The Biphasic Ca^{2+}-Uptake by the Fragmented Sarcoplasmic Reticulum

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The non-equilibrium dialysis has been used for kinetic studies of ATP dependent calcium uptake by the sarcoplasmic reticulum. The uptake displays two phases, which are defined as fast and slow uptake. The former is an exponential function of time, with a half-life time of approximately 15–20 sec, the latter presents the characteristics of an autocatalytic reaction. The fast uptake is characterized by its amplitude, the slow uptake by its rate. Compared with the fast uptake, the slow uptake requires higher amounts of Mg^{2+} or ATP, and is more sensitive to pH variations and aging. The reasons which formerly prevented the resolution of the second phase from the first are discussed. It is concluded that the fast uptake is not a simple binding reaction, and that the slow uptake is more sensitive to changes by the vesicular membrane.

Introduction

When the relaxing activity of the sarcoplasmic reticulum was discovered, a discussion arose whether the Ca^{2+}-sequestering capacity of this subcellular structure is due to ATP-dependent Ca^{2+}-binding, or to an active transport system. More recently, the Ca^{2+}-binding proteins which were isolated and characterized [1–3] were proposed by several authors [4, 5] to take part in the relaxation process. On the other hand, the FSR shows an ATPase activity, and ATP increases dramatically its ability to accumulate calcium, especially in the presence of oxalate or phosphate anions.

Numerous technical problems complicate the observations of Ca^{2+}-uptake or binding. The addition of oxalate to the incubation medium enhances the ability of the vesicles to retain the calcium in the form of oxalate crystals, improving the accuracy of the measurements [6, 7], and permits the demonstration of the existence of an active transport [8]. These advantages are partially offset by the fact that oxalate precipitation reduces the concentration of free calcium in the vesicles at least 2–3 orders of magnitude below normal physiological levels. The observation on the reaction of a reticulum system containing a higher concentration of free calcium in its lumen could provide important information on its behaviour *in vivo*. For this purpose, any separation of the vesicles by centrifugation or ultrafiltration, with or without oxalate, must be avoided. Since movement of calcium regulates *in vivo* muscle contraction and relaxation, an adequate investigation method has the apparently contradictory task not to disturb this potantiality of the Ca to be stored or released, while avoiding any artificial distribution.

The measurement of free calcium present in the medium by the non-equilibrium dialysis method meets these requirements, and for that reason the Colowick's procedure [9] was used with the sarcoplasmic reticulum (unpublished work). The present paper will propose an adaptation of Colowick's method to kinetical purposes, and show some evidence for biphasic kinetics in the calcium uptake by the fragmented sarcoplasmic reticulum.

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Material and Methods

The fragmented sarcoplasmic reticulum was prepared from crayfish tails essentially according to the procedure of MacLennan [10]. The extraction buffer was slightly modified to contain 88 mM KCl, 5 mM imidazol (pH 7.4) and 1 mM dithiothreitol, and was also used when the vesicles were stored at −20 °C.

The protein content was measured by the Biuret method in presence of Na-deoxycholate, and the inorganic phosphorus liberated by the ATPase was determined by the phosphomolybdate method [11]. The radioactive samples were diluted in Bray's solution for liquid scintillation counting.

The apparatus of the non-equilibrium dialysis was described by Colowick and Womack [9]. The circulating buffer contained $MgCl_2$, imidazol and KCl in concentrations corresponding to the incubation medium. When not specified, the latter contained 5 mM $MgCl_2$, 50 mM imidazol (pH 7.45), 100 mM KCl, 0.05 mM labeled $CaCl_2$, 5 mM ATP. The fractions of the free and ATP-bound calcium were determined by the differential dialysis method [12]. The reaction was initiated by the addition of 150 μg vesicular proteins; the final volume was 1 cm^3, the experiments were performed at room temperature.

When used for kinetical purposes, the apparatus had to be calibrated in order to estimate the effect of the dead space. The reaction mixture, without label and without ATP, was introduced into the upper chamber, and the circulating buffer allowed to run. Then, the fraction collector was switched on its minimal timing, in our case 0.1 min, and at a definite time the radioactive Ca^{2+} was added to the reaction mixture. Since mixing in the upper chamber is practically instantaneous, the kinetics of appearance of label in the fractions of the collector can be attributed to the apparatus only. If the procedure is correct, the instantaneous disappearance of the respective substance must show corresponding kinetics. This can be observed when ATP is added to form an ATP complex. If the estimation of this rate by hand on a graph is not satisfying, the observed data may be computerized if it is assumed that the radioactivity measured in a collected fraction is proportional to that which existed a moment before in the lower chamber. This hypothesis is reasonable, provided the following conditions exist: a. The time interval requested for the bulk to join the tubes of the collector must be short (10 – 20 sec). b. The dead space of the tubing must be as small as possible. c. The timing of the collector cannot exceed a certain limit (in our case 0.2 min). The proportionality coefficient is the ratio of the volumes of the aliquot *versus* that of the lower chamber. The amounts of radioactivity X_1 and X_2 measured in the upper and the lower chamber respectively, and the set of equations

$$\frac{dX_2}{dt} = k_{21} X_1 - k_{02} X_2$$

and

$$\frac{dX_1}{dt} = k_{21} X_1$$

permit the calculation of the dialysis rate constant k_{21} and the efflux rate constant from the lower chamber k_{02}. The back diffusion of radioactivity through the membrane is negligible. A complete solution may be found from a set of data with the help of the SAAM 23 program [13, 14].

A similar procedure was used to follow the decrease of the label in the fractions of the collector after addition of the vesicles to the reaction medium.

Results

a. Influence of the medium on the free calcium concentration

As shown in Fig. 1a and 1b, the fraction of added calcium in the free form depends on both total ATP and Mg concentrations. When the ratio

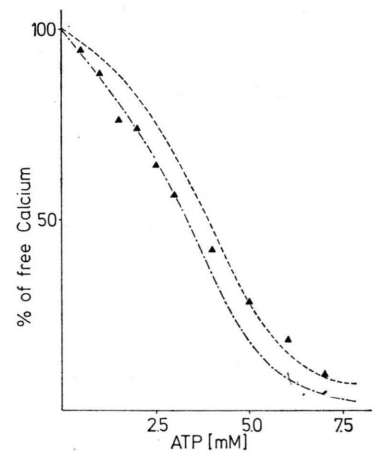

Fig. 1 a. ATP dependence of CaATP complex. The medium contains 5 mM $MgCl_2$, 50 mM Imidazol (pH = 7.45), 100 mM KCl, 0.05 mM $CaCl_2$, and increasing concentrations of ATP. ▲ = experimental values; calculated values based on association constants found in the literature: $-\cdot-\cdot-$ = for $K_{MgATP} = 1.75 \times 10^4\ M^{-1}$ and $K_{CaATP} = 10^4\ M^{-1}$ [16]; $---$ = for $K_{MgATP} = 10^4\ M^{-1}$ and $K_{CaATP} = 0.4 \times 10^4\ M^{-1}$ [35].

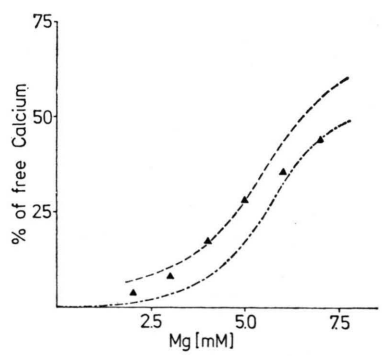

Fig. 1 b. Mg dependence of the CaATP complex. The medium contains 50 mM imidazol (pH = 7.45), 100 mM KCl, 0.05 mM $CaCl_2$, 5 mMATP and increasing concentrations of $MgCl_2$. Symbols as for Fig. 1 a.

of the former to the latter is $\leqq 1$, the dependence is especially strong. Some experiments indicate that the fraction of free calcium increases with lowering pH, which is consistent with the fact that a less ionized ATP has a lower association constant with divalent cations [15].

In the conditions given in the methods section, only $25-30\%$ of the Ca^{2+} is free. For the same conditions, in the presence of ATP, the dialysis rate of the total Ca^{2+} is reduced to 40% of the value observed in the absence of the nucleotide, since the CaATP complex diffuses more slowly. Because of this decrease, the Ca-uptake was always initiated by addition of reticulum to a medium already containing the specified amount of ATP.

Control experiments with aged vesicles or mixtures containing 1 or 2 mM $CaCl_2$ showed no variation in the dialysis rate of the radioactivity on addition of reticulum. Therefore, artefacts, such as a change of viscosity of the reaction mixture when the vesicles are added, are ruled out.

When the apparatus is calibrated, the observed increase and decrease of the radioactivity has an apparent half-life time of about 20 sec, which must be taken in account for estimation of parameters in fast kinetics. However, it is possible to distinguish between fast and slow processes.

b. The kinetics of the uptake

In the curves of Fig. 2 b the decrease of the radioactivity corresponds to an uptake of calcium by the vesicles. It displays two phases. The first phase will be called the fast uptake because it practically terminates one, or one and half minutes after the addition of reticulum to the medium. During this time interval the decrease of the radioactivity is expotential with a half-life time of about $15-20$ sec.

The second phase becomes observable during the second minute after the addition of the vesicles and lasts several minutes until nearly complete disappearance of the radioactivity from the solution. It will be called the slow uptake. The rate coefficient which characterizes the removal of the free ion is steadily increasing, showing the characteristics of an autocatalytic phenomenon.

These complex kinetics are illustrated in Fig. 2 b, for different amounts of vesicles and different experimental conditions. Both phases of calcium uptake were visible in all types of experiments, except

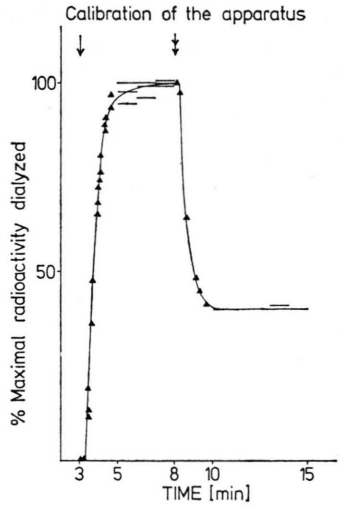

Fig. 2 a. Calibration of the apparatus. The medium contains 5 mM $MgCl_2$, 50 mM imidazol (pH = 7.45), 100 mM KCl and 0.05 mM labeled $CaCl_2$. After 3 min trace amounts of radioactive calcium (↓), and after 8 min 5 mM of ATP (↕) are added).

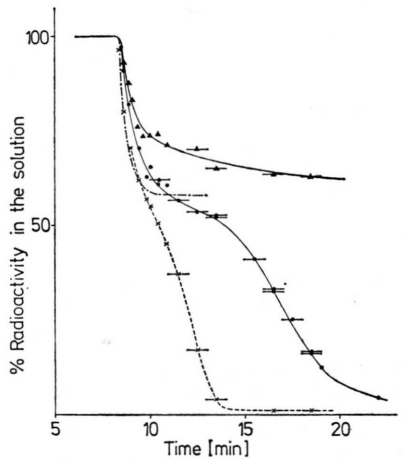

Fig. 2 b. Kinetics of calcium uptake by the fragmented sarcoplasmic reticulum, determined by non-equilibrium dialysis method. After 8 min the standard medium receives: ▲, ———: 100 μg of slightly aged sarcoplasmic vesicles; ●, ———: 150 μg of fresh or freshly thawed vesicles; — · — · —: expected curve if the uptake by 150 μg of fresh reticulum were instantaneous. This curve is adapted to a 42% uptake from the curve shown in Fig. 2 a. ×, — — —: The medium contained in addition 5 mM oxalate and received after 8 min 150 μg of fresh or freshly thawed vesicles.

where the slow uptake was too slow to be detected, for instance when the vesicles are aged or in low concentrations or too rapid to be resolved from the fast uptake, as it is observed in the presence of 5 mM oxalate.

For practical reasons, the decrease of radioactivty which is observed two and half minutes after the addition of the vesicles was considered as corresponding to the fast uptake. This assumption is based on the empirical observation that at this time, for the characteristics of our apparatus, the first phase is nearly ended and the slow uptake is not yet very active; it seems reasonable as long as fast and slow uptake may be resolved.

c. The conditions of the uptake

It was noted that the factors under study have a detectable influence on the size, but not on the rate of the fast uptake, which is too fast to be resolved; the same factors affect the rate, and not the size of the slow uptake, since the latter is practically a non-or-all phenomenon. Moreover, the action of these factors is qualitatively similar, but quantitatively different.

The fast uptake reaches its maximum at very low concentrations of ATP or Mg^{2+}, while the slow uptake increases progressively with higher concentrations. It may be suspected that the fast uptake existing in the absence of added Mg^{2+} is due to endogenous Mg^{2+} (Figs 3 and 4). Both phases are optimally activated around pH 7.0, but the effect of pH variations on the second phase is much more pronounced. Between pH 6.8 and 7.5 the fast uptake parallels the total ATPase activity. The latter has a maximum at pH = 6.6, which is 0.3 and 0.7 μmol/min mg protein respectively for the fresh and the freshly thawed reticulum.

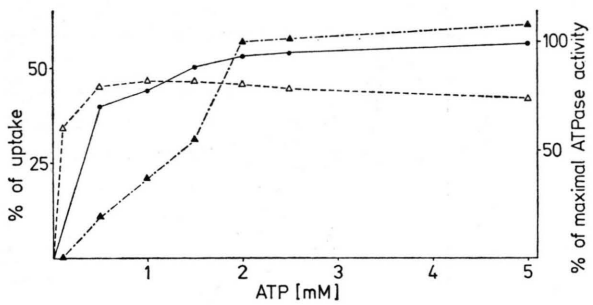

Fig. 3. Activation by ATP of the ATPase activity, the fast and the slow uptake. The medium is that given in the methods section. ●, ———: ATPase activity. △, — — —: percent of calcium accumulated during the fast uptake. ▲, — · — · —: percent of the remaining calcium which is accumulated during the 8 min following the fast uptake. The latter value is an indication of the rate of the slow uptake.

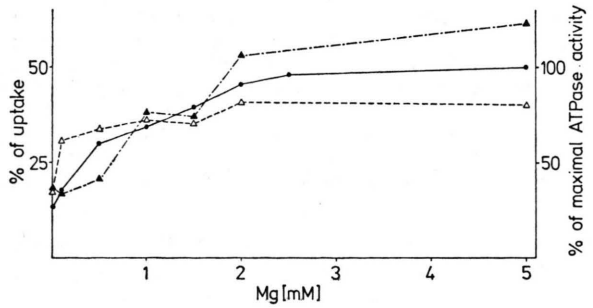

Fig. 4. Activation by Mg of the ATPase activity, the fast and the slow uptake. The medium is that indicated in the methods section. Symbols as for Fig. 3.

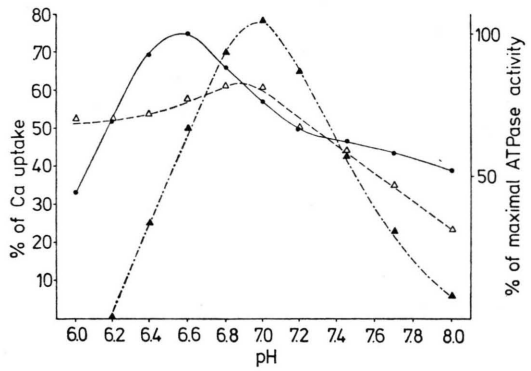

Fig. 5. pH dependence of the ATPase activity, the fast and the slow uptake. The medium is that indicated in the methods section. Symbols as for Fig. 3. The time interval for the slow uptake is 5 min.

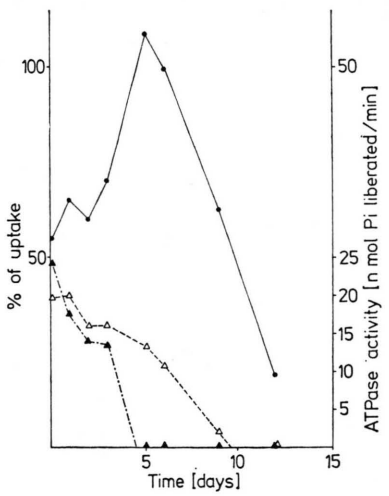

Fig. 6. Influence of aging on the ATPase activity, the fast and the slow uptake. The medium is that indicated in the methods section. Symbols as for Fig. 3. The time interval for the slow uptake is 6 min.

Figs 5, 6 A and 6 B show that the slow uptake, especially at pH 7.45, is extremely sensitive to the state of the vesicles. When the freshly isolated reticulum is stored at 0 °C, a marked alteration is ob-

Table I. Influence of mitochondrial inhibitors on the fast and slow calcium uptake by the sarcoplasmic reticulum. The incubation medium is that described in the methods section.

Inhibitor	Concentration [M]	Concentration of reticulum [$\mu g/cm^3$]	Initial concentration of calcium [μM]	% of fast uptake	% of slow uptake after 5 min
None	—	160	40	53	88
Dicumarol	6×10^{-5}	160	40	38.5	0
Antimycin A	1.4×10^{-7}	160	40	53	85
2,3 dimercapto-1 propanol	10^{-3}	160	40	51.5	95
None	—	150	50	38	60.5
Azide	10^{-3}	150	50	37	55.5
Dinitrophenol	2×10^{-4}	150	50	38.5	57

served at the fourth day: the slow uptake disappears, while the ATPase activity increases to twice its initial value. Later, both total ATPase activity and fast uptake parallel in decay.

As there is no absolute criterion for the purity of a subcellular fraction, several mitochondrial inhibitors were assayed. None of them affects the reticulum, except dicumarol which fully inhibits the slow uptake.

Discussion

The strong dependence of the free Ca^{2+} level on the added [ATP]/[Mg] ratio, when this ratio varies in physiological ranges, can be visualized by non-equilibrium dialysis. Since the Ca-ADP complex is much weaker than the Ca-ATP complex, the cleavage of ATP leads to a partial release of the ion from the nucleotide pool. The theoretical curves are calculated from association constants given for a slightly different medium [15, 16]. This may explain why the experimental relationship deviates from those obtained with these association constants.

The time resolution of non-equilibrium dialysis method is not yet high enough to detect whether a part of the first phase is instantaneous, as proposed by Entman *te al.* [16]. The question is still controversial, since the fast binding is presumably a mixing artefact [17].

Under the experimental conditions applied in the present investigation, the storage capacity of the vesicles can reach 1000 $nmol/mg^{-1}$ protein. This value is considerably higher than most of those found with the conventional method. Since in the experiment the Ca level in the solution is reduced to 2% of its initial value, a high Ca^{2+} gradient exists.

The shape and the half-life time of the fast uptake curve is similar to that of curves obtained in the absence of oxalate by several investigators [17–19], and corresponds to the parameters of component B of the binding as calculated by Entman *et al.* [16]. The fast uptake reaches its maximal activity already at low Mg^{2+} concentrations [20]. Its decrease with increasing pH, as mentioned by several authors [16, 21, 22], is observed only for pH exceeding 7.0.

Entman *te al.* [16] reported the kinetics which are the closest to the curves shown in Fig. 2. Their component B of the "binding", which is comparable in many respects to the fast uptake, is followed by a second phase where the initially slow rate increases and becomes linear. However, they failed, like other investigators, to observe the second phase in absence of oxalate. The commonly used methods involve a number of physical and chemical factors which may obscure the appearance of a two-phase calcium uptake. a. *The physical artefacts.* The calcium accumulated in the absence of precipitating anions is partially lost during separation of the vesicles from the medium on Millipore filters. The recovery is quantitative only when the reticulum is incubated in the presence of 5 mM oxalate (unpublished data). b. *The chemical artefacts.* The presence of oxalate stabilizes the active transport rate by keeping the level of free Ca^{2+} inside the vesicles such that the ion product remains equal to the solubility product. When the level of the external free Ca^{2+} is buffered with EGTA, the relatively stable gradient through the membrane makes it possible to demonstrate the coupling between ATP splitting and Ca^{2+} uptake [23, 24], yet it masks other phenomena. As shown in Fig. 2, both phases cannot be separated in presence of 5 mM oxalate, because the second phase becomes as fast as the first [25]. The

kinetics of the Ca^{2+} uptake has also been followed by measuring light absorbancy changes undergone by Ca-murexide [17, 26–28]. However, this metallochromic indicator shifts the chemical equilibrium by complexing part of the free ion. For instance, Scarpa and Inesi [29] did not detect any formation of Ca·ATP, which should represent about 16% of the total calcium under their experimental conditions, if the association constants for Mg·ATP are 1.7×10^4 and 10^4 [15, 16] respectively. The turbidity monitoring avoids this interference [30], but the change of absorbance is specific for a crystal formation, and cannot detect the accumulation of the free ion.

Other factors must be taken into consideration for a convenient observation of the biphasic uptake. a. *The structure of vesicles.* Compared to the first phase, the slow uptake has higher requirements for Mg^{2+} and ATP, and is more sensitive to variations in pH and aging. The calcium capacity of the vesicles disappears in a few days in absence, but not in presence of oxalate [31], which reduces the energy required for the process by lowering the chemical and the electrical gradient through the membrane. All these statements suggest that the slow uptake requires well preserved vesicular membranes, involving a minimal passive diffusion and optimal coupling between the hydrolysis of ATP and Ca^{2+} accumulation. Among the necessary elements of the structure are the sulfhydril groups [32] whose oxidation is prevented by dithiothreitol [33]. The latter was reported to maintain the pumping ability of the vesicles extracted from the lobster [34], but not from the rabbit. These statements are consistent with our own observations, since dithiothreitol was necessary for the slow uptake by the crayfish reticulum, while results not reported here showed no influence of this reagent on the vesicles extracted from the rabbit muscle according to the procedure of Hasselbach and Makinose [7]. This difference is not understood.

b. *Autocatalytic properties of the slow uptake.* The slow uptake seems to be autocatalytic and is therefore very sensitive to all factors mentioned above. This may explain why the two phases have not yet been observed.

The possibility that the slow uptake is due to mitochondrial fragments is ruled out by the use of specific mitochondrial inhibitors such as azide or dinitrophenol, antimycine or 2,3-dimercapto-1-propanol. If the heterogeneity of the fraction were responsible for the complexity of the uptake, that would suppose that the preparation is made out of two distinct populations. It is not excluded that the fast uptake gives rise to a discontinuous heterogeneity between unloaded and loaded vesicles, the latter would be the only one responsible for the slow uptake. This view is consistent with the observation that the slow uptake is always preceded by the fast uptake, which would structurally modify the vesicles or active them or, as a third possibility, represent the first step of the kinetics of calcium accumulation. According to these considerations, the first phase would be a necessary, but not a sufficient condition for the second phase, since the latter does not always occur. Both phases may also be completely independent from each other, the first phase simply requires less energy. In the present state of knowledge, the only possible conclusion is that the fast uptake, which is not instantaneous, probably represents more than a simple binding, and that the slow uptake is linked to a higher state of organization of the vesicular membrane.

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