Ca^{2+} retained cannot be released by X-537A, which is not surprising since under these conditions the intra- and extravesicular Ca^{2+} concentration would be equal.

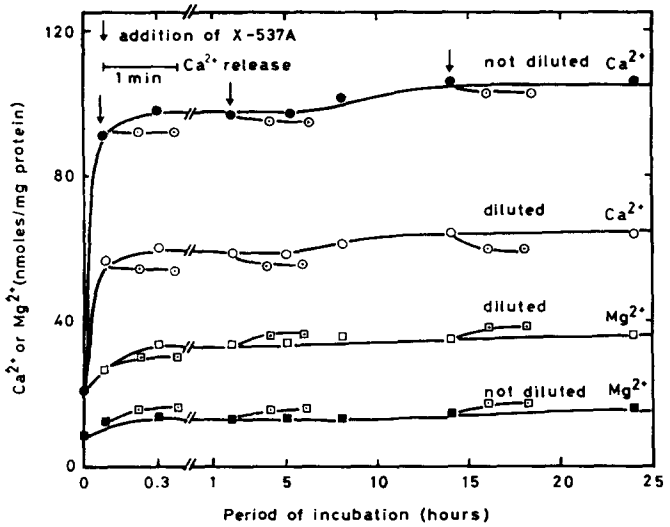

Fig. 6. Passive Ca^{2+} uptake by isolated sarcoplasmic reticulum at low Ca^{2+} in the medium in the presence of phosphate. Reaction medium contained 20 mM Tris/maleate (pH 6.9), 100 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate and 20 mg protein in a total volume of 10 ml. After various periods up to 24 h of incubation at 3 °C, either 0.25 ml of the sarcoploasmic reticulum suspension was directly filtered through Millipore filters, or 1.0 ml was diluted 4-fold with the Ca^{2+}-free reaction medium at room temperature (25 °C), after which samples containing 0.5 mg protein were filtered at 30 s. In parallel experiments, X-537A was added to an aliquot of reaction medium or to the diluted medium to a final concentration of 20 μM and the protein (0.5 mg) was also removed by filtration at 30 and 60 s. Ca^{2+} (●) and Mg^{2+} (■) retained by sarcoplasmic reticulum without dilution of the reaction medium. Ca^{2+} (○) and Mg^{2+} (□) retained by sarcoplasmic reticulum after dilution with Ca^{2+}-free reaction medium. Ca^{2+} (⊙) and Mg^{2+} (⊡) retained by sarcoplasmic reticulum after addition of X-537A.

In Fig. 6 we further show that when the sarcoplasmic reticulum vesicles previously incubated in 4 mM $CaCl_2$ and 5 mM sodium phosphate are diluted 4-fold with the medium without Ca^{2+}, so that the final external Ca^{2+} concentration was 1 mM as in the experiments of the previous section, the amount of Ca^{2+} retained by the membranes during the incubation (about 95 nmol Ca^{2+}/mg protein) was reduced to 55–60 nmol Ca^{2+}/mg protein owing to the exchange with other cations. Most of this Ca^{2+} is bound, since only a small amount could be liberated by X-537A (Fig. 6). The low content of intravesicular free Ca^{2+} observed in these experiments corresponds to the free Ca^{2+} calculated to diffuse into the sarcoplasmic reticulum (16 nmol/mg protein) at equilibrium in the medium of 4.0 mM $CaCl_2$, assuming a water space of 4 μl/mg protein [22].

These results suggest that under conditions which have been utilized for ATP synthesis by passively loaded sarcoplasmic reticulum, Ca^{2+} retained by sarcoplasmic reticulum vesicles represents mostly Ca^{2+} bound to the external side of the membranes which is not released by the ionophore X-537A. We are currently exploring this aspect of the problem.

DISCUSSION

In this work we report that, in the absence of ATP, periods of incubation of sarcoplasmic reticulum of the order of 10–12 h are necessary for a measurable amount of Ca^{2+} to penetrate into the vesicular space (Fig. 1), as determined from the fact that it is only after this period of incubation that X-537A releases Ca^{2+} from sarcoplasmic reticulum vesicles when they are transferred to a low-Ca^{2+} medium. A fraction of Ca^{2+} taken up rapidly by the sarcoplasmic reticulum membranes exchanges with other cations, but it is not released by X-537A, which indicates that this fraction of Ca^{2+} is bound to the external side of the membrane. On the other hand, the Ca^{2+} which gradually penetrates into the vesicles is rapidly released by X-537A, but not by EGTA.

The fraction of intravesicular Ca^{2+} which can be detected under the experimental conditions corresponds to 30–40 nmol Ca^{2+}/mg protein, whereas the Ca^{2+} bound externally is of the order of 50–60 nmol/mg protein. The total binding capacity of sarcoplasmic reticulum membranes is actually much higher, since some of the binding sites are occupied by Mg^{2+} and K^{+} under the experimental conditions. In fact, several Ca^{2+} binding proteins of high capacity have been identified in the sarcoplasmic reticulum membrane, although it is not clear whether they are located on the external or internal sides of the membrane [23–27]. It is of interest that the rate of release of intravesicular Ca^{2+} by EGTA, in the absence of ADP and phosphate, is sufficiently low to permit studying the effect of X-537A after releasing the externally bound Ca^{2+} with EGTA (Fig. 3). Furthermore, approximately the same result is obtained for the value of Ca^{2+} liberated by X-537A whether it is added before or after EGTA, which suggests that about the same fraction of Ca^{2+} is being mobilized in both cases and that it is not affected by EGTA. This fraction of Ca^{2+}, assumed here to be the intravesicular Ca^{2+}, is about 30–40 nmol Ca^{2+}/mg protein, as was determined in the other types of experiments whose results are summarized in Figs 1, 4 and 5.

It should be kept in mind that the vesicles may aggregate during incubation, which may reduce the effective vesicular volume. In fact, if we assume a water space of 4–5 μl/mg protein [22], we can calculate that 80–100 nmol Ca^{2+}/mg protein should be found inside the sarcoplasmic reticulum vesicles after they are equilibrated with 20 mM Ca^{2+}. The fact that we find only 30–40 nmol of intravesicular Ca^{2+} per mg of protein suggests that either some Ca^{2+} is lost during sucrose washing of the filters or the effective volume of the sarcoplasmic reticulum vesicles is in fact lower than the calculated 4–5 μl/mg protein, which appears to vary under several conditions [22].

Inesi et al. [28] report a value of about 160 nmol Ca^{2+}/mg protein for passive loading of sarcoplasmic reticulum with Ca^{2+}, probably because these workers apparently measured the total ^{45}Ca retained in the Millipore filters after filtration, whereas we washed the filters twice with 0.25 M sucrose to ensure elimination of the contaminating Ca^{2+} of the trapped suspending medium. Yamada et al. [13] also washed the filters twice and they found lower values (25–30 nmol Ca^{2+}/mg protein) for passive Ca^{2+} retention by sarcoplasmic reticulum under similar experimental conditions of incubation. Inesi et al. [28] studied the Ca^{2+} efflux from loaded vesicles after diluting the incubation medium in the presence of EGTA which removes the

Ca^{2+} externally bound. On the other hand, Yamada et al. [13] measured the retention of ^{45}Ca by sarcoplasmic reticulum after diluting the radioactive incubation mixture with a medium containing a large amount of cold Ca^{2+}. Since the sarcoplasmic reticulum membrane is relatively impermeable to Ca^{2+}, probably only the externally bound ^{45}Ca exchanged with the Ca^{2+} of the medium, whereas the internal radioactivity should remain unchanged. Thus, both types of experiments are inadequate to study the fraction of Ca^{2+} bound externally which we report here.

Our results are of particular interest in reference to reported studies of ATP synthesis by sarcoplasmic reticulum vesicles passively loaded with Ca^{2+} because of its release by EGTA in the presence of ADP and orthophosphate [12, 13]. It has been reported [12] that a rectilinear relationship exists between the Ca^{2+} concentration in which the sarcoplasmic reticulum is equilibrated during loading and the amount of ATP synthesis, but the Ca^{2+} fraction released, which is responsible for ATP synthesis, has not been determined, although a priori it appears reasonable to suppose that only the intravesicular Ca^{2+} is responsible for ATP synthesis during its efflux. The present approach utilizing X-537A and EGTA as tools to distinguish between the intravesicular and externally bound Ca^{2+} permits relation of the phenomena of transmembrane Ca^{2+} efflux and ATP synthesis, and is applied in a next paper on Ca^{2+} efflux from sarcoplasmic reticulum coupled to ATP synthesis. It is evident from our studies that a large fraction of the Ca^{2+} retained by sarcoplasmic reticulum under conditions of passive uptake is bound to the external side of the membrane and the intravesicular Ca^{2+} which is released by X-537A is essentially free inside the vesicles. We detected a small capacity of the internal side of the membrane to bind Ca^{2+}, compared with the high capacity of the external side, which is compatible with the localization of the calsequestrin on the outer surface of the membrane [4, 22, 25, 27].

The Ca^{2+} binding capacity of sarcoplasmic reticulum in the presence of ATP may be different from that observed under conditions for passive uptake because conformational changes induced by ATP in sarcoplasmic reticulum , demonstrated by spin label techniques [29, 30], may alter the affinity of the internal binding sites of the membrane for Ca^{2+}.

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