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TIME-DEPENDENT CHANGES OF CALCIUM INFLUX AND EFFLUX RATES IN RABBIT SKELETAL MUSCLE SARCOPLASMIC RETICULUM

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Summary

Unfractionated and low buoyant density sarcoplasmic reticulum vesicles released calcium spontaneously after ATP- or acetyl phosphate-supported calcium uptake when internal Ca^{2+} was stabilized by the use of 50 mM phosphate as calcium-precipitating anion. This spontaneous calcium release could not be attributed to falling Ca^{2+} concentration outside the vesicles (Ca_o^{2+}), substrate depletion, ADP accumulation, nonspecific membrane deterioration or the attainment of a high vesicular calcium content. Instead, spontaneous calcium release was directly proportional to Ca_o^{2+} at the time that calcium content was maximal. A causal relationship between high Ca_o^{2+} and spontaneous calcium release was suggested by the finding that elevation of Ca_o^{2+} from less than 1 μM to 3—5 μM increased the rate and extent of calcium release.

The spontaneous calcium release was due both to acceleration of calcium efflux and slowing of calcium influx that was not accompanied by a significant change in the rate of ATP hydrolysis. Neither reversal of the transmembrane KCl gradient nor incubation with cation and proton ionophores abolished the spontaneous calcium release. The persistence of calcium release under conditions where the membrane was permeable to both anions and cations makes it unlikely that this phenomenon is due to a changing transmembrane potential.

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Abbreviations used: Ca_i^{2+}, calcium ion concentrations inside the vesicles; Ca_o^{2+}, calcium ion concentration in the medium outside the vesicles, calculated as described previously [3]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid. As used in this article, 'calcium uptake' and 'calcium release' refer to the rate of net gain or loss of calcium by the vesicles; 'calcium influx' and 'calcium efflux' refer to unidirectional calcium fluxes into and out of the vesicles, respectively.

The similarity between the Ca^{2+} dependence of spontaneous calcium release and of calcium uptake, along with other similarities between these processes, suggest that calcium release is mediated by the calcium pump in these membranes.

Introduction

Vesicles prepared from the sarcoplasmic reticulum of skeletal muscle have been shown to accumulate calcium in the presence of Mg^{2+} and an energy donor such as ATP [1,2]. The amount of calcium accumulated by the vesicles is markedly increased when Ca_i^{2+} is maintained at low levels by the inclusion in the medium of calcium-precipitating anions such as oxalate or phosphate [4], which stabilize Ca_i^{2+} and prevent inhibition of the calcium pump by high internal Ca^{2+} concentrations [5,6]. In a typical calcium uptake reaction, the vesicles fill with calcium at a rate that is determined by Ca_o^{2+} [7]. Spontaneous release of a portion of the accumulated calcium often follows the attainment of maximal calcium content. This phase of calcium release can be seen when reactions are carried out in the absence [8—12] or presence [13] of a calcium-precipitating anion such as oxalate or phosphate. In the presence of a calcium-precipitating anion, calcium release rate increases when a large calcium load, relative to the protein concentration in the reaction mixture, allows Ca_o^{2+} to remain high immediately after calcium content has reached an initial maximum [13].

The present study examines the mechanism responsible for the spontaneous changes in calcium content that occur after calcium content reaches an initial maximum at high levels of Ca_o^{2+}. When ATP is substrate, a complex series of changes in calcium influx and efflux rate occurs such that there is a transient calcium release that is followed by renewed uptake of calcium [14,15]. The spontaneous calcium release, which occurs without a significant alteration in the rate of concomitant ATP hydrolysis, is due to slowing of calcium influx accompanied by a transient acceleration of calcium efflux. The latter appears to be initiated when Ca_o^{2+} is high at the time that calcium content reaches its maximum. Initiation of spontaneous calcium release after addition of micromolar amounts of Ca^{2+} to reactions that had reached maximal calcium content at low Ca_o^{2+} supports the view that high Ca_o^{2+} can transiently accelerate calcium release from sarcoplasmic reticulum vesicles.

Materials and Methods

Preparation of sarcoplasmic reticulum vesicles and measurements of calcium content, calcium uptake, calcium release, calcium influx and calcium efflux were as described previously [3,13,16]. All experiments with acetyl phosphate as substrate were carried out with a vesicle fraction prepared by differential centrifugation and extraction with 0.6 M KCl. In experiments utilizing ATP as substrate, either this preparation or the low buoyant density fraction purified on a 20—60% (w/v) linear sucrose density gradient [17,18] was used. Unless otherwise specified, experimental data were obtained with the unfractionated sarcoplasmic reticulum vesicles. Experiments were carried out in 120 mM KCl, 40 mM histidine buffer (pH 6.8) and 50 mM Tris/phosphate as the calcium-pre-

cipitating anion. In most studies carried out with acetyl phosphate as substrate, $MgCl_2$ was present at a concentration of 5 mM and the temperature was 37°C (standard conditions for acetyl phosphate). Unless otherwise stated, when ATP was substrate, $MgCl_2$ and ATP concentrations were 5 mM, reaction temperature was 25°C, and an ATP-regenerating system (0.15 mg/ml pyruvate kinase and 4.0–5.0 mM phospho*enol*pyruvate) was used to maintain ATP concentration and minimize ADP accumulation (standard conditions for ATP).

Adenosinetriphosphatase activity was measured in the same medium used for calcium uptake by analyzing the pyruvate concentration [19] in filtrates obtained after the vesicles were removed by passage through the Millipore filter. The pyruvate kinase concentration was sufficiently high to ensure that the conversion of phospho*enol*pyruvate to pyruvate was not the rate-limiting step in the ATPase reaction, because when 0.1 mM ADP was added to a reaction mixture lacking vesicles it was converted completely to ATP within 15 s.

In experiments where Ca_o^{2+} was increased during the calcium uptake reaction, 7—8 ml of a complete reaction mixture was transferred to a tube containing 0.10—0.25 ml $^{45}CaCl_2$ of the same specific activity as that used in the original calcium uptake reaction. Radioactivity of unfiltered reaction mixtures was measured before and after the $^{45}CaCl_2$ addition, and appropriate corrections were made for the small (less than 4%) dilution of the $^{45}CaCl_2$ in the initial reaction mixture. Use of $^{45}CaCl_2$ of a single specific activity simplified calculation of vesicular calcium contents before and after elevation of Ca_o^{2+}.

All reagents used were reagent grade, and deionized water was redistilled from glass prior to use. Disodium ATP (Boehringer-Mannheim) was desalted and neutralized with $MgCl_2$ and Tris as described previously [3]. Pyruvate kinase (type III) was purchased from Sigma Chemical Company. Acetyl phosphate and phospho*enol*pyruvate (monopotassium salt) were purchased from Boehringer-Mannheim Company.

Ionophores were added in ethanol such that the final concentration of ethanol in the reaction mixture did not exceed 0.5% (v/v). These low ethanol concentrations were without significant effects on calcium uptake. Gramicidin D and valinomycin were obtained from the Sigma Chemical Company. Nigericin was a generous gift of Dr. Robert Hosley of Eli Lilly Company.

Ionized Ca^{2+} concentrations (Ca_o^{2+}) were calculated to take into account the binding of both Ca^{2+} and Mg^{2+} by ATP [3]. In the presence of 5 mM MgATP, Ca_o^{2+} will be approximately 18% of total $CaCl_2$ because of the binding of Ca^{2+} to ATP^{4-}.

Results

Time-dependent oscillations in calcium content

The calcium content of vesicles incubated with ATP as substrate and 50 mM phosphate as Ca^{2+}-precipitating anion characteristically exhibited time-dependent oscillations when reactions were initiated with more calcium than could be transported into the interior of the vesicles (Fig. 1). The maximum observed calcium capacity, defined as the maximum calcium content during the initial phase of calcium uptake prior to any calcium release, varied among different preparations of vesicles and was approximately 7.8 μmol/mg in the

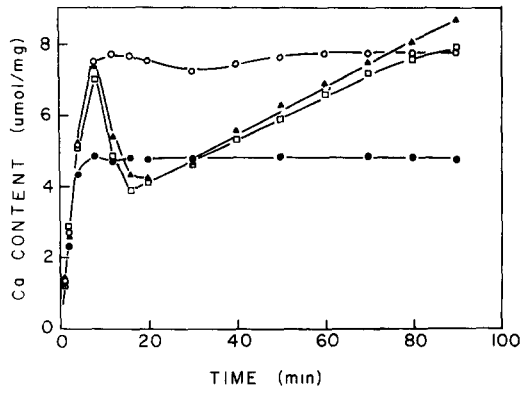

Fig. 1. Effects of varying initial total $CaCl_2$/vesicle ratio (μmol calcium/mg vesicles) on calcium uptake reactions carried out with 6 μg/ml vesicles and 5 mM MgATP as substrate (see Materials and Methods). Initial $CaCl_2$ concentrations of 30, 48, 60 and 98 μM gave Ca_o^{2+} concentrations of 6 μM (●), 9.6 μM (○), 12.0 μM (▲), and 19.6 μM (□). The initial total calcium : protein ratios were 5, 8, 10 and 16.3, respectively.

experiment shown in Fig. 1. Spontaneous calcium release was seen in reactions started with initial calcium : protein ratios of 10 and 16.3 μmol/mg, which exceeded the maximum observed calcium capacity of this preparation. When the initial total calcium : protein ratio was reduced to 5 μmol/mg, calcium content reached a maximum when Ca_o^{2+} was approximately 0.16 μM. Under these conditions, where transport of more than 97% of the added calcium into the vesicles had reduced Ca_o^{2+} to an extremely low level, calcium content did not change significantly during the subsequent hour. An increase in the initial calcium : protein ratio to 8 μmol/mg, which caused the total amount of added calcium to exceed the maximal observed calcium capacity of the vesicles (7.8 μmol/mg), led to the appearance of a transient phase of spontaneous calcium release followed by slow reuptake of virtually all of the released calcium. The lowest level of Ca_o^{2+} during the reaction carried out at the total calcium : protein ratio of 8 μmol/mg was 0.26 μM. When the total calcium : protein ratio was increased further, to 10 and 16.3 μmol/mg (Fig. 1) the rate of spontaneous calcium release became more rapid. In these experiments carried out at high total calcium : protein ratios, Ca_o^{2+} at the time that calcium content reached its initial maximum was 2.6 and 10.1 μM, respectively. Thus, in the experiment depicted in Fig. 1, an increase in initial Ca_o^{2+} from 6.0 to 19.6 μM was associated with a marked acceleration of spontaneous calcium release even though the increase of Ca_o^{2+} in this high range had little effect on calcium uptake velocity [7]. As can be seen in Fig. 1, there was no direct correlation between maximal calcium content and the appearance of spontaneous calcium release. Instead, maximal calcium content immediately prior to spontaneous calcium release at the higher total calcium : protein ratios (10 and 16.3 μmol/mg) was often less than maximal calcium content at lower total calcium : protein ratios (8 μmol/mg) that were associated with little or no spontaneous calcium release (Fig. 1).

Preincubation of the vesicles for 1 h in the complete reaction mixture, except for omission of MgATP, did not significantly influence either initial calcium uptake velocity or the time-dependent changes in calcium content when reactions were subsequently initiated by addition of 5 mM MgATP. The combination of low concentrations of vesicles and high ATP concentrations minimized possible effects of either substrate depletion or ADP accumulation on calcium content. Inclusion of an ATP-regenerating system was without significant effect on either the initial calcium uptake velocity or spontaneous calcium release, although late calcium reuptake was more rapid and more extensive when the ATP-regenerating system was present.

The time-dependent changes in calcium content seen with the unfractionated sarcoplasmic reticulum preparation were also apparent in studies with the fraction of low buoyant density ('light') vesicles purified by density gradient centrifugation. Although both the initial calcium uptake velocity and maximal calcium content of the light vesicular fraction were higher than in the unfractionated preparation, the basic pattern of calcium uptake-calcium release-calcium reuptake was similar.

Effects of Ca_o^{2+} on calcium release

Fig. 1 suggests the existence of a direct relationship between Ca_o^{2+} at the time that the vesicles achieved their maximal calcium content and the subsequent rate of spontaneous calcium release. This relationship was examined by pooling all of 25 measurements made in nine consecutive independent experiments where sufficient data were available to define Ca_o^{2+} during the initial maximum of calcium content. The rate of calcium release, estimated during the period when between 25% and 75% of the spontaneous calcium release took place, was directly proportional to Ca_o^{2+} during the initial maximum of calcium content (Fig. 2). Calcium release rate appeared to reach a maximum when Ca_o^{2+} was above 5—7 μM. This maximum rate of calcium release, which was 0.4 ±

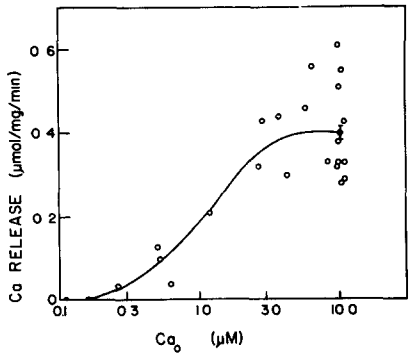

Fig. 2. Relationship between spontaneous calcium release rate (ordinate) and Ca_o^{2+} at the time that the vesicles achieved their maximal calcium content (abcissa). Calcium release rates were measured as described in the text in experiments such as shown in Fig. 1 with 6 μg/ml vesicles, 30—100 μM $CaCl_2$, 5—10 mM $MgCl_2$ and 5 mM ATP in the presence or absence of an ATP-regenerating system. Other conditions were as described for standard conditions for ATP (see Materials and Methods). •, at the right of graph represents the mean ± S.E. for the 11 points where Ca_o^{2+} ranged between 8.4 and 11.0 μM when vesicular calcium content was maximal.

0.04 μmol/mg per min in the range of Ca_o^{2+} between 8.4 and 11 μM, was approximately half of the initial calcium uptake velocity of 0.8—1.0 μmol/mg per min observed under these conditions.

Relationship between calcium release and ATP hydrolysis

To determine whether slowing of the calcium pump ATPase reaction could account for spontaneous calcium release, ATP hydrolysis was determined by measuring the rate of pyruvate liberation. The appearance of the phase of spontaneous calcium release was not accompanied by significant slowing of the rate of ATP hydrolysis (Fig. 3).

The ratio between the initial rates of calcium uptake and concomitant ATP hydrolysis, which was 0.8 in the experiment shown in Fig. 3, ranged between 0.5 and 1.5 in other experiments. The time-dependent changes in calcium content and rate of spontaneous calcium release were similar in the more highly coupled preparations.

Time-dependent changes in calcium influx and calcium efflux velocities

To determine whether the spontaneous calcium release resulted from slowing of calcium influx, acceleration of calcium efflux, or both, unidirectional calcium flux rates [13,16] were measured during calcium uptake reactions as shown in Fig. 4A. Calcium influx slowed progressively during the early period of the reaction (Fig. 4A and B) while, at the same time, calcium efflux rate increased. The initial maximum of calcium content was reached 6—8 min after the start of the reaction, when the increasing rate of calcium efflux became equal to that of the decreasing rate of calcium influx (Fig. 4B). Further acceleration of calcium efflux and slowing of calcium influx were responsible for the early calcium release. An abrupt fall in calcium efflux rate terminated the calcium release phase, and calcium uptake was renewed when calcium efflux rate fell below that of calcium influx (Fig. 4B).

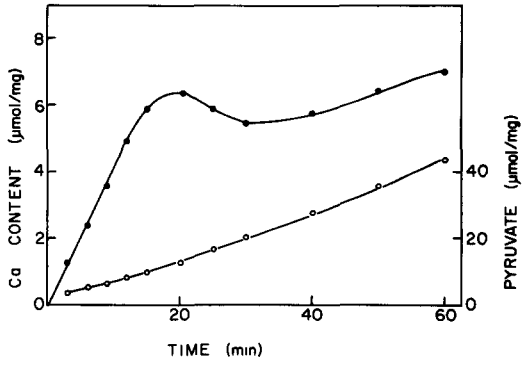

Fig. 3. ATP hydrolysis during a calcium uptake reaction carried out with 6 μg/ml light vesicles and 72 μM $CaCl_2$ under standard conditions for ATP (see Materials and Methods). ATPase activity, determined by the amount of pyruvate present in the filtrate after Millipore filtration (○), was measured concurrently with changing calcium content (●). The intercept of the curve for pyruvate liberation represents the pyruvate contamination in the phospho*enol*pyruvate. There was no significant pyruvate liberation under these conditions in 1 mM EGTA.

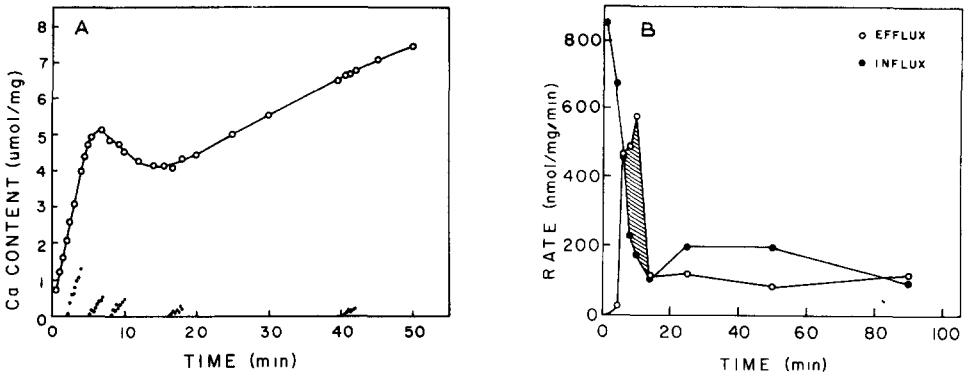

Fig. 4. (A) Changes in calcium influx rates during calcium uptake reactions carried out with 6 μg/ml vesicles and 60 μM $CaCl_2$ under standard conditions for ATP (see Materials and Methods). Changes in calcium content (○) were measured in a reaction initiated with $^{45}CaCl_2$. Calcium influx measurements (●) were initiated by addition of small quantities of carrier-free $^{45}CaCl_2$ at 2, 5, 8, 16, and 40 min to an identical reaction initiated with non-radioactive $CaCl_2$. (B) Time-dependent changes in the rates of calcium influx (●) and efflux (○) in an experiment similar to that shown in (A) except that vesicle concentration was 15 μg/ml and $CaCl_2$ was 108 μM. Calcium influx rates were measured as described in Materials and Methods, and efflux rates were calculated from simultaneous estimates of calcium influx and changes in calcium content as described previously [13,14]. Calcium uptake occurred when calcium influx rates exceeded those of calcium efflux. Calcium release occurred when calcium efflux rates exceeded those of calcium influx (shaded).

Effects of raising Ca_o^{2+} on calcium release

The data in Figs. 1 and 2 suggest that the appearance of spontaneous calcium release is related to a high level of ionized Ca^{2+} in the medium outside the vesicles at the time that the vesicles reach their maximal observed calcium capacity. This interpretation was tested directly by adding a small amount of $CaCl_2$ to calcium-filled vesicles such that Ca_o^{2+} would be increased 2—4 μM from below 1 μM. In the experiment depicted in Fig. 5, where the added $CaCl_2$ raised

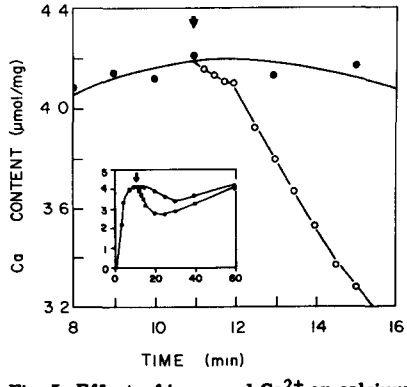

Fig. 5. Effect of increased Ca_o^{2+} on calcium release. Reactions were initiated with 12 μg/ml vesicles and 54 μM $^{45}CaCl_2$ under standard conditions for ATP (see Materials and Methods). At t = 11 min, 7.5 ml of the reaction mixture were transferred to a tube containing 0.28 ml concentrated $^{45}CaCl_2$ of the same specific activity that increased Ca_o^{2+} by 3.3 μM, from 0.6 to 3.9 μM (↓). Calcium contents of the control (●) and transfer (○) vesicles were measured. The inset shows data obtained during the entire time course of the experiment.

Ca_o^{2+} from 0.6 to 3.9 μM, calcium addition was followed by a calcium release that was more rapid and of greater magnitude than occurred spontaneously in the control reaction. The increased rate of calcium release that followed $CaCl_2$ additions when Ca_o^{2+} was below 1 μM at the time that calcium content had reached its maximum occasionally exhibited a slow phase, lasting approximately 1 min, followed by more rapid calcium release (Fig. 5).

In experiments where a low total calcium : protein ratio did not allow the calcium content to reach the maximal observed loading capacity of the vesicles, addition of $CaCl_2$ was followed by renewed calcium uptake [3,20].

Effects of Ca_i^{2+}

Elevation of Ca_i^{2+}, which was accomplished by reducing the concentration of phosphate, the calcium-precipitating anion, slowed initial calcium uptake velocity (Fig. 6) [5,6]. Reduction of phosphate concentration from 50 to 10 mM, which would increase Ca_i^{2+} from approximately 150 to 750 μM [3], both delayed and attenuated the time-dependent changes in calcium content (Fig. 6). In spite of the higher Ca_i^{2+}, spontaneous calcium release was slower at the lower phosphate concentration.

Effects of low ATP and acetyl phosphate

The time-dependent changes in calcium content were attenuated when ATP concentration was lowered from 5 mM to 5 μM (Fig. 7). At the lower ATP concentration, where initial calcium uptake velocity was slower, the initial maximum of calcium content was reached later and less calcium was released prior to calcium reuptake. The rate of the calcium reuptake phase was also slower at the lower ATP concentration (Fig. 7).

In experiments where acetyl phosphate instead of ATP was the energy donor (Fig. 8) no calcium reuptake was observed after spontaneous calcium release. The shape of these curves was not significantly altered by 1 h preincubation of the vesicles in the complete reaction mixture from which only acetyl phosphate was omitted. Reduction of acetyl phosphate concentration, which slowed

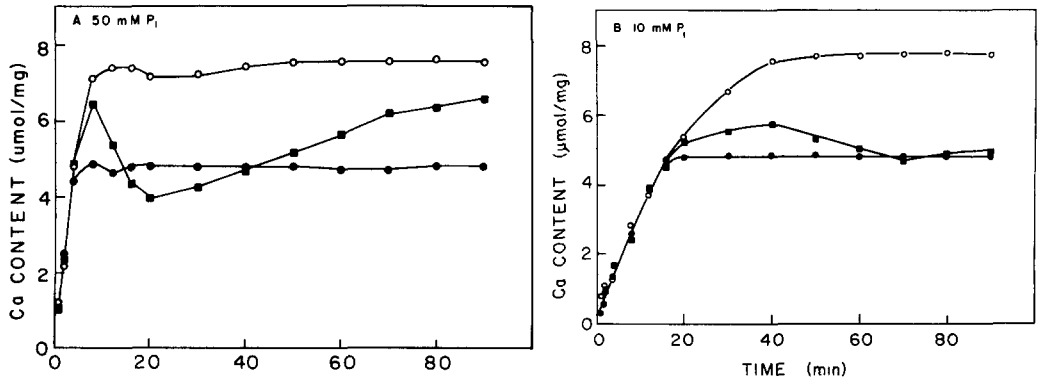

Fig. 6. Effects of internal Ca^{2+} on the time-dependent changes in calcium content. Reactions were carried out under standard conditions for ATP (see Materials and Methods) with 6 μg/ml vesicles and 30 μM (●), 48 μM (○), and 98 μM (■) $CaCl_2$ in 50 mM (A) or 10 mM (B) phosphate.

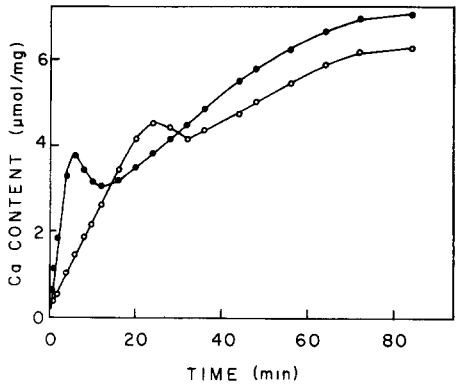

Fig. 7. Effects of ATP concentration on time-dependent changes in calcium content seen with sarcoplasmic reticulum vesicles. •, reaction carried out under standard conditions for ATP (see Materials and Methods) with 6 μg/ml vesicles, 48 μM $CaCl_2$, and 5 mM MgATP. ○, reaction carried out under identical conditions except that total ATP concentration was 5 μM and total $MgCl_2$ was 0.37 mM.

initial calcium uptake velocity, delayed the onset of calcium release. Studies of the unidirectional calcium flux rates during the reaction shown in Fig. 8, demonstrated a time-dependent fall in calcium influx rate and increase in calcium efflux rate (Fig. 8B).

Effects of preincubation with KCl and phosphate

To evaluate the possibility that transmembrane cation or anion fluxes might influence the time-dependent changes in calcium content seen when ATP was substrate, experiments were carried out with light sarcoplasmic reticulum vesicles that had been preincubated for 24 h at a concentration of 2 mg/ml in 0, 120 or 800 mM KCl in the presence or absence of 50 mM phosphate (Fig. 9). These light vesicles, which were obtained from a sucrose density gradient,

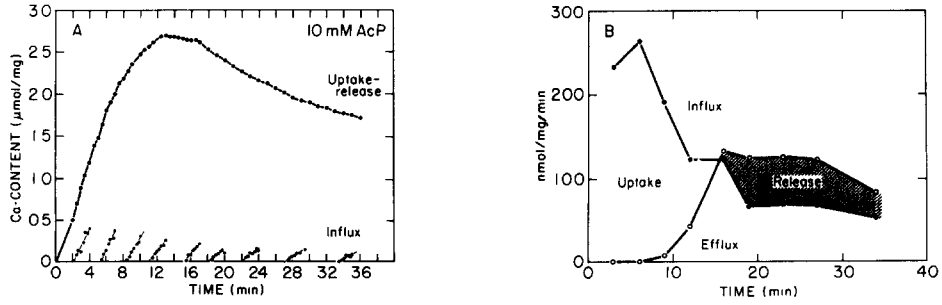

Fig. 8. (A) Changes in calcium influx rates during a calcium uptake reaction carried out with 10 μg/ml vesicles, 70.7 μM $CaCl_2$ under the standard conditions for acetyl phosphate (see Materials and Methods). Changes in calcium content (•) were measured in a reaction initiated with $^{45}CaCl_2$. Calcium influx measurements (○) were initiated by additions of small quantities of carrier-free $^{45}CaCl_2$ at 2, 5, 8, 11, 18, 22, 22, 27 and 33 min to an identical reaction initiated with non-radioactive $^{40}CaCl_2$. (B) Time-dependent changes in the rates of calcium influx (•) and efflux (○) in the experiment shown in (A). The phase of calcium release, which occurred when calcium efflux rate exceeded that of calcium influx, is shaded. See legend to Fig. 4 for methodological details.

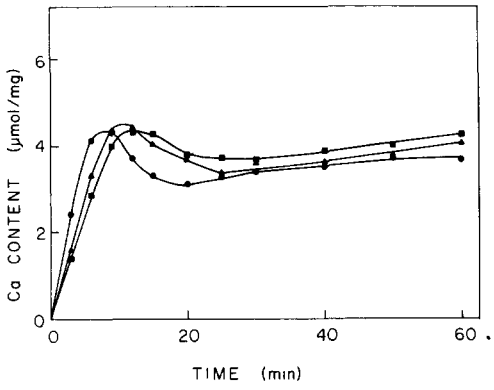

Fig. 9. Effects of preincubating vesicles in various KCl concentrations on time-dependent changes in calcium content. Light vesicles were preincubated in the absence (■), in 120 mM (▲), or in 800 mM (●) KCl for 24 h at 0°C in 12% (w/v) sucrose in the presence of 50 mM Tris/phosphate. Calcium uptake reactions were then carried out with 6 μg/ml vesicles transferred from the preincubation medium into 72 μM $CaCl_2$, 120 mM KCl and 5 mM MgATP under standard conditions for ATP (see Materials and Methods). The volume of the suspension of vesicles transferred was such that the preincubation medium was diluted 1 : 320 in the final reaction mixture.

contained less than 5 μM potassium, as determined by flame photometry. Variation of both the magnitude and direction of the K^+ gradient across the vesicles did not alter the pattern of calcium uptake followed by calcium release and reuptake (Fig. 9).

Effects of cation ionophores

We have previously shown that carbonylcyanide *p*-chloromethoxyphenylhydrazone is without effect on the time-dependent changes in calcium content of sarcoplasmic reticulum vesicles [21]. As Madeira [22] recently suggested that such compounds may not dissipate proton gradients across the sarcoplas-

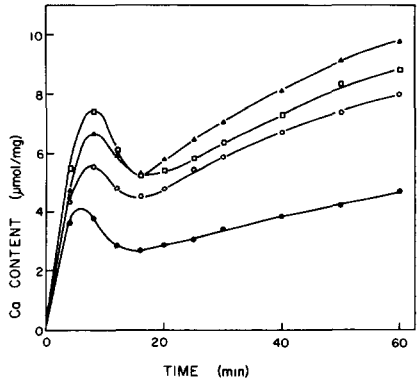

Fig. 10. Effects of ionophores on time-dependent changes in calcium content. Reactions were carried out with 6 μg/ml light vesicles in the absence (●) or presence of gramicidin D plus valinomycin (▲), gramicidin D plus nigericin (○) or valinomycin plus nigericin (□) in a reaction mixture containing 72 μM $CaCl_2$ as described under standard conditions for ATP (see Materials and Methods). Reactions were started by addition of vesicles to the complete reaction mixtures. Ionophores were present at a final concentration of 5 μg/ml.

mic reticulum, we also investigated the effects of ionophore combinations that would render the membranes permeable to protons (nigericin and to a lesser extent, gramicidin D) [23] as well as to monovalent cations (gramicidin D, nigericin and valinomycin). The combination of nigericin and valinomycin should abolish proton gradients through the ability of valinomycin to promote K^+-K^+ exchange and of nigericin to facilitate the exchange of protons with the now permeant K^+. While different combinations of these ionophores in the reaction mixture stimulated calcium uptake (Fig. 10), none of the ionophore combinations abolished the time-dependent changes in calcium content.

Discussion

The present findings confirm previous reports of a spontaneous calcium release following the initial uptake of calcium into sarcoplasmic reticulum vesicles [8—13]. Variations of experimental conditions that slowed initial calcium uptake rate tended to slow the phase of spontaneous calcium release. A similar relationship was documented by Sorenson and deMeis [12].

The complex time-dependent changes in calcium content seen with ATP as substrate did not depend on whether the calcium uptake reaction was started by addition of ATP, Ca^{2+}, or the vesicles to an otherwise complete reaction mixture. Preincubation of the vesicles in the presence of ATP or Ca^{2+} for 1 h did not alter the time of appearance of the spontaneous calcium release so that it is unlikely that spontaneous calcium release is due to nonspecific deterioration of the vesicles, such as might result from the action of proteases or lipases. Furthermore, whereas calcium efflux through a nonspecific leak would be expected to increase when Ca_i^{2+} is elevated, the spontaneous calcium release described in the present report was slowed by elevation of Ca_i^{2+} (Fig. 6). Our previous studies show that increasing Ca_i^{2+} also decreases calcium permeability (defined as the ratio calcium efflux rate/Ca_i^{2+}) at the time of the initial maximum of calcium content [16].

A possible role for depletion of high-energy phosphate substrates in causing the spontaneous release of calcium can be excluded by the finding that an ATP-regenerating system did not significantly reduce calcium release. Furthermore, a 1000-fold reduction in ATP concentration both delayed and slowed the spontaneous calcium release (Fig. 7). Similarly, when acetyl phosphate was substrate, reduction in the concentration of this substrate delayed the phase of calcium release. Perhaps the most convincing evidence that the early phase of calcium release did not result from substrate depletion is found in the appearance of a late phase of calcium reuptake when ATP was the energy donor (Fig. 1).

It is possible that the observed calcium release was initiated by rupture of some of the vesicles when the amount of the calcium phosphate precipitate exceeds the volume within the vesicles. However, the absolute volume of the calcium phosphate precipitate can occupy only a small fraction of the total intravesicular volume. Assuming the calcium phosphate precipitate to be in the form of $Ca_3(PO_4)_2$, a calcium content of 7 μmol/mg will represent 2.3 μmol of the tribasic calcium phosphate/mg protein. This precipitate, which has a molecular weight of 310 and a density of 3.14 g/ml, would have a mass of $7.2 \cdot 10^{-4}$

g/mg of protein that can occupy a volume of 0.2 μl/mg of protein. This represents only 3—4% of the intravesicular volume, which is approximately 5—7 μl/mg [7,24], so that mechanical rupture of a fraction of the vesicles caused by the calcium phosphate precipitate appears unlikely to explain the release of up to half of the accumulated calcium (Fig. 1). The inclusion of a calcium-precipitating anion (50 mM phosphate) can be expected to maintain a reasonably constant Ca^{2+} concentration inside the vesicles [4,7], so that spontaneous calcium release cannot be attributed to a rise in Ca_i^{2+}. As shown in Figs. 1 and 6, calcium release does not necessarily occur when high calcium contents are reached, making it unlikely that calcium release is due either to rupture of some of the vesicles or to the attainment of a high level of Ca_i^{2+}.

Fractionation of the sarcoplasmic reticulum vesicles on a sucrose density gradient did not alter the biphasic curve of calcium uptake seen when ATP was substrate, so that it is unlikely that the early and late uptake phases reflect differences between two populations of vesicles derived from longitudinal and terminal cisternae regions of the sarcoplasmic reticulum [17,18]. The possibility that 'inside-out' sarcoplasmic reticulum vesicles contribute significantly to the ATP hydrolysis documented in this report could be excluded by freeze-fracture electron microscopic studies of these preparations. These studies indicate that less than 1% of the vesicles contain the approximately 90 Å diameter membrane particles on their outer fracture faces (MacAlister, T. and Louis, C.F., unpublished data).

The appearance of spontaneous calcium release is due both to a decline in calcium influx rate and an increase in calcium efflux rate (Fig. 4). Slowing of calcium influx rate during the initial phase of calcium uptake was seen after translocation of a significant amount of calcium to the interior of the vesicles, but at the time that calcium influx rate slowed Ca_o^{2+} remained at levels that would saturate almost completely the calcium pump [25]. Weber et al. [26] and Barlogie [27] also observed a fall in calcium influx rate as vesicles filled with calcium, and Weber [6] found that preloading of the vesicles with calcium decreased ATPase activity when the vesicles were subsequently exposed to Ca^{2+}. The finding that the rate of ATP hydrolysis remained constant throughout the period when calcium influx rate was decreasing (Fig. 3) indicates that the slowing of calcium influx was not due to inhibition of the calcium pump, but instead represents an uncoupling of ATP hydrolysis and calcium transport.

It is unlikely that the calcium release observed in this study reflected complete reversal of the calcium pump because the latter would be impossible in experiments carried out in the absence of adenine nucleotides (Fig. 8). Transient acceleration of calcium efflux in the presence of ATP also cannot be attributed to complete reversal of the calcium pump as ADP concentrations were minimized by an ATP-regenerating system. In addition, calcium release was observed in the presence of high levels of Ca^{2+} and ATP in the external medium, both of which markedly inhibit the incorporation of P_i into ADP during complete calcium pump reversal [28—31].

Changing transmembrane K^+ fluxes appear not to be causally related to the time-dependent changes in calcium influx and efflux rates because the spontaneous calcium release was not abolished in the virtual absence of both internal and external K^+ (Fig. 9). The finding that these time-dependent changes in

calcium fluxes were only minimally affected by preincubation in 800 mM KCl prior to calcium uptake in 120 mM KCl, or by preincubation in the absence of KCl prior to calcium uptake in 120 mM KCl (Fig. 9), provides further evidence that neither K^+ fluxes nor associated charge movements in either direction across the membrane are primarily responsible for these time-dependent changes. A role for anion fluxes in causing spontaneous calcium release is also unlikely as this phenomenon is seen in reactions carried out in the absence [8—12] and presence of 50 mM phosphate, to which the vesicles are highly permeable [32]. The finding that the time-dependent changes in calcium uptake and release are seen after incubation of the vesicles with KCl plus monovalent cation ionophores such as gramicidin or valinomycin in the presence of the permeant anions, chloride and phosphate (Fig. 10), provides strong evidence that the changing calcium flux rates are not due to changing transmembrane potentials, such as suggested by Zimniac and Racker [33]. Treatment of the membranes with ionophore combinations that would be expected to render the vesicles permeable to protons also failed to abolish these time-dependent changes in calcium content (Fig. 10) [21].

Additions of $CaCl_2$ that elevate Ca_o^{2+} from less than 1 μM to 3—5 μM at the time that calcium content reached an initial maximum induced the earlier appearance of a calcium release that was more rapid than occurred spontaneously at the lower Ca_o^{2+} (Fig. 5). This phenomenon is similar in some ways to the Ca^{2+}-induced calcium release observed in the sarcoplasmic reticulum of skinned muscle fibers [34]. The Ca^{2+} concentration range over which elevation of Ca_o^{2+} accelerates calcium release in sarcoplasmic reticulum vesicles is similar to that at which calcium release is induced in the more intact preparations. However, the rates of calcium release observed in the isolated vesicles are much slower.

The spontaneous calcium release documented in the present report was associated with a high Ca^{2+} concentration outside the vesicles after the initial maximum of calcium content was reached (Fig. 2). A similar relationship between high Ca_o^{2+} and spontaneous calcium release has been noted in studies carried out in the absence of a calcium-precipitating anion [10]. A semilogarithmic plot of the relationship between Ca_o^{2+} at the time that calcium content reached an initial maximum and spontaneous calcium release rate appears sigmoid (Fig. 2). Calcium release rate appears to reach a maximum when Ca_o^{2+} exceeds 4—6 μM, and is half-maximal when Ca_o^{2+} is approximately 1.0 μM. This latter value is similar to the Ca_o^{2+} at which initial calcium uptake velocity is half-maximal [7,16].

The present findings extend our earlier observation that the control of calcium efflux from sarcoplasmic reticulum vesicles exhibits a number of similarities to that of calcium influx via the calcium pump [16]. The Ca^{2+} dependence of spontaneous calcium release is similar to that of the calcium pump and, as noted previously in the case of calcium efflux [13,16], there is a general parallel between effects of changing experimental conditions on the velocities of calcium uptake and of spontaneous calcium release. While the present studies do not define the nature of the mechanism that induces the spontaneous calcium release of calcium from sarcoplasmic reticulum vesicles, it appears likely that this spontaneous calcium release is mediated by the calcium pump protein present in these membranes.

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