Luminal Calcium Regulates Calcium Release in Triads Isolated from Frog and Rabbit Skeletal Muscle

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ABSTRACT Triads isolated from frog and rabbit skeletal muscle were equilibrated with different external [Ca^{2+}], ranging from 0.025 to 10 mM. Vesicular calcium increased with external [Ca^{2+}] as the sum of a linear plus a saturable component; the latter, which vanished after calsequestrin removal, displayed B_{max} values of 182 and 132 nmol of calcium/mg of protein, with K_d values of 1.21 and 1.14 mM in frog and rabbit vesicles, respectively. The effect of luminal [Ca^{2+}] on release kinetics in triads from frog and rabbit skeletal muscle was investigated, triggering release with 2 mM ATP, pCa 5, pH 6.8. In triads from frog, release rate constant (k) values increased sixfold after increasing luminal [Ca^{2+}] from 0.025 to 3 mM. In triads from rabbit, k values increased 20-fold when luminal [Ca^{2+}] increased from 0.05 to 0.7 mM. In both preparations, k values remained relatively constant (10–12 s^{-1}) at higher luminal [Ca^{2+}], with a small decrease at 10 mM. Initial release rates increased with luminal [Ca^{2+}] in both preparations; in triads from rabbit the increase was hyperbolic, and in triads from frogs the increase was sigmoidal. These results indicate that, although triads from frog and rabbit respond differently, in both preparations luminal [Ca^{2+}] has a distinctive effect on release, presumably by regulating sarcoplasmic reticulum calcium channels.

INTRODUCTION

Calcium release from sarcoplasmic reticulum (SR) in skeletal muscle is mediated by the ryanodine receptor-calcium release channels that have been identified with the foot proteins located in the SR terminal cisternae regions (Fleischer and Inui, 1989). Calcium release has been extensively investigated in vitro, measuring calcium fluxes from SR vesicles isolated mostly from mammalian (rabbit) skeletal muscle. These studies have shown that calcium release is activated by micromolar [Ca^{2+}] and by mM [ATP] and caffeine, and is inhibited by mM [Mg^{2+}], acidic pH, and micromolar ruthenium red (Smith et al., 1985, 1986; Meissner, 1984; Meissner et al., 1986; Sumbilla and Inesi, 1987; Moutin and Dupont, 1988; Ikemoto et al., 1989). These effects are all exerted from the cytoplasmic side of the channel.

There are a limited number of studies, all of them carried out in mammalian muscle, describing regulation of calcium release and of calcium channel opening probability (P_o) by luminal calcium. In SR vesicles isolated from rabbit skeletal muscle, a study of calcium release kinetics describes a regulatory effect of intraluminal [Ca^{2+}] on release rate constants, after triggering release with caffeine (Ikemoto et al., 1989). Likewise, SR vesicles isolated from pig skeletal muscle require a threshold luminal [Ca^{2+}] for calcium-induced calcium release (Nelson and Nelson, 1990), suggesting the presence of luminal regulatory sites.

Studies of the effect of luminal (*trans*) calcium on SR calcium channels incorporated in bilayers have produced contradictory results. It was reported that increasing *trans* calcium from the μM to the mM range decreased P_o in channels from rabbit skeletal muscle and produced a permanent closure of the channels present in pig skeletal muscle SR (Ma et al., 1988; Fill et al., 1990). Contrary to these findings, a 60% increase in P_o on increasing *trans* calcium from 50 μM to 10 mM was recently described for the purified ryanodine receptors from rabbit reconstituted in liposomes and fused with planar lipid bilayers (Tripathy and Meissner, 1994). The effects of luminal calcium on P_o may be due to calcium binding to luminal regulatory sites of the channel protein and may depend also on the mechanism of channel activation, as indicated by studies with the calcium release channels of rabbit skeletal (Herrmann-Frank, 1993) and sheep cardiac SR (Sitsapesan and Williams, 1994).

In amphibian skeletal muscle, however, there are no reports regarding the effect of luminal calcium on calcium channel behavior or calcium release. The two ryanodine receptor isoforms present in amphibian skeletal muscle (Olivares et al., 1991; Oyamada et al., 1994) respond differently to changes in *cis* [Ca^{2+}] (Murayama and Ogawa, 1992). Whether the presence of two isoforms also affects regulation by luminal [Ca^{2+}] of calcium release in SR isolated from frog skeletal muscle is not known. Thus, to analyze eventual differences in SR from frog, we studied the role of luminal calcium on calcium release kinetics in triads isolated from amphibian skeletal muscle, and we compared this preparation with triads isolated from mammalian skeletal muscle. We found that changes in luminal [Ca^{2+}] had distinct effects on the rate constants of calcium release and on the initial rates of release in both triad preparations. Although in both systems the release rate constants increased markedly in response to changes in luminal calcium concentration, the

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Abbreviations used: EGTA, ethyleneglycolbis(aminoethylether)tetraacetic acid; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; MOPS, (3-[*N*-morpholino]-propane sulfonic acid; SR, sarcoplasmic reticulum.

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changes were more striking in triads from rabbit than in triads from frog. The initial rates of release increased with luminal calcium in a hyperbolic fashion in triads from rabbit, and in sigmoidal fashion in triads from frog, suggesting intrinsic differences between both species. A partial preliminary report of some of these results has been presented elsewhere (Prieto et al., 1994).

MATERIALS AND METHODS

Preparation of triads

Triads were isolated from frog (*Caudiverbera caudiverbera*) and from rabbit skeletal muscle in the presence of a combination of protease inhibitors, following a procedure described in detail in previous work (Hidalgo et al., 1993). Triads were stored at −80°C for up to 1 month.

To remove calsequestrin, triads were incubated (0.5 mg protein/ml) in 0.15 M sucrose, 1 mM EGTA, 20 mM Tris/HCl, pH 8.0, for 30 min at 20°C. Vesicles were overlaid on a two layer discontinuous sucrose gradient, and were sedimented at 100,000 × *g*; triads depleted of calsequestrin were collected as a band from the 35–60% sucrose interface.

Calcium loading at equilibrium

Triads (0.6 mg of protein/ml) were incubated in loading solutions containing 0.1 M KCl, 40 mM MOPS, pH 6.8, and different concentrations of $CaCl_2$ plus $^{45}CaCl_2$ (5–15 mCi/mmol). After equilibration for 2 h at 18–20°C, 0.05 ml of the vesicle containing solution were diluted in 1 ml of the same loading solution without radioactive calcium, and were filtered through Millipore filters (AA, 0.8 μm). The filters were washed with 5 ml of nonradioactive loading solution, and their radioactivity was determined in a liquid scintillation counter. Nonspecific calcium binding was determined in the presence of 0.3 μg/ml ionophore A23187.

Calcium release studies

The release of the accumulated calcium was induced at 22°C with a solution containing 2 mM ATP, pCa 5, pH 6.8, in a fast filtration system (Biologic) as described elsewhere (Moutin and Dupont, 1988; Donoso and Hidalgo, 1993).

Determination of the intravesicular volume

Triads (rabbit) were diluted to 8 mg/ml in a solution containing 0.12 M sucrose, 10 mM histidine, pH 6.8, and either 2 μCi of [^{3}H]inulin or 5 μCi of [^{14}C]glycerol in a total volume of 180 μl. Vesicles were incubated for 30 min at 20°C and were sedimented at 100,000 × *g* in a Beckman airfuge. The supernatant was carefully removed, and the amount of radioactivity associated with the pellet was determined in a liquid scintillation counter. The intravesicular volume was calculated as the difference between the distribution volumes of [^{14}C]glycerol (total volume) and [^{3}H]inulin (extravesicular volume).

Other procedures

Gel electrophoresis was performed as described by Laemmli (1970). Protein was determined according to Hartree (1972) using bovine serum albumin as standard. Free [Ca^{2+}] was calculated with a computer program (Goldstein, 1979) using the binding constants for EGTA, HEDTA, and ATP from Martell and Smith (1974). However, to ensure accuracy free [Ca^{2+}] was always measured with a calcium electrode.

Materials

All reagents used were of analytical grade. Protease inhibitors (Leupeptin, Pepstatin A, benzamidine and phenylmethylsulfonyl fluoride) were obtained from Sigma Chemical Co. (St. Louis, MO); $^{45}CaCl_2$ and [^{14}C]glycerol were obtained from DuPont-New England Nuclear Corp. (Wilmington, DE), and [^{3}H]inulin from Amersham Corp. (LaJolla, CA).

RESULTS

Calcium equilibration in the triads

The amount of calcium taken up at equilibrium by triads isolated from frog skeletal muscle, measured as a function of extravesicular [Ca^{2+}], exhibited two components (Fig. 1 *A*): a saturable component that displayed a B_{max} value of 182

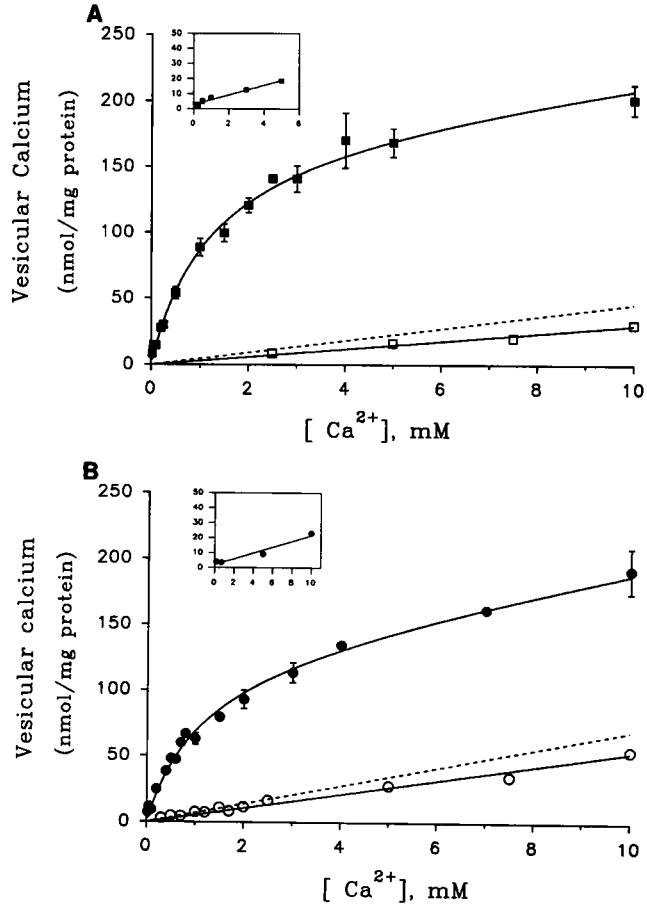

FIGURE 1 Calcium taken up at equilibrium by triads isolated from frog (*A*) and rabbit (*B*) skeletal muscle. Triads were incubated in varying external [Ca^{2+}], and the amount of calcium taken up at equilibrium by the vesicles was determined as described in Materials and Methods. Filled symbols represent the Mean ± SE of three to six determinations. Open symbols represent the amount of calcium taken up by the vesicles after treatment with ionophore A23187, which corresponds to nonspecific binding. The dashed line depicts the amount of nonspecifically bound calcium plus the calcium that is free in the intravesicular lumen. The best fit to the experimental points (——) was given by the functions: vesicular calcium (nmoles/mg) = $(182c/(1.21 + c)) + 4.5c$ (frog) and vesicular calcium (nmoles/mg) = $(132c/(1.14 + c)) + 6.9c$ (rabbit), where c is the external calcium concentration in mM. The insets to *A* and *B* show vesicular calcium as a function of external calcium concentration in triads devoid of calsequestrin.

nmol/mg of protein with a $K_{0.5}$ of 1.21 mM (Table 1), and a linear component that was adjusted as described below. Triads from rabbit featured a similar behavior (Fig. 1 *B*), with a linear plus a saturable component with a B_{max} value of 132 nmol/mg protein and a $K_{0.5}$ of 1.14 mM (Table 1).

Origin of the saturable component

As evidenced by their gel electrophoretic pattern (Fig. 2), both triad preparations displayed similar calsequestrin contents, corresponding to 22–26% of the total protein, as indicated by the respective scans of their SDS-gels. To investigate whether the saturable component found in both triad preparations corresponds to intravesicular calcium binding to calsequestrin, the only luminal triad protein that has a large calcium binding capacity, we extracted calsequestrin from the triads and measured calcium accumulation. After incubation with EGTA, pH 8.0, both triad preparations released most of their calsequestrin (Fig. 2) and lost their saturable calcium binding component (Fig. 1, *A* and *B*, *insets*). Thus, we conclude that calsequestrin is the primary source of luminal calcium binding.

Origin of the linear component

The linear, nonsaturable binding component, on the other hand, should be given by the sum of the intravesicular free [Ca^{2+}] plus the calcium nonspecifically bound to internal and external vesicular sites. The intravesicular volume of triads (see Materials and Methods) was 1.6 μl/mg protein. This value, which is in the range reported in the literature for isolated SR vesicles (Duggan and Martonosi, 1970), should originate a linear component for free intravesicular calcium with a slope of 1.6 nmol/mg protein/mM [Ca^{2+}].

To determine nonspecific binding, we measured calcium bound at equilibrium after adding ionophore A23187 during the entire equilibration period. We found that calcium binding to vesicles treated with A23187 followed a linear function, with a slope of 2.9 nmol/mg protein/mM [Ca^{2+}] in triads from frog (Fig. 1 *A*, *open squares*) and a slope of 5.3 nmol/mg prot/mM [Ca^{2+}] in triads from rabbit (Fig. 1 *B*, *open circles*). To calculate the total slope of the linear component, we added to these values the value of the slope of the free intravesicular component (1.6 nmol/mg protein/mM [Ca^{2+}]). The calculated values for the combined slope of the linear component were 4.5 and 6.9 nmol/mg protein/mM [Ca^{2+}] for triads from frog and rabbit, respectively. These values were used in the nonlinear fits used to generate the curves shown in Fig. 1, *A* and *B*, which produced the respective saturable components.

TABLE 1 Calcium binding properties

	Calsequestrin		Saturable component*	
	K_d (mM)	B_{max} (nmol/mg)	$K_{0.5}$ (mM)	B_{max} (nmol/mg)
Rabbit	1.16‡	830§	1.14 ± 0.10	132 ± 4
Frog	0.905¶	1200¶	1.21 ± 0.13	182 ± 6

*Data taken from Fig. 1. Values are given ± SE of the best non-linear fit of the experimental points to the equation described in the legend to Fig. 1.
‡Ikemoto et al. (1974).
§Damiani et al. (1986).
¶Volpe and Simon (1991).

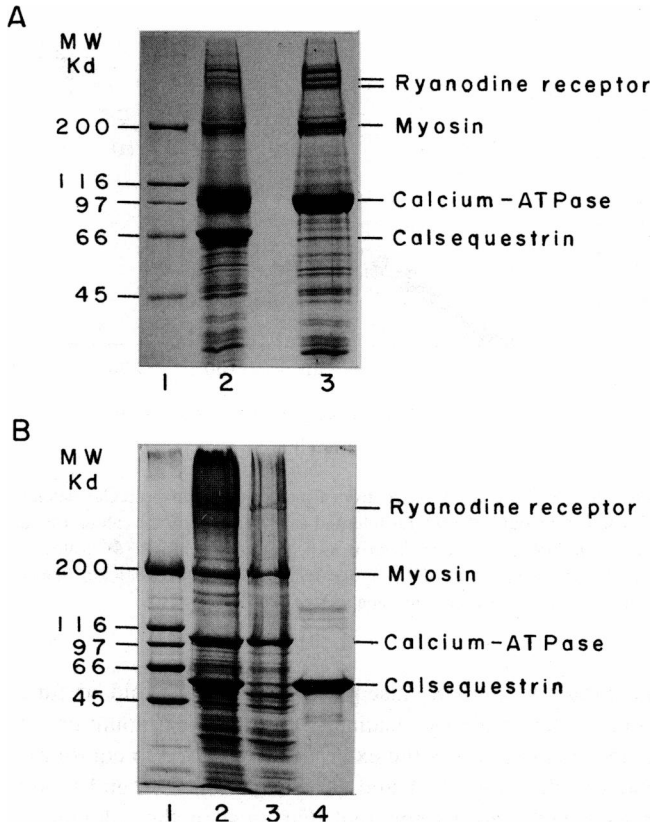

FIGURE 2 SDS-polyacrylamide electrophoresis of triads isolated from frog (*A*) and rabbit (*B*) skeletal muscle. Linear polyacrylamide gradient gels were stained with Coomassie blue. After scanning the gels, a calsequestrin content (%) of 26.4 ± 1.5 (3) in triads from frog, and of 22.2 ± 4.1 (3) in triads from rabbit, was obtained (Mean ± SD). (*A*) Lane 1, molecular weight standards; lane 2, triads from frog; lane 3, the same triads after incubation in 1 mM EGTA, pH 8.0. (*B*) Lane 1, molecular weight standards; lane 2, triads from rabbit; lane 3, the same triads after incubation with 1 mM EGTA, pH 8.0; lane 4, calsequestrin, extracted from rabbit skeletal muscle and purified by chromatography on phenyl-sepharose columns as described previously (Cala and Jones, 1983).

Calcium release as a function of vesicular calcium

Calcium release was induced with 2 mM ATP at pCa 5, pH 6.8, 22°C. As shown previously, in these conditions triad vesicles equilibrated in 3 mM $CaCl_2$ release 70% of the accumulated calcium, with rate constants of 10–14 s^{-1} (Donoso and Hidalgo, 1993).

Vesicles passively equilibrated with varying external [Ca^{2+}] released the same constant proportion (70%) of the total calcium taken up regardless of the amount of calcium equilibrated inside the vesicles (Fig. 3). This finding suggests that a fraction (30%) of the vesicles present in both triad

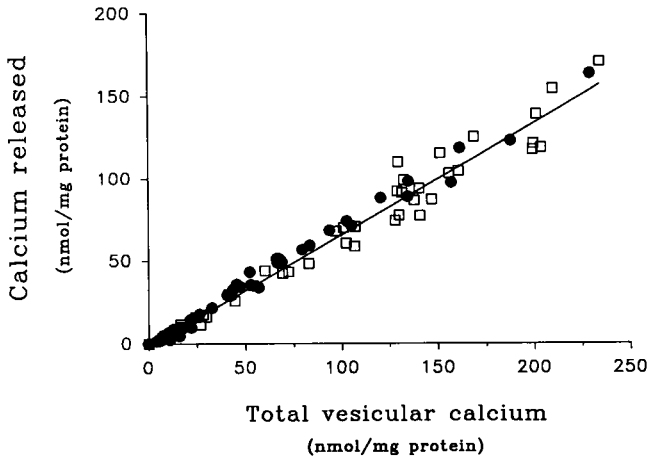

FIGURE 3 Calcium released from triads at different vesicular calcium loads. The amount of calcium released as a function of the calcium taken up at equilibrium in triads from frog (□) and from rabbit (●) follows a straight line, with a slope of 0.7 nmol calcium released/nmol calcium loaded, according to linear regression analysis of the data.

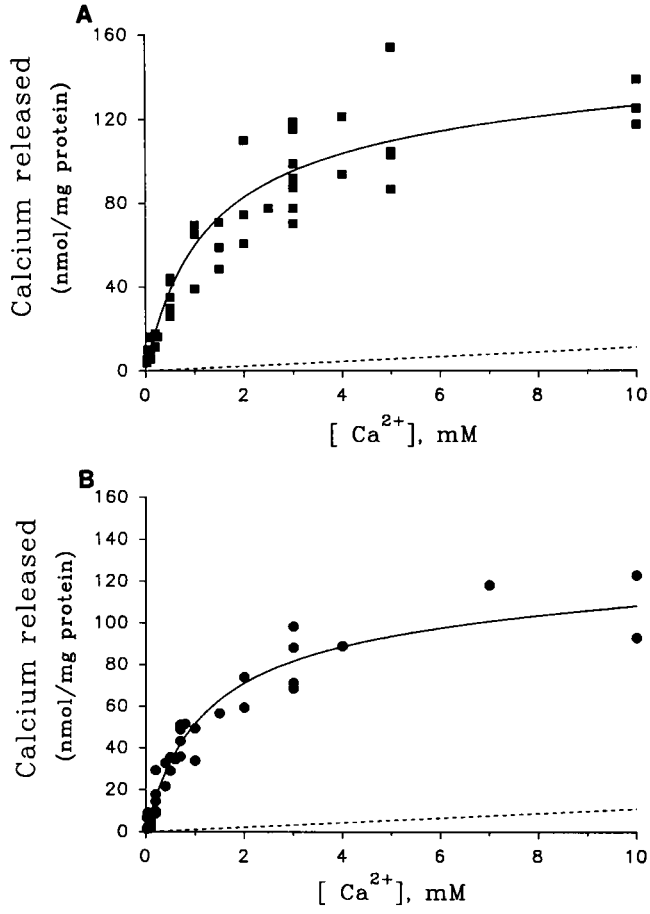

FIGURE 4 Calcium released as a function of the calcium concentration in the incubation solution. (*A*) Triads from frog; (*B*) triads from rabbit. The solid lines correspond to the expression $f(c) = B_{max}c/(K_{0.5} + c) + 1.1c$, which was obtained assuming that 70% of the total luminal $[Ca^{2+}]$ (Fig. 1) was released. Values for frog were B_{max} = 127.4 nmol/mg; $K_{0.5}$ = 1.21 mM. Values for rabbit were B_{max} = 92.4 nmol/mg protein; $K_{0.5}$ = 1.14 mM.

preparations contain calsequestrin but are devoid of functional calcium release channels. Plotting the amount of calcium released versus the external $[Ca^{2+}]$ used to equilibrate the vesicles (Fig. 4, *A* and *B*, triads from frog and rabbit, respectively) makes apparent that most of the calcium released originated from the saturable component (*solid lines*), because the amount of free intravesicular calcium (*dashed lines*) even at 10 mM $[Ca^{2+}]$ represented a small fraction (<10%) of the total calcium released. Because we have presented evidence showing that the saturable component originates from calcium binding to calsequestrin, these results indicate that before its release from the vesicular lumen calcium must first dissociate from calsequestrin.

Effect of luminal $[Ca^{2+}]$ on the rate constants of calcium release

There is no information concerning the effect of luminal calcium on calcium release in amphibian skeletal muscle. Hence, we measured ATP-induced calcium release in triads isolated from frog skeletal muscle having varying luminal $[Ca^{2+}]$. Release kinetics obtained with frog membranes were then compared under the same experimental conditions with those obtained with rabbit membranes. Release rate constants were calculated from the exponential decay curves of accumulated calcium with time, as illustrated in Fig. 5 for vesicles equilibrated with 1 mM $[Ca^{2+}]$.

Changes in luminal $[Ca^{2+}]$ had marked effects on release rate constants in both triad preparations. In triads from frog, rate constant values increased from a value of $k = 1.45 \pm 0.16\ s^{-1}$ in vesicles equilibrated with 0.025 mM $[Ca^{2+}]$, the lowest concentration used, to a value of $k = 9.1 \pm 0.65\ s^{-1}$ in vesicles equilibrated in 3 mM $[Ca^{2+}]$ (Fig. 6 *A*). However, it is noteworthy that increasing $[Ca^{2+}]$ 10-fold, from 0.025 to 0.25 mM produced almost no change in k. Further increasing luminal $[Ca^{2+}]$ resulted in rate constant values ranging up to $11.7 \pm 1.65\ s^{-1}$ at 5 mM $[Ca^{2+}]$; lower values were observed at 10 mM $[Ca^{2+}]$, with $k = 6.4 \pm 0.75\ s^{-1}$.

The effect of luminal $[Ca^{2+}]$ on the rate constants of calcium release was even more remarkable in triads from rabbit (Fig. 6 *B*); a 20-fold increase after a 14-fold increase in luminal $[Ca^{2+}]$ was observed, from a value of $k = 0.55 \pm 0.28\ s^{-1}$ at 0.05 mM $[Ca^{2+}]$, to a value of $k = 11.8 \pm 1.35\ s^{-1}$ at 0.7 mM $[Ca^{2+}]$. In comparison, rate constants in frog membranes increased only sixfold in response to a 120-fold increase in luminal $[Ca^{2+}]$. Further raising the luminal $[Ca^{2+}]$ resulted in rate constants of release with values in the range of 8–10 s^{-1}.

The lines in Fig. 6, *A* and *B* were generated by the best fit to the experimental points given by the theoretical equations relating rate constants with luminal $[Ca^{2+}]$ presented in detail in Discussion.

It may be argued that the decrease in rate constants observed in vesicles containing μM luminal $[Ca^{2+}]$ was caused by the diminished chemical gradient for calcium present in these conditions, although formally rate constants are independent of the existing chemical gradients. To investigate

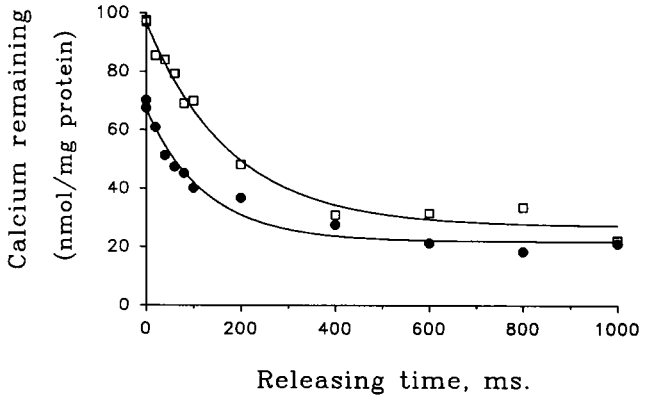

FIGURE 5 ATP-induced calcium release in triads. Vesicles were equilibrated with 1 mM [Ca^{2+}], and release was induced with 2 mM ATP as stated under Materials and Methods. Calcium remaining in the vesicles (N) decayed as a single exponential plus an offset such that $N(t) = N(0)\exp(-kt) + B$. Values obtained in the frog (□) were $N(0)$ = 70 nmol calcium/mg protein; k = 5.7 s^{-1}; B = 26.7 nmol calcium/mg protein, and in the rabbit (●) $N(0)$ = 45.3 nmol calcium/mg protein; k = 8.1 s^{-1}; B = 21.8 nmol calcium/mg protein.

this possibility, we carried out experiments in triad vesicles from frog equilibrated at 0.1 mM luminal [Ca^{2+}], using releasing solutions containing 2 mM ATP, pH 6.8, and either pCa 5 (ratio $[Ca^{2+}]_i/[Ca^{2+}]_e$ = 10) or pCa 7 (ratio $[Ca^{2+}]_i/[Ca^{2+}]_e$ = 1000). We chose triads from frog to carry out these experiments because we have previously shown that in this preparation raising external calcium from pCa 7 to pCa 5 did not affect the rate constants of ATP-induced calcium release (Donoso and Hidalgo, 1993). As shown in Table 2, despite the very different chemical gradients present, the rate constants had the same values at pCa 5 and at pCa 7. These results rule out effects of [Ca^{2+}] gradients in the observed changes in rate constants.

Effect of luminal [Ca^{2+}] on the initial rates of calcium release

Vesicular calcium (N) decreased with time following the equation $N(t) = N(0)\exp(-kt)$. From this expression, the initial rate of calcium release can be calculated as

$$v_i = (dN/dt)_{t\to 0} = -kN(0).$$

The analysis of initial rates as a function of luminal calcium showed that in both systems initial rates increased with increasing luminal [Ca^{2+}], and saturated in the mM [Ca^{2+}] range (Fig. 7). However, although the data in triads from rabbit were well fitted to a simple hyperbolic function, the initial rates of release in triads from frog increased with luminal [Ca^{2+}] following a sigmoidal function, with a Hill coefficient of 1.8.

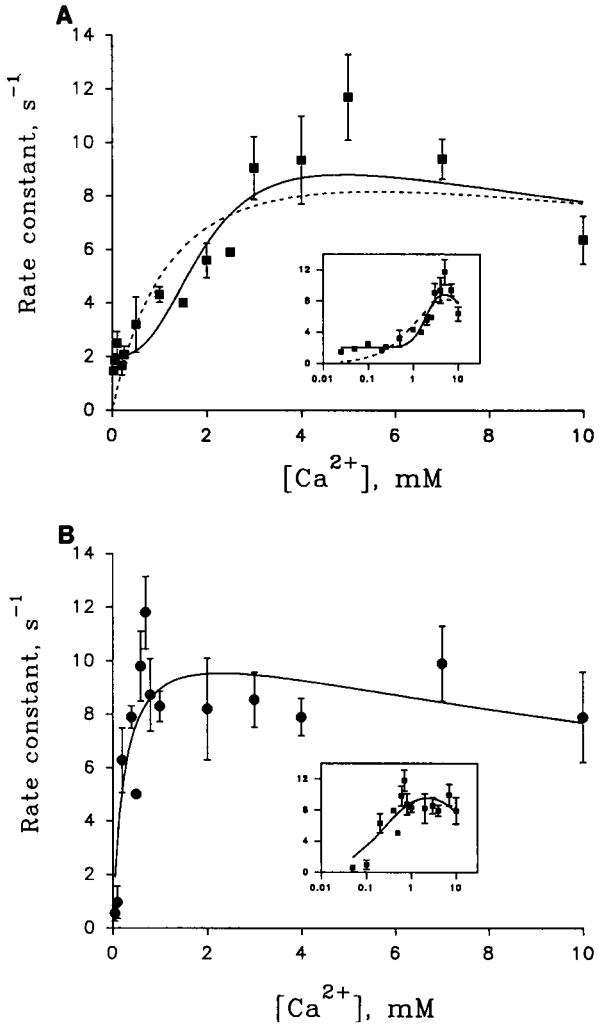

FIGURE 6 Rate constants of calcium release as a function of the concentration of calcium present in the incubation solution used to equilibrate the vesicles. (A) Triads from frog; (B) triads from rabbit. Each point represents the Mean ± SE of two to six independent determinations. The dashed line in A and the solid line in B were fitted to the equation (see text):

$$k = [AN\{\gamma_{max}c/(K_m + c)\}\{P_{o_{max}}c/(K_o + c)\}]/c$$

where A = $3RT/4\pi z^2F^2r^3$, r is the vesicular radius, N is the number of channels per vesicle, γ_{max} is the maximal value of conductance (100 pS) for a channel with a K_m = 20 mM; $P_{o_{max}}$, maximal value for channel open probability, and c, luminal calcium concentration. In triads from frog, the best fit gave $AN\gamma_{max}P_{o_{max}} = 2.69 \times 10^2$ mM s^{-1} and K_o = 1.6 mM. In triads from rabbit, the best fit gave the numerical values for $AN\gamma_{max}P_{o_{max}} = 2.37 \times 10^2$ mM s^{-1} and K_o = 0.26 mM. The solid line in A was fitted to the equation (see text):

$$k = A'c^n/[(K_m + c)(K_o + c^n)] + B/(K_m + c) \quad (2)$$

where $A' = AN_1\gamma_{max}(P_{o_{max}})$ and $B = AN_2\gamma_{max}(P_{o_{max}})_2$. N_1 and N_2 stand for the number of channel isoforms per vesicle, each with its own $P_{o_{max}}$ value. Using the value of K_m = 20 mM and γ_{max} = 100 pS, the best fit gave the following values for the parameters: A' = 196 mM s^{-1}, n = 2.6, K_o = 6.6 mM, and B = 40.7 mM s^{-1}.

DISCUSSION

Calcium equilibration results

Calsequestrin is the main intrareticular calcium-binding protein of skeletal muscle SR. Because of its high calcium-binding capacity and its intermediate affinity, calsequestrin has been proposed to act as an intrareticular calcium buffer (Fleischer and Inui, 1989).

TABLE 2 Effect of extravesicular and luminal [Ca^{2+}] on the rate constants (k) of calcium release in triads isolated from frog skeletal muscle

	Luminal [Ca^{2+}]	
	0.1 mM	3 mM
Releasing solution*	k (s^{-1})	k (s^{-1})
pCa 7	2.45 ± 0.66‡	9.9 ± 1.6 (5)
pCa 5	2.48 ± 0.24 (5)	9.1 ± 0.65 (4)

Values are given as Mean ± SE; the number of determinations is in parentheses.

*The releasing solution had in addition 2 mM ATP, 20 mM HEPES/Tris, pH 6.8.

‡The error represents the calculated error obtained from the nonlinear exponential fit to one set of experimental values.

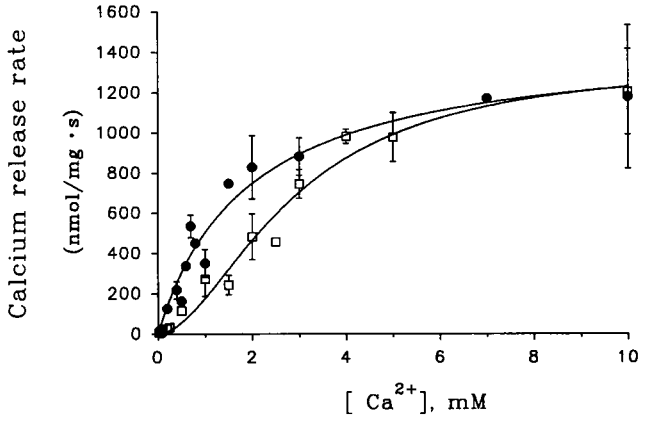

FIGURE 7 Initial rates of calcium release (v_i) as a function of the concentration of calcium present in the incubation solution used to equilibrate the vesicles. (□) triads from frog, (●) triads from rabbit. The best fit to the points (——) was given by: $v_i = 1360c^{1.8}/(6.7 + c^{1.8})$ for triads from frog and by: $v_i = 1458c/(1.9 + c)$ for triads from rabbit.

The results presented in this report, showing that triad vesicles depleted of calsequestrin lost the saturable component of calcium binding at equilibrium, indicate that this component stems from calcium binding to calsequestrin. The concordance between the $K_{0.5}$ values of calcium binding found in our experiments and the K_d values for calcium binding to calsequestrin isolated from frog (Volpe and Simon, 1991) and rabbit skeletal muscle (Ikemoto et al., 1974), all measured under similar ionic conditions (0.1 M KCl), further supports this conclusion (Table 1).

Both triad preparations displayed similar calsequestrin contents, 22–26% of the total protein. Thus, the higher B_{max} values for calcium-binding obtained in triads from frog presumably reflect the larger calcium binding capacity of calsequestrin from frog muscle (Table 1). Furthermore, from the respective B_{max} values of the saturable components, a calsequestrin content of 15–16% of the total protein can be calculated for both preparations. These values are somewhat lower than the contents given by the gel scans. It is possible that the B_{max} for calsequestrin inside the vesicles, where calsequestrin forms a network anchored to the terminal cisternae (Franzini-Armstrong et al., 1987), is somewhat lower than in solution.

The present results show that in the range of luminal [Ca^{2+}] investigated most of the calcium present in the vesicles must first dissociate from calsequestrin before being released. We have found that calcium appears to dissociate from calsequestrin in solution in the microsecond time range,[1] much faster than the millisecond time range of the calcium release responses. Furthermore, vesicles equilibrated with calcium in the presence of A23187 did not exhibit a saturable calcium-binding component (Fig. 1), indicating that calcium bound to calsequestrin dissociated during the filter washing period and was lost from the vesicles. It remains to be established whether in vivo most of the calcium present in the SR lumen is free (Volpe and Simon, 1991) or bound to calsequestrin, and whether calcium dissociation from calsequestrin in vivo is as fast as in solution.

Calcium release experiments

Several studies using different experimental approaches have indicated that luminal calcium regulates calcium release from skeletal muscle SR. However, contradictory results have been reported. In skinned fibers, a critical level of calcium load in SR is required to observe calcium-induced calcium release (Endo, 1977). In heavy SR vesicles isolated from rabbit skeletal muscle, fast release kinetic studies of caffeine-triggered calcium release indicate that rate constants change markedly on raising luminal [Ca^{2+}] (Ikemoto et al., 1989). Furthermore, heavy SR vesicles from pig skeletal muscle, actively loaded in the presence of ATP, release calcium only after reaching a threshold value of luminal [Ca^{2+}] (Nelson and Nelson, 1990), suggesting the presence of intraluminal regulatory sites for calcium release. An increase in channel opening when increasing luminal [Ca^{2+}] would be consistent with the above reports. Studies with single SR calcium channels incorporated in bilayers, however, have produced contradictory results, because inhibitory (Ma et al., 1988; Fill et al., 1990) and stimulatory effects (Tripathy and Meissner, 1994) of luminal calcium on P_o have been reported.

In this work we have found that luminal [Ca^{2+}] changed markedly the rate constants and the initial rates of ATP-induced calcium release in triads from frog and rabbit skeletal muscle. However, the two vesicular preparations showed

[1] After calcium-binding, calsequestrin experiences extensive conformational changes (Ikemoto et al., 1974; Ostwald et al., 1974) that cause changes in its intrinsic fluorescence, and undergoes aggregation (He et al., 1993). We have determined the time course of calcium association and dissociation from calsequestrin by measuring its intrinsic fluorescence in a stopped-flow system after mixing calcium-saturated calsequestrin with EGTA-containing solutions, or after mixing calcium-free calsequestrin with calcium-containing solutions. We found that the change in intrinsic fluorescence that occurs concomitantly with calcium dissociation or association to calsequestrin (Ikemoto et al., 1974) took place in less than 1 ms, the resolution time of the stopped-flow system (Prieto et al., 1994).

some significant differences in their responses. The rate constants in triads from rabbit increased 20-fold when luminal [Ca^{2+}] increased 14-fold, from 0.05 to 0.7 mM. In triads from frog, in contrast, k values increased only sixfold after increasing 120-fold luminal [Ca^{2+}], from 0.025 to 3 mM. Furthermore, in the lowest range of luminal [Ca^{2+}] studied, 0.025 to 0.25 mM, triads from frog exhibited almost no variations in rate constants, with values $\geq 1.5\ s^{-1}$ (see Fig. 6 *A*, *inset*), whereas triads from rabbit showed a steady increase in rate constants in this luminal [Ca^{2+}] range. Likewise, although initial release rates increased with luminal [Ca^{2+}] in both preparations, in triads from rabbit the increase was hyperbolic, and in triads from frog the increase was sigmoidal (Fig. 7).

These observations indicate that triads from frog and rabbit respond differently to changes in luminal [Ca^{2+}]. It is known that SR calcium release channels from frog display two types of calcium dependence to changes in *cis* calcium concentration (Bull and Marengo, 1993), and that the two calcium channel isoforms isolated from frog skeletal muscle SR and reconstituted in lipid bilayers behave differently to changes in *cis* [Ca^{2+}] (Murayama and Ogawa, 1992). Hence, it is conceivable that the two isoforms may also respond differently to changes in luminal [Ca^{2+}], as discussed below. Studies with the isolated isoforms are needed to test this point.

Data analysis of rate constant changes

To test whether the observed changes in rate constants as a function of luminal calcium can be ascribed solely to the properties of the calcium release channels present in the vesicles, we have applied the following theoretical treatment to our results. The relationship between the rate constant for the flux of an ion from a single vesicle and the properties of the channels that mediate the flux is given by the expression derived from constant field theory (Miller, 1984),

$$k = \frac{3NRT\gamma P_o}{4\pi z^2F^2r^3c} = \frac{AN\gamma P_o}{c}, \quad (1)$$

where $A = (3RT/4\pi z^2F^2r^3)$, R, T, z, and F have their usual meaning, r is the vesicular radius, N is the number of channels per vesicle, γ is the single channel conductance, P_o is the probability of the channel of being in its open state, and c is the luminal ion concentration.

Triads from rabbit

The conductance of most ion channels, and in particular of the calcium release channels from rabbit skeletal muscle (Smith et al., 1986), changes as a function of ion concentration according to the expression

$$\gamma = \frac{\gamma_{max}c}{K_m + c}. \quad (2)$$

Values of $K_m = 20$ mM (Meissner, 1984; Smith et al., 1986) and $\gamma_{max} = 100$ pS (Smith et al., 1986) have been reported for calcium. Replacing Eq. 2 in Eq. 1 originates the expression

$$k = \frac{AN\gamma_{max}P_o}{K_m + c}. \quad (3)$$

According to Eq. 3, k values should decrease with increasing luminal calcium if P_o decreased as reported (Ma et al., 1988; Fill et al., 1990). But the experimental findings of Ikemoto et al. (1989) and of this work indicate that k values increase markedly with luminal calcium. Thus, to explain this behavior of k with c according to equation [3], P_o should increase with luminal calcium, in agreement with the recent findings of Tripathy and Meissner (1994). We propose that P_o increases hyperbolically with luminal [Ca^{2+}], such that

$$P_o = \frac{P_{o_{max}}c}{K_o + c}. \quad (4)$$

This equation was selected because the experimental points were well fitted to the expression that results from replacing P_o as given by Eq. 4 into Eq. 3:

$$k = \frac{AN\gamma_{max}P_{o_{max}}c}{(K_o + c)(K_m + c)}. \quad (5)$$

For SR vesicles in triads with a vesicular radius $r = 100$ nm (Mitchell et al., 1983), at 20°C a value of $A = 1.56 \times 10^{13}$ mM s^{-1} S^{-1} was calculated.

Equation [5] can be expressed as

$$k = \frac{A'c}{(K_m + c)(K_o + c)}, \quad (6)$$

where $A' = AN\gamma_{max}P_{o_{max}}$. The nonlinear fit of the experimental points to Eq. 6 yielded the solid line drawn in Fig. 6 *B* for triads from rabbit and predicted the large initial increase and the subsequent decrease of k values with luminal [Ca^{2+}] observed in this triad preparation. Furthermore, the theoretical fit gave values of $A' = 2.37 \times 10^2$ mM s^{-1}, and of $K_o = 0.26$ mM. Because $A' = AN\gamma_{max}P_{o_{max}}$, substituting $A = 1.56 \times 10^{13}$ mM s^{-1} S^{-1} and $\gamma_{max} = 100$ pS, it follows that $NP_{o_{max}} = 0.15$. If $N = 1$, then $P_{o_{max}} = 0.15$, and if $N = 2$, $P_{o_{max}} = 0.075$. These are reasonable values for both parameters, justifying the theoretical approach followed.

Triads from frog

For the SR channels present in frog skeletal muscle, there are no published data for variations in either conductance or P_o with luminal [Ca^{2+}]. Assuming that the conductance changes as in SR channels from rabbit with the same value of $K_m = 20$ mM for calcium, the theoretical fit of the experimental points to Eq. 6 originated the curve shown in Fig. 6 *A* (*dashed line*), with the parameters $A' = 2.69 \times 10^2$ mM s^{-1}, and $K_o = 1.6$ mM. Because $A' = AN\gamma_{max}P_{o_{max}}$, replacing $A = 1.56 \times 10^{13}$ mM s^{-1} S^{-1} and $\gamma_{max} = 100$ pS (Suarez-Isla et al., 1988), it follows that $NP_{o_{max}} = 0.17$. If $N = 1$, then $P_{o_{max}} = 0.17$.

However, the best fit to the experimental k values in triads from frog was less adequate than in triads from rabbit, indicating once again that the two vesicular preparations behave differently. A modified version of Eq. 6, considering release through two independent channels, each with its own P_o vs. c function, one of them sigmoidal and the other independent of c (Eq. 7), and both of them with the same hyperbolic function for γ as that described above in Eq. 2 for the rabbit isoform, gave a better fit to the data (Fig. 6 *A*, *solid line*).

$$k = k_1 + k_2$$

$$k_1 = \frac{A'c^n}{(K_m + c)(K_o + c^n)} \quad \text{where} \quad A' = AN_1\gamma_{max}(P_{o_{max}})_1$$

$$k_2 = \frac{B}{K_m + c} \quad \text{where} \quad B = AN_2\gamma_{max}(P_{o_{max}})_2.$$

N_1 and N_2 stand for the number of channel isoforms per vesicle, each with its own $P_{o_{max}}$ value. The overall expression for k as a function of c is

$$k = \frac{A'c^n}{(K_m + c)(K_o + c^n)} + \frac{B}{K_m + c} \qquad (7)$$

The best fit for k_1, using the value of K_m = 20 mM and γ_{max} = 100 pS, gave the following values for the parameters: A' = 196 mM s^{-1}, n = 2.6 and K_o = 6.6 mM. From these values, the product $N_1P_{o_{max}}$ = 0.13.

The best fit for k_2, using again the value of K_m = 20 mM and γ_{max} = 100 pS, gave the value for the parameter B = 40.7 mM s^{-1}. From this value, the product $N_2(P_{o_{max}})_2$ = 0.03, indicating either that the hypothetical channels that originate k_2 are present at a very low density of channels per vesicle or have a low open probability.

Because there is insufficient experimental information on the channel properties of the two isolated isoforms of the ryanodine receptors of frog muscle, this approach is discussed here only as a possible predictive model of how luminal calcium may influence k values in SR from frog skeletal muscle.

From this theoretical analysis, we propose that the properties of the calcium release channels present in the vesicles can account solely for the observed experimental behavior of the rate constants. Whether luminal calcium controls directly channels properties or whether this effect is mediated by calsequestrin should be investigated further, because a role of calsequestrin in controlling calcium release in vesicles has been proposed (Ikemoto et al., 1989, 1991; Gilchrist et al., 1992), and it has been reported that SR calcium channel open probability increases after calsequestrin addition to the *trans* side in the presence of mM luminal [Ca^{2+}] (Kawasaki and Kasai, 1994).

CONCLUSIONS

The main finding of this work is that luminal [Ca^{2+}] controls calcium release kinetics in triads isolated from frog and rabbit skeletal muscle, albeit with some clear-cut differences. Using the theoretical relationship between the rate constant for the flux of an ion from a single vesicle and the properties of the channels that mediate the flux (Miller, 1984), we were able to generate curves that predicted the experimental changes of rate constants with luminal [Ca^{2+}]. If in the intact fibers release rate constants vary with luminal [Ca^{2+}] as they do in vesicles, models of calcium efflux from SR should take this into account if the physiological conditions are to be reproduced.

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REFERENCES

Bull, R., and J. J. Marengo. 1993. Sarcoplasmic reticulum release channels from frog skeletal muscle display two types of calcium dependence. *FEBS Lett.* 331:223–227.

Cala, S. E., and L. R. Jones. 1983. Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca^{2+}-dependent elution from phenyl-sepharose. *J. Biol. Chem.* 258: 11932–11936.

Damiani, E., S. Salvatori, F. Zorzato, and A. Margreth. 1986. Characteristics of skeletal muscle calsequestrin: comparison of mammalian, amphibian and avian muscles. *J. Muscle Res. Cell Motil.* 7:435–445.

Donoso, P., and C. Hidalgo. 1993. pH-Sensitive calcium release in triads from frog skeletal muscle. Rapid filtration studies. *J. Biol. Chem.* 268: 25432–25438.

Duggan, P. F., and A. Martonosi. 1970. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol* 56:147–167.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* 57:71–108.

Fill, M., R. Coronado, J. R. Mickelson, J. Vilven, J. Ma, B. A. Jacobson, and C. F. Louis. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 50:471–475.

Fleischer, S., and M. Inui. 1989. Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18:333–364.

Franzini-Armstrong, C., L. J. Kenney, and E. Varriano-Marston. 1987. The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study. *J. Cell Biol.* 105:49–56.

Gilchrist, J. S. C., A. N. Belcastro, and S. Katz. 1992. Intraluminal Ca^{2+} dependence of Ca^{2+} and ryanodine-mediated regulation of skeletal muscle sarcoplasmic reticulum Ca^{2+} release. *J. Biol. Chem.* 267: 20850–20856.

Goldstein, D. A. 1979. Calculation of the concentrations of free cations and cation-ligand complexes in solutions containing multiple divalent cations and ligands. *Biophys. J.* 26:235–242.

Hartree, E. F. 1972. Determination of proteins: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48: 422–427.

He, Z., A. K. Dunker, C. R., Wesson, and W. R. Trumble. 1993. Ca^{2+}-induced folding and aggregation of skeletal muscle sarcoplasmic reticulum calsequestrin. The involvement of trifluoperazine-binding sites. *J. Biol. Chem.* 268:24635–24641.

Herrmann-Frank, A. 1993. Regulation of the ryanodine receptor/Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by luminal calcium. *Biophys. J.* 64:152*a*. (Abstr.)

Hidalgo, C., J. Jorquera, V. Tapia, and P. Donoso. 1993. Triads and transverse tubules isolated from frog skeletal muscle contain high levels of inositol 1,4,5-trisphosphate. *J. Biol. Chem.* 268:15111–15117.

Ikemoto, N., B. Antoniu, J. J. Kang, L. G. Mészáros, and M. Ronjat. 1991. Intravesicular calcium transient during calcium release from sarcoplasmic reticulum. *Biochemistry.* 30:5230–5237.

Ikemoto, N., B. Nagy, G. M. Bhatnagar, and J. Gergely. 1974. Studies on a metal-binding protein of the sarcoplasmic reticulum. *J. Biol. Chem.* 249:2357–2365.

Ikemoto, N., M. Ronjat, L. G. Mészáros, and M. Koshita. 1989. Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochemistry.* 28:6764–6771.

Kawasaki, T., and M. Kasai. 1994. Regulation of calcium channels in sarcoplasmic reticulum by calsequestrin. *Biochem. Biophys. Res. Commun.* 199:1120–1127.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.* 227:680–685.

Ma, J., M. Fill, C. M. Knudson, K. P. Campbell, and R. Coronado. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science.* 242:99–102.

Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants, Vol. 1. Plenum Press, New York.

Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. *J. Biol. Chem.* 259:2365–2374.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Miller, C. 1984. Ion channels in liposomes. *Ann. Rev. Physiol.* 46:549–558.

Mitchell, R. D., A. Saito, P. Palade, and S. Fleischer. 1983. Morphology of isolated triads. *J. Cell Biol.* 96:1017–1029.

Moutin, M. J., and Y. Dupont. 1988. Rapid filtration studies of Ca^{2+}-induced Ca^{2+} release from skeletal sarcoplasmic reticulum. *J. Biol. Chem.* 263: 4228–423.

Murayama, T., and Y. Ogawa. 1992. Purification and characterization of two ryanodine-binding protein isoforms from sarcoplasmic reticulum of bullfrog skeletal muscle. *J. Biochem.* 112:514–522.

Nelson, T. E., and K. E. Nelson. 1990. Intra- and extraluminal sarcoplasmic reticulum membrane regulatory sites for Ca^{2+}-induced Ca^{2+} release. *FEBS Lett.* 263:292–294.

Olivares, E. B., D. J. Tanksley, J. A. Airey, C. F. Beck, Y. Ouyang, T. J. Deerinck, M. H. Ellisman, and J. L. Sutko. 1991. Nonmammalian vertebrate skeletal muscles express two triad junctional foot protein isoforms. *Biophys. J.* 59:1153–1163.

Ostwald, T. J., D. H. MacLennan, and K. J. Dorrington. 1974. Effects of cation binding on the conformation of calsequestrin and the high affinity calcium-binding protein of sarcoplasmic reticulum. *J. Biol. Chem.* 249: 5867–5871.

Oyamada, H., T. Murayama, T. Takagi, M. Iino, N. Iwabe, T. Miyata, Y. Ogawa, and M. Endo. 1994. Primary structure and distribution of ryanodine-binding protein isoforms of the bullfrog skeletal muscle. *J. Biol. Chem.* 269:17206–17214.

Prieto, H., P. Donoso, P. Rodriguez, and C. Hidalgo. 1994. Intraluminal calcium affects markedly calcium release rates in triads from rabbit but not from frog. *Biophys. J.* 66:339*a*. (Abstr.)

Sitsapesan, R., and A. J. Williams. 1994. Regulation of the gating of the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel by luminal Ca^{2+} *J. Membr. Biol.* 137:215–226.

Smith, J. S., R. Coronado, and G. Meissner. 1985. Sarcoplasmic reticulum contains adenine nucleotide activated calcium channels. *Nature.* 316:446–449.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single-channel calcium and barium currents of large and small conductance from sarcoplasmic reticulum. *Biophys. J.* 50:921–928.

Suarez-Isla, B. A., V. Irribarra, A. Oberhauser, L. Larralde, R. Bull, C. Hidalgo, and E. Jaimovich. 1988. Inositol (1,4,5)-trisphosphate activates a calcium channel in isolated sarcoplasmic reticulum membranes. *Biophys. J.* 54:737–741.

Sumbilla, C., and G. Inesi. 1987. Rapid filtration measurements of Ca^{2+} release from sarcoplasmic reticulum vesicles. *FEBS Lett.* 210:31–36.

Tripathy, A., and G. Meissner. 1994. Effect of SR lumenal Ca^{2+} on the open probability of the skeletal muscle ryanodine receptor/Ca^{2+} release channel. *Biophys. J.* 66:416*a*. (Abstr.)

Volpe, P., and B. J. Simon. 1991. The bulk of Ca^{2+} released to the myoplasm is free in the sarcoplasmic reticulum and does not unbind from calsequestrin. *FEBS Lett.* 278:274–278.