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DIFFERENTIATION BETWEEN Ca^{2+} TRANSPORT AND ATP-INDUCED Ca^{2+} BINDING BY SARCOPLASMIC RETICULUM *

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Summary

The Ca^{2+} actively accumulated by sarcoplasmic reticulum isolated from skeletal muscle is composed of two fractions; one represented by intravesicular free Ca^{2+} and another represented by Ca^{2+} selectively bound to the membranes. Both of these Ca^{2+} fractions depend on ATP, although it is not clear whether ATP hydrolysis is essential for accumulation of the second Ca^{2+} fraction. The existence of the membrane-bound Ca^{2+} induced by ATP is clearly shown in experiments in which the Ca^{2+} retention by sarcoplasmic reticulum is measured in the presence and in the absence of X-537A, a Ca^{2+} ionophore, which makes the membrane permeable to Ca^{2+}. Thus, in the presence of X-537A all Ca^{2+} accumulated due to ATP is bound to the membranes. This membrane-bound Ca^{2+} represents about 30 nmol/mg protein in the range of external p*Ca* values of 7 to 3.5. The magnitude of this Ca^{2+} fraction is slightly higher whether or not the experiments are performed in the presence of oxalate, which greatly increased the intravesicular Ca^{2+} accumulation. Furthermore, taking advantage of the impermeability of sarcoplasmic reticulum to EGTA, it is possible to show the existence of the membrane-bound Ca^{2+} as a distinct fraction from that which exists intravesicularly.

Introduction

The capacity of the sarcoplasmic reticulum to accumulate Ca^{2+} is the main event responsible for controlling muscle contraction [1,2]. However, the state

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

of the Ca^{2+} actively accumulated by the reticulum is still controversial and several possibilities have been described: (a) the Ca^{2+} is translocated across the membrane and is deposited in a free form inside the vesicles [1,3,4], equilibrating with binding sites [5—7]; (b) the Ca^{2+} ions are selectively bound to the membranes in the presence of ATP [8—14].

Numerous technical problems have made it difficult to discern between Ca^{2+} uptake and Ca^{2+} binding by sarcoplasmic reticulum. In the presence of oxalate, an active transport of Ca^{2+} appears to be evident [3,5,15,16]. The Ca^{2+} is taken up by a process dependent on the $(Mg^{2+} + Ca^{2+})$-ATPase activity and is retained in the form of oxalate crystals which have been identified by electron microscope examinations [17—19]. However, in the absence of precipitating agents it is not clear whether the Ca^{2+} is transported into the vesicles or whether ATP induces selective binding of Ca^{2+} to the membranes.

In this work we measured ATP-dependent Ca^{2+} uptake by sarcoplasmic reticulum under conditions which prevent the accumulation of intravesicular free Ca^{2+} (X-537A present), and we utilized EGTA to distinguish the fraction of Ca^{2+} which is retained at the external side of the membrane.

The results indicate that part of the Ca^{2+} actively taken up by sarcoplasmic reticulum is externally bound to the membranes and part is transported into the vesicles. Thus, selective binding of Ca^{2+} induced by ATP could be differentiated from the Ca^{2+} transport process.

Materials and Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum was isolated from rabbit white skeletal muscle as previously described [20].

Active uptake and binding of Ca^{2+} by isolated sarcoplasmic reticulum

Sarcoplasmic reticulum (0.5 mg/ml) was incubated at 35°C in a medium containing 10 mM Tris-maleate, 5 mM $MgCl_2$, 50 mM KCl, 40 μM ionophore X-537A (if present), 1 mM EGTA, various concentrations of $CaCl_2$ and 2 mM ATP in a total volume of 2.0 ml at the pH value of 7.0. After 60 s of reaction, 0.5 mg of protein was removed from the medium by the Millipore filtration technique [15] and Ca^{2+} analyses were performed in both filters and filtrates as described below. Controlling tests were carried out under the same conditions but in the absence of ATP.

Ca^{2+} release from sarcoplasmic reticulum vesicles promoted by EGTA and X-537A

Active Ca^{2+} uptake by sarcoplasmic reticulum (0.5 mg/ml) was carried out at 35°C in a medium containing 10 mM Tris-maleate, 5 mM $MgCl_2$, 50 mM KCl, 5 mM potassium oxalate (if present), 0.15 mM $CaCl_2$ and 2 mM ATP in a total volume of 4.0 ml at pH 7.0. After 60 s of reaction, 0.5 mg of protein was removed from the medium by Millipore filtration, and the reaction vessel was transferred to one of several water baths equilibrated at several temperatures between 8 and 50°C. The Ca^{2+} release was initiated by adding 3 mM EGTA and, 30 s later, a new aliquot containing 0.5 mg of protein was withdrawn from

the medium and filtered through Millipore filters. In some experiments, the Ca^{2+} release was carried out under the same conditions, but the vesicles were previously loaded with Ca^{2+} at several temperatures, so that different amounts of Ca^{2+} were accumulated by the reticulum for 10 s or 60 s of uptake reaction in the absence or in the presence of oxalate, respectively.

The amount of Ca^{2+} externally bound to the membranes was determined by subtracting the Ca^{2+} retained in the presence of EGTA from that retained in its absence at temperatures under 40°C. At higher temperatures, the intravesicular Ca^{2+} is also released, but below 40°C the rate of release is very slow so that we could thus differentiate between intravesicular Ca^{2+} and Ca^{2+} bound to the outside of the vesicles.

Another group of experiments was performed at 35°C in the absence of oxalate in a medium otherwise as described above. After 60 and 90 s of uptake reaction, 0.5 mg of protein were removed from the medium by the Millipore filtration technique [15]. Then, 40 μM of X-537A was added and after 30 and 60 s new aliquots containing 0.5 mg of protein were removed from the medium and 3 mM EGTA was added. Finally, aliquots of 0.5 mg of protein were also filtered for Ca^{2+} analysis. In parallel experiments, EGTA was added before X-537A. The ionophore X-537A releases the intravesicular Ca^{2+}, whereas EGTA removes predominantly the external Ca^{2+}.

Analysis of Ca^{2+} and of protein

The analysis of Ca^{2+} retained by sarcoplasmic reticulum vesicles was determined by the Millipore filtration technique [15] as described above. The Millipore filters (HA, 0.45 μM) retaining the protein were washed twice by filtering, each time, 1.0 ml of 0.25 M sucrose. Finally, they were immersed in 2.5 ml of a solution containing 2% trichloroacetic acid and 0.5% La^{3+} and, after vigorous agitation, Ca^{2+} was analysed in this solution by measuring absorption in a Perkin Elmer Spectrophotometer, Model 305.

In some experiments, Ca^{2+} analysis in the filtrates was also performed after adjusting concentrations of trichloroacetic acid and La^{3+} as indicated above.

The protein was measured by the biuret method [21] using bovine serum albumin as a standard.

Reagents

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

Results

Selective binding of Ca^{2+} induced by ATP in sarcoplasmic reticulum membranes

As was reported before [11], at certain p*Ca* values ATP promotes binding of Ca^{2+} to sarcoplasmic reticulum membranes in exchange for other cations, so that it has been doubtful whether the Ca^{2+} accumulated in the absence of precipitating agents is due to an active transport into the vesicles or to a selective binding to the membranes.

In this work we tentatively differentiate the existence of these processes in sarcoplasmic reticulum by studying active Ca^{2+} uptake and Ca^{2+} release under various experimental conditions.

Fig. 1 shows that active Ca^{2+} uptake depends on the Ca^{2+} concentrations in the medium reaching maximal values of about 155 nmol/mg protein at the p*Ca* value of 6.2. By using the ionophore X-537A, which increases the Ca^{2+} permeability of the membrane, we studied the effect of ATP on the retention of Ca^{2+} by sarcoplasmic reticulum under conditions which do not permit intravesicular Ca^{2+} accumulation. Indeed, the Ca^{2+} retention of the vesicles was greatly reduced in the presence of the ionophore. However, significant amounts of Ca^{2+} were retained as compared with those observed under the same conditions but in the absence of ATP. This fraction of Ca^{2+} retained in the presence of ATP and X-537A is probably bound to the membranes and represents about 30 nmol/mg protein in the range of external p*Ca* values of 7 to 3.5. It appears therefore that besides an active transport process, ATP promotes selective binding of Ca^{2+} to the sarcoplasmic reticulum membranes.

Differentiation between external Ca^{2+} binding and intravesicular Ca^{2+} accumulation by sarcoplasmic reticulum

Since controversial interpretation has been reported about the state and the localization of the Ca^{2+} actively stored by sarcoplasmic reticulum, it appears of

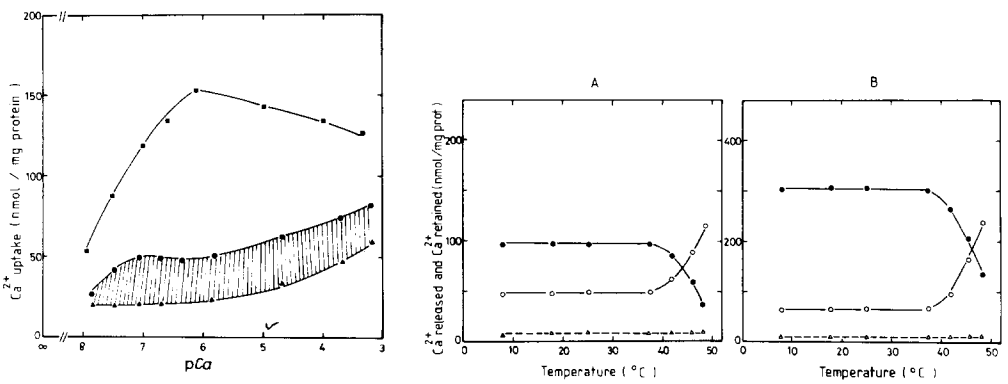

Fig. 1. Effect of p*Ca* on the active Ca^{2+} uptake by sarcoplasmic reticulum. The reticulum vesicles (0.5 mg/ml) were incubated in a medium containing 10 mM Tris-maleate, 5 mM $MgCl_2$, 50 mM KCl, 40 μM of X-537A (if present), 1 mM EGTA and various concentrations of $CaCl_2$ in a total volume of 2 ml at pH 7.0. The uptake reaction was started by adding 2 mM ATP and, 60 s later, 0.5 mg of protein was removed from the medium by the Millipore filtration technique. A control in the absence of ATP was carried out under the same conditions. ■, Ca^{2+} retained in the presence of ATP and in the absence of X-537A; ●, Ca^{2+} retained in the presence of ATP and X-537A; ▲, Ca^{2+} retained in the absence of ATP and in the presence of X-537A. The shaded portion of the graph represents ATP-dependent Ca^{2+} binding.

Fig. 2. Effect of temperature on the EGTA-induced Ca^{2+} release from loaded sarcoplasmic reticulum vesicles. Ca^{2+} uptake by sarcoplasmic reticulum was carried out at 35°C, in the absence (A) or in the presence (B) of oxalate as described in the text. After 60 s of reaction, 1.0 ml aliquot containing 0.5 mg of protein was filtered through Millipore filters and the remaining reaction medium was equilibrated for 2 min at various temperatures. Then, 3 mM EGTA was added and a new aliquot containing 0.5 mg of protein was removed 30 s later and filtered. In control experiments, the reticulum was incubated in the absence of ATP. Ca^{2+} retained (●———●) and Ca^{2+} released (○———○) after EGTA addition to actively loaded vesicles in the presence of ATP. The sum of both fractions represents the total Ca^{2+} previously taken up. (△- - - - -△) Ca^{2+} released by EGTA from unloaded vesicles (in the absence of ATP).

interest to differentiate between the amount of Ca^{2+} which is bound externally to the membranes and the amount which is retained intravesicularly.

Fig. 2 shows that, between 8 and 40°C, the addition of EGTA induces liberation of about 40—45 or 60 nmol Ca^{2+}/mg protein from vesicles preloaded with Ca^{2+} at 35°C in the absence (A) or in the presence (B) of oxalate, respectively. In both cases, the efflux of Ca^{2+} is significantly increased only at temperatures above 40°C, which indicates that at high temperatures, another fraction of the accumulated Ca^{2+} is accessible to EGTA. In contrast, over the range of temperatures studied, only small amounts of Ca^{2+} (about 10 nmol/mg protein) were released by EGTA from reticulum vesicles incubated in the absence of ATP. This is reasonable because, as we observe in Fig. 3, there is little passive binding at the Ca^{2+} concentrations used in these experiments.

Fig. 3 shows that the EGTA-induced Ca^{2+} release does not depend on the amount of Ca^{2+} previously accumulated by sarcoplasmic reticulum. The different loading of the reticulum vesicles was obtained by measuring uptake reaction at various temperatures for 10 s in the absence of oxalate (A) or for 60 s in its presence (B). The amount of Ca^{2+} accumulated for those periods increases with the temperature up to about 37°C. Above 40°C, the Ca^{2+} taken up sharply declines, especially when the reaction is performed in the absence of oxalate. Addition of EGTA after loading the vesicles at the various temperatures causes release of a constant fraction of Ca^{2+}, which indicates that the fraction of Ca^{2+} accessible to EGTA has the same value in spite of differing amounts of Ca^{2+} having been taken up by the vesicles. In the presence of oxalate (Fig. 3B) the EGTA-induced Ca^{2+} release was higher at temperatures above 40°C, which

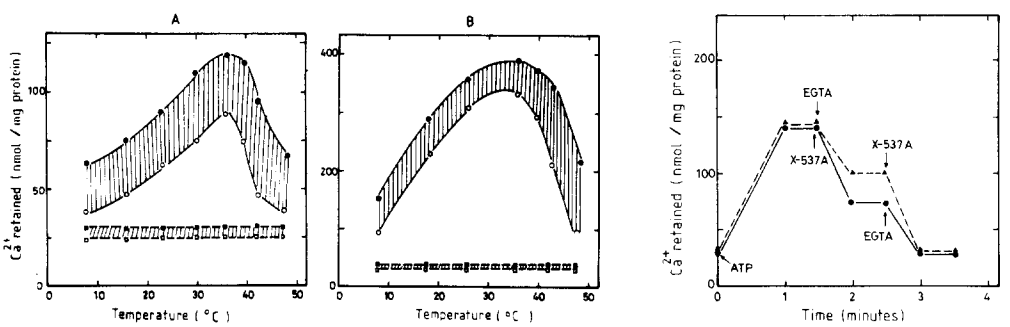

Fig. 3. Effect of temperature on the EGTA-induced Ca^{2+} release from sarcoplasmic reticulum vesicles containing different amounts of Ca^{2+} accumulated. Ca^{2+} uptake by sarcoplasmic reticulum was carried out for 10 s in the absence of oxalate (A) or for 60 s in its presence (B), at various temperatures between 8 and 50°C as described in the text. The analysis of Ca^{2+} release by EGTA was performed as described in Fig. 2. ●, active Ca^{2+} uptake; ○, Ca^{2+} remaining after EGTA addition; ■, passive Ca^{2+} binding; □, intrinsic Ca^{2+}. The shaded portions of the graph represent the Ca^{2+} released by EGTA. Solid line, ATP present; dashed line, ATP absent.

Fig. 4. Release of specific Ca^{2+} fractions from sarcoplasmic reticulum by X-537A and EGTA. The vesicles were actively loaded with Ca^{2+} at 35°C as described in the text. After 60 s of reaction, 40 μM X-537A and 3 mM EGTA, or vice-versa, were added to a final concentration in the medium of 40 μM and 3 mM, respectively. Before and after addition of these compounds, aliquots containing 0.5 mg of protein were taken and were filtered through Millipore filters. ●, Ca^{2+} retained by the reticulum before and after addition of first X-537A and then EGTA. ▲, Ca^{2+} retained by the reticulum after addition of first EGTA and then X-537A.

reflects that, during the uptake reaction at high temperatures, some Ca^{2+} was retained inside the vesicles in the precipitated form. In contrast, at these temperatures no Ca^{2+} appears to be accumulated inside the vesicles in the absence of oxalate and, therefore, only the external Ca^{2+} was released by EGTA (Fig. 3A). These observations indicate that the ATP-dependent Ca^{2+} bound, which is released by 3 mM EGTA, is independent of the total Ca^{2+} accumulated.

The results suggest that the Ca^{2+} taken up by sarcoplasmic reticulum in the presence of ATP is retained in two pools. One which is easily accessible to EGTA and another which is accessible only at high temperatures and probably represents intravesicular Ca^{2+} which flows out due to leakiness of the membranes.

The intravesicular Ca^{2+} fraction can also be released by ionophore X-537A. This ionophore increases the membrane permeability to Ca^{2+} so that intravesicular and extravesicular free Ca^{2+} concentrations equilibrate rapidly [22]. The results depicted in Fig. 4 show that distinct fractions of Ca^{2+} are released by EGTA and X-537A, respectively. The sarcoplasmic reticulum vesicles were actively loaded with Ca^{2+} at 35°C in the absence of oxalate. After uptake, the addition of X-537A (40 μM) released about 70 nmol of Ca^{2+} per mg of protein whereas about 40 nmol/mg protein were further released by addition of EGTA (3 mM). As we observed in preliminary experiments, 40 μM X-537A is enough to support maximal ionophore-mediated Ca^{2+} release.

Results previously reported by other investigators using optical methods [22,23] showed that X-537A completely releases the Ca^{2+} actively accumulated by sarcoplasmic reticulum. However, some retention of Ca^{2+} was detected by isotopic measurements in the presence of 20 μM X-537A [22]. These discrepancies may be due to the lower sensitivity of the optical methods to detect the small fraction of Ca^{2+} selectively bound to the membranes in the presence of ATP.

The results reported here permit us to differentiate between the intravesicular Ca^{2+} and the Ca^{2+} which is externally bound to the membranes. Indeed, addition of X-537A after the external Ca^{2+} was liberated by EGTA (Fig. 4) caused further release of about 70—75 nmol Ca^{2+} from the intravesicular region. This fraction of Ca^{2+} probably includes the Ca^{2+} which is free inside the vesicles and also that which is internally bound to the membranes. Therefore, it is concluded that a significant fraction (30 nmol/mg protein) of the Ca^{2+} actively taken up by sarcoplasmic reticulum in the absence of oxalate is bound to the external surface of the membrane, whereas about 70—75 nmol/mg protein is transported into the vesicles. In the presence of oxalate the amount of intravesicular Ca^{2+} is greatly increased, but the amount of externally bound Ca^{2+} is only slightly higher (Figs. 2 and 3).

Discussion

Several observations previously reported [8—14] indicate that the Ca^{2+} accumulated by isolated sarcoplasmic reticulum is mostly bound to the membranes. However, the bulk of the experimental results has been interpreted as evidence that the Ca^{2+} is actively transported at the expense of ATP, which is hydro-

lysed in the process [1,3,15,16,24]. As an alternative hypothesis it was considered that, probably, Ca^{2+} is first transported and then bound to the internal side of the membranes [5—7].

The results reported here show that besides an active Ca^{2+} transport, ATP induces selective binding of Ca^{2+} to the sarcoplasmic reticulum (Fig. 1). In the presence of X-537A there is no intravesicular Ca^{2+} accumulation but a small fraction of Ca^{2+} is retained by the membranes due to ATP (Fig. 1). These results are in agreement with those of Scarpa et al. [22] who observed marked reduction of the active Ca^{2+} uptake in the presence of X-537A, although a small remaining fraction was detected by the isotopic method but not by the murexide method. Taking advantage of the impermeability of the sarcoplasmic reticulum membrane to EGTA [5], we could differentiate the intravesicular Ca^{2+} accumulated from that externally retained in the presence of ATP. We found that EGTA easily removes the Ca^{2+} which is externally bound to the membranes, whereas the intravesicular Ca^{2+} is only accessible when the membranes are made permeable to Ca^{2+}. When the membranes are incubated at temperatures below 40°C, the EGTA-induced Ca^{2+} release does not depend on the temperature (Fig. 2), which indicates that this release does not involve transmembrane flux of Ca^{2+}. We observed before [25] that the Ca^{2+} efflux is mediated by ADP and this fraction of Ca^{2+}, in turn, is temperature-dependent and is accompanied by ATP synthesis by reversal of the Ca^{2+} pump. In contrast, when the membranes are kept at high temperatures (over 40°C), a larger fraction of Ca^{2+} easily flows out from the vesicles, which suggests that the membranes have become leaky and that the intravesicular Ca^{2+} is released under these conditions. The amount of Ca^{2+} released by EGTA at temperatures below 40°C remains constant even when different amounts of Ca^{2+} have been accumulated during the uptake reaction at various temperatures, which indicates that, indeed, only extravesicular Ca^{2+} is removed from the vesicles by EGTA (Fig. 3). At higher temperatures (over 40°C), the amount of Ca^{2+} taken up decreases, probably because leakiness of the membrane occurs and Ca^{2+} can not be accumulated unless it is precipitated in the presence of oxalate. Furthermore, most of the Ca^{2+} which we found externally bound to the membranes is dependent on the presence of ATP, because in its absence negligible amounts of Ca^{2+} are passively bound by the reticulum at the low Ca^{2+} concentrations used.

These results strongly suggest that Ca^{2+} storage by sarcoplasmic reticulum includes two distinct fractions differentially localized in the vesicles. Indeed, we also observed (Fig. 4) that from the total Ca^{2+} retained by sarcoplasmic reticulum in the absence of oxalate (approx. 145 nmol/mg protein), about 40 nmol were released by EGTA and about 70—75 nmol were released by X-537A. Probably, part of the Ca^{2+} transported into the vesicles is also internally bound to the membranes, since the Ca^{2+} concentration within the vesicles would be sufficient to saturate all binding sites of the internal side of the membrane.

The binding capacity of the reticulum membranes has been extensively studied by several investigators and it has been shown that, under passive conditions, both sides of the membrane bind Ca^{2+}, although with different capacities and affinities [20,26]. However, as we observed here, passive Ca^{2+} binding is negligible at the Ca^{2+} concentrations needed to obtain maximal active uptake of Ca^{2+} by sarcoplasmic reticulum. Therefore, most of the Ca^{2+} which we

found bound to the external side of the membranes (Figs. 2, 3 and 4) is dependent on the presence of ATP. Carvalho and Leo [11] suggested that ATP increases the affinity of the membrane binding sites for Ca^{2+}. These conclusions were further supported by Steinberg et al. [27] who found that phospholipids play an important role in ATP-dependent Ca^{2+} binding. They suggested that there is an ATP-dependent Ca^{2+} binding which differs from the active oxalate-dependent Ca^{2+} transport. Katz and Repke [28] also suggested that Ca^{2+} binding is effected by a limited number of high affinity sites and probably represents a process independent of the Ca^{2+} uptake. Our results support this view and suggest that ATP-dependent Ca^{2+} binding to the membrane occurs in the presence and in the absence of oxalate.

In summary, two pools of Ca^{2+} taken up at expense of ATP exist in sarcoplasmic reticulum. In the first pool, Ca^{2+} is externally bound to the membranes, whereas in the second one Ca^{2+} is inside the vesicles where it probably is in equilibrium with internal binding sites. Both phenomena need ATP, but in one case Ca^{2+} is selectively bound to sarcoplasmic reticulum membranes by a process which probably involves conformational changes induced by ATP [14] and in the other case Ca^{2+} is transported by a process which depends on the ATPase activity [1,3,15,16,24]. The relationship between these two processes is not clear. However, it appears that both processes, which have been formulated as alternatives for active Ca^{2+} uptake, exist in sarcoplasmic reticulum and probably play a role in the muscle contraction.

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