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FLUORIMETRIC MONITORING OF CALCIUM BINDING TO SARCOPLASMIC RETICULUM MEMBRANES

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Summary

1. Chlorotetracycline fluorescence was utilized to monitor Ca^{2+} binding by sarcoplasmic reticulum membranes, isolated from rabbit skeletal muscle, during active transport. Transport of Ca^{2+} is accompanied by increased chlorotetracycline fluorescence and binding, and X-537 A releases both Ca^{2+} and chlorotetracycline from the sarcoplasmic reticulum membranes.

2. Transport of Ca^{2+} in the presence of 5 mM oxalate is accompanied first by an increase in chlorotetracycline fluorescence, which is lower than that of the control without oxalate, and subsequently declines after it reaches a maximal value when about 150 nmol of Ca^{2+} are accumulated. The fluorescence reaches a minimal value 1—2 min after adding ATP, when Ca^{2+} accumulation is still occurring. In the presence of 5 mM phosphate, the fluorescence signal is also lower than it is in the absence of Ca^{2+} precipitating agents, as is expected if the fluorescence signal reflects Ca^{2+} bound to the membranes.

3. The ionophore X-537 A added after initiation of Ca^{2+} accumulation in the presence of oxalate does not cause detectable release of accumulated Ca^{2+}, but induces a decrease in chlorotetracycline fluorescence. If added before Ca^{2+} transport begins, X-537 A inhibits Ca^{2+} accumulation as well. The passive binding of Ca^{2+} or Mg^{2+} and the chlorotetracycline fluorescence is increased by X-537 A at concentrations above 40 μM.

4. The results illustrate the usefulness of the technique in determining transient alterations in the state of accumulated Ca^{2+} in biological systems, provided that experimental conditions which obviate the interference of other divalent cations are utilized.

Introduction

The Ca^{2+} accumulated by sarcoplasmic reticulum in the presence of ATP is retained free within the sarcoplasmic reticulum vesicles and is also bound to the

sarcoplasmic reticulum membranes [1—5], but it is technically difficult to distinguish between the two forms of Ca^{2+} accumulated. We showed previously [7] that transport of Ca^{2+} by sarcoplasmic reticulum membranes in the presence of chlorotetracycline is accompanied by an increase in the binding of chlorotetracycline to the membranes, which parallels an increase in chlorotetracycline fluorescence [6—10]. Therefore, chlorotetracycline can be utilized to monitor Ca^{2+} binding to sarcoplasmic reticulum and other biological membranes. Thus, chlorotetracycline has recently been utilized to detect binding of Ca^{2+} to sarcoplasmic reticulum [6—10] mitochondria [11—13], erythrocyte ghosts [14], and nerve [14].

In an aqueous medium the affinity of chlorotetracycline for Ca^{2+} is lower than it is for Mg^{2+} [15,16], but the affinity of chlorotetracycline for Ca^{2+} is selectively increased if the reaction occurs in a non-polar medium [15]. Thus, chlorotetracycline is preferentially transferred to biological membranes which selectively retain Ca^{2+}. Although other diamagnetic cations also interact with chlorotetracycline to a smaller extent it is possible to devise experimental conditions which obviate interference from other cations [6—12].

When Ca^{2+} is transported across biological membranes into vesicular spaces, such as occurs in isolated sarcoplasmic reticulum, it is expected that the Ca^{2+} retained free or precipitated in the vesicular spaces will be detected by chlorotetracycline fluorescence only transiently during its transport across the membrane while it interacts with the membrane components.

In the present study we measured the fluorescence signal of chlorotetracycline during Ca^{2+} transport by sarcoplasmic reticulum under conditions in which most Ca^{2+} is expected to be bound to the membranes, and also in the presence of oxalate or phosphate which precipitate the transported Ca^{2+} within vesicles. This fluorescent technique was also employed to monitor the release of Ca^{2+} from sarcoplasmic reticulum by X-537 A under various conditions. We conclude that the method is of particular interest to monitor transient changes in the state of Ca^{2+} in the sarcoplasmic reticulum vesicles.

Methods

Biological material

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle as described previously [5] using 0.1 M KCl plus 10 mM Tris-maleate (pH 7.0) as the isolation medium. Protein concentration was determined by the biuret method [17] using standards of bovine serum albumin.

Fluorescence measurements and Ca^{2+} uptake by sarcoplasmic reticulum

Incubations of sarcoplasmic reticulum membranes under conditions of Ca^{2+} uptake were performed in media of the composition described in the legends of the figures. Samples of these media (2.5 ml) were used to follow continuously the change in the fluorescence of chlorotetracycline. Other samples (2.5 ml) were filtered at the desired time intervals through "Millipore" filters, and the filters were eluted in 2 ml of 4% trichloroacetic acid plus 1% La^{3+}, and Ca^{2+} or Mg^{2+} was measured by atomic absorption spectroscopy with a Perkin-Elmer 305 spectrophotomer using standards of $CaCl_2$ or $MgCl_2$ solutions containing 4% trichloroacetic acid plus 1% La^{3+}.

Binding of chlorotetracycline to sarcoplasmic reticulum membranes

The binding of chlorotetracycline to sarcoplasmic reticulum membranes was determined by a method described previously [18], with some modifications. The membranes were incubated (0.4 mg/ml) in centrifuge tubes containing 10 ml of medium of the composition described in the legend of Fig. 1. The suspensions were centrifuged at 105 000 × *g* for 1 h at 0°C, and an aliquot (0.1 ml) of the supernatant solution was transferred to the spectrofluorometer cuvette containing 2.5 ml of 95% ethanol plus 50 mM $MgCl_2$. The bound chlorotetracycline was calculated by difference between the total chlorotetracycline added and the free chlorotetracycline remaining in the supernatant, which was measured by fluorescence.

Instrumentation and reagents

Fluorescence measurements were performed using a Hitachi-Perkin-Elmer MPF-3 spectrofluorometer using excitation at 380 nm and emission at 530 nm, with a cut-off filter of 390 nm in the emission path. The spectrofluorometer was calibrated before and during the experiments with 25 μl of a freshly prepared 1 mM chlorotetracycline solution in 2.5 ml of 95% ethanol plus 50 mM $MgCl_2$.

Chlorotetracycline was purchased from Sigma Chemical Company. The antibiotic X-537 A was generously supplied by Dr. J. Berger from Hoffman-La Roche. All the other reagents were of analytical grade.

Results

Changes in the fluorescence and binding of chlorotetracycline during Ca^{2+} uptake by sarcoplasmic reticulum

The results shown in Fig. 1 demonstrate that the addition of ATP to sarcoplasmic reticulum incubated under conditions which permit Ca^{2+} uptake causes

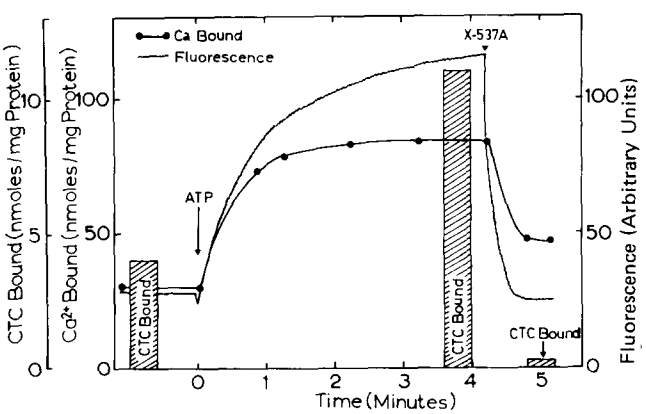

Fig. 1. Fluorescence changes and binding of chlorotetracycline to sarcoplasmic reticulum membranes during Ca^{2+} uptake. The incubation medium contained 0.35 M sucrose, 5 mM histidine-HCl at pH 6.75, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μM chlorotetracycline and 0.4 mg of sarcoplasmic reticulum protein per ml. Additions to the medium: ATP, 1 mM; X-537 A, 40 μM. The shaded bars represent the binding of chlorotetracycline measured before addition of ATP, at maximal Ca^{2+} uptake after adding ATP, and after addition of X-537 A [7].

an increase in the fluorescence of chlorotetracycline as reported in earlier studies [6—9]. It was suggested previously [8,9] that this increase in fluorescence is due to Ca^{2+} uptake, and in Fig. 1 we show that in fact the increase in chlorotetracycline fluorescence nearly parallels the accumulation of Ca^{2+} by the membranes, so that 4 min after ATP addition the fluorescence signal corresponds to about 85 nmol of Ca^{2+} per mg of protein, which was the maximal value obtained under the experimental conditions. Actually the fluorescence continues to increase somewhat after maximal Ca^{2+} accumulation, which is not readily explainable.

The binding of chlorotetracycline to the membranes is also represented in Fig. 1. The binding increases from 4 nmol of chlorotetracycline per mg of protein to 11 nmol of chlorotetracycline per mg of protein after addition of ATP.

Addition of X-537 A, a Ca^{2+} ionophore, 4 min after adding ATP, induced the release of all bound chlorotetracycline and the fluorescence decreased to the initial value before adding ATP, even though about 45 nmol of Ca^{2+} per mg of protein are retained by sarcoplasmic reticulum membranes. We observed previously [6,7] that, in the presence of Mg^{2+} (1—2 mM), X-537 A does not release all the Ca^{2+} taken up due to the addition of ATP, probably because some X-537 A added combines with Mg^{2+}.

Effect of permeant anions (oxalate and phosphate) on the ATP induced increase in fluorescence of chlorotetracycline and on calcium uptake by sarcoplasmic reticulum

The results summarized in Fig. 2 represent the effect of oxalate and phosphate on the fluorescence of chlorotetracycline and on the Ca^{2+} uptake by sarcoplasmic reticulum membranes. Fig. 2A shows that the inclusion of 5 mM

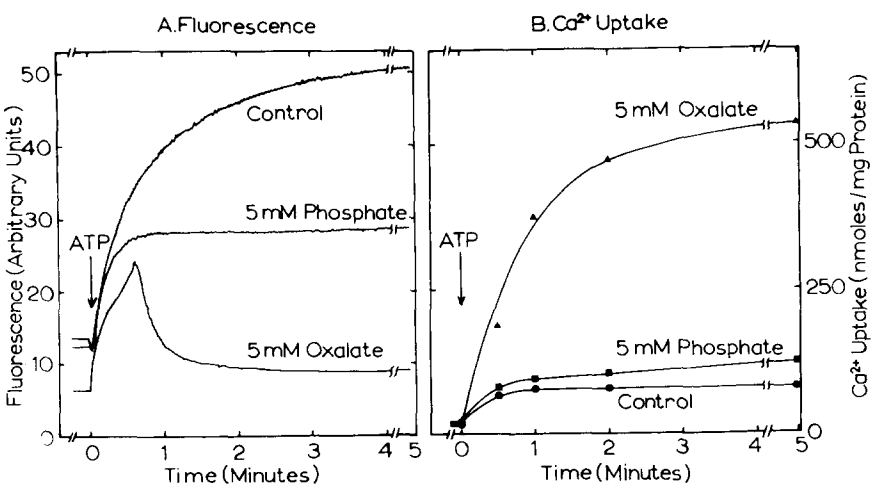

Fig. 2. Effect of phosphate and oxalate on the ATP induced increase in the fluorescence of chlorotetracycline (A) and on the Ca^{2+} uptake (B) by sarcoplasmic reticulum membranes. The incubation medium was identical to that described in the legend of Fig. 1, except that 5 mM potassium phosphate or potassium oxalate was present, as indicated in the traces, and the reaction was started by adding 1 mM ATP plus 0.2 mM $CaCl_2$. The fluorescence was followed continuously, and samples for Ca^{2+} uptake were withdrawn at the times indicated and filtered as described in Methods.

oxalate or phosphate in the assay media causes a decrease in the chlorotetracycline fluorescence signal in comparison to the fluorescence observed in the control experiment in which the permeant anions were omitted. The addition of 5 mM phosphate decreases the fluorescence signal, but a constant maximal value is maintained with time as shown in Fig. 2A. However, in the presence of 5 mM oxalate, a maximal fluorescence signal is attained within 1 min after adding ATP, when about 150 nmol of Ca^{2+} per mg of protein were accumulated, and then the fluorescence spontaneously decreases until a low steady-state level is attained.

The Ca^{2+} taken up by the sarcoplasmic reticulum membranes in the experiments of Fig. 2A is shown in Fig. 2B. One can see that in the absence of permeant anions the amount of Ca^{2+} taken up was of about 80 nmol Ca^{2+} per mg of protein, and the addition of either 5 mM phosphate or oxalate caused an increase of the amount of Ca^{2+} taken up by the membranes. In the presence of phosphate the maximal uptake, measured 5 min after adding ATP, was about 150 nmol Ca^{2+} per mg of protein, whereas in the presence of 5 mM oxalate all Ca^{2+} added to the medium (500 nmol/mg of protein) was taken up within 5 min after adding ATP. Fig. 2B shows that in the presence of 5 mM oxalate both the amount and rate of Ca^{2+} uptake are increased, in relation to the control experiment.

These results show that in the presence of precipitating agents the fluorescence signal of chlorotetracycline does not reflect total Ca^{2+} accumulated and that the spontaneous decrease in fluorescence, which occurs in the presence of oxalate, takes place while the Ca^{2+} retained by sarcoplasmic reticulum is increasing (Fig. 2A and B). Similar results were previously reported for Ca^{2+} and Mg^{2+} accumulation by mitochondrial membranes when phosphate was the permeant anion present [12].

In Fig. 3 we show the changes in the fluorescence of chlorotetracycline

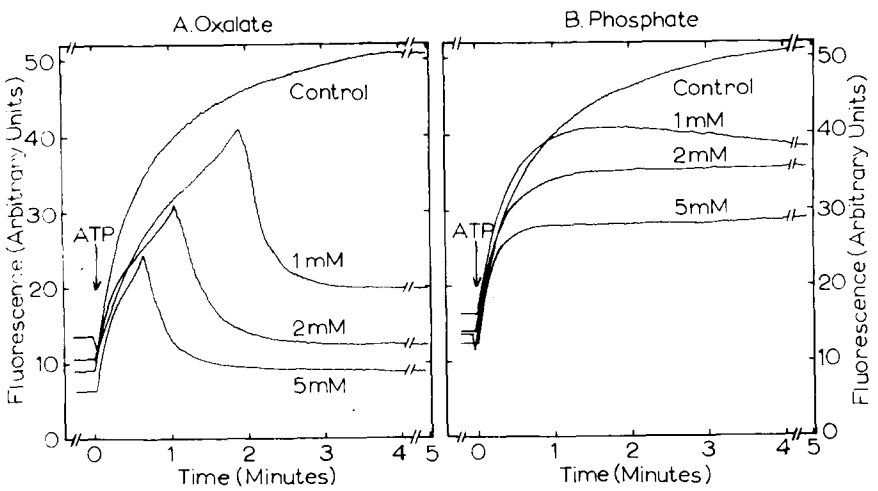

Fig. 3. Fluorescence of chlorotetracycline during Ca^{2+} uptake by sarcoplasmic reticulum vesicles in the presence of various concentrations of oxalate (A) or phosphate (B). Reaction conditions were the same as described in the legend of Fig. 1, except that the potassium oxalate or potassium phosphate was varied from 0 to 5 mM.

which occur during Ca^{2+} uptake in the presence of various concentrations (0—5 mM) of oxalate (Fig. 3A) or phosphate (Fig. 3B). Again, oxalate caused a reduction of fluorescence, specially in the case of 5 mM oxalate. For lower oxalate concentrations, the decrease in fluorescence was less accentuated, and the point of spontaneous decrease in the fluorescence signal was delayed in time. This probably suggests that in the presence of lower oxalate concentrations the rate of Ca^{2+} uptake is lower than it is at higher concentrations, and it takes longer to reach the amount of Ca^{2+} taken up which is necessary to cause precipitation of calcium-oxalate inside the vesicles.

Fig. 3A also shows that the steady-state fluorescence level observed within 3 to 4 min after adding ATP decreases with increasing oxalate concentrations. The data presented in Fig. 3B show that increasing concentrations of phosphate are also effective in decreasing the fluorescence signal of chlorotetracycline accompaining Ca^{2+} uptake by sarcoplasmic reticulum, as was described for oxalate. However, whereas in the case of oxalate there is a critical point at which the fluorescence begins to decline, in the presence of phosphate the maximal fluorescence signal attained for each phosphate concentration studied is maintained constant once it is reached.

The fluorescence decrease shown in Figs. 2 and 3 in the presence of oxalate probably reflects Ca^{2+} release from the membrane binding sites in spite of the larger amounts of Ca^{2+} being accumulated by the membrane vesicles.

Effects of X-537 A

The results summarized in Fig. 4 are instructive in elucidating further the mechanism of Ca^{2+} uptake and release by sarcoplasmic reticulum membranes in the presence of oxalate. Fig. 4A shows the changes in the chlorotetracycline fluorescence accompaning Ca^{2+} uptake by sarcoplasmic reticulum in the presence of 5 mM oxalate. In the control experiment (Fig. 4A), we observed an

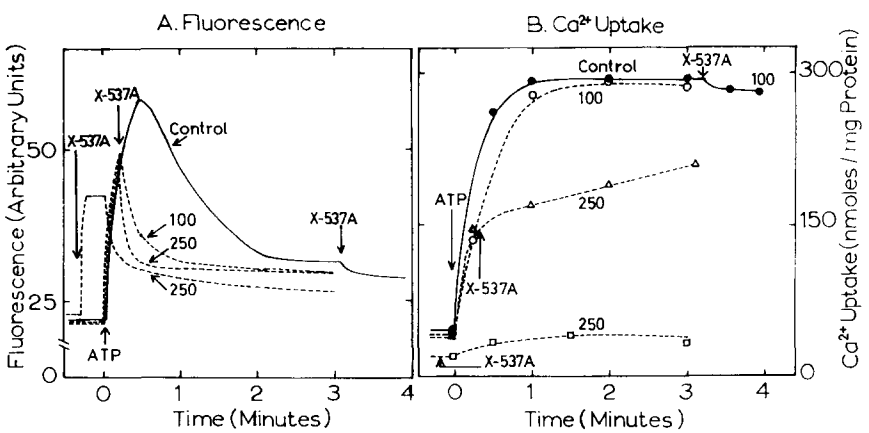

Fig. 4. Effect of X-537 A on the fluorescence of chlorotetracycline (A) and Ca^{2+} uptake (B) by sacroplasmic reticulum membranes measured in the presence of 5 mM oxalate. Incubation medium was the same as described in the legend of Fig. 1, except that 5 mM oxalate was present in all the experiments. In the control experiment, X-537 A (100 nmol/mg of protein) was added at the end of Ca^{2+} uptake. In the other experiments, X-537 A was added, during or before Ca^{2+} uptake was initiated by ATP, in the concentrations indicated in the trace expressed in nmol of X-537 A added per mg of protein [7].

increase in fluorescence of chlorotetracycline after initiating Ca^{2+} uptake by addition of ATP, and then a spontaneous drop in the fluorescence signal takes place. Addition of X-537 A (100 nmol/mg of protein) 3 min after addition of ATP causes only a slight decrease in the fluorescence of chlorotetracycline. In Fig. 4B the actual Ca^{2+} uptake and release occurring in the experiments of Fig. 4A is represented. One can see that in the control experiment the uptake reaches about 300 nmol/mg of protein, and the addition of X-537 A, after reaching maximal Ca^{2+} uptake, does not release a significant amount of Ca^{2+} from the vesicles.

Addition of 100 nmol of X-537 A per mg of protein, at 20 s. after adding ATP, inhibits the fluorescence signal of chlorotetracycline (Fig. 4A), but does not inhibit the Ca^{2+} uptake significantly (Fig. 4B), whereas 250 nmoles of X-537 A per mg of protein have a pronounced effect on the Ca^{2+} uptake and on the chlorotetracycline fluorescence. If added before ATP, X-537 A (250 nmol/mg protein) is effective in inhibiting completely Ca^{2+} uptake as well as the fluorescence signal (Fig. 4A and B).

The observation that the alterations in chlorotetracycline fluorescence do not always parallel the alterations in Ca^{2+} uptake when oxalate is present obviously is releated to the fact that chlorotetracycline fluorescence apparently reflects only membrane bound Ca^{2+} [6—9], whereas in the presence of precipitating agents retention of Ca^{2+} by sarcoplasmic reticulum vesicles is due mainly to precipitation of calcium-oxalate inside the vesicles. This aspect is further considered in Discussion.

An apparent anomaly occurs in Fig. 4A for the curve which describes the alteration in fluorescence when 250 nmol of X-537 A are added before adding ATP. The mere addition of X-537 A increases the fluorescence signal, which could not readily be explained, particularly since X-537 A does not have a direct effect on chlorotetracycline fluorescence. We reasoned that the addition of X-537 A, before addition of ATP, increases the binding of divalent cations to the membranes, which would in turn increase the fluorescence. Since Mg^{2+} is the prevalent cation in the medium, under the conditions of the experiment, there would be an increase in membrane bound Mg^{2+} (as well as some Ca^{2+}). We tested this supposition by measuring the effect of X-537 A on the passive binding of Ca^{2+} and Mg^{2+} by sarcoplasmic reticulum membranes when 2 mM Ca^{2+} or Mg^{2+} was present. The results obtained are reported in Fig. 5.

In Fig. 5A we observe that increasing concentrations of X-537 A, between 40 and 280 μM, increase the fluorescence of chlorotetracycline in the sarcoplasmic reticulum membranes, and the effect of X-537 A is more acentuated, for the higher X-537 A concentrations, in the presence of 2 mM Ca^{2+} than it is in the presence of 2 mM Mg^{2+}. In Fig. 5B we observe that the effect of X-537 A in increasing the fluorescence of chlorotetracycline in the membranes is related to an increase in the membrane bound Ca^{2+} or Mg^{2+}. It is evident from Fig. 5 that an enhancement of Ca^{2+} binding from 120 nmol to 270 nmol per mg protein is effective in increasing the chlorotetracycline fluorescence from 10 to 170 fluorescence units, whereas in the presence of the same added Mg^{2+} concentration (2 mM), the binding of Mg^{2+} increases from 70 nmol to 210 nmol of Mg^{2+} per mg of protein, and this increase in Mg^{2+} binding corresponds to an enhancement of fluorescence of chlorotetracycline from 20 to 80 fluorescence

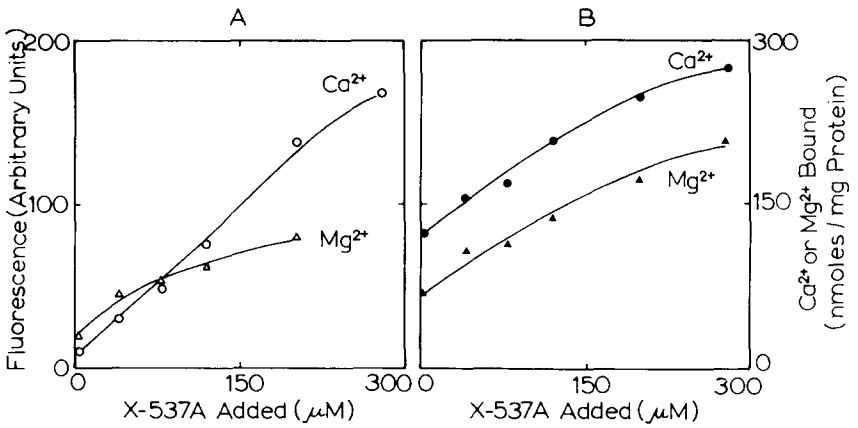

Fig. 5. Effect of X-537 A on the fluorescence of chlorotetracycline (A) and on the Ca^{2+} or Mg^{2+} binding (B) to sarcoplasmic reticulum membranes. The medium contained 0.35 M sucrose, 5 mM histidine-HCl at pH 6.75, 2 mM Ca^{2+} or Mg^{2+}, 10 μM chlorotetracycline, 1 mg sarcoplasmic reticulum protein/2.5 ml, and the X-537 A concentration was varied as indicated on the abscissa. The fluorescence values are corrected for the fluorescence of chlorotetracycline in the absence of sarcoplasmic reticulum membranes and for changes in fluorescence due to X-537 A additions to this blank. Ca^{2+} and Mg^{2+} binding was determined by filtration of the membrane suspensions through "Millipore" filters and by measuring the amount of Ca^{2+} or Mg^{2+} retained in the filters by the procedure described in Methods.

units. Therefore, the same increase in the binding of divalent cation promotes an increase in chlorotetracycline fluorescence which in the case of Ca^{2+} is at least twice as high as in the case of Mg^{2+}. This observation is in agreement with previous observations [6,7] that membrane bound Ca^{2+} has higher affinity for chlorotetracycline than the membrane bound Mg^{2+}. Results not reported in Fig. 5 show that 280 μM X-537 A causes an increase in chlorotetracycline binding from 2.5 to 12 nmol/mg of protein in the presence of 2 mM Mg^{2+} and from 6.0 to 18 nmol/mg of protein in the presence of 2 mM Ca^{2+}, under the conditions reported in the legend of Fig. 5.

Discussion

The results show that Ca^{2+} transport by isolated sarcoplasmic reticulum can be monitored by continuously measuring the fluorescent signal of chlorotetracycline, and that the increase in fluorescence observed after adding ATP is accompanied by an increase in the binding of chlorotetracycline by the sarcoplasmic reticulum membranes (Fig. 1). The existing evidence suggests that the increase in fluorescence, under the conditions of the experiments, reflects an increase in Ca^{2+} binding, and it is not a measure of free or precipitated Ca^{2+} in the vesicles [6—9]. Thus, it is possible to choose conditions in which the total Ca^{2+} taken up by the vesicles is high, in the presence of oxalate or phosphate, but the fluorescence signal is low (Figs. 2 and 3).

Thus, the Ca^{2+} uptake observed in the presence of oxalate can be followed by measuring the increase in chlorotetracycline only during the early stages of Ca^{2+} uptake, before intravesicular precipitation of calcium oxalate occurs. After precipitation begins, the Ca^{2+} continues to be taken up, but the fluorescence drops to a steady-state level which depends on the concentration of oxalate

(Figs. 2 and 3). The drop in fluorescence can be explained if we assume that calcium oxalate begins to precipiate only after "supersaturation" of oxalate with Ca^{2+}; i.e., the intravesicular concentration of free Ca^{2+} temporarily reaches a value which exceeds the solubility product of calcium oxalate, so that the intravesicular Ca^{2+} binding sites are transiently in equilibrium with a concentration of Ca^{2+} higher than that which persists at the steady state, after calcium oxlate begins to precipitate. Once precipitation of calcium oxalate begins, the free Ca^{2+} inside the vesicles decreases and a new equilibrium state is reached between the free Ca^{2+} and the Ca^{2+} binding sites on the membranes.

Apparently the precipitation of calcium phosphate occurs more rapidly than the precipitation of calcium oxalate, and, therefore, no excess free Ca^{2+}, over that determined by the solubility product for calcium phosphate, is accumulated inside the vesicles, and maximal fluorescence once reached remains constant for a particular phosphate concentration (Fig. 3). However, as in the case of oxalate, the chlorotetracycline fluorescence reflects only membrane bound Ca^{2+} and cannot be utilized to measure total Ca^{2+} uptake.

After Ca^{2+} is accumulated in sarcoplasmic reticulum vesicles in the form of calcium oxalate precipitate, X-537 A does not release the Ca^{2+} accumulated (Fig. 4), and similar results have been reported for cardiac muscle [19]. If X-537 A is added to sarcoplasmic reticulum vesicles before ATP, or after ATP but before precipitation of Ca^{2+} oxalate begins, as detected by the decrease in chlorotetracycline fluorescence, Ca^{2+} accumulation is either totally or partially inhibited, respectively (Fig. 4), at a concentration of X-537 A of 250 nmol per mg of protein.

It is of interest that if X-537 A added after ATP, but before calcium oxalate precipitation begins, causes a decrease in chlorotetracycline fluorescence although Ca^{2+} continues to be transported, even if at a slower rate. Our interpretation of these results (Fig. 4) is that the X-537 A makes the membrane partially leaky to Ca^{2+}, but if this leakiness is initiated after a critical concentration of intravesicular Ca^{2+} is reached, it is sufficiently compensated by the ratio of Ca^{2+} transport, so that Ca^{2+} oxalate can still begin to precipitate. In the presence of oxalate, the Ca^{2+} gradient is greatly minimized so that some leakiness of the membrane is tolerated in the presence of oxalate without completely impeding the precipitation of intravesicular calcium oxalate, although it will occur at a slower rate.

The ionophore, X-537 A, has been widely used as a tool to allow Ca^{2+} to pass easily across biological membranes [19,20]. In our experience, X-537 A increases the passive binding of Ca^{2+} and Mg^{2+} by sarcoplasmic reticulum. The effect is significant only at relatively high concentration of X-537 A and it is higher for Ca^{2+} than for Mg^{2+} (Fig. 5). The concentrations of X-537 A normally utilized in biochemical studies are of the order of 20 μM [19—21] which has only a relatively small effect on passive binding of Ca^{2+} or Mg^{2+} (Fig. 5). At higher concentrations, one has to take into consideration the effect of X-537 A on the passive binding of Ca^{2+} and Mg^{2+}.

We were able to monitor continuously the Ca^{2+} binding by sarcoplasmic reticulum under various conditions utilizing chlorotetracycline as a fluorescent probe which is sensitive to Ca^{2+} in the membrane phase, but not to Ca^{2+} which precipitates intravesicularly. The results illustrate the usefulness of the techni-

que in tackling the important problem of determining the distribution and the state of accumulated Ca^{2+} in biological sistems. Our studies further permitted us to conclude that in the presence of oxalate the concentration of free Ca^{2+} inside the sarcoplasmic reticulum vesicles transiently exceeds the value which exists at equilibrium after calcium oxalate precipitates inside the sarcoplasmic reticulum vesicles.

Acknowledgements

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References

1 Hasselbach, W. (1964) Progr. Biophys. 14, 167—222
2 Martonosi, A. (1971) Biomembranes 14, 191—256
3 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329—369
4 Inesi, G. (1972) Ann. Rev. Biophys. Bioenerg. 1, 191—210
5 Carvalho, A.P. and Leo, B. (1967) J. Gen. Physiol. 50, 1327—1352
6 Carvalho, C.M. and Carvalho, A.P. (1976) Ciencia Biol. (Portugal) 2, 223—238
7 Carvalho, C.M. and Carvalho, A.P. (1976) Ciencia Biol. (Portugal) 2, 239—253
8 Casswell, A.H. and Warren, S. (1972) Biochem. Biophys. Res. Commun. 46, 1757—1763
9 Casswell, A.H. and Pressman, B.C. (1972) Biochem. Biophys. Res. Commun. 49, 292—298
10 Lau, Y.H., Chiu, T.H., Casswell, A.H. and Potter, L.T. (1974) Biochem. Biophys. Res. Commun. 61, 510—516
11 Casswell, A.H. (1972) J. Membrane Biol. 7, 345—364
12 Schuster, S.M. and Olson, M.S. (1974) J. Biol. Chem. 249, 7151—7158
13 Casswell, A.H. and Hutchison, J.D. (1971) Biochem. Biophys. Res. Commun. 42, 43—49
14 Hallet, M., Schneider, A.S. and Carbone, E. (1972) J. Membrane. Biol. 10, 31—44
15 Casswell, A.H. and Hutchison, J.D. (1971) Biochem. Biophys. Res. Commun. 43, 625—630
16 Silva, J.J.R.F. and Dias, M.H.M. (1972) Rev. Port. Quim. 14, 159—169
17 Layne, E. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 3, pp. 447—451
18 Schuster, S.M. and Olson, M.S. (1973) J. Biol. Chem. 248, 8370—8377
19 Entman, M.C., Gillette, P.C., Wallick, E.T., Pressman, B.C. and Schwartz, A. (1972) Biochem. Biophys. Res. Commun. 48, 847—857
20 Vale, M.G.P. (1974) Ciencia Biol. (Portugal) 2, 161—170
21 Scarpa, A., Baldassare, J. and Inesi, G. (1972) J. Gen. Physiol. 60, 735—749