Dependence of Ionophore- and Caffeine-induced Calcium Release from Sarcoplasmic Reticulum Vesicles on External and Internal Calcium Ion Concentrations*

(Received for publication, September 21, 1976)

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The effects of the ionophore, X537A, and caffeine on ATP-dependent calcium transport by fragmented sarcoplasmic reticulum were studied in the absence (calcium storage) or presence (calcium uptake) of calcium-precipitating anions. The ionophore caused rapid calcium release after calcium storage, the final level of calcium storage being the same whether a given concentration of X537A was added prior to initiation of the reaction or after calcium storage had reached a steady state. Although 10 to 12 $\mu$M X537A caused approximately 90% inhibition of oxalate-supported calcium uptake when added prior to the start of the reaction, this ionophore concentration caused only a small calcium release when added after a calcium oxalate precipitate had formed within the vesicles, and only slight inhibition of calcium uptake velocity when added during the calcium uptake reaction. When low initial calcium loads limited calcium uptake to 0.4 nmol of calcium/mg of protein, subsequent calcium additions in the absence of the ionophore led to renewed calcium uptake. Uptake of the subsequent calcium additions was not significantly inhibited by 10 to 12 $\mu$M X537A.

These phenomena are most readily understood in terms of constraints imposed by fixed $C_{a}$ (calcium ion concentration inside the vesicles) on the pump-leak situation in sarcoplasmic reticulum vesicles containing a large amount of an insoluble calcium precipitate, where most of the calcium is within the vesicles and $C_{a}$ is maintained at a relatively low level. These constraints restrict calcium loss after calcium permeability is increased because calcium release can end when the calcium pump is stimulated by the increased $C_{a}$ (calcium concentration outside the vesicles) so as to compensate for the increased efflux rate. In contrast, an increased permeability in vesicles that have stored calcium in the absence of a calcium-precipitating ion causes a much larger portion of the internal calcium store to be released. Under these conditions calcium storage capacity is low so that release of stored calcium is less able to raise $C_{a}$ to levels where the calcium pump can compensate for the increased efflux rate.

The constraints imposed by anion-supported calcium uptake explain the finding that more calcium is released by X537A or caffeine when these agents are added at higher levels of $C_{a}$, and that more calcium leaves the vesicles in response to a given increase in calcium permeability at higher $C_{a}$. Although such calcium release is amplified by increased $C_{a}$, the amplification is attributable to the constraints described above and does not represent a “calcium-triggered calcium release.”

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Sarcoplasmic reticulum vesicles actively transport calcium in the presence of Mg$^{2+}$ and ATP. When the ionized Ca$^{2+}$ concentration in the external medium is in the micromolar range, the vesicles rapidly accumulate ~100 nmol of calcium/mg of protein (3-5). In the presence of calcium-precipitating anions such as oxalate and phosphate, which can lead to formation of calcium precipitates within the sarcoplasmic reticulum of partially disrupted muscle cells (6, 7), the vesicles take up a much larger amount of calcium (8-10). These anions, by stabilizing Ca$^{2+}$ increase net calcium transport by maintaining Ca$^{2+}$ concentration inside the vesicles at a low level, thereby reducing an inhibitory effect of high internal calcium on the transport process (11, 12).

The present report examines the ability of the calcium-precipitating anions, oxalate and phosphate, to modify calcium release from sarcoplasmic reticulum vesicles that is induced by agents believed to increase membrane permeability. Although these anions have proven extremely useful in the analysis of the mechanism of calcium transport by the sarcoplasmic reticulum, the stabilization of Ca$^{2+}$ is shown to impose...
important constraints upon the pump-leak properties of these membranes. One of these constraints allows high Ca to amplify the calcium release that follows a given increase in calcium permeability when Ca is held constant. It is concluded that variable effects of X537A and caffeine to induce calcium release from sarcoplasmic reticulum vesicles arise largely from these constraints on the balance between calcium pumping into the vesicles and the calcium efflux induced by an increased leak.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the method of Harigaya and Schwartz (13), in which the muscle was homogenized with 10 mM sodium bicarbonate in a Waring Blendor, or by the method of deMeis and Hasselbach (14).

Calcium transport was measured in the absence and calcium uptake in the presence of various concentrations of oxalate and phosphate. All reaction mixtures, unless otherwise stated, contained 0.12 M KCl, 40 mM histidine buffer, pH 6.8, and 5 mM MgATP. The reaction mixtures were incubated for 5 min prior to the addition of "CaCl₂ or "CaCl₂/EGTA buffer (see Ref. 15 for relevant equations), unless otherwise noted. Samples for calcium transport in the absence of oxalate were usually taken after 2 min (16). Calcium uptake velocities in the presence of oxalate or phosphate were calculated from several samples taken at time intervals appropriate to establish initial calcium uptake velocity. Samples were filtered through filter type AH (0.45 μm pore size) Millipore filters mounted in Swinny adapters. A Micromedic automatic pipette or a Clay-Adams micropipette was used to deliver 50-μl aliquots of the filtrate into a polyethylene vial along with 5 to 10 ml of Bray’s solution for counting in a Packard liquid scintillation spectrometer. All studies were carried out at 25°C.

When calcium release was measured, the vesicles were loaded with "Ca in the presence or absence of the calcium-precipitating anions. Aliquots of the reaction mixtures were transferred to predetermined times to separate tubes containing, in 1/10 the volume, either X537A or EGTA. The 1% dilution of the reaction mixtures could be ignored in the analysis of these data and the small amount of EGTA used to solubilize the X537A, which never exceeded 20 mM, had no detectable effects (17). The transfer caused a slight (3 to 8%) apparent decrease in the amount of calcium associated with the microsomes in experiments carried out in the absence of calcium-precipitating anions, due most likely to exchange with traces of monovalent cations. Where this artifact represented a significant fraction of the observed calcium release, appropriate corrections were made by transferring a similar aliquot of the reaction mixture to a control tube containing neither ionophore nor EGTA. Samples were taken concurrently from the "control" and "release" reaction mixtures, filtered through Millipore filters and analyzed as described above. Where EGTA or X537A were added to a single preparation of vesicles, the EGTA was always added first because a precipitate was observed when concentrated EGTA solutions were mixed with concentrated ethanolic solutions of X537A. This precipitate could be avoided when the concentrated EGTA was diluted in the reaction mixture prior to addition of the concentrated X537A solution.

Protein concentration was determined by the biuret method with bovine serum albumin as a standard.

All reagents used were reagent grade. In some experiments, ATP, purchased as the disodium salt from Sigma Chemical Co., was deionized with Dowex 50W-X8 and neutralized with MgCl₂ and Tris. Distilled water was deionized and redistilled from glass prior to use. X537A was a gift from Hoffman-La Roche Laboratories.

RESULTS

Comparison of X537A Effects on Calcium Storage and Oxalate-supported Calcium Uptake

Effects on Calcium Storage in Absence of Calcium-precipitating Anions

Concentration and Time Dependence—Calcium storage (in the absence of oxalate) was inhibited by low X537A concentrations when the vesicles were incubated with the ionophore for 5 min prior to the addition of calcium. In accord with earlier findings (18), inhibition of calcium storage increased with increasing ionophore concentration, being half-maximal at approximately 6 μM X537A. The exposure of the vesicles to X537A for 5 min permitted development of the full inhibitory effect, no change in the extent of inhibition being seen when the time of incubation with the ionophore was varied between 1 and 30 min.

Effects on Cₐ₀—The extent of inhibition of calcium storage by 25 μM X537A was not influenced by varying C₀, and kinetic analysis of the Ca⁺ dependence of the inhibitory effects of 15 μM X537A showed that inhibition by the ionophore was noncompetitive with Ca (Fig. 1). The extrapolated value for maximal calcium storage decreased by approximately 75%, from 122 to 31 nmol/mg of protein, whereas the apparent Kₐ₀ did not change significantly.

Ability of X537A to Induce Calcium Release—Vesicles loaded with calcium in the presence of MgATP, but in the absence of calcium-precipitating anions, promptly released calcium when X537A was added. This X537A-induced calcium release, like that of the initial calcium storage reaction, was too rapid to be measured by the Millipore filtration technique. As previously reported (18), the quantity of calcium released was dependent on X537A concentration and did not increase significantly when the time of exposure to the ionophore was prolonged.

The final level of calcium storage was the same whether the ionophore was added before, or after, calcium storage had reached steady state (Fig. 2). The velocity of ionophore-induced calcium release was much more rapid than that seen when Ca⁺ concentration in the medium was lowered with EGTA (Fig. 3).

Effects on Oxalate-supported Calcium Uptake

Concentration and Time Dependence—Calcium uptake velocity was inhibited by X537A when the ionophore was added to the reaction mixture 5 min prior to the addition of calcium. Slightly lower concentrations of the ionophore (3 μM) were needed for half-maximal inhibition of calcium uptake velocity
than for calcium storage capacity, as reported previously for cardiac microsomes (19). Inhibition of calcium uptake velocity was maximal after 5-min incubation with the ionophore.

Effects of Ca\textsuperscript{2+} — The extent of inhibition of calcium uptake velocity was not influenced by varying Ca\textsuperscript{2+} (Table I).

Ability of X537A to Induce Calcium Release — Addition of 12 \( \mu M \) X537A to vesicles after oxalate-supported calcium uptake had reached a steady state caused a small amount of calcium to be released (Fig. 4). This small calcium release, approximately 100 nmol/mg of protein under the conditions of this experiment, was transient; after approximately 60 s the released calcium was again taken up by the microsomes. In contrast, the same concentration of X537A added to the reaction mixture prior to initiation of the calcium transport reaction caused almost complete inhibition of subsequent calcium uptake (Fig. 4). Addition of 12 \( \mu M \) X537A at various times during the initial uptake reaction slowed calcium uptake velocity without affecting calcium uptake capacity significantly (Fig. 5).

Reduction of X537A-induced Inhibition by Prior Calcium Uptake — The ability of prior calcium uptake to reduce the effects of X537A could be shown when the ionophore was added to a reaction mixture after a small amount of oxalate-supported calcium uptake had taken place. In studies where calcium uptake was limited by the amount of added CaCl\(_2\) to a maximum of 0.4 \( \mu \)mol of calcium/mg of protein, 12 \( \mu M \) X537A caused only the previously described transient calcium release (Fig. 6). Subsequent addition of CaCl\(_2\) after a limited calcium uptake had reached steady state induced further calcium uptake (Fig. 6, Arrow A) in the control reaction. No significant inhibition of subsequent calcium uptake was seen when CaCl\(_2\) was added to the X537A-containing reaction mixture either during (Fig. 6, Arrow B) or after (Fig. 6, Arrow C) the transient calcium release.

Reduction of Ca\textsuperscript{2+} concentration in the medium by millimolar concentrations of EGTA after oxalate-supported calcium uptake had reached steady state caused a slow calcium

**Table I**

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} concentration (( \mu M ))</th>
<th>Calcium uptake velocity (( \mu mol/min ))</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>0.176</td>
<td>0.087</td>
</tr>
<tr>
<td>0.30</td>
<td>0.215</td>
<td>0.119</td>
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<tr>
<td>0.42</td>
<td>0.303</td>
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</tr>
<tr>
<td>0.56</td>
<td>0.436</td>
<td>0.223</td>
</tr>
<tr>
<td>0.72</td>
<td>0.625</td>
<td>0.327</td>
</tr>
<tr>
<td>1.32</td>
<td>0.977</td>
<td>0.452</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effects of X537A on the final level of calcium stored by vesicles in the absence of oxalate. A reaction mixture for calcium storage by 0.10 mg of protein/ml incubated with 10 \( \mu M \) X537A for 5 min before addition of 3 \( \mu M \) Ca\textsuperscript{2+} (25 \( \mu M \) CaCl\(_2\) and 15 \( \mu M \) EGTA) (○) is compared with a control reaction mixture incubated without X537A (○). At t = 4 min (arrow), X537A (10 \( \mu M \) final concentration) was added to an aliquot of the control reaction (▲).

**Fig. 3.** Comparison of the effects of X537A and EGTA on calcium release after calcium storage in the absence of oxalate. Calcium storage was measured with 0.137 mg of protein/ml and 10 \( \mu M \) Ca\textsuperscript{2+} (○). Calcium release was initiated by 10 \( \mu M \) X537A (▲) or 2.5 mM EGTA (●).

**Fig. 4.** Effect of time of X537A addition on oxalate-supported calcium uptake. Vesicles (12 \( \mu g \) of protein/ml) were incubated under standard conditions with 12 \( \mu M \) X537A for 5 min before calcium uptake was initiated by addition of 10 \( \mu M \) Ca\textsuperscript{2+} (▲). A control reaction was carried out in the absence of the ionophore (●), and an aliquot of the latter was transferred at t = 4 min to a tube containing X537A (final concentration 12 \( \mu M \)) for measurement of X537A-induced calcium release (○).
Fig. 5. Effects of X537A added at various times during oxalate-supported calcium uptake. A control reaction was carried out with 10 μg of protein/ml and 10 μM Ca²⁺ (●). At 1 min (A), 2 min (B), and 4 min (C), 5-ml aliquots of the control reaction mixture were added to 50 μl of X537A (final concentration 12 μM) in separate tubes and subsequent calcium uptake was measured (○).

Fig. 6. Effect of X537A on subsequent calcium uptake after limited calcium uptake had taken place. A limited calcium uptake of 0.4 μmol/mg of protein was allowed to take place by use of 12 μg of protein/ml and only 5 μM ⁴⁰CaCl₂ (●). At t = 30 s (A), 5 ml of this control reaction mixture was transferred to a tube containing 50 μl of ⁴⁰CaCl₂ of the same specific activity (final concentration 50 μM) and subsequent calcium uptake was measured (●). At the same time, 18 ml of the control mixture were transferred to a tube containing 0.18 ml of X537A (final concentration, 12 μM) and calcium release was measured (○). At t = 1 min (B) and t = 4 min (C), 5 ml of the X537A-containing reaction mixture were transferred to tubes containing 50 μl of ⁴⁰CaCl₂ (final concentration 50 μM) of the same specific activity and subsequent calcium uptake was measured (△, □).

**Release.** The ability of X537A to induce calcium release was increased only slightly after addition of a high EGTA concentration which lowered Cai to approximately 0.01 μM (Fig. 7).

**Effects of Prior Incubation of Vesicles with X537A**

Vesicles were exposed to X537A and then washed prior to measurement of calcium storage and uptake to determine whether inhibition was due to a direct effect of X537A upon the membranes, and to exclude possible effects of changes in the composition of the medium such as could result from complexing of cations by the ionophore. Brief (5 min) exposure to 10 μM X537A followed by washing caused significant inhibition of both subsequent calcium storage capacity and calcium uptake velocity (Table II).

**Mechanism of X537A-induced Calcium Release**

In view of evidence that calcium efflux in sarcoplasmic reticulum vesicles can occur through reversal of the calcium pump (20, 21) and that incorporation of the ATPase protein into these membranes increases passive calcium permeability (22), studies of X537A-induced calcium efflux were carried out under conditions where ADP, which is essential for pump reversal (20) was absent. In studies where calcium uptake was initiated with acetyl phosphate, rather than ATP, i.e. where ADP lack prevents pump reversal, the appearance of marked effects of X537A to promote calcium release (Fig. 8) supports the view that the effects of the ionophore are independent of an action to promote calcium efflux by way of the calcium pump. The greater calcium release seen in the presence of acetyl phosphate than ATP is attributable to the much slower rate of calcium transport into the vesicles when acetyl phosphate instead of ATP is the energy donor (14).

**Relationship between Ca₂⁺ and Transmembrane Calcium Fluxes**

**Pump-Leak Properties of Sarcoplasmic Reticulum Vesicles which Contain Large Stores of Calcium at Constant Cai**

Effects of Cai on Calcium Influx and Efflux Rates - Curves relating Ca₂⁺ to the rate of calcium influx via the calcium pump of the sarcoplasmic reticulum can also characterize the dependence of calcium efflux rate on Cai under steady state conditions where calcium influx and calcium efflux rates are equal at the end of a calcium uptake reaction. One such curve is shown in Fig. 9, which is based on published data for the Ca₂⁺ dependence of calcium uptake velocity in 2.5 mM oxalate (16, see also Table I). Because the rate of calcium influx, an active transport process that is mediated by the calcium pump, increases with increasing Cai (11, 16), the steady state rate of calcium efflux at any given level of Cai also increases with increasing Cai if the calcium pump is not slowed when the vesicles reach their "capacity" to take up calcium. Calcium influx and efflux rates are thus predicted to depend on Cai, at the time that calcium uptake reaches a steady state where there is neither calcium uptake nor calcium release. Like calcium influx rate, that of calcium efflux at the steady state should be increased at higher levels of Cai in the micromolar range (Fig. 9). (The actual efflux rates during these steady states and their implications in terms of the dependence of calcium permeability on Cai are addressed in the following paper of this series.) If Fig. 9 accurately describes the depend-

As used in the article, "calcium uptake" and "calcium release" refer to the net gain or loss of calcium by the vesicles; "calcium influx" and "calcium efflux" refer to rates of unidirectional fluxes into and out of the vesicles, respectively.
Calcium Release from Sarcoplasmic Reticulum

Fig. 7. Calcium release after oxalate-supported calcium uptake induced by 12 μM X537A (○), 3 mM EGTA (■), and both X537A and EGTA together (□). Calcium uptake was measured with 9 μg of protein/ml and 10 μM Ca^{2+} in 2.5 mM Tris oxalate (○) as described under "Materials and Methods."

Table II

Effect of prior exposure of vesicles to X537A followed by washing on subsequent calcium transport

<table>
<thead>
<tr>
<th>Calcium transport</th>
<th>Oxalate absent</th>
<th>Oxalate present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg</td>
<td>nmol/mg/min</td>
</tr>
<tr>
<td>Control</td>
<td>56</td>
<td>350</td>
</tr>
<tr>
<td>10 μM X537A</td>
<td>34</td>
<td>220</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>39</td>
<td>31</td>
</tr>
</tbody>
</table>

Fig. 8. Dependence of X537A-induced calcium release on substrate during calcium uptake. Calcium release was studied after calcium uptake in 5 mM ATP (○, ●) or 2 mM acetyl phosphate (ACP) (■, □), 500 μg of protein/ml, 50 μM "CaCl,, and 5 mM MgCl, in the presence of 10 mM phosphate as described under "Materials and Methods." In the case of acetyl phosphate-supported calcium uptake, the initial calcium uptake reaction was allowed to take place at 33°. At t = 15 min, both reaction mixtures were brought to 25° and X537A was added to an aliquot of each at a final concentration of 10 μM (○, □). Samples were also taken from reaction mixtures to which no ionophore was added (●, ■).

Fig. 9. Relationship between Ca^{2+} concentration (Ca_{ox}) and calcium influx or calcium eflux rate when calcium uptake in 2.5 mM oxalate reaches steady state. The dotted arrows labeled A and B represent the effects of an agent that increases calcium eflux rate 4-fold after calcium uptake in 2.5 mM oxalate has reached a steady state at Ca_{ox} concentrations of 0.5 μM (A) and 0.1 μM (B) (discussed in text).

The operation of the constraints that result from the relationships shown in Figs. 9 and 10 are illustrated by the following:

Constraints Imposed by Anion-supported Calcium Uptake—The amount of calcium release following an increase in the "leakiness" of sarcoplasmic reticulum vesicles is constrained in protocols where calcium-precipitating anions (e.g. oxalate or phosphate) stabilize Ca_{ox} and where the vesicles are presented with relatively low calcium loads. The low total calcium/protein ratios cause a large portion of the added calcium, often more than 95%, to be transported into the vesicles at the same time that calcium uptake reaches steady state. The operation of these constraints can be understood in terms of the relationship between Ca_{ox} and calcium flux rates shown in Fig. 9, and by examination of the relationship between Ca_{ox} and the Ca^{2+} concentration gradient, Ca_{ox}/Ca, (Fig. 10).

The Ca^{2+} concentration gradient, Ca_{ox}/Ca, at any concentration of Ca_{ox}, is inversely proportional to Ca_{ox} (Fig. 10). The hyperbolic relationships shown in Fig. 10 describe the pump-leak properties of sarcoplasmic reticulum vesicles that are mandated when experimental design holds Ca_{ox} constant and only Ca_{ox} is allowed to vary. These constraints cause the curve relating Ca_{ox} to the Ca^{2+} concentration gradient to be hyperbolic because the term Ca_{ox} appears directly on the abscissa and its reciprocal appears on the ordinate, while the term Ca_{ox} on the ordinate is a constant. The two hyperbolic lines in Fig. 10 are constructed by simple calculation of the ratio Ca_{ox}/Ca, at two levels of Ca_{ox}, 8 μM (2.5 mM oxalate) and 20 μM (1.0 mM oxalate) and various levels of Ca_{ox}. These low Ca_{ox} levels maintain the Ca^{2+} concentration gradient well below its maximum, which is greater than 3000 (10, 23). Experimental determination of these Ca^{2+} concentration gradients is made possible by the use of calcium-precipitating anions which allow Ca_{ox} to be calculated from the solubility products of the corresponding calcium precipitate. Direct analysis of the composition of protein-free filtrates allows calculation of Ca_{ox}. The curves in Fig. 10 indicate that when increasing amounts of calcium are presented to vesicles that have only a limited "capacity" to take up calcium, the Ca^{2+} concentration gradient becomes reduced as the steady state level of Ca_{ox} increases.

The operation of the constraints that result from the relationships shown in Figs. 9 and 10 are illustrated by the follow-
ing examples, which show the way in which varying Ca and Ca influence the amount of the calcium release caused by an increased calcium permeability of sarcoplasmic reticulum vesicles.

Predicted Effects of Ca on Ionophore-induced Calcium Release — When steady states are reached at a Ca of 8 μM (solid line in Figs. 9 and 10), calcium influx and efflux rates in 2.5 mM oxalate will be 0.07 μmol/mg of protein/min at a Ca of 0.1 μM, 0.3 μmol/mg of protein/min at a Ca of 0.5 μM, and 1.2 μmol/mg of protein/min at a Ca of 1.5 μM. These values, which will be used in subsequent calculations (see below) are based on the calcium uptake measurements in Table I and in Ref. 16.

The "leakiness" of these vesicles can be described by a calcium permeability coefficient, K, that is calculated most simply from the relationship:

$$\text{Calcium efflux} = K_c \times \text{Ca}$$  \hspace{1cm} (1)

According to Equation 1, $K_c$ is 0.0375 min⁻¹, where calcium efflux rate is 0.3 μmol/mg of protein/min and Ca is 8 μM (2.5 mM oxalate), at a Ca of 0.5 μM.

Calcium permeability appears to be increased approximately 4-fold by 10 μM X537A as this ionophore concentration reduces calcium storage capacity in the absence of calcium-precipitating agents to approximately one-fourth of its initial level. If this value is correct, 10 μM X537A will increase $K_c$ in the above example from 0.0375 to 0.150 min⁻¹, thereby causing efflux rate at the constant Ca (8 μM) to increase 4-fold to 1.2 μmol/mg of protein/min. As a result, calcium will be released from the vesicles into the surrounding medium until the increased Ca, accelerates calcium influx via the pump and causes a new steady state to be reached when calcium influx and efflux rates again become equal. The ability of calcium release to restore a new steady state is made possible by the large amount of the calcium precipitate within the vesicles. According to previous data regarding the Ca²⁺ dependence of the calcium pump (Table I, 16), the new steady state in the above example, where calcium influx and efflux rates are 1.2 μmol/mg of protein/min, would be reached at a Ca of approximately 1.5 μM. This response to X537A is depicted by the dotted arrow labeled A in Figs. 9 and 10, which predicts that the new steady state will be reached after calcium release from the vesicles amounts to 1.0 μmol of calcium/liter of the reaction mixture raises Ca to 1.5 μM. This calcium release, expressed in terms of the concentration of vesicles, would be approximately 100 nmol/mg of protein at a protein concentration of 10 μg/ml.

If the same 4-fold increase in calcium efflux is produced by the addition of 10 μM X537A at a lower initial Ca, of 0.1 μM, where at steady state both calcium influx and calcium efflux rates are 0.07 μmol/mg of protein/min (Table I, 16), the new efflux rate will be increased 4-fold to 0.28 μmol/mg of protein/min. This corresponds to a rise in $K_c$ from 0.00875 to 0.035 min⁻¹. Under these conditions, the new steady state reached when calcium release from the vesicles stimulates the calcium pump will occur when both calcium influx and efflux rates are 0.28 μmol/mg of protein/min, i.e. when Ca rises to 0.4 μM (dotted arrow B in Figs. 1 and 2). To achieve this higher level of Ca, net calcium efflux from the vesicles must be only 0.3 μmol/liter of the reaction mixture (30 nmol/mg of protein at a protein concentration of 10 μg/ml) as depicted by the dotted arrow labeled B in Figs. 9 and 10. The initial level of Ca thus determines the amount of calcium that must be released from the vesicles after a given increase in permeability. The higher the initial Ca, the more calcium must be released from the internal calcium precipitate into the medium outside the vesicles to restore a steady state through stimulation of the calcium pump.

The amount of calcium release will depend on Ca, as long as the vesicles contain most of the calcium in the reaction mixture and the Ca²⁺ concentration gradient remains below the maximum that can be achieved by the ATP-dependent calcium pump. If Ca is sufficiently high or Ca, so low that this gradient approaches its maximum to 2000 to 4000 (10, 23), then the hyperbolic relationships shown in Fig. 10 will not hold as, for example, in the case of calcium storage in the absence of the calcium-precipitating anions. Furthermore, a new steady state can be restored as shown in Fig. 10 only when the calcium store in the vesicles is sufficient to raise Ca, to levels which allow calcium influx rate via the calcium pump to equal the new, higher rate of calcium efflux. This will not usually be true for calcium storage in the absence of oxalate or phosphate, where only a small amount of calcium is retained in the vesicles.

Predicted Effects of Ca on Ionophore-induced Calcium Release — The properties of the pump-leak system operating under the constraints described above also predict that the amount of X537A-induced calcium release increases with increasing Ca. If, for example, the 4-fold increase in calcium permeability described above is brought about by the addition of the ionophore where the initial value of $K_c$ in Equation 1 is 0.00875 and Ca is 20 μM (1 mM oxalate), the response will be altered from that shown by the dotted arrow B in Fig. 10 (which describes the response at a Ca of 8 μM) to that shown by the dotted arrow B'. If the rate of calcium influx was independent of Ca, the initial steady state in the absence of the ionophore would be reached where calcium efflux and calcium influx rates are 0.175 μmol/mg of protein/min (0.00875 min⁻¹) times 20 μM Ca). Increasing Ca in this range has a slight inhibitory effect on the calcium pump (11, 12), so that an increase in Ca, from 8 to 20 μM will inhibit calcium influx rate about 20% (11). Steady state rates of calcium influx and cal-

FIG. 10. Plots of the relationship between the Ca²⁺ concentration gradient (Ca/Ca) and Ca, where Ca, is maintained constant at 8 μM (2.5 mM oxalate, — — ) and 20 μM (1.0 mM oxalate, — — ). The dotted arrows labeled A and B represent the effects of an agent that increases calcium permeability 4-fold as described in Fig. 9. The dotted arrow labeled B' represents the effects of the same permeability changes shown by Arrow B under conditions where Ca, = 20 μM (discussed in text).
Calcium Release from Sarcoplasmic Reticulum

Calcium efflux at any level of Ca, will, therefore, be lower where Ca is 20 μM than where Ca is 8 μM. An initial Ca, of 0.3 μM at this steady state has been calculated from the data in Table I and Ref. 16, which reflects the 20% inhibition of calcium uptake rate when Ca, is increased from 8 to 20 μM. Under the latter conditions, a 4-fold increase in K, will increase calcium efflux rate from 0.175 to 0.7 μmol/mg of protein/min. According to the data in Table I and Ref. 16, modified to take into account the 20% inhibition of the calcium pump at a higher Ca, the new steady state in which calcium influx rate equals the higher rate of calcium efflux would occur at a Ca, of approximately 1.1 μM (dotted arrow B' in Fig. 10). The calcium release needed to achieve this higher Ca, of 0.6 μmol/liter, is more than twice that brought about by the same increase in K, at the lower level of Ca, (dotted arrow B in Fig. 10). The initial level of Ca, thus determines the amount of calcium that must be released from the vesicles after a given increase in calcium permeability. The higher the initial Ca, the more calcium will be released.

The foregoing analysis does not explain an effect of increasing Ca, to augment calcium release following a given increase in calcium permeability at any initial level of Ca,. It has been shown, however, that the Ca** dependence of initial calcium uptake velocity varies with oxalate concentration such that the apparent K, decreases when the oxalate level is decreased (24). This effect would reduce the ability of higher levels of Ca, to stimulate the calcium pump following a given increase in calcium permeability in the presence of 1 mM oxalate, compared to that in 2.5 mM oxalate. The resulting alteration in the curve shown in Fig. 9 to one showing evidence of saturation by Ca, in the micromolar range, coupled with the inhibition of the calcium pump at higher levels of Ca, (see above), would reduce the ability of the calcium released by an increase in calcium permeability to stimulate calcium influx. In this way, a given increase in calcium permeability at a higher level of Ca, would cause a greater calcium release than the same increase in calcium permeability at low Ca, at any given level of Ca,.

Pump-Leak Properties of Sarcoplasmic Vesicles after Calcium Storage in Absence of Calcium-precipitating Anions

The response to agents such as X537A after calcium storage differs from that described above in the case of oxalate-supported calcium uptake. In the latter, the ability of internal calcium stores to increase Ca, can allow stimulation of the calcium pump to restore a new steady state after only a small amount of calcium is released. In the case of calcium storage, however, the calcium content of the vesicles is much less and the levels of Ca, are much greater at any initial level of Ca,. For this reason, the ability of the vesicles to compensate for an increased calcium efflux rate by increasing calcium influx via the ATP-dependent calcium pump through increased Ca, is severely limited.

Effects of Ca, and Ca, on Ionophore- and Caffeine-induced Calcium Release

Dependence of Calcium Release after Oxalate-supported Calcium Uptake on X537A Concentration—The effects of varying X537A concentration on the release of calcium after calcium uptake in 2.5 mM oxalate had reached a steady state were examined at two protein concentrations (Fig. 11). At the lower protein concentration of 7.6 μg/ml (Fig. 11A), the total amount of added 45CaCl2 (50 μM) allowed a maximum calcium uptake of 6.6 μmol/mg of protein, which exceeded the capacity of this preparation to take up calcium. At the steady state of calcium uptake by these vesicles, Ca, at the time of X537A addition ranged between 9.3 and 3.1 μM for the four reactions shown in Fig. 11A. Increasing X537A concentration from 5 to 40 μM increased the amount of calcium released, as shown by the shaded area in the figure. At the lower ionophore concentrations, net calcium release was transient, whereas higher concentrations of X537A caused calcium release to be sustained. When this membrane preparation was examined under identical conditions at a higher protein concentration of 13.7 μmol/ml, the maximal possible calcium uptake of 3.65 pmol/mg of protein was less than the capacity of these vesicles, which was approximately 5 μmol/mg of protein (Fig. 11A). As a result, calcium uptake reached a steady state when Ca, fell to 0.11 μM in the four reactions shown in Fig. 11B. Under these conditions, increasing X537A concentration from 5 to 40 μM again induced a calcium release that was small and transient at the lower ionophore concentrations, and larger and sustained when X537A concentrations exceeded approximately 90 μM. At all X537A concentrations, however, the amount of calcium released initially was less in the studies carried out at the higher protein concentration. The effect of protein concentration cannot be attributed solely to the changing X537A to protein ratio as it was found that the inhibitory effects of 4 μM X537A on the initial rate of oxalate-supported calcium uptake decreased by only 25% when protein concentration was increased 10-fold, from 9 to 90 μg/ml. The quantitative differences between the effects of X537A seen in Fig. 11, A and B, are, instead, attributable primarily to differences in the levels of Ca, at the time of X537A addition (see above).

Effect of Ca, on X537A-induced Calcium Release—Comparison of the shaded areas in Fig. 11, A and B. Both of which utilize similar scales, shows that the total amount of calcium released initially at the higher protein concentration (Fig. 11B) was much less than at the lower protein concentration (Fig. 11A). The possibility that the decreased amount of calcium released at the higher protein concentration reflected a reduction in X537A/protein ratio was excluded by comparing the effects of varying protein and ionophore concentrations under conditions where Ca, varied in a manner similar to that shown in Fig. 11, A and B. The data in Fig. 12 show that at the higher protein concentration of 18 μg/ml, the amount of calcium release induced by 5 μM X537A was approximately 15 nmol/mg of protein after 30 s, whereas doubling the X537A concentration to 10 μM increased the amount of calcium released to only approximately 25 nmol/mg in 30 s. At the lower protein concentration of 9 μg/ml, on the other hand, 5 μM X537A caused a release of approximately 80 nmol of calcium/mg after 30 s. In the experiment shown in Fig. 12, Ca, at the time of X537A addition was 0.11 μM at the higher protein concentration, and 1.7 μM at the lower protein concentration. This experiment, like that shown in Fig. 11, indicates that the decrease in X537A-induced calcium release at higher protein concentrations is due to the lower level of Ca, at the time of X537A addition, which occurs when increasing protein concentration allows the vesicles to reduce Ca, to a lower level.

The dependence of the extent of X537A-induced calcium release on Ca, could also be shown when Ca, was lowered by EGTA immediately before addition of the ionophore. Low concentrations of EGTA added to the reaction mixture 30 s prior to the transfer of an aliquot to the tube containing the ionophore significantly reduced the extent of X537A-induced calcium release (Fig. 13). As was the case when Ca, was reduced by decreasing the total calcium/protein ratio, a de-
FIG. 11. Dependence of calcium release on X537A concentration. 
A single preparation was used to study calcium release at protein 
concentrations of 7.6 µg/ml (A) and 13.7 µg/ml (B). Calcium uptake 
was initiated by addition of “CaCl₂ to a final concentration of 50 µM 
in the presence of 2.5 mM oxalate at t = 0 as described under 
“Materials and Methods.” At t = 8 min, a 5-ml aliquot of the reaction 
mixture was transferred to a tube containing 25 µl of an ethanolic

cr. In ionophore-induced calcium release occurred even 
though the Ca⁺⁺ concentration gradient across the vesicles, 
Ca/Caₐ, was increased due to the lowered Caₐ. (The level of 
Caₐ in the experiments shown in Figs. 11 and 12 was held at 8 
µM by the 2.5 mM oxalate, and in Fig. 13 at 4 µM by the 5 mM 
oxalate.)

The findings in all of 12 consecutive experiments where 
calcium release was induced with 10 µM X537A after calcium 
uptake in 2.5 or 5.0 mM oxalate had reached a steady state are 
plotted in Fig. 14. An inverse relationship between Caₐ and 
the amount of calcium released is clearly seen. The line in Fig. 
14, which was calculated by assuming that the ionophore 
caused a 4-fold increase in calcium permeability, as described 
earlier, illustrates the general correspondence of the extent of 
net calcium release predicted by the analysis presented earlier 
in this report to the data obtained in studies of the effects of 10 
µM X537A on oxalate-supported calcium uptake. The agreement 
between measured calcium release and that predicted by 
this analysis is reasonably close, even though the data in Fig. 
14 include measurements carried out over a range of protein 
concentrations at both 2.5 and 5.0 mM oxalate. Calcium up-
take velocity is minimally influenced by changing oxalate 
concentration in this range (11).

Effects of Caₐ on X537A-induced Calcium Release—The 

concentration of calcium within the sarcoplasmic vesicles can 
be varied by allowing calcium uptake to proceed in the presence 
of various concentrations of oxalate or phosphate. In the 
present studies, the levels of Caₐ that would be reached in the 
presence of various concentrations of the calcium-precipitating 
anions were first estimated in a series of pilot runs that 
allowed more complex experiments to be carried out in which 
the effects of different levels of Caₐ could be compared at 
similar Caₐ concentrations. Typical experimental findings are 
shown in Fig. 15 where Caₐ at the time of X537A addition 
varied between 0.1 and 1.8 µM. This example was chosen 
because it illustrates the consistent finding that the amount of 
calcium released after X537A addition increased with increasing 
Caₐ in a manner that is independent of the effects of Caₐ 
described above.

The dependence of X537A-induced calcium release on Caₐ is 
pinned as the observed response in Caₐ after X537A addition 
following calcium uptake in 10 to 50 mM phosphate in Fig. 16. 
This experiment, in which Caₐ ranges between 750 µM (at 10 
mM phosphate) and 150 µM (at 50 mM phosphate), illustrates 
the typical finding that calcium release became greater as the 
concentration of the calcium-precipitating anion was lowered. 
This situation, where X537A-induced calcium release in-
creases as the Ca⁺⁺ concentration gradient is increased by
FIG. 12. Effects of varying protein and X537A concentrations on X537A-induced calcium release. Calcium release from sarcoplasmic reticulum vesicles at two protein concentrations was induced by 5 µM X537A (Panels A and C) or 10 µM X537A (Panel B). Symbols are the same as in Fig. 11. Conditions were chosen so that the X537A/protein ratios in Panels B and C were the same, whereas the Ca concentration at the time of X537A addition (t = 8 min) varied as shown at the left-hand side of each panel. Experimental conditions were as described under "Materials and Methods" and the legend to Fig. 11.

Increasing Ca, can be contrasted to that described in the preceding section where calcium release decreases when the Ca²⁺ concentration gradient is increased by lowering Ca²⁺.

The increasing amount of X537A-induced calcium release seen as Ca was increased (tabulated at the right in Fig. 16) caused an increase in the final, steady state, Ca concentration reached at the conclusion of the X537A-induced calcium release. The maximum Ca²⁺ concentration ratios, Ca/Ca₀, across the membrane vesicles prior to the addition of X537A for the experiment shown in Fig. 16 are tabulated in Table III, along with the calcium concentration ratios at the steady state reached after X537A addition.

The values for the concentration ratios prior to X537A addition shown in Table III are not accurate measurements of the maximal concentrating ability of these sarcoplasmic reticulum vesicles as Caₐ in the control reactions continued to decrease slowly between 10 and 20 min after the reactions were started, reaching levels that gave concentration ratios up to 4800 (data not shown). Because precautions were not taken in this study to allow control reactions to reach steady state, the data in Table III (and elsewhere in the present report) cannot be used to assess the maximal concentrating ability of these preparations.

Effects of Caffeine — It is well known that caffeine can cause calcium release from the sarcoplasmic reticulum of mammalian muscle (25), although these effects have been seen only at high caffeine concentrations and are somewhat variable. In studies of canine cardiac sarcoplasmic reticulum,³ for exam-

³ A. Failor and A. M. Katz, unpublished observations.

FIG. 13. Effect of EGTA addition on X537A-induced calcium release. Calcium uptake was initiated as described under "Materials and Methods" by addition of 6CaCl₂ to a final concentration of 50 µM in the presence of 5 mM oxalate at t = 0. Protein concentration was 6 pg/ml. Data are plotted as total calcium content in A. At t = 8 min, a portion of the reaction mixture was transferred to a tube containing sufficient EGTA to give a final EGTA concentration of 10 µM. (Total calcium outside the membranes at this time was 0.5 µM.) Calcium contents of both control (— — —) and EGTA-containing (— — —) reaction mixtures were then followed. At t = 8½ min, X537A (final concentration = 10 µM) was added to an aliquot of the EGTA-containing reaction mixture (O—O, lower panel) and to a separate control reaction mixture to which no EGTA had been added (O—O, upper panel of A). At t = 12 min X537A was added to another aliquot of both EGTA-containing and control reaction mixtures. The second X537A addition, therefore represented a repeat of the first. Concentrations of Ca₀ at the times of the first and second X537A additions to the control reaction were 1.23 and 1.49 µM, respectively, whereas Ca₀ at the time of X537A addition to the EGTA-containing reaction mixtures were below 0.1 µM. Net calcium release from control (O) and EGTA-containing (■) reaction mixtures are plotted in B as mean ± the range of the calculated differences for the X537A-induced calcium releases at t = 8½ and t = 12 min.
Calcium Release from Sarcoplasmic Reticulum

FIG. 14. Dependence of extent of X537A-induced calcium release on Ca, concentration at the time of X537A addition. All data from 12 consecutive experiments where the effects of 10 µM X537A on maximum calcium release were measured and plotted. Experimental protocols were as described under "Materials and Methods" and in the legend to Fig. 11. Protein concentrations ranged between 5 and 18 µg/ml in the presence of 50 µM 4CaCl₂. The line in this figure has been drawn according to the discussion in the text.

FIG. 15. Effect of varying Ca, on X537A-induced calcium release. Calcium uptake was initiated in 5.0 mM oxalate (6 µg of protein/ml, ●), 1.0 mM oxalate (10 µg of protein/ml, ○), and 50 mM phosphate (6.8 µg of protein/ml, △) in 50 µM 4CaCl₂ as described under "Materials and Methods." These different protein concentrations were chosen so as to allow Ca, to reach steady state at approximately 1 µM. Actual values of Ca, at the times of addition of 10 µM X537A are given in the figure. Calcium release was measured after X537A addition at t = 8 min for the experiment with 5 mM oxalate (○) and at t = 10 min for those with 1 mM oxalate (●) and 50 mM phosphate (△).

FIG. 16. Effects of varying phosphate concentration on X537A-induced increase in Ca, release. Calcium release was induced with 10 µM X537A 10 min after initiation of calcium uptake with 250 µg of protein/ml, 50 µM 4CaCl₂ and 10 (●), 15 (○), 20 (×), 25 (+), 30 (■), and 50 (□) mM phosphate as described under "Materials and Methods." Also tabulated are Ca, and net calcium release, the latter having been calculated from the change in calcium content.

TABLE III

<table>
<thead>
<tr>
<th>Phosphate concentration</th>
<th>Before X537A</th>
<th>After X537A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cₐ₀ (µM)</td>
<td>Cₐ₀ (µM)</td>
</tr>
<tr>
<td></td>
<td>Concentration ratio</td>
<td>Concentration ratio</td>
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<tr>
<td>mM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>10</td>
<td>750</td>
<td>0.28</td>
</tr>
<tr>
<td>15</td>
<td>500</td>
<td>0.19</td>
</tr>
<tr>
<td>20</td>
<td>375</td>
<td>0.16</td>
</tr>
<tr>
<td>25</td>
<td>300</td>
<td>0.14</td>
</tr>
<tr>
<td>30</td>
<td>250</td>
<td>0.11</td>
</tr>
<tr>
<td>50</td>
<td>150</td>
<td>0.10</td>
</tr>
</tbody>
</table>

after phosphate-supported calcium uptake under conditions where Cₐ₀ was high, above 1 µM, whereas when Cₐ₀ was less than 1 µM, virtually no calcium release was seen (Fig. 17). Transient calcium release following caffeine addition was also noted in frog sarcoplasmic reticulum by Ogawa (91), who suggested that the transient nature of the caffeine effect might reflect an action of the drug to promote a "calcium-triggered calcium release." In view of the findings with X537A discussed above, however, it is much more likely that the transient calcium release following addition of caffeine to sarcoplasmic reticulum vesicles is due to the fact that the initial rate of calcium release induced by caffeine initially exceeds that at which the calcium is pumped back into the vesicles by the calcium pump. Similarly, the reported finding that caffeine reduces calcium uptake capacity less than it slows calcium uptake rate (25) is in accord with the effects of X537A reported in the present paper. Reduction of total calcium uptake capacity by caffeine at high but not low Ca, (Fig. 5 of Ref. 25) is similar to the effects documented in Fig. 17 of the present paper. This evidence that caffeine acts in a manner similar to X537A, an agent that increases calcium efflux, can account for the failure of caffeine to inhibit ATPase activity and its ability to reduce the coupling ratio between calcium transport and ATP hydrolysis (26).
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FIG. 17. Effects of Ca\(^{2+}\) on caffeine-induced calcium release. Following calcium uptake by 50 \(\mu\)g of protein/ml in 50 \(\mu\)M \(^{45}\)CaCl\(_2\) (\(\bullet\)) or 100 \(\mu\)M \(^{45}\)CaCl\(_2\) (\(\bigcirc\)) and 50 mM phosphate, measured as described under "Materials and Methods," caffeine was added at \(t = 15\) min to a final concentration of 5 mM. Subsequent calcium release was followed for both the lower (\(\bullet\)) and higher (\(\bigcirc\)) calcium concentration. The level of Ca\(^{2+}\) at the time of caffeine addition is indicated on the figure.

The potency of caffeine to induce calcium release was studied with protocols designed so as to magnify the effects of a leak-producing agent, i.e. where both Ca\(^{2+}\) and Ca\(^{2+}\) were relatively high. This was accomplished by examining the effects of caffeine with 50 mM phosphate as the calcium-precipitating agent, and with a slight excess of total calcium relative to protein. Under these conditions, concentrations of caffeine as low as 0.25 mM evoked significant calcium release (Fig. 18).

DISCUSSION

The present findings regarding the ability of low concentrations of X537A to inhibit calcium transport by vesicles derived from the sarcoplasmic reticulum are in accord with earlier studies in both skeletal (18, 27, 28) and cardiac (19) preparations. In view of the broad specificity of X537A (19, 27, 29), the possibility that inhibition of calcium transport is due to an interaction between the free ionophore and cations in the reaction mixture was excluded by the finding that washing of the vesicles after brief exposure to X537A does not prevent the inhibition of subsequently measured calcium transport activities (Table II). The failure of washing to reverse the X537A effects indicates that the ionophore, which is poorly soluble in aqueous solutions, remains bound to the membranes.

The inhibitory effects of X537A on calcium storage in the absence of calcium-precipitating anions are not dependent on the Ca\(^{2+}\) concentration in the medium and the ionophore causes no significant change in the Ca\(^{2+}\) concentration at which calcium storage capacity is half-maximal (Fig. 1). Similarly, the extent of inhibition by X537A of oxalate-supported calcium uptake velocity is not influenced by Ca\(^{2+}\) (Table I).

The finding that the final level of calcium storage is the same whether X537A is added prior to or after the addition of calcium (Fig. 2) is in accord with a previous report (18) and is consistent with the view that the ionophore acts to render the vesicles permeable to calcium. However, the effects of X537A added to an oxalate-supported calcium uptake medium after the addition of calcium differ markedly from those seen when the ionophore is added prior to initiation of the calcium uptake reaction (Fig. 4). Incubation of the vesicles in 10 to 12 \(\mu\)M X537A prior to the addition of calcium caused subsequent calcium uptake to be inhibited almost completely, whereas addition of the same final ionophore concentration after completion of the calcium uptake reaction caused only a transient release of a small amount of calcium as seen in Fig. 4. Similar effects have been seen in cardiac preparations (19).

The effects of a calcium oxalate precipitate within the vesicles on the response of added X537A are shown in different ways in Figs. 5 and 6. In the experiment shown in Fig. 5, the ionophore was added at various times after the calcium uptake reaction had been started. Although the ionophore slows the reaction, calcium uptake continues to virtually the same level as that reached in control reactions even though this concentration of X537A, when added prior to the start of the calcium uptake reaction, completely inhibits calcium uptake (Fig. 4). Because the extent to which calcium uptake is inhibited by X537A is not influenced by Ca\(^{2+}\) in the range between 0.24 and 1.32 \(\mu\)M (Table I), preservation of the ability to transport calcium in the presence of the ionophore cannot be attributed to the reduction in Ca\(^{2+}\) that occurs when the vesicles take up calcium. The effect of prior calcium oxalate precipitation within the vesicles is also seen when additional calcium is added after the vesicles have been preloaded with a small amount of calcium oxalate to approximately 15 to 20% of their...
C calcium permeability is increased at high Ca++. Although X537A- and caffeine-induced calcium release are thus amplified by increased Ca++, this amplification does not represent a "calcium-triggered calcium release." Instead, the amplification of calcium release by increasing Ca++ can be attributed solely to the constraints described above.

The present findings indicate that the constraints imposed upon the pump-leak situation in sarcoplasmic reticulum, and especially the amplification of calcium release by increasing Ca++, must be considered in studies of the actions of agents that increase the calcium permeability of the membranes. Recognition of these constraints also permits a clearer understanding of spontaneous changes in the calcium permeability of these membranes (31).

Acknowledgment—The valuable technical assistance of Miss Susan Corkedale in many of these experiments is gratefully acknowledged.

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Calcium uptake can continue in the presence of X537A, are not altogether unexpected as it is well established that the ionophore does not inhibit the calcium pump ATPase (18, 19, 28).

Inhibition of the effects of X537A on calcium release after Ca++ is stabilized at a low level by calcium oxalate precipitation cannot be explained on the basis that the precipitate within the vesicles is slow to dissolve, and so is retained on the Millipore filters used to measure calcium uptake, because destruction of the membranes with lipid solvents causes rapid calcium release from these calcium oxalate precipitates (30). Furthermore, prior calcium oxalate precipitation not only inhibits ionophore-induced calcium release, but also allows subsequent addition to this precipitate to take place in the presence of the ionophore.

The ability of a calcium oxalate precipitate within the vesicles to inhibit calcium release cannot be attributed to the ability of the high internal oxalate concentration to maintain internal Ca++ concentration at a level sufficiently low so as to prevent significant calcium efflux when permeability is increased by the ionophore. Because the solubility product for calcium oxalate is approximately $2 \times 10^{-9}$ M under the conditions of these studies, the Ca++ concentration inside the vesicles will be fixed at 8 $\mu$M by 2.5 mM oxalate, so that in experiments where calcium uptake is limited by a low calcium/protein gradient (Fig. 6), over 90% of the calcium enters the vesicles to produce significant calcium concentration gradients across the membranes. Furthermore, calcium release by X537A after oxalate-supported calcium uptake is not markedly accelerated when Ca++ is lowered to approximately 0.01 $\mu$M by the addition of high EGTA concentrations (Fig. 7).

Modification of the response of sarcoplasmic reticulum vesicles to X537A and caffeine when calcium precipitates form within the vesicles is explained most easily by the constraints imposed by fixed Ca++ in vesicles that contain large amounts of an insoluble calcium precipitate. When Ca++ is maintained constant under conditions where most of the calcium is within the vesicles, and where the calcium concentration gradient, Ca++/Ca-, is less than the maximum that can be achieved by the calcium pump, these agents cause a transient calcium release that ends when the calcium pump is stimulated by the increased Ca++, so as to compensate for the increased efflux rate. Thus, where calcium stores within the vesicles are high relative to the amount of calcium outside the vesicles, only a small portion of the internal calcium is released by X537A (Fig. 4) or caffeine (Fig. 17). In contrast, when similar amounts of X537A are added after calcium storage in the absence of calcium-precipitating ions (Fig. 2), a much larger fraction of the internal calcium is released. The absolute quantity of calcium released by the ionophore after calcium storage is, however, less than after anion-supported calcium uptake, due to the lower calcium storage capacity of the vesicles (16) and the much higher Ca++ levels. As a result, release of even a large fraction of the calcium stored in the absence of calcium-precipitating anions cannot raise Ca++ to levels that allow the calcium pump to compensate for the increased absolute rate of calcium efflux that accompanies a given increase in calcium permeability.

The constraints described above, which are imposed by large internal calcium stores and a constant Ca++, require that more calcium be released to achieve a new steady state when calcium permeability is increased at high Ca++. Although X537A- and caffeine-induced calcium release are thus amplified by increased Ca++, this amplification does not represent a "calcium-triggered calcium release." Instead, the amplification of calcium release by increasing Ca++ can be attributed solely to the constraints described above.

The present findings indicate that the constraints imposed upon the pump-leak situation in sarcoplasmic reticulum, and especially the amplification of calcium release by increasing Ca++, must be considered in studies of the actions of agents that increase the calcium permeability of the membranes. Recognition of these constraints also permits a clearer understanding of spontaneous changes in the calcium permeability of these membranes (31).
Dependence of ionophore- and caffeine-induced calcium release from sarcoplasmic reticulum vesicles on external and internal calcium ion concentrations.

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