Reversible Loss of Cooperative Calcium Ion Binding by Sarcoplasmic Reticulum Calcium Adenosinetriphosphatase†

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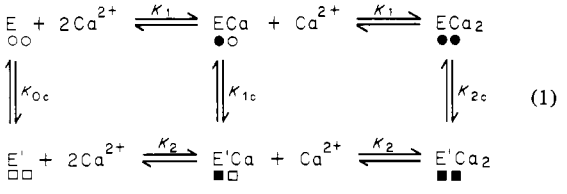
ABSTRACT: Incubation of rabbit skeletal muscle sarcoplasmic reticulum vesicles in solutions of very low [Ca^{2+}] caused Ca^{2+} to bind noncooperatively, as determined by the dependence of the intrinsic tryptophan fluorescence intensity on added increments of Ca^{2+}. Cooperative Ca^{2+} binding was obtained if the ATPase was incubated in [Ca^{2+}] high enough (25 μM) to saturate the two high-affinity Ca^{2+} binding sites and then titrated with [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. The cooperative binding had an apparent association constant of 6.3×10^6 M^{-1} and a Hill coefficient of 2.6; these constants for the noncooperative binding case were 5.0×10^5 M^{-1} and 1.2, respectively. The transitions from the noncooperative to the cooperative Ca^{2+} binding forms of the enzyme were slow compared to the time required for Ca^{2+} binding to reach equilibrium. Thus, it appears that sarcoplasmic reticulum CaATPase is a hysteretic enzyme. Intrinsic association constants for Ca^{2+} binding and equilibrium constants for the transitions between the two forms in low and high [Ca^{2+}] were estimated from analyses of a general scheme for cooperative and noncooperative binding.

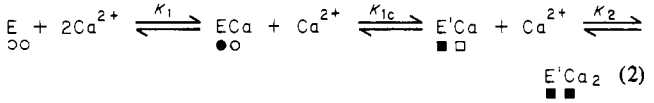
Sarcoplasmic reticulum (SR)[1] CaATPase activity is activated by Ca^{2+} binding in the micromolar concentration range (Ebashi & Lipmann, 1962; Hasselbach & Makinose, 1962; Weber et al., 1966; Martonosi & Feretos, 1964). Presumably the Ca^{2+} that binds at the high-affinity sites is also transported. Many measurements of the apparent association constant for Ca^{2+} and SR CaATPase have shown that it is about 2×10^6 M^{-1} at 25 °C in about 0.1 M KCl (Carvalho, 1966; Chevallier & Butow, 1971; Meissner et al., 1973; Ikemoto, 1974; Dupont, 1976; Kalbitzer et al., 1978; Inesi et al., 1980; Verjovski-Almeida & Silva, 1981). There also seems to be general agreement that at higher temperatures two Ca^{2+} are transported per ATP hydrolyzed (Hasselbach & Makinose, 1963; Weber et al., 1966; Yamada et al., 1970; Deamer, 1973). In itself, Ca^{2+} binding is an important step in the mechanism of chemiosmotic energy transduction by SR. Earlier reports of Ca^{2+} binding suggested it was noncooperative (Meissner, 1973; Kalbitzer et al., 1978; Dupont, 1976); but recently, it was shown by direct measurements of Ca^{2+} bound to SR vesicles and by electron spin resonance measurements of the response of spin-labeled SR to Ca^{2+} that there is strong positive cooperativity between two Ca^{2+} binding sites (Inesi et al., 1980). The cooperativity was subsequently confirmed by using fluorescence techniques (Verjovski-Almeida & Silva, 1981).

A general scheme for Ca^{2+} binding to SR with two identical Ca^{2+} binding sites that can bind cooperatively or noncooperatively is shown in eq 1, in which E and E′ are the ATPases



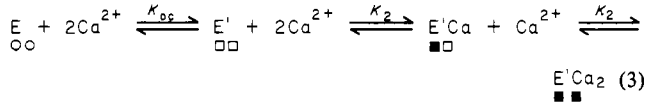
(1)

that can bind two Ca^{2+} in a noncooperative or cooperative manner, K_1 and K_2 are association constants for Ca^{2+} binding, and K_{0c}, K_{1c}, and K_{2c} are equilibrium constants for transitions between E and E′ with 0, 1, and 2 bound Ca^{2+}, respectively. The symbols below E (○, ●) and E′ (□, ■) are meant to represent empty and Ca^{2+}-filled noncooperative and cooperative binding sites. Two pathways from the general scheme in eq 1 have been used to analyze the high-affinity cooperative Ca^{2+} binding to SR (Inesi et al., 1980; Verjovski-Almeida & Silva, 1981). The first is given in eq 2, which has the first



(2)

Ca^{2+} binding to E, followed by a conformational change to E′Ca which has higher affinity sites. In this equation, when $K_{1c}K_2$ is larger than K_1, there is positive cooperativity. The second scheme is given in eq 3, which has the ATPase in



(3)

equilibrium with the conformation that binds Ca^{2+} cooperatively before any Ca^{2+} binds. When $K_2 > K_{0c}K_2$, there is positive cooperativity.

In either case, when there is strong positive cooperativity, species containing one Ca^{2+} usually are not detectable. Typically, one then either determines an apparent association constant, K_{app}, from the free [Ca^{2+}] at the transition midpoint or fits an expression related to eq 2 or 3 to the binding curve to get values for parameters composed of K_1, K_{0c}, K_{1c}, and K_2. A problem is that K_1, K_{0c}, and K_{1c} usually are not independent of K_2 in the well-defined region of the binding curve, making it very difficult to obtain unique values for more than one parameter. For a discussion of this and an example of an

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[1] Abbreviations: SR, sarcoplasmic reticulum; ATP, adenosine 5′-triphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; CaATPase, (Ca^{2+},Mg^{2+})-ATPase from rabbit skeletal muscle sarcoplasmic reticulum; n_H, Hill coefficient; $pCa(^1/_2)$, the negative logarithm of the free Ca^{2+} concentration at the midpoint of a titration curve; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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exception, see Gutfreund (1972).

Reported here are measurements of fluorescence-detected Ca^{2+} binding to SR CaATPase in the absence of ATP under conditions that foster either cooperative or noncooperative binding. Straightforward analysis of the noncooperative case gives $K_1 = 5 \times 10^5$ M^{-1}. The cooperative case may be complicated by hysteretic behavior. Analyses were done by using eq 2 and 3 to estimate values for K_1, K_{0c}, and K_{2c}.

Experimental Procedures

Proteins and Chemicals. Vesicles of rabbit skeletal muscle SR were prepared by the method of Eletr & Inesi (1972) and stored at 0 °C in 30% sucrose solution for no longer than 2 days before a measurement was made. Gel electrophoresis in the presence of 1% sodium dodecyl sulfate indicated that over 70% of the protein stained by Coomassie Brilliant Blue or fluorescamine (Sigma Chemical Co.) appeared in the band corresponding to 110000 amu, presumably the ATPase (Eletr & Inesi, 1972). Protein concentration was determined by the biuret method or the Folin test and compared to standards of bovine serum albumin without correction. ATPase concentration was taken to be 70% of the total protein concentration. All chemicals were reagent grade or better. The concentration of free Ca^{2+} was determined from the concentrations of total $CaCl_2$ and EGTA by using an effective dissociation constant of 2.46×10^{-7} M calculated from the tables in Martell & Smith (1974) for pH 7.

ATPase Activity and Ca^{2+} Uptake. ATPase activities were determined by measuring orthophosphate production by using the method of Lin & Morales (1977) or Murphy (1981). Vesicles were made permeable to Ca^{2+} with the ionophore A23187 (Calbiochem-Behring Corp.). Ca^{2+} uptakes were measured by the Millipore filtration method (Martonosi & Feretos, 1964). The ratios of Ca^{2+} taken up to ATP hydrolyzed were calculated from uptake and activity data obtained on identical solutions containing oxalate. The exact conditions are given in Table II.

Fluorescence Measurements. Tryptophan fluorescence intensities were measured with a Perkin-Elmer MPF-44B fluorospectrophotometer in the ratio mode. Irradiation was at 295 nm to minimize the contribution from tyrosine residues. Emission intensity was measured at 335 nm without correcting for Raman scattering. The exciting light was polarized at 90° with respect to the plane of the exciting and emitted light, and the emitted light passed thru a 310-nm cutoff filter to minimize the effect of Rayleigh scattered light. Temperature was maintained to ±0.1 °C and measured in the cuvette with a mercury thermometer. A typical titration required 15 min.

Results

Conditions were chosen to optimize the stability of the fluorescence emission due to tryptophan. The fluorescence from suspensions of SR vesicles was found to be sensitive to temperature and pH; careful control of these parameters was required. Figure 1 shows the fluorescence intensity vs. time for vesicles in 150 mM Mops, 50 mM KCl, 5 mM $MgCl_2$, and 25 μM $CaCl_2$, pH 7.0 at 25 °C, before and after addition of 1 mM EGTA. The difference in emission intensity was about 4% of the total fluorescence intensity, in agreement with the original results of Dupont (1976). The intensity was stable for at least 20 min, without significant drift, as long as the temperature and pH were kept constant. The effect of EGTA was complete within minutes and completely reversed by adding Ca^{2+}. The total change in fluorescence intensity was the same magnitude if the SR was incubated in EGTA and then treated with excess $CaCl_2$. Lowering the concentration

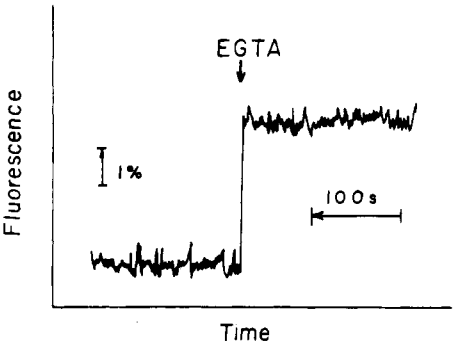

FIGURE 1: Tryptophan fluorescence intensity vs. time is shown for a 1–2 μM vesicular SR suspension in 150 mM Mops, 50 mM KCl, 5 mM $MgCl_2$, and 25 μM $CaCl_2$, pH 7.0 at 25 °C, before and after a very small volume of EGTA is added to give 1 mM EGTA. Irradiation at 295 nm passed thru a 90° polarizer. Emission at 335 nm was detected, amplified, and displayed on the recorder such that a 20% change in total fluorescence gave a full-scale deflection. Excitation and emission slits were set to require a minimum of amplification; typical band-pass widths were 4–8 nm.

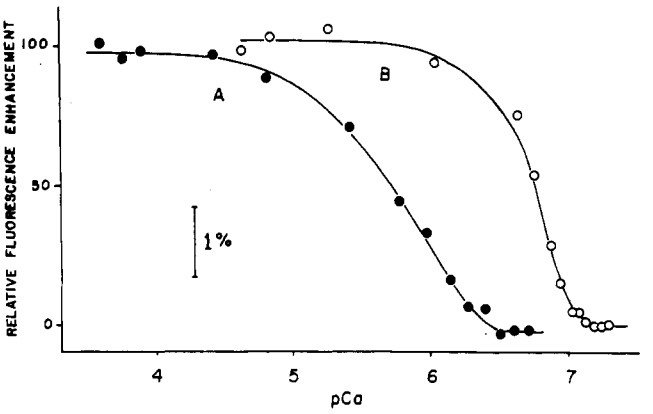

FIGURE 2: EGTA titrations of 25 μM Ca-incubated SR (B) and Ca^{2+} titrations of 1 mM EGTA-incubated SR (A) were monitored by fluorescence as described in Figure 1. The changes in fluorescence were corrected for dilution due to the added aliquots of Ca^{2+} or EGTA and normalized to 100% for the total transition. The free [Ca^{2+}] was determined from the total added [Ca^{2+}] and [EGTA] by using an effective K_a for CaEGTA of 4.07×10^6 M^{-1}, as calculated from data in Martell & Smith (1974).

of buffer down to 20 mM Mops at pH 7.0 did not appear to affect the change in fluorescence. However, as others have observed (Guillian et al., 1980), the scatter in the data increased with lower buffer concentrations, so almost all experiments were done in 150 mM buffer. Reducing the concentrations of EGTA and Ca^{2+} from the 1 mM range to the 50 μM range did not alter the total change in fluorescence, and the concentration of free Ca^{2+} at which the midpoint of a transition occurred was shifted only slightly upward. The intensity of light scattered at 90° was monitored to check for possible settling or aggregation of the dilute suspensions of vesicles. Preequilibrated Ca^{2+}-free and Ca^{2+}-saturated solutions had scattering intensities that were constant with time (not shown), indicating that the vesicles were not settling or aggregating appreciably.

Ca^{2+} Binding. When SR vesicles in solutions at 25 °C containing 25 μM added Ca^{2+} were titrated with EGTA, the dependency of the tryptophan emission on free [Ca^{2+}] indicated that the binding of Ca^{2+} was cooperative. From the data in Figure 2, the free [Ca^{2+}] at the midpoint of a curve drawn through the points was determined to be $pCa(^1/_2) = 6.8$, and the Hill coefficient (n_H) was 2.6, as summarized in Table I. The K_{app} (=6.3×10^6 M^{-1}) is somewhat larger than previous

Table I: Calcium Binding to SR

incubated in[a]	titrated with	$pCa(^1/_2)$	n_H	K_{app} (M^{-1})
25 μM Ca^{2+}	EGTA	6.8 ± 0.1	2.6 ± 0.5	6.3×10^6
1 mM EGTA	Ca^{2+}	5.7 ± 0.2	1.2 ± 0.3	5.0×10^5
1 mM EGTA then 25 μM Ca^{2+}	EGTA	6.8 ± 0.4	2.5 ± 0.6	
25 μM Ca^{2+} then 1 mM EGTA	Ca^{2+}	5.6 ± 0.3	0.9 ± 0.4	

[a] Solutions of SR vesicles in 50 mM KCl, 150 mM Mops, and 5 mM $MgCl_2$ at 25 °C, pH 7.0, plus either 25 μM Ca^{2+} or 1 mM EGTA were incubated 1 h. These solutions were then either diluted in an identical buffer to obtain 1–2 μM CaATPase and titrated (see Figure 2) or diluted in the other buffer and incubated 1 h and then titrated. The Ca^{2+} concentration at the midpoint of the transition (n_H) was determined from averages of three to seven titrations. K_{app} was calculated from $\log K_{app} = pCa(^1/_2)$.

Table II: SR ATPase Activities and Ca^{2+} Uptake[a]

	activity			
incubated in	ionophore A23187	oxalate	EGTA	Ca^{2+}/ ATP
25 μM Ca^{2+}	7.7 ± 0.3	4.4 ± 0.4	0.4 ± 0.2	1.8
1 mM EGTA	6.7 ± 0.8	4.3 ± 0.3	0.4 ± 0.2	1.7
control	6.7 ± 0.4	5.3 ± 0.3	0.3 ± 0.2	1.7

[a] SR was incubated as described in Table I and then assayed for ATPase activity in 2 mM ATP, 10 mM $MgCl_2$, 80 mM KCl, and 20 mM Mops (pH 6.8) at 37 °C with added 1 mM $CaCl_2$, 1 mM EGTA, and 1/1000 by weight Ca^{2+} ionophore A23187 or 1 mM EGTA, and 4 mM oxalate or 1 mM EGTA, corresponding to the three center columns above. Ca^{2+} uptakes using $^{45}Ca^{2+}$ were determined for the given oxalate conditions, and the ratios of Ca^{2+} taken up to ATP hydrolyzed are given in the fifth column. Activities are given as μmol of P_i (mg of SR)$^{-1}$ min^{-1}.

measurements of Ca^{2+} affinity [$(1–3) \times 10^6$ M^{-1}], and n_H is also slightly larger than values obtained recently (1.8–2.2). Nearly identical results were obtained at 4 °C (not shown). The asymmetry of the titration curves was consistently observed.

A striking difference in the [Ca^{2+}] dependence of the fluorescence was observed if the vesicles were first incubated in 1 mM EGTA and then titrated with Ca^{2+}. The total change was the same, but the midpoint of the transition was shifted to $pCa(^1/_2) = 5.7$, corresponding to $K_{app} = 5.0 \times 10^5$ M^{-1}, and n_H was reduced to 1.2 (Figure 2). The loss of cooperativity and the greater than 10-fold reduction in the apparent association constant were fully reversible by adjusting the free [Ca^{2+}]. Preincubation in 1 mM EGTA, followed by incubation in 25 μM Ca^{2+} before titration with EGTA, gave results (Table I) that were indistinguishable from those obtained with Ca^{2+}-incubated SR. The duration of the incubations was 30–180 min with no observable differences in the titration curves. No attempt was made to establish a minimum time required for the transition between the cooperative and noncooperative forms.

ATPase Activities and Ca^{2+} Uptake. The effect of the EGTA incubation on ATPase activity was investigated by comparing EGTA-incubated SR to Ca^{2+}-incubated SR. A small decrease (about 12%) was observed for the Ca^{2+}-activated ATPase activity of the EGTA-incubated SR vesicles in the presence of the Ca^{2+} ionophore A23187 (Table II). No significant loss of Ca^{2+}-activated ATPase activity was observed in the presence of oxalate, nor was there any observable difference in the "basic" (no Ca^{2+}) ATPase activities of the two preparations. Ca^{2+} uptake was measured for conditions identical with those used to obtain the ATPase activities in the presence of oxalate. The ratio of Ca^{2+} removed from the solution to ATP hydrolyzed was unchanged. These results (Table II) all indicate that the incubation in EGTA does not impair the Ca^{2+} pumping or Ca^{2+}-activated ATPase activity of the vesicles.

Discussion

The values for the apparent association constant (6.3×10^6 M^{-1}) and Hill coefficient (2.6) for Ca^{2+} binding to high-affinity sites on SR vesicles obtained when Ca^{2+}-saturated ATPase is titrated with EGTA are in reasonable agreement with published values, indicating that the measurements are reliable. In the present and cited cases demonstrating cooperative binding (Inesi et al., 1980; Verjovski-Almeida & Silva, 1981), Ca^{2+}-containing SR solutions had various amounts of EGTA added to obtain the desired free [Ca^{2+}] for a titration curve, and strong cooperativity was observed for about two Ca^{2+} ions.

It is clear from Figure 2 and Table I that incubating SR vesicles in solutions with very low [Ca^{2+}] leads to a reduction in Ca^{2+} affinity and a loss of cooperativity, while retaining the full fluorescence response to Ca^{2+} binding. The reduction and loss are completely reversible, depending only on free [Ca^{2+}]. Thus, this is a convenient way to uncouple the two Ca^{2+} binding sites and investigate noncooperative binding under controlled conditions. It seems reasonable to expect that there are other ways to induce the ATPase to bind Ca^{2+} noncooperatively, which may explain the mixed reports of cooperative and noncooperative binding.

The unimpaired ATPase activity and Ca^{2+} uptake indicate either that incubation of the SR in EGTA has no effect on the enzymatic properties of the Ca^{2+} pump or that any effects are rapidly reversed under the high [Ca^{2+}] and [ATP] employed in the assays. Distinguishing between the two possibilities is difficult because Ca^{2+}-activated ATPase activity measurements require [Ca^{2+}] high enough to saturate the Ca^{2+} binding sites. It would be surprising if the cooperative Ca^{2+} binding were unrelated to the ratio of Ca^{2+} taken up per ATP hydrolyzed, since the [Ca^{2+}] dependence of the ATPase activity indicates a cooperative effect of two Ca^{2+} (deMeis & Hasselbach, 1971). In either case, it is clear that for the low concentrations used to titrate the Ca^{2+}-free vesicles the binding is noncooperative and is adequately described by the upper line in the scheme in eq 1. This result indicates that the reactions $E \rightarrow E'$, $ECa \rightarrow E'Ca$, and $ECa_2 \rightarrow E'Ca_2$ are not occurring or do not proceed rapidly enough to affect the Ca^{2+} binding during a titration under the conditions used here. It is the slowness of these transitions from the noncooperative to the cooperative binding form that gives the curves in Figure 2 their hysteresis-like appearance.

These results may be related to a study by Dupont & Leigh (1978) on the rates of Ca^{2+} association and dissociation with SR ATPase. They mixed EGTA-incubated SR with Ca^{2+} and Ca^{2+}-incubated SR with EGTA to obtain k_{on} and k_{off}, using fluorescence to detect the changes. They interpreted the biphasic increase of fluorescence intensity with time from k_{on} experiments as evidence for fast binding followed by a subsequent slower conformational change. A possibly related, but different, effect of low [Ca^{2+}] was recently reported for SR vesicles incubated in 5 mM EGTA with no added $MgCl_2$ (McIntosh & Berman, 1978; Diamond et al., 1980). In that case, the ATPase activity and Ca^{2+} uptake were irreversibly lost. Addition of a Ca^{2+} ionophore to the incubation mixture led to the irreversible loss of a very high affinity Ca^{2+} binding site. In the present study, only high-affinity ($\sim 10^6$ M^{-1}) sites are modified, and the effect is completely reversible. This may be due to the high added [$MgCl_2$] or the absence of any Ca^{2+}

ionophore in the incubation mixture.

The main point of this report is that SR vesicles can exist in forms that bind Ca^{2+} either cooperatively or noncooperatively. In addition, if the scheme in eq 1 is taken as a model for both kinds of binding, the values for K_{app} in Table I can be used to calculate or estimate values for some of the K's in the scheme. The noncooperative binding case is straightforward, and the top line in eq 1 is adequate. Since n_H is close to 1, $K_{app} = K_1 = 5 \times 10^5\ M^{-1}$. This value is about 4-fold lower than the values reported earlier for noncooperative binding (Meissner, 1973).

Analysis of the cooperative binding case is not as simple. Equation 1 is based in part on the assumption that there are two identical Ca^{2+} binding sites. Two points need to be made regarding this assumption. First, it is possible that the K_{app} and n_H values for cooperative binding in Table I, which appear somewhat larger than the literature values, are in fact larger. If n_H is larger than 2, eq 1 is not applicable, but the experimental error in the measurements is too large at this time to require $n_H > 2$. Second, there is some evidence that detergent-solubilized SR ATPase *monomer* binds two Ca^{2+} cooperatively (Verjovski-Almeida & Silva, 1981) and has its ATPase activity activated by Ca^{2+} cooperatively (Moller et al., 1980; Murphy et al., 1982). It seems unlikely that two sites on a monomer would be identical. However, neither point demands the use of a scheme more complicated than eq 1.

The scheme can be somewhat simplified by recalling that K_{2c} is large and the rate of the reaction $ECa_2 \rightarrow E'Ca_2$ is slow, so the reaction $E'Ca_2 \rightarrow ECa_2$ must be very slow. Thus, the equilibrium described by K_{2c} can be ignored in analyzing the cooperative titration data. Further simplification requires assuming the reactions described by either K_{0c} or K_{1c} can be ignored. In the former case, eq 2 is obtained. Then, $K_1K_{1c}K_2 = 4.0 \times 10^{13}\ M^{-2} = K_{app}^2$ for the cooperative case, and using the value for K_1 from above, one calculates $K_{1c}K_2 = 8 \times 10^7\ M^{-1}$ as the effective association constant for the second Ca^{2+} bound, which is near that obtained by Inesi et al. (1980), who also used eq 2.

If the alternative assumption is made (e.g., K_{1c} is ignored), the scheme in eq 3 is obtained. Now, $K_{0c}K_2^2 = 4.0 \times 10^{13}\ M^{-2} = K_{app}^2$ for the cooperative case. Since the scheme is now a simple loop, it is also true that $K_{0c}K_2^2 = K_{2c}K_1^2$, and one can calculate $K_{2c} = 159$. An upper limit can be set on K_{0c}: since n_H is close to 1 for the noncooperative titration, $K_{0c} \leq 0.1$. If it were larger, the bottom line in eq 1 would contribute significantly. Thus, a lower limit can be set for the intrinsic association constant for the second Ca^{2+}, $K_2 \geq 2 \times 10^6\ M^{-1}$. In this scheme, the ratio of the effective association constants, $K_2/(K_{0c}K_2)$, is >10. At present, a rational choice between the models in eq 2 and 3 cannot be made.

The observation that SR CaATPase is hysteretic seems to be the first made for a membrane-bound enzyme. As discussed by Frieden (1979), enzymes that possess this property of "memory" are usually regulatory systems. Neet & Ainslie (1980) and Frieden (1979) pointed out two physiological uses of this property. One is creating or enhancing cooperativity for the enzyme reaction, and the other is damping an oscillatory mechanism, so that it matches the reaction it controls. One can speculate that a lag time between Ca^{2+} release and its cooperative binding and uptake by SR would ensure that the cytoplasmic $[Ca^{2+}]$ became high enough to activate contraction. For such a mechanism to be applicable, the in vivo rate of interconversion would have to be much faster than the apparent in vitro rate. In any case, instantaneous triggering of Ca^{2+} uptake when $[Ca^{2+}]$ reaches $\sim 10^{-6}$ M near the SR does not seem desirable.

Acknowledgments

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