Control of Calcium Efflux from Sarcoplasmic Reticulum Vesicles by External Calcium*

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Factors modifying calcium efflux from rabbit skeletal muscle sarcoplasmic reticulum vesicles were examined after phosphate-supported calcium uptake reached steady state. Increasing external Ca++ promoted calcium efflux by a mechanism that exhibited Michaelis-Menten kinetics; Vmax at 25° with 5 mM MgATP as substrate and 50 mM phosphate as calcium-precipitating anion was ~400 nmol/mg/min and the level of Ca++ outside the vesicles at which calcium efflux velocity is half-maximal, Kca, was 0.8 to 1.0 μM. Replacing 120 mM KCl with 120 mM NaCl did not appreciably change calcium efflux. Elevation of Mg++ in the range 0.10 to 1.13 mM markedly inhibited calcium efflux, Mg++ acting as a competitive inhibitor with Ca++ for binding to external sites. This inhibitory effect of Mg++ resembles that observed for "Ca++-triggered calcium release" in "skinned" muscle fibers. Variations of the calcium ion concentration inside the microsomal vesicles (Ca,) in the range 150 to 750 μM altered the maximal calcium efflux velocity but did not significantly influence Kca, and reduction of Ca, to 4 to 8 μM by replacing phosphate with 2.5 to 5 mM oxalate as the calcium-precipitating anion did not lower Kca, suggesting that the interaction of calcium-sensitive sites on the external surface of the membranes with Ca++ is independent of the interaction of the internal sites with Ca++. Substitution of acetyl phosphate for ATP increased Kca approximately 10-fold, but did not abolish the ability of increasing external Ca++ to promote calcium efflux. The calcium-activated calcium efflux thus cannot be attributed to reversal of the calcium pump, which is inhibited by high external Ca++ and ATP, and requires ADP. A number of similarities were found between factors that influence calcium transport via the calcium pump and those that modify calcium efflux.

Contraction in fast skeletal muscle is initiated when calcium is released from the sarcoplasmic reticulum into the cytosol (1) by a process that allows calcium to flow out of a region of high calcium concentration within this membrane system (2, 3). The mechanism that initiates this calcium flux is not known, but may involve an increase in the calcium permeability of the sarcoplasmic reticulum initiated by an electrical potential change across these membranes (4, 5) or by an increased Ca++ concentration at their external surface ("Ca++-triggered calcium release") (6, 7).

Studies of purified sarcoplasmic reticulum vesicles indicate that calcium efflux1 is increased when Ca, is elevated (8-11), and that calcium permeability at any given level of Ca, increases with increasing Ca, but decreases with increasing Ca, (12, 13). The dependence of calcium efflux on Ca, and Ca, is characterized further in the present study, and the effects of Mg++ and replacement of K+ by Na+ are described. The concentration of Ca, that promotes half-maximal calcium efflux velocity is shown to be independent of the level of Ca, suggesting that calcium-sensitive regulatory sites at the inside and outside of these membranes interact independently with Ca++. Similarities between the properties of calcium efflux from, and calcium transport into these vesicles, and similar shifts in the concentration at which calcium uptake and efflux velocities are half maximal when ATP is replaced with acetyl phosphate are consistent with the view that the calcium pump can also mediate the calcium efflux that is promoted by high Ca,.

METHODS

Preparation of sarcoplasmic reticulum vesicles and measurements of calcium uptake, calcium release, calcium influx, and calcium efflux were as described previously (8, 12, 13). Experiments were carried out in 0.12 M KCl or NaCl and 40 mM histidine buffer (pH 6.8). The sodium concentration of reaction mixtures to which NaCl was not added was well below 1 mM, as determined by flame photometry. In studies of the Mg++-dependence of calcium efflux, various concentrations of MgCl2 were added to ATP (final concentration 5 mM) and pH adjusted to 6.8 prior to use. Ionized Mg++ concentrations were calculated from equations described previously (14). Temperature was 25° for all studies in which ATP was used as substrate, and 37° or 25° where acetyl phosphate was substrate.

1 As used in this article, calcium influx and calcium efflux refer to rates of unidirectional calcium fluxes into and out of the vesicles, respectively. Calcium uptake and calcium release refer to the rate of net gain or loss of calcium by the vesicles.

The abbreviations used are: Ca, calcium ion concentration in the medium outside the microsomal vesicles, calculated as described previously (8); Ca, calcium ion concentration inside the microsomal vesicles, Kca, Ca, level at which calcium efflux velocity is half-maximal.
RESULTS

Effects of Varying $Ca_i$ at Constant $Ca_o$—We found previously that calcium permeability coefficients, calculated by dividing calcium efflux velocity by $Ca_i$ (the "driving force"), were inversely proportional to the $Ca^2+$ concentration gradient $Ca_i/ Ca_o$ (12, 13). The mechanism underlying this relationship was examined further in the present study by extending the range of $Ca_i$ over which calcium efflux velocity at constant $Ca_o$ was measured. The dependence of calcium efflux velocity on $Ca_i$ in the range between 0.1 and 10 $\mu M$ exhibited Michaelis-Menten kinetics, the double reciprocal plots of $1/v$ versus $1/Ca$ being linear (Fig. 1). Values for $K_{Ca}$ and $V_{max}$ were estimated in this and subsequent experiments by a computer calculation that used a linear least squares fit to double reciprocal plots such as shown in Fig. 1.

Effects of Replacement of KCl with NaCl—In light of evidence that passive calcium fluxes across the plasma membranes of both the heart (15) and the squid axon (16) can be modified by changing $Na^+$ concentration, the effect of replacing 120 mM KCl with 120 mM NaCl was examined in calcium phosphate-loaded vesicles where initial calcium uptake was carried out in 5 mM MgATP. No differences in the $Ca^2+$-dependence of calcium efflux were seen when reaction mixtures containing either of these alkali metal salts were compared (Fig. 2). When the results of four independent experiments were pooled and analyzed by plotting the logarithm of the calcium permeability coefficient versus the logarithm of the ratio $Ca_i/ Ca_o$ (12, 13), the lines drawn by the method of least mean squares did not differ significantly (Fig. 3).

Effects of $Mg^2+$—Elevation of $Mg^2+$ in the range 0.10 to 1.13 mM inhibited calcium efflux in experiments where total ATP concentration was maintained at 5 mM, and MgATP concentration varied between 3.9 and 4.9 mM (Fig. 4). The dependence of calcium efflux velocity on $Mg^{2+}$, estimated by interpolation of curves such as those shown in Fig. 4 to 0.2 $\mu M$ $Ca_o$, indicates that elevation of $Mg^{2+}$ concentration from 0.1 to 1.13 mM reduces calcium efflux velocity by approximately two-thirds (Fig. 5).

The data shown in Fig. 4 suggest that elevation of $Mg^{2+}$ increases the $K_{Ca}$ for calcium efflux. This impression was supported by double reciprocal plots of the effects of $Ca_o$ on calcium efflux at various levels of $Mg^{2+}$. The data in Fig. 6:

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**Fig. 1.** Double reciprocal plot of the dependence of calcium efflux velocity on $Ca_o$. Reactions were carried out as described under "Methods" in 5 mM MgATP with 50 mM phosphate as the calcium-precipitating anion. Protein concentration was 20 $\mu g$/ml. $V_{max}$ in this experiment was 400 nmol/mg/min and $K_{Ca}$ was 0.8 $\mu M$.

**Fig. 2.** Effects of $Na^+$ and $K^+$ on calcium efflux velocity. Reactions were carried out as described under "Methods" in 5 mM MgATP with 50 mM phosphate as the calcium-precipitating anion in the presence of 120 mM KCl or 120 mM NaCl. Protein concentration was 10 $\mu g$/ml.

**Fig. 3.** Effects of $Na^+$ and $K^+$ on calcium permeability coefficient ($K_{Ca}$). Reactions were carried out as described in the legend to Fig. 2 in the presence of 120 mM KCl (closed symbols) or 120 mM NaCl (open symbols). Each symbol corresponds to one of four independent experiments. Lines were computed by the method of least mean squares.

**Fig. 4.** Effects of $Mg^{2+}$ on the dependence of calcium efflux velocity on $Ca_o$. Reactions were carried out as described under "Methods" in 5 mM ATP and 4.5 mM MgCl$_2$ ($Mg^{2+} = 0.18$ mM), 5.0 mM MgCl$_2$ ($Mg^{2+} = 0.37$ mM), and 5.5 mM MgCl$_2$ ($Mg^{2+} = 0.70$ mM). The calcium-precipitating anion was 50 mM phosphate and protein concentration was 10 $\mu g$/ml.
Effects of Mg2+ on calcium efflux velocity at Ca2+ = 0.2 μM. Each symbol represents the data from one of four independent experiments carried out as described in the legend to Fig. 4. MgCl2 concentrations ranged between 4.0 and 6.0 mM in the presence of 5 mM ATP.

Effects of Varying Ca2+—In our previous studies, we reported that calcium efflux velocity did not increase in proportion to elevation of Ca2+, the driving force for calcium efflux. As a result, calcium permeability coefficients, calculated as efflux velocity divided by Ca2+, decreased with increasing Ca2+ (12, 13). The interrelationship between the effects of Ca2+ and Ca2+ were examined in experiments such as that shown in Fig. 7, where the dependence of calcium efflux velocity on Ca2+ was examined at two levels of Ca2+. A 40 to 50% decrease in Vmax at the higher Ca2+ was consistently seen (Table I), indicating that the calcium permeability coefficient was markedly reduced by elevation of Ca2+. No appreciable change in the Kc for calcium efflux accompanied a 5-fold elevation of Ca2+ (Table I). In studies where oxalate instead of phosphate was used as the calcium-precipitating anion, reduction of Ca2+ to 4 or 8 μM also did not significantly reduce the Kc for calcium efflux, which remained at or above 1 μM.

Effects of ATP and Acetyl-P (AcP) on the dependence of calcium efflux velocity on Ca2+. Reactions were carried out as described under "Methods" in 5 mM MgATP at 25°C (○) or 2 mM acetyl-P + 5 mM MgCl2 at 37°C (●). The calcium-precipitating anion was 50 mM phosphate and protein concentration was 20 μg/ml. Arrows indicate values for Kc.

Effects of Replacement of ATP with Acetyl Phosphates—The effects of Ca2+ on calcium efflux velocity were significantly altered when acetyl-P replaced ATP as the energy donor. As shown in Fig. 8, Kc increased approximately 10-fold in acetyl-P, whereas Vmax decreased 2- to 3-fold. These marked differences in Kc were also apparent when the reactions with acetyl-P were carried out at 25°C. The increased Kc seen with acetyl phosphate cannot be attributed to the higher Mg2+ affinity of acetyl-P as reduction of MgCl2 to 0.37 mM in the presence of acetyl-P caused an increase in Kc.
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DISCUSSION

The present findings confirm previous reports (9-13) that calcium efflux from sarcoplasmic reticulum vesicles is increased by increasing Ca\textsubscript{a}, and our earlier observations that calcium permeability coefficients are reduced by increasing Ca\textsubscript{a} (12, 13). Establishment of an electrical potential across these membranes by the Ca\textsuperscript{2+} concentration gradient, Ca\textsubscript{a}/Ca\textsubscript{i}, does not readily explain these effects as the ability of raising Ca\textsubscript{a} to increase calcium efflux and calcium permeability at a constant Ca\textsubscript{a} resembles a saturable process (Figs. 1 and 7). Instead, the present findings suggest that Ca\textsubscript{a} increases calcium efflux by binding to sites on the external surface of the membrane. The inhibitory effects of high Ca\textsubscript{a}, which resemble those observed in the case of the calcium transport into the vesicles via the calcium pump (17, 18), can be attributed to an action on Ca\textsuperscript{2+}-binding sites on the interior of the membrane (see below).

The putative Ca\textsuperscript{2+}-binding sites on the inside of these membranes do not appear to be the same as those on the outside, as evidenced by the failure of a 5-fold increase in Ca\textsubscript{a} from 150 to 750 μM, brought about by reduction in phosphate concentration from 50 to 10 μM, to modify the apparent Ca\textsuperscript{2+} affinity of the external Ca\textsuperscript{2+}-binding site (Fig. 7, Table D). Furthermore, replacement of phosphate with 5 mM oxalate, which lowers Ca\textsubscript{a} to a level of approximately 4 μM did not reduce K\textsubscript{Ca}. The present findings thus do not support the interpretation that calcium efflux is mediated by a carrier with a single Ca\textsuperscript{2+}-binding site that can move between the interior and exterior of the membrane. Instead, they suggest the existence of at least two sites that can respond independently to changes in Ca\textsuperscript{2+} concentrations at either side of the membrane.

The absence of detectable effects of replacing 120 mM KCl with 120 mM NaCl indicates that the calcium permeability control mechanism observed in the sarcoplasmic reticulum is significantly different from that which has been postulated to effect sodium-calcium exchange in the plasma membrane of various tissues (15, 16). The physiological properties of this sodium-calcium exchange can most simply be explained by a receptor which binds and transports either one calcium or two sodium ions, depending on the relative concentration of Ca\textsuperscript{2+} and Na\textsuperscript{+} at either side of the membrane. In the case of the present calcium efflux mechanism, however, elevation of Na\textsuperscript{+} from <1 mM to 120 mM had no detectable effects on calcium efflux (Fig. 2) or the calcium permeability coefficient (Fig. 3).

The inhibitory effect of raising Mg\textsuperscript{2+} concentration in the millimolar range (Figs. 4 and 5) is unlikely to have arisen from changes in the level of MgATP, which ranged between 3.9 and 4.9 mM in these studies. It is possible that the effects associated with high Mg\textsuperscript{2+} could reflect a concomitant reduction in free ATP, which was 1.1 mM at the lower Mg\textsuperscript{2+} concentration and 0.1 mM at the higher level of Mg\textsuperscript{2+}, but the kinetic properties of the Mg\textsuperscript{2+} effect suggest instead that this ion competes with Ca\textsuperscript{2+} for binding to an external site in these membranes. A similar competition between Ca\textsuperscript{2+} and high concentrations of Mg\textsuperscript{2+} has been observed for the formation of the phosphorylated ATPase intermediate and phosphoprotein decomposition (19) and for the activation of the calcium pump ATPase (20), and evidence has been presented that both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} can occupy cation-binding sites in these membranes (21).

The inhibitory effects of Mg\textsuperscript{2+} observed in this study of calcium efflux from skeletal muscle sarcoplasmic reticulum vesicles are similar to those noted in the case of "skinned" skeletal muscle fibers. In the latter, elevation of Mg\textsuperscript{2+} from 0.05 to 0.9 mM markedly inhibits Ca\textsuperscript{2+}-triggered calcium release (22). A similar inhibitory effect of Mg\textsuperscript{2+} on calcium efflux from rat cardiac sarcoplasmic reticulum vesicles has also been noted by Dunnett and Nayler (23). The similarities between the effect of Mg\textsuperscript{2+} to inhibit the ability of elevated Ca\textsubscript{a} to promote calcium efflux from sarcoplasmic reticulum vesicles, and the inhibitory effect of Mg\textsuperscript{2+} on Ca\textsuperscript{2+}-triggered calcium release in the skinned fiber suggest that these two procedures may reflect the operation of a common mechanism.

Calcium efflux from the sarcoplasmic reticulum has previously been observed to be coupled to ATP synthesis (pump reversal) under conditions where Ca\textsubscript{a} is high and Ca\textsubscript{i} is low. This process, in which osmotic energy derived from the downhill flux of calcium is used for ATP synthesis (23-27), is inhibited by high Ca\textsubscript{a} (23, 26, 27) and by high levels of ATP in the surrounding medium (27). In contrast, the calcium efflux described in the present report is stimulated by high Ca\textsubscript{a} (Fig. 1) and is more rapid in the presence of ATP than in acetyl-P (Fig. 8). Conclusive evidence that this calcium efflux does not represent pump reversal is shown in Fig. 8, where stimulation of calcium efflux by increasing Ca\textsubscript{a} is seen when theophylline is the energy donor. As these conditions exclude ADP formation during the initial calcium uptake reaction, calcium efflux cannot be coupled to the resynthesis of ATP.

Stimulation of both calcium influx and efflux by the addition of a second portion of calcium to sarcoplasmic reticulum vesicles that had previously taken up a small amount of calcium was described by Makinose (11), who also found that this effect of increasing Ca\textsubscript{a} was accompanied by a reduced rate of P\textsubscript{i} incorporation in the NTP fraction. Makinose also found that ADP, which promotes NTP-NDP exchange, stimulated both calcium influx and efflux (11), an effect which we have recently confirmed (4). The conditions used previously to demonstrate the stimulation of calcium efflux by high Ca\textsubscript{a} allow for a considerable degree of calcium-calcium exchange. Similarly, the present measurements of calcium efflux represent almost exclusively an exchange between internal and external calcium in vesicles that had reached a steady state of calcium content. Although a portion of the calcium efflux described here may be coupled to concurrent influx of this cation, we have demonstrated a rapid and extensive calcium release after the calcium pump is inhibited by addition of ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid to the reaction mixture (13). Thus, while the calcium efflux described in the present report may represent, in part, a coupled calcium-calcium exchange, a large fraction of this calcium efflux appears to be independent of concurrent calcium influx.

During the course of these studies, a number of similarities were observed between factors which affect calcium efflux from sarcoplasmic reticulum vesicles and those known to influence the rate of active calcium transport into these vesicles. Resemblances between calcium efflux and initial calcium uptake velocities include similarities in the K\textsubscript{Ca}, in the inhibitory effects of high Ca\textsubscript{a}, and in the ability of Mg\textsuperscript{2+} to compete with Ca\textsuperscript{2+} for an external site on these membranes. In addition, acetyl-P has been found to increase the K\textsubscript{Ca} for calcium uptake to a level approximately 10 times higher than that seen when ATP is the energy donor (28), a finding which resembles qualitatively that shown in Fig. 8. Additional evidence that an ion pump can also mediate passive ion fluxes has been ob-

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1. J. Dunnett and W. Nayler, personal communication.
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...tained in studies of the sodium pump of the plasma membrane, which can, under some conditions, catalyze passive potassium fluxes (29, 30). Furthermore, incorporation of the ATPase protein of the sarcoplasmic reticulum into phospholipid vesicles is accompanied by a marked increase in calcium permeability (31). The present findings are, therefore, consistent with the hypothesis that the calcium efflux from sarcoplasmic reticulum vesicles which is activated by high Ca\(^{2+}\) may involve the calcium pump.

**REFERENCES**

Control of calcium efflux from sarcoplasmic reticulum vesicles by external calcium.
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