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STATE OF TRANSLOCATED Ca^{2+} BY SARCOPLASMIC RETICULUM INFERRED FROM KINETIC ANALYSIS OF CALCIUM OXALATE PRECIPITATION

VÍTOR M.C. MADEIRA

Centro de Biologia Cellular, Departamento de Zoologia, 3049 Coimbra Codex (Portugal)

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Uptake of Ca^{2+} by sarcoplasmic reticulum in the presence of oxalate displays biphasic kinetics. An initial phase of normal uptake is followed by a second phase coincident with precipitation of calcium oxalate inside the vesicles. The precipitation rate induced by actively transported Ca^{2+} is depressed by increasing the added Ca^{2+} concentration. This correlates linearly with the reciprocal of precipitation rate. Therefore, a maximal limit rate could be extrapolated at zero Ca^{2+} (V_0). The rate of precipitation, also a function of added amount protein, gives a linear correlation in a double reciprocal plot. Thus, it was possible to estimate the maximal precipitation rate occurring at infinite protein concentration (V^{∞}). With the combined extrapolated values a maximal expected precipitation rate could be calculated (V_0^{∞}). Kinetics of calcium oxalate precipitation was studied in the absence of calcium uptake and empirical equations relating the rate of precipitation with the added Ca^{2+} were established. Entering V_0^{∞} in the equations, an internal free Ca^{2+} concentration of approx. 2.5 mM was estimated. Additionally, it is shown that the ionophore X-537A does not supress the Ca^{2+} uptake, if added during the oxalate-dependent phase, albeit the uptake proceeds at a slower rate after the release of approx. 70 nmol Ca^{2+}/mg protein. This amount presumably equals the internal free Ca^{2+} not sequestered by oxalate, producing a maximal concentration approx. 14 mM. Taking into account low affinity binding of internal binding sites and the transmembrane Ca^{2+} gradients built up during the uptake of Ca^{2+}, values of free Ca^{2+} ranging from 3 to 6 mM, approaching those estimated by the precipitation analysis, could be estimated.

Introduction

Sarcoplasmic reticulum membranes accumulate Ca^{2+} by an active process coupled to the hydrolysis of ATP catalysed by the Ca^{2+}-translocating ATPase [1,2]. This energetic accumulation depends on the physical intactness of the membrane vesicles and on the activity of the Ca^{2+} pump [3]. Disrupted membranes do not sequester Ca^{2+} although they may still exhibit a very high activity [4]. Ca^{2+} accumulated by sarcoplasmic reticulum vesicles leaks out when the membranes are disrupted or Ca^{2+} ionophores are added. At the steady state of Ca^{2+} accumulation, the low net ATP hydrolysis is regulated by the transmembrane Ca^{2+} gradient [4]. The discharge of the gradient by ionophores and disrupting agents releases the ATPase from a controlled inhibitory condition to a state of high uncoupled activity maintained as long as ATP is present [4]. The controlled condition is a function of the Ca^{2+} gradient before saturation with Ca^{2+} occurs, and also a function of the internal Ca^{2+} concentration, at saturation [4,5]. Therefore, these findings exclude the possibility that accumulated Ca^{2+} is bound by an ATP-dependent binding mechanism [6,7], but, rather, support the postulate that Ca^{2+} is translocated into the interior of sarcoplasmic reticulum vesicles, where it remains

largely as free Ca^{2+} [3,8]. Concurring with this hypothesis is the fact that the vesicles are competent to generate transmembrane Ca^{2+} gradients greater than 3000 [9], even when the transport proceeds in the presence of oxalate which precipitates Ca^{2+} in the form of calcium oxalate deposits in the vesicular lumen [10].

Ca^{2+}-precipitating anions have been utilized in studies of Ca^{2+} transport to enhance the amount of accumulated Ca^{2+} by lowering the intravesicular calcium activity [1,2,11]. Thus, Ca^{2+} release due to membrane leakage and ATPase inhibition at Ca^{2+} saturation are avoided. Oxalate and other trapping anions are not actively transported [1,11] and it is generally assumed that they passively cross the membrane following actively translocated Ca^{2+}. However, we have recently shown that oxalate has to be protonated, at the expense of protons ejected by the Ca^{2+} pump, before it can cross the membranes [12]. When enough oxalate has crossed the membrane, calcium oxalate precipitation taking place inside the vesicles can be monitored by turbidimetric techniques. Since the rate of precipitation depends on the concentration of free Ca^{2+} inside the vesicles, we could infer the internal Ca^{2+} concentration built up by the Ca^{2+}-translocating ATPase system.

Materials and Methods

Fragmented sarcoplasmic reticulum was isolated as described elsewhere [13]. Ca^{2+}-ATPase in preparations routinely assayed by SDS-polyacrylamide gel electrophoresis amounted to, at least, 80% of total protein.

The uptake of Ca^{2+} was followed with a Ca^{2+} electrode of the neutral carrier type supplied by Dr. Simon (Laboratorium für Organische Chemie, Zurich). Incubation media at 25°C (2.5 ml) contained 50 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 10 mM Tris-maleate (pH 6.9) and other components, as and if stated. The amounts of vesicular protein and $CaCl_2$ were as referred to in the legends to the figures. The uptake reaction was initiated by adding 0.4 mM Mg-ATP.

Turbidity changes were monitored by recording the apparent absorbance changes in a Spectronic 20 spectrophotometer with a modified cell and cell holder to permit thermostabilization (25°C) and continuous magnetic stirring. When more precise measurements, with improved time resolution, were required (Fig. 1), a split-beam spectrophotometer was used. The uptake of Ca^{2+} was initiated by adding Ca^{2+} simultaneously to both cuvettes (by two operators), but oxalate was omitted in reference.

Results and Discussion

Kinetics of calcium oxalate precipitation during Ca^{2+} uptake

The Ca^{2+} uptake in the presence of oxalate has two distinct phases [12,14]. The initial phase taking place during a short period is independent on the presence of oxalate and is kinetically identical to the profile of Ca^{2+} uptake observed in the absence of oxalate (Fig. 1 and Ref. 12). When

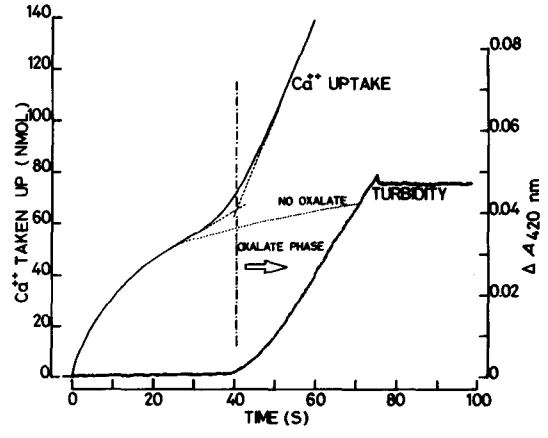

Fig. 1. Uptake of Ca^{2+} and precipitation profile of calcium oxalate in sarcoplasmic reticulum vesicles. Ca^{2+} uptake was monitored with the Ca^{2+} electrode and precipitation was followed by light scattering. The reactions were carried out in 2.5 ml medium, at 25°C and pH 7.35, containing 0.5 mg sarcoplasmic reticulum. For technical reasons, the uptake of Ca^{2+} was initiated by adding 0.4 mM Mg-ATP in electrode measurement or 0.12 mM $CaCl_2$ in turbidity recording. The following events are worth of note: (a) Ca^{2+} uptake has two distinct phases, the initial phase being not dependent on oxalate presence (oxalate was absent in experiment represented by dotted trace); (b) turbidity development has a lag period of 40 s; (c) a second phase of Ca^{2+} uptake (oxalate-dependent phase) occurs simultaneously with turbidity development due to Ca^{2+} precipitation inside the vesicles. The Ca^{2+} uptake (electrode recording) could not be followed to completion since the electrode response becomes erratic when the total Ca^{2+} concentration in medium falls below 10^{-6} M.

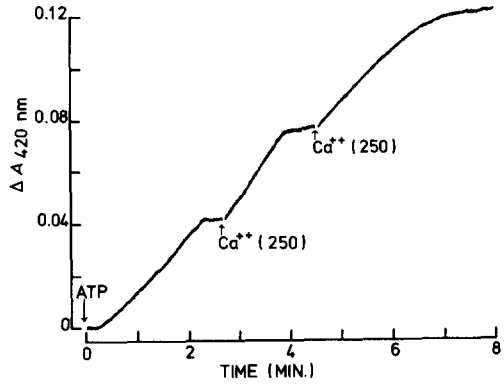

Fig. 2. Cycles of turbidity development during Ca^{2+} uptake and calcium oxalate precipitation. The reactions were carried out in 2.5 ml medium at 25°C and pH 6.9, containing 0.2 mg sarcoplasmic reticulum protein. The first cycle was initiated by adding Mg-ATP (1 mM) to the medium supplemented before with 500 nmol $CaCl_2$. Note the initial lag period lasting for about 15 s. The other cycles elicited by adding pulses of $CaCl_2$ (250 nmol each) do not show lag period. Note also that the rate of the second cycle is higher than that observed during the first cycle. The rate of the 3rd cycle tends to decrease progressively with time due to partial inactivation of vesicle population as consequence of membrane damage induced by calcium oxalate crystals.

oxalate is present, a second phase, distinct from the initial, takes place, beginning after a period whose length is dependent on the pH of medium (Fig. 1 and Ref. 12). When the pH is decreased, the onset of the oxalate-dependent phase is anticipated and the two phases are no more distinguishable at pH values approaching 6.0 [12].

The oxalate-dependent phase of Ca^{2+} uptake is accompanied by Ca^{2+} precipitation inside the vesicles conveniently monitored by light scattering recordings (Fig. 1 and Ref. 15). Fig. 1 shows that the precipitation of calcium oxalate coincides with the oxalate-dependent phase. The precipitation profile also shows a lag period (Fig. 1) dependent on the pH [12]. As previously discussed more exhaustively, the precipitation reaction begins only after oxalate has been transported to the inside of the vesicles. This transport takes place after oxalate protonation at the expense of protons ejected by the Ca^{2+} pump during the uptake of Ca^{2+} and simultaneous hydrolysis of ATP. Once protonated, oxalate can be transferred across the membrane and reaches the vesicular lumen. Then, the precipitation of calcium oxalate can take place.

A considerable time-lag can only be noticed during an initial course of uptake. If a second course of Ca^{2+} uptake is elicited by adding a new pulse of $CaCl_2$, the precipitation reaction takes place immediately without lag phase (Fig. 2). Therefore, it is concluded that, after the first course of uptake, the free Ca^{2+} concentration is high enough to cause precipitation with the oxalate accumulated in the vesicle. Since the free Ca^{2+} would then be in equilibrium with calcium oxalate crystals, the precipitation reaction would take place without a lag phase.

The precipitation rate is dependent on the concentration of added Ca^{2+}. It is apparent from Fig. 2 that the precipitation rate tends to increase when external Ca^{2+} is being exhausted, since the profile deviates progressively from linearity towards an increased rate. It is also apparent from Fig. 2 that a second precipitation course, after addition of half the initial amount of Ca^{2+}, occurs at a higher rate. The rate of precipitation, however, is slowed down when the maximal capacity of the system is approached (Fig. 2). This occurrence is most probably due to partial inactivation of the vesicle population as a consequence of membrane damage induced physical constraints imposed by the crystals and needles of calcium oxalate which presumably disrupt the vesicles. Since the population of active vesicles is reduced, the apparent specific rate of calcium oxalate precipitation is decreased.

The rate of calcium oxalate precipitation as affected by the protein concentration

As expected, the rate of calcium oxalate precipitation inside the vesicles, during the uptake of Ca^{2+}, depends on the amount of added vesicles (inset of Fig. 3). For high concentrations of vesicles, the rate tends to deviate from linearity and progressively increases with time to a maximal value which was taken for calculation. This behaviour is related to the decrease of Ca^{2+} concentration in the medium, as already discussed above and further detailed below. Taking the maximal precipitation rates, the plot of Fig. 3 was drawn. This plot permits to extrapolate the maximal velocity of calcium oxalate precipitation if the protein concentration were infinite (V^{∞}). This was achieved by extrapolating the line to the ordinate, thus permitting the estimation of a value V^{∞} of approx.

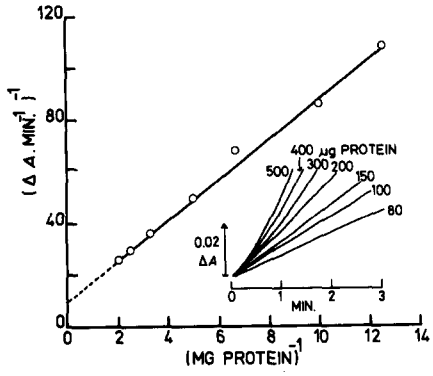

Fig. 3. Calcium oxalate precipitation rate during Ca^{2+} uptake as a function of sarcoplasmic reticulum vesicle concentration. Media (2.5 ml, 25°C, pH 6.9) contained 600 nmol $CaCl_2$. Reactions were initiated by adding 1 mM Mg-ATP. Lag periods are not represented. Maximal precipitation rates were calculated from the inset experiment and then plotted in a double reciprocal plot from which a rate for infinite protein concentration (V^{∞}) was estimated (ordinate intersection), as 0.1 ΔA/min.

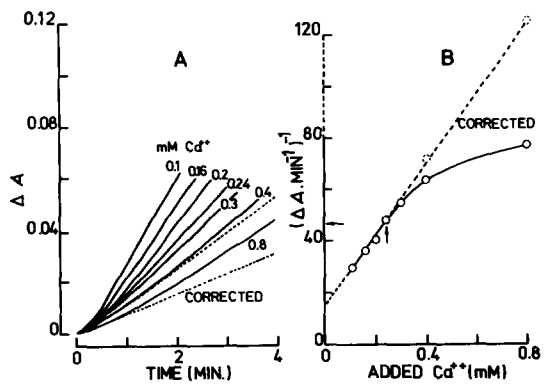

Fig. 4. Calcium oxalate precipitation rate during Ca^{2+} uptake as a function of added Ca^{2+} concentration. Media (2.5 ml, 25°C, pH 6.9) contained 0.2 mg sarcoplasmic reticulum protein. Reactions were initiated by adding 1 mM Mg-ATP to media supplemented with $CaCl_2$ as stated in the traces. Recordings corresponding to added $CaCl_2$ equal or higher than 0.4 mM needed correction due to calcium oxalate spontaneous precipitation in the medium not related with Ca^{2+} uptake. Corrections were done by taking into account changes in apparent absorbance in the absence of ATP. Corrected recordings are depicted by dashed lines. Part B is a semireciprocal plot were the reciprocals of ΔA rates are presented against the added Ca^{2+} concentration. The plot permitted the extrapolation of a limit maximal theoretical precipitation rate (ordinate intersection) corresponding to approx. 0.065 ΔA/min. Position of arrows denote the rate obtained for 600 nmol $CaCl_2$ (0.021 ΔA/min) which is only 1/3 of the theoretical maximal rate.

0.1 ΔA/min. This normalized value is very consistent from preparation to preparation being always very similar when the amount of Ca^{2+} added is 600 nmol (0.24 mM). However, this value depends on the added concentration of Ca^{2+}. Therefore, we have further normalized V^{∞} taking into account the added Ca^{2+}, as described below.

Rate of calcium oxalate precipitation during active transport as a function of added calcium

As already discussed above in brief, precipitation of calcium oxalate inside the vesicles is dependent upon the concentration of added Ca^{2+}. The rate decreases with increasing Ca^{2+} in the medium (Fig. 4). It was possible to linearly approach this dependence, when the reciprocal of precipitation rate is plotted against added Ca^{2+} (Fig. 4B). The plot deviates from linearity only when added $CaCl_2$ equals or exceeds 0.4 mM. This occurrence is a consequence of 'passive' calcium precipitation in external medium due to the high calcium concentrations which spontaneously initiate precipitation. Therefore, the turbidity change in medium due to 'passive' precipitation adds to that caused inside the vesicles by Ca^{2+} transport. However, the 'passive' extra precipitation could be corrected after carrying similar experiments in the absence of ATP. It should be noted that only experiments corresponding to 0.8 and 0.4 mM added Ca^{2+} needed correction, since lower Ca^{2+} did not produce any artefacts during the experimental time-course. When the correction was done, a dashed curve approaching linearity could be obtained (Fig. 4B). We could then extrapolate a maximal limit rate of precipitation (V_0) corresponding to about 0.065 ΔA/min, when the amount of protein in medium is 0.2 mg. This amount of protein was chosen to permit accurate estimation of precipitation rates, avoiding extreme values which could include major errors.

From data in Fig. 4B it is possible to predict (arrows) that a rate of 0.021 ΔA/min would be obtained, if the amount of added Ca^{2+} were 600 nmol (0.24 mM), as in experiments referred to in the previous section. This concentration of Ca^{2+} produces a rate of about 1/3 (0.021/0.065) of the theoretical maximal expected at the zero limit of

Ca^{2+} concentration. Therefore, the maximal expected rate at infinite protein concentration and zero Ca^{2+} (V_0^{∞}) can be predicted as 0.3 ΔA/min (3×0.1). This value is used below to estimate the maximal free Ca^{2+} concentration inside the vesicles by comparing it with a parameter obtained in practical conditions for the precipitation of calcium oxalate itself.

Kinetics of calcium oxalate precipitation in the absence of calcium uptake

The 'passive' precipitation of calcium oxalate has been followed by monitoring the light scattering of buffered solutions containing oxalate, to which $CaCl_2$ was added to initiate the precipitation. The profiles of Ca^{2+} precipitation depend on the amount of added Ca^{2+} and sarcoplasmic reticulum protein present in medium (Fig. 5). Added protein improves the measurements in the sense that the results become more reproducible and the turbidity development becomes linear with time. Significant deviations from linearity are observed in absence of added protein (Fig. 5, for 1 mM $CaCl_2$) and the results do not reproduce well. It appears that the precipitation profile depends very much on random formation of nucleation sites which trigger the precipitation process. Probably, the nucleation sites form randomly in number, geometry and time, so that quantitative measurements are difficult to be carried out. However, when protein is present, nucleation sites apparently form in a more ordered way, since the kinetic profiles are reproducible and maintain linearity over an extended period, in the same range as the time elapsed during the measurement of Ca^{2+} uptake reported in previous sections.

Protein in medium tends to decrease the rate of calcium oxalate precipitation and maximal effects are observed when 1 mg of protein is added. Further addition of protein has no longer any detectable effect. Therefore, it is assumed that any additional increase in protein concentration would not alter the rate of precipitation.

For standard evaluation of the precipitation rates, 0.5 mg of protein was included in the medium and the added calcium changed from 0.4 to 2 mM (Fig. 6). Development of turbidity is practically linear with time, thus, permitting precise estimations of precipitation rates described in Fig. 6. The precipitation rate, against the concentration of added Ca^{2+}, exhibits an exponential profile. The rate increases moderately with Ca^{2+} concentration up to 1.2 mM (Fig. 7A); above this concentration, a dramatic increase in precipitation rate is noticed and it was not technically feasible to explore Ca^{2+} concentrations higher than 2 mM. Above this concentration, turbidity development is so fast that it could not be accurately estimated.

Data in Fig. 7A were manipulated in the form of the semilog plot of Fig. 7B. This linear plot fits

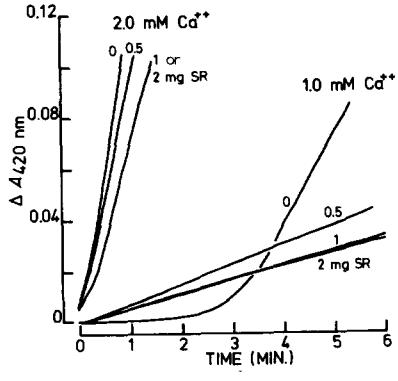

Fig. 5. Kinetics of 'passive' calcium oxalate precipitation as a function of protein in medium. Media were identical to those used for Ca^{2+} uptake except no ATP was added. Addition of sarcoplasmic reticulum protein affects the precipitation kinetics. Thus, when protein was added, turbidity change is linear with time and reproducible results are obtained. Maximal effect of protein is observed at 1 mg.

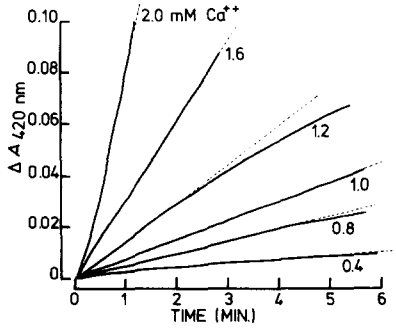

Fig. 6. Kinetics of 'passive' calcium oxalate precipitation as a function of added $CaCl_2$. Media (2.5 ml, 25°C, pH 6.9) contained 0.5 mg of protein. Precipitation reactions were initiated by adding $CaCl_2$ as stated on traces. Rates as defined by dashed lines were taken for further processing of results shown in next figure.

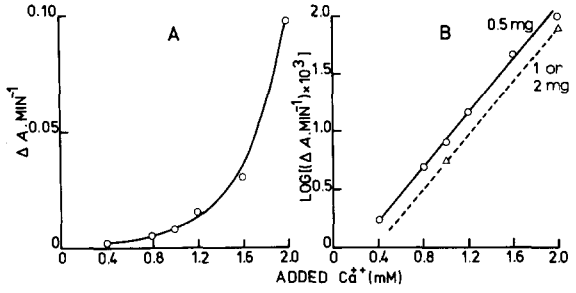

Fig. 7. Rate of calcium oxalate precipitation as function of added $CaCl_2$. Results of preceding figure were reprocessed in this form. Dependence of precipitation rate on added Ca^{2+} is apparently an exponential function giving an apparent linear relationship in the semilog plot of part B. Increasing the amount of protein has the effect of displacing the line to a parallel position. From the lines, empirical equations (Eqns. 1, 2 and 3) were derived (see text).

the equation:

$$\log V = \log V_0 + K\,[Ca^{2+}] \qquad (1)$$

where V states for the rate of precipitation, V_0 the limit rate (ordinate intercept), K, the slope and $[Ca^{2+}]$, the added Ca^{2+} concentration. After calculation of V_0 and K, the following expression applies:

$$\log V = -0.245 + 1.159\,[Ca^{2+}] \qquad (2)$$

or, for high protein concentration in the medium (actually 1 or 2 mg), as shown in the parallel dashed line of Fig. 7B

$$\log V = -0.420 + 1.159\,[Ca^{2+}] \qquad (3)$$

Therefore, protein in medium affects only the ordinate intercept (limit rate), but not the slope.

Activity of Ca^{2+} translocated by the Ca^{2+} pump

Ca^{2+} uptake proceeds via a transmembrane transport mechanism instead of an ATP-supported Ca^{2+} binding [3]. Membrane fragments produced either by mechanical means or by the use of detergents, are unable to store Ca^{2+}, although the fragments still retain high ATP splitting activity sensitive to calcium ions [4]; on the other hand, Ca^{2+} ionophores and detergents release Ca^{2+} previously accumulated by the pumping process. Concurring with the translocation mechanism are the findings that only asymmetrically reconstituted vesicles are competent to accumulate Ca^{2+} [16]. Furthermore, direct observations of deposits of calcium oxalate or phosphate in the luminal space [10] provided additional evidence that Ca^{2+} has to be translocated across the entire membrane thickness, before reaching the internal vesicular space.

Early observations of Ca^{2+} transport in the presence of oxalate permitted to calculate transmembrane Ca^{2+} gradients ($Ca^{2+}_{in}/Ca^{2+}_{out}$) ranging from 3000 to 6000 [9], since free Ca^{2+} concentration may reach values outside as low as 10^{-8} M, and values inside approaching 10^{-4} M imposed by the solubility product of calcium oxalate [9]. Therefore, the Ca^{2+} pump is able to generate a corresponding energy to sustain such a gradient [3]. Apparently, the Ca^{2+} gradient, which is a function of internal and external Ca^{2+} concentrations, is the principal parameter controlling the activity of the pump at the steady state, before saturation of sarcoplasmic reticulum with respect to Ca^{2+} occurs [4]. Presumably, the Ca^{2+} pump, in the absence of oxalate, shall be competent to generate transmembrane Ca^{2+} gradients similar do those calculated in its presence. Therefore, minimal internal Ca^{2+} concentrations ranging from 3 to 6 mM may build up when the external Ca^{2+} is lowered to 10^{-6} M which is easily accomplished by the pump.

As discussed in previous sections, the maximal theoretical rate of calcium oxalate precipitation inside the vesicles, as consequence of Ca^{2+} translocation, at extrapolated infinite protein and zero Ca^{2+} (limit rate), corresponds to about 0.3 ΔA/min. Entering this value in the Equations 2 and 3, Ca^{2+} concentrations ranging from 2.35 mM (0.5 mg sarcoplasmic reticulum added) to 2.50 mM (high protein) could be calculated. Therefore, Ca^{2+} concentrations of this order of magnitude would theoretically induce precipitation rates inside the vesicles equivalent to the maximal calculated values. We realize that these are normalized values for ideal conditions different from those used in most practical work. Therefore, the values must be taken as maxima allowable.

In a previous report [12], we have shown that the ionophore lasalocid (formerly X-537A) is only effective in preventing Ca^{2+} uptake with oxalate, when added before the uptake is initiated or dur-

ing the initial phase kinetically indistinct from the uptake in absence of oxalate. Lasalocid added during the oxalate-dependent phase does not suppress the uptake; nevertheless, the uptake proceeds at a slower rate after the release of a limited amount of Ca^{2+} [12]. The amount of released Ca^{2+} is similar in both phases of Ca^{2+} uptake and amounts to about 70 nmol/mg protein in the experiment described in Fig. 8. Presumably, this equals the amount of free Ca^{2+} not sequestered by oxalate in the luminal space. Taking the specific luminal volume as 5 μl/mg protein (volume accessible to tritiated water), calculated free Ca^{2+} is approx. 14 mM, a value considerably higher than that inferable from precipitation studies. Therefore, free Ca^{2+} concentrations higher than 2.5 mM may actually build up inside the sarcoplasmic reticulum, as consequence of Ca^{2+} translocation.

In the absence of oxalate, most preparations can accumulate 150 nmol Ca^{2+}/mg protein which would produce a concentration of approx. 30 mM if all the translocated Ca^{2+} remained free inside the vesicles. Careful titration of the internal binding sites [17] permitted the estimation of a maximal binding of 30 nmol/mg protein to low affinity sites ($K_d \approx 1$ mM). These sites have been assigned to the ATPase moiety facing the inside of the vesicles [16] and are probably identical to the inhibition sites described by Ikemoto [18]. Therefore, the storage of 150 nmol/mg protein would produce internal Ca^{2+} concentrations exceeding 15 mM.

In conclusion, the range of internal free Ca^{2+} concentrations infered from calcium oxalate precipitation studies and calcium release experiments (2.5 to 14 mM) is consistent with the capacity of transport, binding activity of internal sites and transmembrane Ca^{2+} gradients built up by the Ca^{2+} pump.

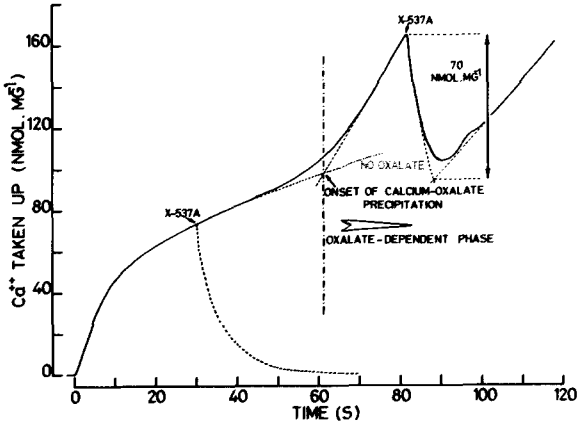

Fig. 8. Effect of the ionophore X-537A on Ca^{2+} uptake profile in the presence of oxalate. The uptake was carried out at pH 7.43 and 25°C in standard media containing 0.12 mM $CaCl_2$ and 0.5 mg protein. The uptake reaction was initiated by adding 0.4 mM Mg-ATP. When X-537A (25 μM) is added before the oxalate-dependent phase of uptake, Ca^{2+} is released and further net uptake is abolished, an effect identical in to that in the absence of oxalate. However, when the ionophore is added at the oxalate-dependent phase, Ca^{2+} is also released (70 nmol/mg protein, here), but net Ca^{2+} uptake is further observed, although at a decreased rate.

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