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Calcium oxalate and calcium phosphate capacities of cardiac sarcoplasmic reticulum

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Both oxalate-supported and phosphate-supported calcium uptake by canine cardiac sarcoplasmic reticulum initially increase linearly with time but fall to a steady-state level within 20 min. The departure from linearity could be due to a decrease in influx or to an increase in efflux of calcium. Because Ca^{2+}-ATPase activity is linear, a decrease in the influx of calcium is an unlikely cause of the non-linear calcium uptake curves. A possible cause of an increase in calcium efflux is rupture of the vesicles. This hypothesis was tested by investigating the amount of calcium which could be released upon addition of 5 mM EGTA. The amount of rapidly releasable calcium was zero until a threshold calcium uptake of about 4–6 μmol calcium oxalate or calcium phosphate per mg was reached. After that point the rapidly releasable calcium continued to increase with calcium oxalate to reach more than 23 μmol / mg, but stayed constant at about 0.7 μmol / mg for calcium phosphate. The rapidly releasable calcium was attributed to calcium oxalate or calcium phosphate crystals externalized by vesicle rupture. The differences in the amounts of rapidly releasable calcium were attributed to different kinetics of calcium phosphate and calcium oxalate dissolution. Addition of ryanodine caused a marked increase in the threshold for rapidly releasable calcium oxalate. Transmission electron micrographs showed that vesicles can become filled with calcium oxalate crystals, but the vesicles were heterogeneous with respect to their size and their sensitivity to ryanodine. These observations support the hypothesis that calcium oxalate and calcium phosphate capacities are limited by vesicle rupture and that ryanodine increases the capacity by closing a calcium channel in a subpopulation of vesicles that otherwise would not accumulate calcium.

Introduction

Calcium-precipitating anions have long been used in studies of calcium transport by sarcoplasmic reticulum in order to augment the amount of calcium accumulated [1,2]. One current hypothesis for this augmentation of calcium uptake is that the oxalate enters the vesicle after being protonated by the Ca^{2+}-ATPase [3] and that the transported calcium increases in concentration until calcium oxalate precipitation occurs [4]. Since the early addition of ionophore X537A or A23187 completely release the accumulated calcium [3,5], apparently it is not immediately precipitated as calcium oxalate. After further incubation, calcium oxalate crystallization begins, and these early crystals seed crystallization in the intravesicular space [5,20]. During the linear phase of calcium uptake, the Ca^{2+}-ATPase pumps calcium inward against a calcium gradient and the transported calcium either precipitates as calcium oxalate or

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

leaves the vesicle by passive efflux. When passive efflux is increased by the ionophore A23187, the calcium uptake rate is decreased because there is competition between precipitation and passive efflux [5]. This hypothesis explains the differential sensitivity of calcium uptake for A23187 when the ionophore is added before or after uptake has begun [5,21,22]. If the above description of anion-supported calcium uptake is correct, then what limits the extent of calcium uptake? The Ca^{2+}-ATPase activity during the linear phase of calcium uptake ought to be determined by the intravesicular and extravesicular conditions. The intravesicular calcium concentration should rise sufficiently to inhibit Ca^{2+}-ATPase activity such that the rate of calcium transport is very nearly matched by the rate of calcium oxalate precipitation. This follows from the observation that passive efflux is quite low compared to the rate of calcium uptake [5,6]. As long as suitable conditions persist, the Ca^{2+}-ATPase should continue to transport calcium. As originally proposed by Makinose and Hasselbach [40], it thus appears that the intravesicular volume would be the limiting factor in calcium uptake [41,42]. Calcium uptake would proceed until the vesicles broke due to the accumulation of calcium oxalate or calcium phosphate crystals.

We have investigated the calcium-oxalate and calcium-phosphate capacity of cardiac sarcoplasmic reticulum because this may have implications for studies on calcium release using precipitating anions [7–16] and for studies using capacity as a measure of the amount of sarcoplasmic reticulum in tissues [17,39,42].

Methods

Sarcoplasmic reticulum preparation. Cardiac sarcoplasmic reticulum vesicles were obtained from minced canine ventricles as described previously [18]. The sarcoplasmic reticulum vesicles were stored at −20°C in 30% sucrose, 20 mM Tris buffer (pH 7.0). The protein concentration was estimated by the method of Lowry et al. [19] using serum albumin as a standard.

Calcium oxalate crystallization and dissolution. The kinetics of calcium-oxalate crystallization and dissolution were monitored using ^{45}Ca and filtration through 0.45 μm Millipore filters. The amount of calcium oxalate crystals were calculated from the total radioactivity in the reaction bath and the radioactivity remaining in the filtrate. Results obtained by this method were reproducible. The reaction bath contained 100 mM KCl, 20 mM imidazole buffer (pH 7.0), 10 mM potassium oxalate, 0.2 mM $CaCl_2$ with about 0.1 μCi ^{45}Ca per ml reaction bath. The crystallization reaction was begun by adding 0.04 ml of 0.05 M $CaCl_2$ to a 9.96 ml reaction bath previously pre-equilibrated to 37°C. The crystallization reaction was complete by 15 min (Fig. 1). Dissolution of the crystals was tested by adding various concentrations of EGTA 10 min after starting the crystallization reaction.

Calcium phosphate crystallization and dissolution. The kinetics of calcium phosphate crystallization and dissolution were also followed by the Millipore filtration method using ^{45}Ca. In this case the reaction medium (37°C) contained 100 mM KCl, 20 mM imidazole, 50 mM sodium phosphate (pH 7.0), and a final concentration of 1.5 mM $CaCl_2$ and 0.75 μCi ^{45}Ca per ml. For the dissolution experiments, 0.667 ml of the suspended calcium phosphate crystals at 37°C were added to 4.333 ml reaction medium at 37°C so the final concentrations were 0.2 mM total calcium, 100 mM KCl, 20 mM imidazole (pH 7.0), 50 mM sodium phosphate, 5 mM $MgCl_2$, 5 mM Na_2ATP and various concentrations of EGTA or $^{45}CaCl_2$. When $^{45}CaCl_2$ was added, the specific activity of the calcium was adjusted to match the specific activity of the previously formed calcium phosphate crystals. This was done to ensure that any exchange between free calcium and precipitated calcium would be undetectable in these experiments.

Calcium oxalate uptake by cardiac sarcoplasmic reticulum. Calcium oxalate uptake by cardiac sarcoplasmic reticulum vesicles was followed by Millipore filtration. Vesicles (5–60 μg/ml) were preincubated for 4 min in a bath at 37°C containing 112.9 mM KCl, 22.7 mM imidazole (pH 7.0), with and without 564 μM ryanodine. The 4 min pre-incubation was necessary in order to observe maximal stimulation of uptake by ryanodine [29]. The uptake reaction was begun by the rapid sequential addition of $MgCl_2$ plus Na_2ATP, potassium oxalate and $CaCl_2$ all previously equi-

librated to 37°C. The clock was started upon addition of $CaCl_2$. Final reaction conditions were 100 mM KCl, 10 mM imidazole (pH 7.0), 10 mM potassium oxalate, 0.2 mM $CaCl_2$ with 0.1 μCi ^{45}Ca/ml, 5 mM $MgCl_2$, 5 mM Na_2ATP and 500 μM ryanodine, when added. The reaction bath generally was 10 ml and 0.15 ml aliquots were taken at various times for filtration through 0.45 μm Millipore filters. Aliquots (100 μl) of the reaction bath and filtrates were counted for ^{45}Ca by liquid scintillation spectrometry.

Phosphate liberation by cardiac sarcoplasmic reticulum. The amount of inorganic phosphate liberated from ATP by cardiac sarcoplasmic reticulum was measured from aliquots of filtrates of a reaction bath identical to that used for calcium uptake. The inorganic phosphate was measured by the colorimetric assay described by Penney [38].

Calcium phosphate uptake by cardiac sarcoplasmic reticulum. Phosphate-supported calcium uptake was measured similarly to oxalate supported calcium uptake except that the final reaction conditions were 37°C, 100 mM KCl, 20 mM imidazole (pH 7.0), 100 mM sodium phosphate, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM Na_2ATP and 500 μM ryanodine, when added. The cardiac sarcoplasmic reticulum vesicles were pre-incubated for 4 min in both the presence and absence of ryanodine.

Release of calcium oxalate or calcium phosphate accumulated by sarcoplasmic reticulum. Release of calcium oxalate or calcium phosphate was measured after addition of 5 mM EGTA (pH 7.0), with or without 2 μM A23187. An aliquot (1 ml) of the reaction bath described above was removed to a second incubation vessel maintained at 37°C. At the appropriate times, 5 μl 1 M EGTA was added and 0.15 ml aliquots were then filtered at various times. To determine whether the accumulated calcium oxalate or calcium phosphate was intravesicular, the same experiments were performed except 2 μl 1 mM A23187 in DMSO were added immediately preceding the EGTA.

Electron microscopy of oxalate-loaded vesicles. Cardiac sarcoplasmic reticulum vesicles were loaded at 37°C in a reaction bath containing 100 mM KCl, 20 mM imidazole buffer (pH 7.0), 10 mM potassium oxalate, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.226 mg/ml cardiac sarcoplasmic reticulum protein, with or without 500 μM ryanodine. The total calcium oxalate load was obtained by varying the amount of $CaCl_2$ added. The $CaCl_2$ was added in 0.2 mM increments at timed intervals determined by previously measured calcium uptake rates. The interval was 0.45 min in the absence of ryanodine when calcium uptake was 3.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, and 0.25 min in the presence of ryanodine when calcium uptake rate was 6.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Millipore filtration performed at the end of the loading period confirmed that essentially all of the added calcium was taken up.

A drop of the suspension of loaded vesicles was placed on copper grids coated with 0.5% formvar in ethylenedichloride. The drop was allowed to stand for 1–2 min and then excess fluid was removed by absorption with filter paper. The vesicles adhering to the formvar-coated grids were negatively stained by application of 1% sodium phosphotungstic acid (pH 7.0) for 1 min. Excess stain was absorbed with filter paper and the vesicles were then washed with a drop of distilled water which was removed immediately after application. After drying, grids were photographed using an Hitachi HU-12 transmission electron microscope with 75 keV beam.

Materials

Na_2ATP was purchased from P&L Biochemicals and was used without further purification. A23187 was from Calbiochem. ^{45}Ca was purchased from New England Nuclear. All other chemicals were reagent grade. Ryanodine was purchased from S.P. Penick Corp.

Results

In order to understand the determinants of calcium oxalate and calcium phosphate capacities, we believe it is necessary to have some understanding of the kinetics of calcium oxalate and calcium phosphate crystal formation and dissolution. These kinetics, for example, are responsible for the different sensitivity of calcium uptake rate to the ionophore A23187 when the ionophore is added before or after calcium uptake has begun [5,22]. We used Millipore filtration to investigate crys-

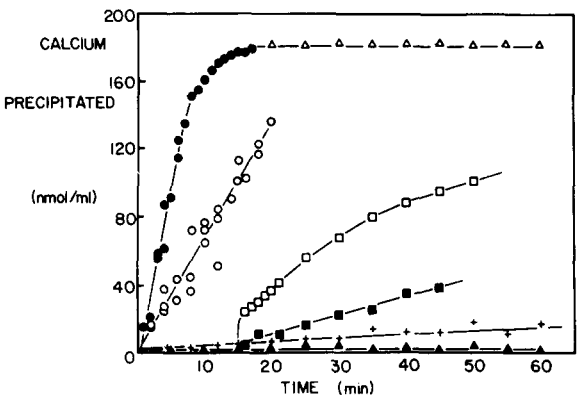

Fig. 1. Kinetics of calcium oxalate crystallization and seeding. The amount of calcium oxalate crystals was determined by Millipore filtration through 0.45 μm filters as described in Methods. Crystallization was conducted at 37°C in a reaction bath containing 100 mM KCl, 20 mM imidazole buffer (pH 7.0), 10 mM potassium oxalate, 0.2 mM $CaCl_2$ and 0.1 μCi ^{45}Ca/ml (●). Separate reactions included 5 mM $MgCl_2$ added before addition of 0.2 mM $CaCl_2$ (○); 5 mM Na_2ATP (pH 7.0) added before addition of 0.2 mM $CaCl_2$ (+); 5 mM $MgCl_2$ plus 5 mM Na_2ATP added before addition of 0.2 mM $CaCl_2$ (▲) or 15 min after addition of $CaCl_2$ (△). In other reactions, pre-formed calcium oxalate crystals were added to a bath so that the final concentrations were 100 mM KCl, 20 mM imidazole, 10 mM potassium oxalate, 0.2 mM uncrystallized $CaCl_2$, 5 mM $MgCl_2$ and 5 mM Na_2ATP. The calcium oxalate crystals were made so that the specific activity of the crystals was the same as the added calcium. The amount of added calcium oxalate crystals was 250 nmol/ml (□) or 62.5 nmol/ml (■).

tallization of calcium oxalate and calcium phosphate. Although this method agrees with results using light-scattering methods [5], both methods may not detect formation of very small microcrystals. Nevertheless, because we monitored sarcoplasmic reticulum uptake using Millipore filtration, this method should correct for artifacts of crystallization which might occur in our experiments.

When calcium and oxalate are incubated under the conditions of our standard reaction bath in the absence of cardiac sarcoplasmic reticulum, no detectable crystallization occurs within one hour (Fig. 1). When both Mg and ATP were omitted from the reaction bath, rapid calcium oxalate crystallization occurs which is complete in about 10–15 min. About 90% of the calcium is retained by the Millipore filters when total $CaCl_2$ is 0.2 mM and potassium oxalate is 10 mM. In unpublished results, we have shown that the rate and extent of crystallization depends on both the calcium and oxalate concentrations, in agreement with other results [4]. When Mg^{2+} plus ATP are added after the crystallization of calcium oxalate, no dissolution is observed for 50 min. In other studies, calcium oxalate crystallization was slowed by the sole addition of 5 mM $MgCl_2$ or 5 mM Na_2ATP (pH 7.0), but ATP was more effective in inhibiting crystallization. Thus it appears that ATP is essential for inhibition of calcium oxalate crystallization in our standard uptake reaction.

From the above results, it appears possible to have two apparently stable solutions of identical composition except calcium oxalate is soluble in one but crystallized in the other. At least one of these solutions is metastable. Since overnight incubation of the complete reaction bath including MgATP results in calcium oxalate crystallization, it is the bath initially containing MgATP which is metastable. There are several possible explanations for these results. First, MgATP may prevent nucleation of the calcium oxalate crystals. Second, MgATP may prevent the growth of the crystals by adsorbing to the surface of the nuclei. Third, MgATP may slow crystal growth by complexing Mg with oxalate and complexing ATP with calcium to lower the effective concentration of the precipitating ions. To determine the possible effects on nucleation, we added various volumes of a suspension of calcium oxalate crystals which had an identical specific activity as the calcium present in a bath containing MgATP. As shown in Fig. 1, seed crystals caused an increase in calcium oxalate crystallization which was related to the amount of seed crystals added. This result suggests that rupture of sarcoplasmic reticulum vesicles, which would exposure calcium oxalate crystals to the extravesicular uptake solution, could cause extravesicular calcium oxalate crystallization which would appear as calcium uptake in the absence of real calcium uptake mediated by the sarcoplasmic reticulum calcium pump.

Although 5 mM MgATP does not dissolve preformed calcium oxalate crystals, these crystals can be readily dissolved by EGTA (Fig. 2). When the EGTA concentration is increased, the rate of dissolution of the calcium oxalate crystals is also increased. At 5 mM EGTA, 0.18 mM calcium

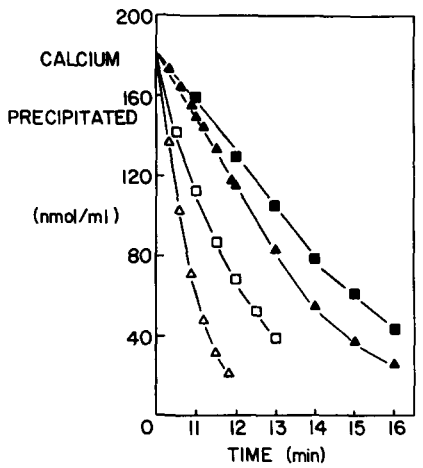

Fig. 2. Kinetics of calcium oxalate dissolution. Formation of crystals was identical to that described in Fig. 1. Dissolution of calcium oxalate crystals occurred after addition of EGTA at a final concentration of 1 mM (□, ■) or 5 mM (△, ▲). Addition of 5 mM $MgCl_2$ plus 5 mM Na_2ATP (pH 7.0), caused a decrease in the rate of calcium oxalate dissolution (▲, ■).

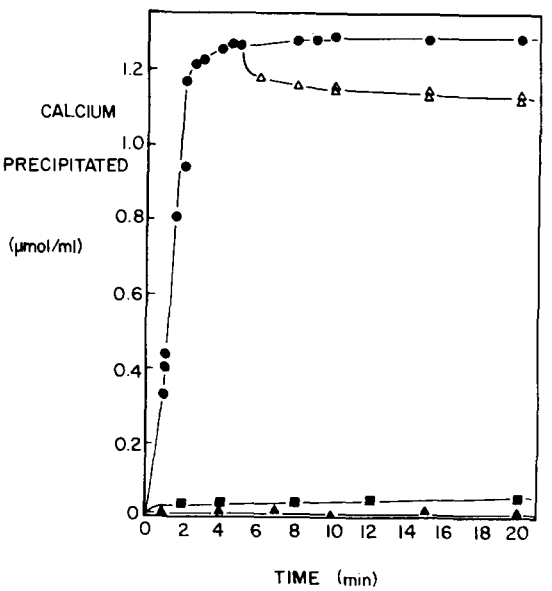

Fig. 3. Kinetics of calcium phosphate crystallization. The reaction medium was kept at 37°C and contained 1.5 mM $CaCl_2$, 50 mM NaH_2PO_4, 100 mM KCl, 20 mM imidazole (pH 7.0), with (▲, △) and without (●) 5 mM $MgCl_2$ and 5 mM Na_2ATP. The MgATP was added before (▲) or 5 min after (△) beginning the crystallization reaction. A separate reaction was performed in which which the calcium concentration was 0.75 mM and MgATP was omitted (■).

oxalate crystals dissolve nearly completely in 2 min (Fig. 2). Adding 5 mM MgATP significantly slowed this reaction. This effect of ATP may be similar to the inhibition of hydroxyapatite dissolution by ATP [43]. The results suggest that addition of EGTA to ruptured vesicles should dissolve the calcium oxalate crystals exposed to the EGTA.

The kinetics of crystallization and dissolution were also investigated with phosphate. The crystallization of calcium phosphate is shown in Fig. 3. In this case the calcium concentration was 1.5 mM and phosphate was 50 mM. These high concentrations were necessary as only a little crystallization was observed when calcium was 0.75 mM or less. As with oxalate, no crystallization was observed when 5 mM MgATP was present prior to formation of the crystals. When 5 mM MgATP was added after the crystals were formed, dissolution was slow. The initial rapid drop in calcium phosphate upon addition of MgATP could be an artifact caused by ^{45}Ca-^{40}Ca exchange. The MgATP we used was contaminated by approx. 7 μmol Ca^{2+} per mmol MgATP, so that 5 mM MgATP added about 35 μM calcium. Complete exchange of this amount of calcium would produce a large part of the rapid drop after adding MgATP as shown in Fig. 3. Because the calcium requirement for crystallization is so much higher for 50 mM phosphate compared to 10 mM oxalate, it is likely that rupture of the vesicles with subsequent exposure of the calcium phosphate crystals would result in dissolution of the calcium phosphate crystals rather than a seeding of further crystallization.

The rate of dissolution of calcium phosphate crystals is shown in Fig. 4. These crystals dissolve very rapidly when the free calcium is maintained very low by the addition of 5 mM EGTA. As the free calcium concentration increases, the rate of dissolution decreases (Fig. 4). When the crystals were diluted into a medium identical to the uptake solution but containing no added calcium (non-precipitated calcium was about 30 μM), dissolution was about 50% complete in 3 min. The initial rapid drop in the curves may be partly due to an exchange of crystal ^{45}Ca with solution ^{40}Ca present as a contaminant of MgATP.

Using the above studies as background, we investigated calcium oxalate uptake by cardiac sarcoplasmic reticulum vesicles in the presence

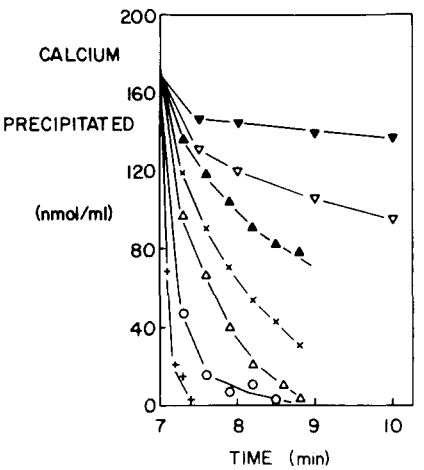

Fig. 4. Kinetics of calcium phosphate dissolution. Calcium phosphate crystals were formed as described in the legend to Fig. 3. The rate of dissolution was estimated as described in Methods by adding pre-formed crystals to solutions containing final EGTA concentrations of 0 mM (▽); 0.1 mM (▲); 0.25 (×); 0.50 mM (△); 1.25 mM (○); and 5.0 mM (+). In another reaction, preformed calcium phosphate crystals were diluted into a medium containing 100 μM $^{45}CaCl_2$ having a specific radioactivity identical to the specific radioactivity of the calcium phosphate crystals (▼). The amount of calcium added was 200 nmol/ml. about 170 nmol/ml was present as calcium phosphate crystals while 30 nmol/ml was free as determined by Millipore filtration.

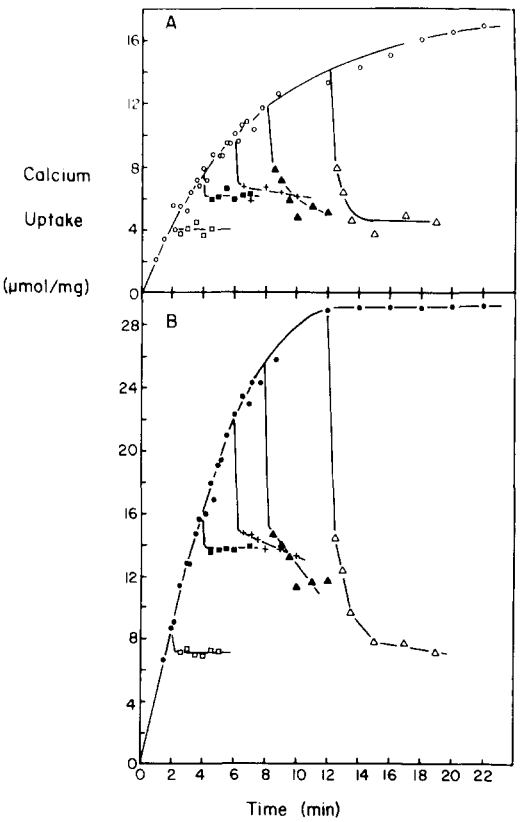

Fig. 5. Oxalate-supported calcium uptake by cardiac sarcoplasmic reticulum vesicles in the absence (A, ○) and presence (B, ●) of 500 μM ryanodine. Reaction conditions are described in Methods. The concentration of cardiac sarcoplasmic reticulum protein was 5.7 μg/ml. The amount of rapidly releasable calcium was estimated by adding 5 mM EGTA at 2 min (□); 4 min (■); 6 min (+); 8 min (▲); or 12 min (△).

and absence of ryanodine. Ryanodine is a neutral alkaloid isolated from *Ryania speciosa* which is postulated to close calcium channels in sarcoplasmic reticulum [23–29]. The calcium oxalate uptake shown in Fig. 5 represents data from three separate baths using a single preparation of cardiac sarcoplasmic reticulum vesicles, but they were reproducible with other preparations as well. In the absence of ryanodine, calcium uptake appeared to be curvilinear over almost its entire course, and a well-defined capacity was not reached. At the last time sampled, calcium uptake exceeded 16 μmol/mg (Fig. 5A). In the presence of 500 μM ryanodine, the initial calcium uptake rate was markedly stimulated and an apparently well-defined capacity was reached at 29.1 μmol/mg. The total available calcium in this experiment was 35.4 μmol/mg, so only 82.2% of the 0.2 mM calcium was taken up in the presence of ryanodine (Fig. 5B).

To determine if the non-linear calcium uptake curve was due to a decrease in calcium influx, we measured both calcium uptake and calcium-dependent ATPase activity in the same reaction. The results (Fig. 6) show that calcium uptake is non-linear while phosphate liberation remains linear over the same time-course. This result suggests that calcium influx does not decrease with time of incubation.

An alternate explanation of the non-linear uptake curve is an increased efflux brought about by rupture of the vesicles. Since our preliminary studies indicated that EGTA could rapidly dissolve calcium oxalate crystals, we added 5 mM EGTA at various times during the uptake reaction to determine the amount of rapidly releasable calcium. In these experiments, dissolution of external calcium oxalate would appear as a net calcium

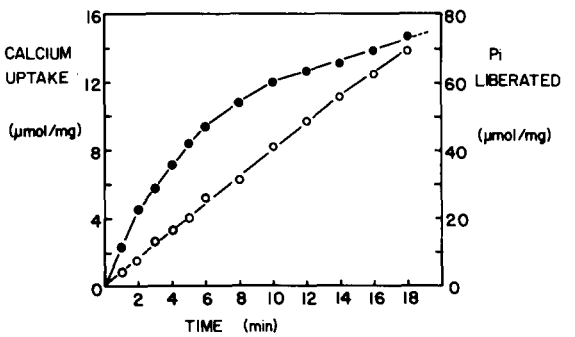

Fig. 6. Oxalate-supported calcium uptake and simultaneous calcium-dependent phosphate liberation from ATP by cardiac sarcoplasmic reticulum vesicles. Reaction conditions were as described in Methods. The cardiac sarcoplasmic reticulum protein concentration was 8.1 μg/ml. Ca uptake (●); P_i liberation (○).

'release'. When EGTA was added early in the reaction (2 min) calcium uptake ceased but there was little calcium release. When EGTA was added 12 min after beginning uptake, there was a large and rapid calcium release followed by a slower release (Fig. 5). If the calcium uptake represents intravesicular calcium, then these releases occur at rates approximating 30 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. In the presence of ryanodine, both calcium uptake and the amount of rapidly releasable calcium was increased (Fig. 5).

There are at least three possible explanations for the results shown in Figs. 5 and 6. First, there is an unknown, time-dependent, spontaneous increase in the permeability of the sarcoplasmic reticulum vesicles [23]; second, EGTA induces the release of an intravesicular pool of calcium; and third, EGTA dissolves calcium oxalate crystals

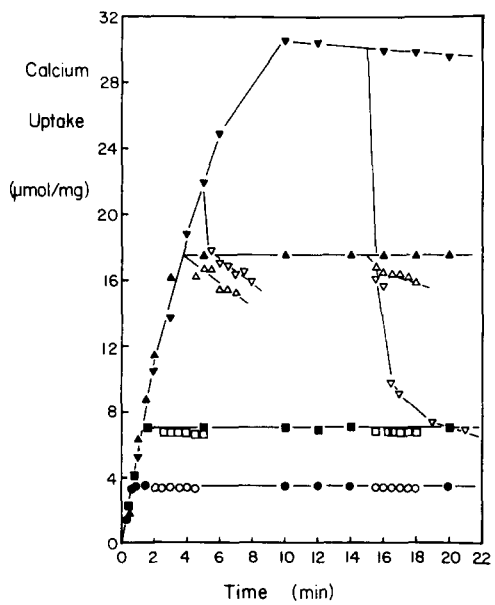

Fig. 7. Time dependence of EGTA-induced release of calcium from cardiac sarcoplasmic reticulum vesicles. Calcium uptake was measured as described in Methods using a final concentration of 500 μM ryanodine. The total available calcium was 3.54 μmol/mg (●, ○); 7.08 μmol/mg (■, □); 17.7 μmol/mg (▲, △) and 35.4 μmol/mg (▼, ▽) brought about by changing the sarcoplasmic reticulum protein concentration as 56 μg/ml (●); 28 μg/ml (■); 11.3 μg/ml (▲); and 5.7 μg/ml (▼). EGTA induced calcium release was tested by adding 5 mM EGTA at various times (open symbols).

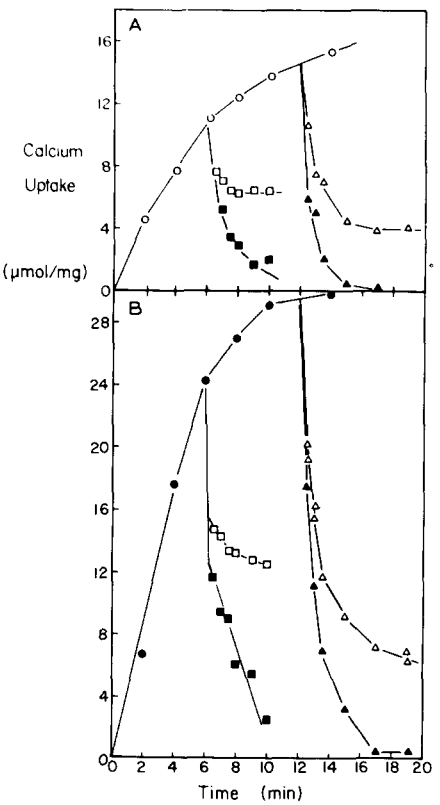

Fig. 8. Intravesicular versus extravesicular calcium oxalate. Oxalate-supported calcium uptake by cardiac sarcoplasmic reticulum vesicle was determined as described in the legend to Fig. 5. Extravesicular calcium oxalate was estimated by the calcium readily released by addition of 5 mM EGTA at 6 (□) and 12 (△) min after beginning the uptake reaction. In separate reactions, the EGTA was accompanied by 2 μM A23187 at 6 (■) and 12 (▲) min after starting calcium uptake. (A) No ryanodine present (○). (B) 500 μM ryanodine present (●).

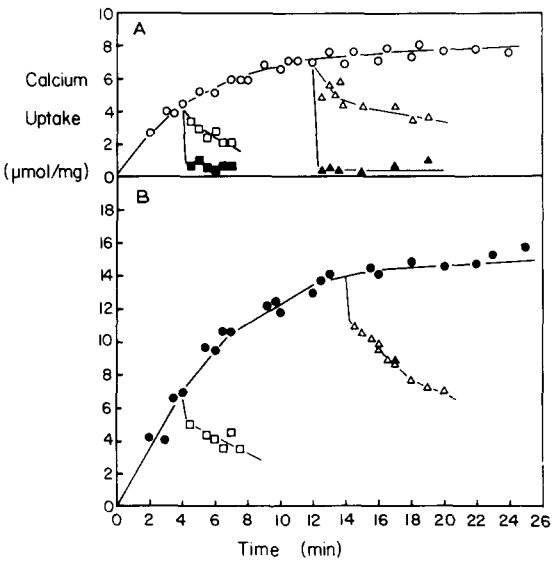

Fig. 9. Phosphate-supported calcium uptake by cardiac sarcoplasmic reticulum vesicles in the absence (A, ○) and presence (B, ●) of 500 μM ryanodine. Reaction conditions are described in Methods. The amount of rapidly releasable calcium was estimated by adding 5 mM EGTA at 4 min (□), and at 12 or 14 min (△) after beginning the uptake reaction. In some cases, 2 μM A23187 was also added (■, ▲) to show that the calcium could be completely released. Total available calcium was 17.7 μmol/mg and the sarcoplasmic reticulum protein concentration was 11.3 μg/ml in the absence of ryanodine; in the presence of ryanodine, the sarcoplasmic reticulum protein concentration was 5.7 μg/ml and total calcium was 35.4 μmol/mg. The sarcoplasmic reticulum preparation was the same as that used for Fig. 5.

externalized by vesicle rupture. To test the first possibility, we added EGTA at different times after loading the vesicles to various levels. The loads were varied by limiting the available calcium. With calcium uptake of 3.5 or 7.0 μmol/mg, in the presence of ryanodine, there was no noticeable release of calcium whether 5 mM EGTA was added after 2 or 15 min of calcium uptake (Fig. 7). With high loads of calcium, achieved when the available calcium exceeded the capacity, there was a greater release of calcium when EGTA was added after 15 min of uptake compared to when EGTA was added after 4 min of uptake. The rapid release of calcium by EGTA, then, is not strictly time-dependent but depends on the continued availability of calcium.

To ascertain whether or not the calcium retained after adding 5 mM EGTA was intravesicular, experiments were performed in which EGTA was added along with A23187. The results (Fig. 8) show that only a portion of the calcium uptake can be released by EGTA while all of the calcium oxalate can be released by EGTA plus A23187. This observation supports the hypothesis that the rapidly released calcium is extravesicular while the calcium slowly released by EGTA is intravesicular.

The experiments described above for oxalate-supported calcium uptake were repeated for phosphate-supported calcium uptake. The results (Fig. 9) show that phosphate-supported up-

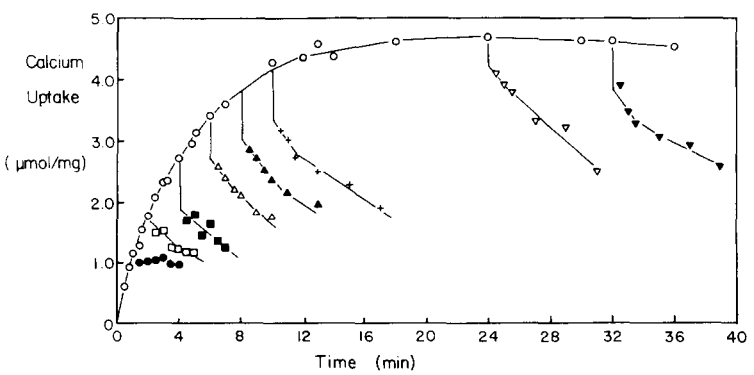

Fig. 10. Phosphate-supported calcium uptake by cardiac sarcoplasmic reticulum. Reaction conditions are described in Methods. Total calcium was 9.61 μmol/mg and the sarcoplasmic reticulum protein concentration was 21 μg/ml. Rapidly releasable calcium was estimated by adding 5 mM EGTA at 1.0 (●); 2.0 (□); 4.0 (■); 6.0 (△); 8.0 (▲); 10 (+); 24 (▽) and 32 (▼) min after beginning the uptake reaction.

take is less than oxalate supported calcium uptake both in the presence and absence of ryanodine. The same cardiac sarcoplasmic reticulum preparation was used for Fig. 5 and Fig. 9 specifically to allow this comparison. As with oxalate, all of the calcium phosphate can be released rapidly upon simultaneous addition of EGTA plus A23187, whereas only a portion is released following addition of EGTA (Fig. 9).

The phosphate-supported calcium uptake in the absence of ryanodine was investigated more throughly and the results are presented in Fig. 10.

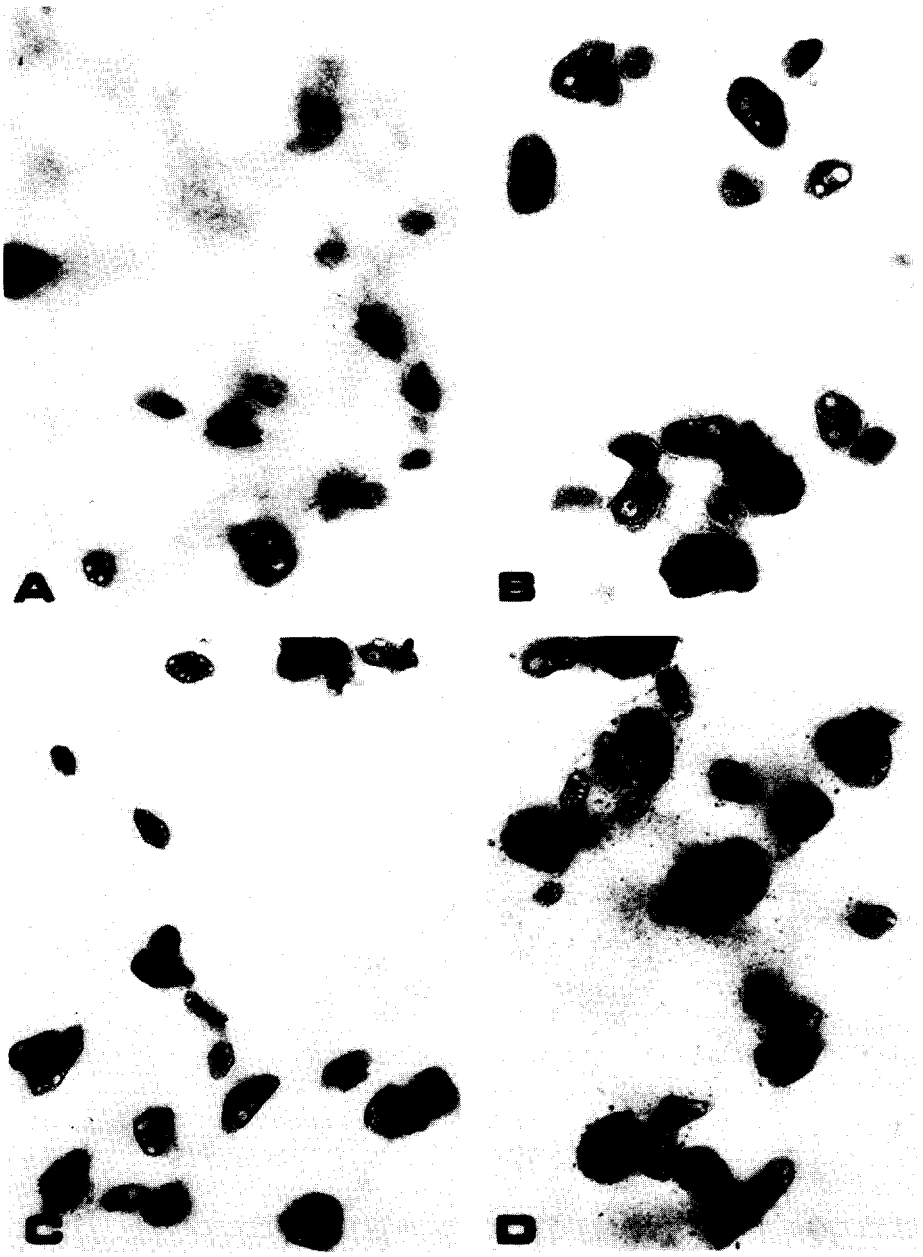

Fig. 11. Electron micrographs of cardiac sarcoplasmic reticulum vesicles loaded with calcium oxalate. Vesicles were loaded in the absence (A, B) and presence (C, D) of 500 μM ryanodine. The calcium loads were 1.8 μmol/mg (A); 3.6 μmol/mg (B, C) or 7.1 μmol/mg (D). Negative staining was performed as described in Methods. Micrographs shown are $\times$45000.

When 5 mM EGTA is added early in the uptake reaction (1 min) a subsequent calcium efflux is very slow. At 2 min, calcium efflux is faster but there is still no rapid initial efflux phase. At 4 min and all times thereafter, 5 mM EGTA caused a rapid efflux of about 0.7 μmol/mg in less than 0.2 min, followed by a slower release of calcium phosphate at a rate of about 0.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The lack of substantial efflux at 1 min of uptake suggests that all of the calcium phosphate is retained by tightly sealed vesicles. The efflux of 0.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at 2 min of uptake suggests that some vesicles have become hyperpermeable, perhaps because of mechanical stress caused by the intravesicular crystals. The rapid phase of release which first appears at 4 min could be attributed to dissolution of extravesicular calcium phosphate crystals.

If vesicle rupture limits calcium oxalate uptake, then vesicles should be nearly full of crystals at high calcium oxalate loads. To test this, we obtained electron micrographs of cardiac sarcoplasmic reticulum vesicles loaded to different levels in the presence or absence of 500 μM ryanodine. When vesicles were loaded to only 1.8 μmol/mg in the absence of ryanodine (Fig. 11A) relatively few vesicles were loaded. However, a significant fraction of the volume of the loaded vesicles was occupied by calcium oxalate crystals. Other vesicles had only a few crystals while others were virtually empty. When vesicles were loaded to 3.6 μmol/mg (Fig. 11B), a larger fraction of the vesicles had crystals but again the load varied from quite full, to half-full to empty. When vesicles were loaded in the presence of ryanodine to 3.6 μmol/mg (Fig. 11C), most of the vesicles contained some crystals but the load varied considerably. Loading to 7.1 μmol/mg in the presence of ryanodine resulted in fuller vesicles although some vesicles were half-full or less than half-full (Fig. 11D). The vesicles shown in Fig. 11 were from the same preparation as those used for Figs. 5 and 9 in order to allow direct comparison.

Discussion

The present results show that the progress of oxalate- and phosphate-supported calcium uptake is curvilinear and that the deviation from linearity occurs at different calcium loads depending on the anion used to support uptake and on the presence or absence of ryanodine. The non-linearity of oxalate-supported uptake was earlier shown in homogenates of rat skeletal muscle, where there was a rather sharp break in the curve [17]. One of the reasons for conducting the experiments reported herein was to try to explain these progress curves, and to define the capacity of cardiac sarcoplasmic reticulum when oxalate or phosphate is used.

The decrease in the rate of calcium uptake with time as shown in Figs. 5 and 9 may be due to a decrease in calcium influx or an increase in calcium efflux. A decrease in calcium influx could be due to a decrease in the turnover rate of the calcium pump or to a decrease in the coupling ratio. An increase in calcium efflux could be due to the opening of a calcium channel, an increase in the permeability of the membrane or a generalized rupture of the membrane due to the accumulation of massive amounts of intravesicular calcium oxalate or calcium phosphate crystals.

The non-linear calcium uptake is not due to a decrease in turnover of the Ca^{2+}-ATPase (Fig. 6) nor to a increase in efflux through the ryanodine-sensitive channel, because these curves were still non-linear in the presence of ryanodine (Fig. 5). We offer evidence that suggests that rupture of the vesicles is responsible for the non-linear curves and that non-simultaneous rupture makes the capacity of the sarcoplasmic reticulum poorly defined.

We have used EGTA to distinguish between intravesicular and extravesicular calcium. As shown by preliminary studies with pre-formed crystals, EGTA caused a rapid dissolution of both calcium oxalate and calcium phosphate. When added to cardiac sarcoplasmic reticulum, EGTA caused a rapid release of calcium which was dependent on the calcium load, but not on the time of incubation. Further, all of the calcium could be released only if the calcium ionophore, A23187, was also included. These results suggest that EGTA can be used to distinguish between intravesicular and extravesicular calcium. The extravesicular calcium can be rapidly 'released', or dissolved, by EGTA alone while the intravesicular calcium is only slowly released by EGTA. Rapid release of the intravesicular calcium requires EGTA and

A23187 in sufficient concentration.

Some explanation needs to be given concerning the large values for extravesicular calcium when oxalate is used (Fig. 5) compared to when phosphate is used (Fig. 9). The preliminary results using pre-formed crystals show that calcium oxalate crystals (0.2 mM) are stable under our reaction conditions with no EGTA (Fig. 1) whereas 0.2 mM calcium phosphate crystals dissolve under our conditions (Fig. 4). Thus, when oxalate is used, rupture of vesicles would result in the accumulation of extravesicular calcium oxalate crystals. When phosphate is used, rupture of vesicles would result in externalization of calcium phosphate crystals which would then dissolve. If rupture of the vesicles occurs continuously, there will always be some calcium phosphate crystals newly externalized which have not yet dissolved. Thus, there should be some readily dissolvable calcium phosphate, but this amount should be less than the readily dissolvable calcium oxalate.

If calcium uptake and vesicle rupture were uniform and homogeneous, then the uptake curves should show marked discontinuities. The smoothness of the curves suggests there is considerable heterogeneity in our cardiac sarcoplasmic reticulum preparation.

The heterogeneity of our cardiac sarcoplasmic reticulum preparation is supported by our observations using electron microscopy (Fig. 11), where heterogeneity with respect to size and calcium oxalate accumulation was apparent. It has been previously reported that skeletal sarcoplasmic reticulum is heterogeneous with respect to calcium phosphate loading [30]. This has been confirmed using oxalate for skinned skeletal muscle fibers [42] and isolated cardiac sarcoplasmic reticulum [31]. The increase in intravesicular calcium oxalate or calcium phosphate in the presence of ryanodine (Figs. 5 and 9) and the electron micrographs suggest that ryanodine increases the number of vesicles which participate in calcium uptake. This agrees with the findings of Jones and Cala [27] that cardiac sarcoplasmic reticulum contains subpopulations of vesicles that differ in their sensitivity to ryanodine.

Differential sensitivity to ryanodine and differences in size are probably not the only types of heterogeneity in these vesicles. As shown in Fig. 11, the vesicles were not uniformly loaded even in the presence of ryanodine. Although a number of types of heterogeneity could explain these results, we cannot describe this heterogeneity further except to say some vesicles load slower than others.

If vesicles are poised on the verge of breaking, the final breach should be provided by an increment of growth in the intravesicular calcium oxalate or calcium phosphate crystal. In this way, vesicles may not break unless there is turnover of the Ca^{2+}-ATPase. This idea allows a full explanation of Fig. 7 obtained in the presence of ryanodine. When the supply of calcium limits uptake to a level at which no vesicles are full (below 12 μmol/mg) uptake is so complete that activator calcium is removed. The Ca^{2+}-ATPase slows to basal levels and very little release of calcium occurs because intravesicular free calcium is low. When the supply of calcium limits uptake to 17.7 μmol/mg, uptake is still complete so the Ca^{2+}-ATPase is low. Addition of EGTA, however, produces a significant passive efflux. This could be attributed to vesicles which are somewhat strained but not broken. When uptake is allowed to proceed further, addition of EGTA dissolves frankly extravesicular calcium oxalate followed by a slower efflux phase due to hyperpermeable but not broken vesicles.

If the internal volume limits calcium uptake, then some estimate of the capacity can be calculated from the magnitude of the volume and the density of the crystals which are formed there. Published values for the enclosed volume of skeletal sarcoplasmic reticulum include: 2.9 μl/mg using ^3H_2O [32]; 1.3 μl/mg by [^{14}C]sucrose [33]; 3.0 μl/mg using [^{14}C]glucose and [^{3}H]choline [34]; 4 μl/mg by [^{14}C]insulin exclusion [35]; 1.5 μl/mg by [^{14}C]sucrose [36]; 4.4 μl/mg by subtraction of the membrane volume from the ^{14}C-EDTA exclusion volume [37]; and 1.4 μl/mg by [^{3}H]mannitol in cardiac sarcoplasmic reticulum [18]. For the purposes of the following argument we use an enclosed volume of 1.5 μl/mg since this is close to the only published value for cardiac sarcoplasmic reticulum. Using a density of calcium oxalate crystals of 2.2 g/ml, the total enclosed volume corresponds to a calcium load of 25.8 μmol/mg. There are several possible forms of calcium phosphate crystals and we cannot eliminate any of

them a priori. The total enclosed volume corresponds to a calcium load of 21.4 μmol/mg for $Ca(PO_3)_2$; 20.1 μmol/mg for $Ca(HPO_4) \cdot 2H_2O$; 13.1 μmol/mg for $Ca(H_2PO_4)_2 \cdot H_2O$; and 45.6 μmol/mg for $Ca_3(PO_4)$.

The apparent capacity for calcium oxalate actually exceeds this calculated maximum (Figs. 5 and 7), but the intravesicular capacity, as determined by the calcium retained after EGTA addition, peaks at 16–18 μmol/mg in the presence of ryanodine. Uptake curves in the presence of ryanodine must be used for comparison with the calculated capacity because otherwise only a fraction of the vesicles participate in uptake (Fig. 11). Much of the extravesicular calcium was once intravesicular, and we should discount only that portion of the extravesicular calcium which resulted from seeding of the reaction bath. Results with pre-formed calcium oxalate crystals suggest that seeding is relatively slow compared to uptake. According to Fig. 1, and using a protein concentration of 5.7 μg/ml as in Figs. 5 and 7, addition of 11 μmol calcium oxalate per mg sarcoplasmic reticulum protein will cause extravesicular precipitation of 2.4 μmol/mg calcium oxalate in 10 min. Thus, by this approximation, most of the extravesicular calcium during oxalate-supported uptake was probably originally intravesicular. The overall calcium oxalate capacity calculated from an intravesicular volume of 1.5 μl/mg is in reasonable agreement with the observed uptake curves.

The calculated capacity for calcium phosphate varies from 13 to 45 μmol/mg, depending upon the type of calcium phosphate crystal which is assumed to form. The apparent capacity, in the presence of ryanodine, peaked at about 15 μmol/mg but only 11 μmol/mg or so was intravesicular. However, as pointed out earlier, it is likely that externalized calcium phosphate crystals were dissolved upon exposure to the reaction bath, and the amount of these crystals should be added to the observed intravesicular content to arrive at a capacity. At present we do not know how much externalized calcium phosphate dissolved by the time calcium uptake leveled off. However, if the initial uptake remained linear, the uptake at 14 min (Fig. 9B) would have been 23 μmol/mg. At 14 min of uptake, 11 μmol/mg was intravesicular and about 3 μmol/mg was extravesicular as determined by dissolution with EGTA. It is possible that another 5–10 μmol/mg had dissolved between 4 and 14 min of incubation, because some extravesicular calcium was evident at 4 min incubation and the dissolution rate under these bath conditions was about $0.7\,\mu mol \cdot mg^{-1} \cdot min^{-1}$ (Fig. 4 and assuming 5.7 μg/ml cardiac sarcoplasmic reticulum protein ml). These calculations are consistent with the idea that the volume of the sarcoplasmic reticulum vesicles limits calcium uptake, but that the entire capacity is not observed due to the heterogeneity of the vesicles and consequent nonsimultaneous vesicle rupture.

Although these results are consistent with the hypothesis that vesicle rupture limits oxalate and phosphate-supported calcium uptake, there are alternate possible explanations and possible sources of artefacts which need to be considered. For example, it is possible that the surface of the sarcoplasmic reticulum vesicles could act as nucleation sites for calcium oxalate or calcium phosphate crystallization. However, apparent calcium uptake is not observed in the presence of sarcoplasmic reticulum vesicles under our assay conditions when A23187 is included to prevent calcium accumulation [5]. Thus passive nucleation due to the sarcoplasmic reticulum vesicles is probably not a source of artifacts in these studies. Second, it has been shown that alkaline EDTA or EGTA treatment causes extraction of some proteins from skeletal sarcoplasmic reticulum with consequent changes in permeability [35]. Thus, it is possible that the extravesicular calcium was not initially present but was made accessible to EGTA by an EGTA-induced rupture of the vesicles. However, the extraction of proteins by EGTA was not observed at pH 7.0 [35] and it is difficult to see how EGTA would not make all vesicles hyperpermeable. Thirdly, if vesicles rupture and release accumulated calcium, then the Ca^{2+}-ATPase activity should increase due to the lack of inhibition by internal calcium. However, previous results show that the Ca^{2+}-ATPase rate in the presence of 10 mM oxalate is not stimulated by increasing permeability with A23187 in cardiac sarcoplasmic reticulum, while it is stimulated in skeletal sarcoplasmic reticulum [5]. Thus the data with the Ca^{2+}-ATPase rate are consistent with the hypothesis

that calcium uptake decreases with time due to rupture of the vesicles.

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