# Intravesicular Calcium Transient during Calcium Release from Sarcoplasmic Reticulum†

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ABSTRACT: The time course of changes in the intravesicular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in terminal cisternal sarcoplasmic reticulum vesicles upon the induction of $Ca^{2+}$ release was investigated by using tetramethylmurexide (TMX) as an intravesicular $Ca^{2+}$ probe. Upon the addition of polylysine at the concentration that led to the maximum rate of $Ca^{2+}$ release, $[Ca^{2+}]_i$ decreased monotonically in parallel with $Ca^{2+}$ release. Upon induction of $Ca^{2+}$ release by lower concentrations of polylysine, $[Ca^{2+}]_i$ first increased above the resting level, followed by a decrease well below it. The release triggers polylysine, and caffeine brought about dissociation of calcium that bound to a nonvesicular membrane segment consisting of the junctional face membrane and calsequestrin bound to it, as monitored with TMX. No $Ca^{2+}$ dissociation from calsequestrin-free junctional face membranes or from the dissociated calsequestrin was produced by release triggers, but upon reassociation of the dissociated calsequestrin and the junctional face membrane, $Ca^{2+}$ dissociation by triggers was restored. On the basis of these results, we propose that the release triggers elicit a signal in the junctional face membrane, presumably in the foot protein moiety, which is then transmitted to calsequestrin, leading to the dissociation of the bound calcium; and in SR vesicles, to the transient increase of $[Ca^{2+}]_i$, and subsequently release across the membrane.

Most of the previous studies of $Ca^{2+}$ release from sarcoplasmic reticulum (SR)[1] (reviews: Endo, 1977; Martonosi, 1984; Ikemoto et al., 1989a,b; Fleischer & Inui, 1989) have been carried out by monitoring either the extravesicular free $Ca^{2+}$ concentration or the total amount of intravesicular calcium, without a thorough investigation of the intravesicular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) changes during $Ca^{2+}$ release from SR. A large portion of the transported $Ca^{2+}$ is bound to the lumenal $Ca^{2+}$ binding proteins such as calsequestrin (Ikemoto et al., 1989b), and the calcium must be dissociated prior to release across the membrane. Therefore, it is anticipated that there might be rather complex changes in the $[Ca^{2+}]_i$ during $Ca^{2+}$ release from SR.

This study was initiated in an attempt to directly monitor the changes in $[Ca^{2+}]_i$ during $Ca^{2+}$ release. As shown here, upon induction of $Ca^{2+}$ release by release-inducing reagents (or release triggers) such as polylysine (Cifuentes et al., 1989) and caffeine, there is a transient increase of $[Ca^{2+}]_i$ prior to the decrease of $[Ca^{2+}]_i$ due to $Ca^{2+}$ efflux across the SR membrane, as monitored with the intravesicularly loaded $Ca^{2+}$ probe tetramethylmurexide (TMX). Addition of release triggers to the membrane segment consisting of the junctional face membrane and calsequestrin resulted in partial dissociation of calcium that had been bound to the complex. The dissociated $Ca^{2+}$ must have originated from calsequestrin, since no $Ca^{2+}$ dissociation occurred from the junctional face membrane alone. The junctional face membrane seems to be required for the induction of the dissociation of the calsequestrin-bound calcium, since no release trigger dependent dissociation of $Ca^{2+}$ occurred from the free calsequestrin. $Ca^{2+}$ dissociation could be restored upon reconstitution of the complex from the JFM and purified calsequestrin. On the basis of these results, we propose that the $Ca^{2+}$ release triggering signal, which is elicited in the foot protein moiety of the terminal cisternal SR membrane, is transmitted to calsequestrin, leading to rapid dissociation of the calcium bound to it. In the sealed SR vesicles, this leads to the transient increase of $[Ca^{2+}]_i$, with a subsequent release of $Ca^{2+}{}_i$ across the SR membrane.

## Experimental Procedures

*Preparation of SR.* The heavy fraction of SR was prepared from rabbit leg and back muscles (fast twitch muscle) by differential centrifugation as described previously (Kim et al., 1983).

*Dissociation and Reassociation of the JFM–Calsequestrin Complex.* The junctional face membrane (JFM) was prepared essentially according to the method described by Costello et al. (1988), and dissociation and reassociation of the complex were performed as follows. The quantities described in the following procedures are those contained in each tube in a TL100.3 rotor of a Beckman TL-100 ultracentrifuge (usually 1 preparation involved 6–12 rotor tubes). A 3-mL volume of heavy SR vesicles (4.0 mg/mL) was suspended by homogenization in a standard solution (0.3 M sucrose, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 0.8 μg/mL antipain, 2 μg/mL trypsin inhibitor, and 20 mM MES, pH 6.8) with added Triton X-100 (8 mg/mL). The insoluble fraction which contained the JFM–calsequestrin complex was collected by centrifugation at 55 000 rpm for 10 min and

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HSR, heavy fraction of sarcoplasmic reticulum; MES, 2-(*N*-morpholino)ethanesulfonic acid; SR, sarcoplasmic reticulum; TMX, tetramethylmurexide.

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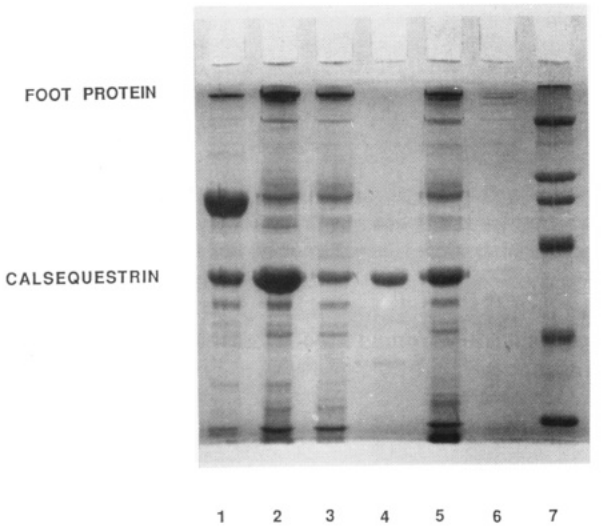


FIGURE 1: Gel electrophoretograms of various fractions of the JFM–calsequestrin complex used for $Ca^{2+}$ dissociation experiments. Lane 1, SR used for solubilization (40 $\mu$g of protein); lane 2, isolated JFM–calsequestrin complex (28 $\mu$g); lane 3, calsequestrin-free JFM (16 $\mu$g); lane 4, dissociated calsequestrin (7.8 $\mu$g); lane 5, reconstituted JFM–calsequestrin complex (21 $\mu$g); lane 6, control; lane 7, molecular weight standards. These fractions (lanes 2–5) were prepared by the sequential treatments of SR and homogenized in the same volume, as described under Experimental Procedures, and were subjected to electrophoresis in a 8% polyacrylamide slab gel. The control sample shown in lane 6 was obtained by centrifugation of the dissociated calsequestrin fraction in the same way as done for reconstitution except that no JFM was added, followed by homogenization in the same volume as for the other fractions; note that no calsequestrin sedimented if no JFM was present. The molecular weight standards (lane 7) consisted of myosin ($M_r$ = 205 000), $\beta$-galactosidase (116 000), phosphorylase B (97 400), bovine albumin (66 000), egg albumin (45 000), and carbonic anhydrase (29 000).

homogenized in 0.2 mL of the standard solution (usually 4–5 mg). This fraction is designated as isolated JFM–calsequestrin complex. To dissociate the attached calsequestrin from the JFM, 0.2 mL of the isolated JFM–calsequestrin complex was diluted to 1 mL (4–5 mg/mL) with a standard solution containing 0.5 M NaCl (final concentration). After centrifugation, the sedimentable fraction was homogenized in a 0.2-mL volume of standard solution (1.4–2.4 mg); the fraction is designated as calsequestrin-free JFM. The supernatant fractions containing the dissociated calsequestrin were pooled, and the protein was concentrated by centrifugation in a microconcentrator, Centricon 10 (Amicon), to 6.0 mg $mL^{-1}$ (dissociated calsequestrin). For reassociation of the dissociated calsequestrin with the calsequestrin-free JFM, 0.6 mL of the concentrated supernatant fraction was added to 0.2 mL of the calsequestrin-free JFM at [NaCl] = 0.5 M. The [NaCl] was reduced to 0.1 M by dilution with the standard solution, and the reconstituted JFM–calsequestrin complex was collected by centrifugation, and homogenized in a 0.2-mL volume of the standard solution (2.2–3.2 mg).

The gel electrophoretic patterns of these fractions are shown in Figure 1. The isolated JFM–calsequestrin complex contains a considerable amount of calsequestrin [Figure 1, lane 2; cf. Costello et al. (1986, 1988) and Ikemoto et al. (1989a,b)]. As reported previously (Ikemoto et al., 1989a,b), upon treatment of the JFM–calsequestrin complex with 0.5 M NaCl, a large portion of the attached calsequestrin was dissociated from the JFM; after centrifugation, the supernatant fraction contained primarily the dissociated calsequestrin (Figure 1, lane 4) and the sedimentable fraction the JFM with little calsequestrin, viz., the calsequestrin-free JFM (Figure 1, lane 3). When the calsequestrin-free JFM was mixed with the dissociated calsequestrin in 0.5 M NaCl and then the [NaCl] was reduced to 0.1 M by dilution, followed by sedimentation, the sedimented fraction contained the reconstituted JFM–calsequestrin complex (Figure 1, lane 5).

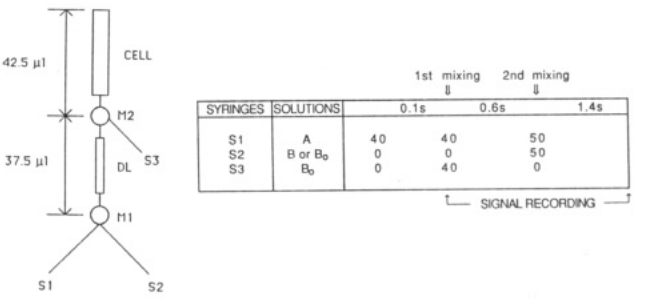


FIGURE 2: Diagrammatic representation of the three-syringe stopped-flow apparatus and the mixing schedule used for sequential monitoring of the intravesicular $[Ca^{2+}]$ levels of SR before and after the induction of $Ca^{2+}$ release. The numbers in the table indicate the volume (in microliters) of each solution to be delivered at each drive. DL, delay line; $M_1$, $M_2$, mixers; $S_1$, $S_2$, and $S_3$, syringes. For the compositions of solution A, B, $B_0$, see Experimental Procedures.

*Stopped-Flow Spectrometry of the Intravesicular $Ca^{2+}$ Transient during $Ca^{2+}$ Release.* Of different types of $Ca^{2+}$-sensitive dyes, tetramethylmurexide (TMX) appeared to be most suitable as an intravesicular $Ca^{2+}$ probe for monitoring rapid changes of $[Ca^{2+}]_i$ of SR. As described by Ohnishi (1978), TMX shows a linear response to submillimolar $Ca^{2+}$ and is relatively specific for $Ca^{2+}$, less sensitive to $Mg^{2+}$, and virtually insensitive to monovalent ions and pH. Importantly, TMX could be readily introduced into the intravesicular space of SR during $Ca^{2+}$ uptake as shown under Results.

For the uptake of $Ca^{2+}$ and TMX, the HSR (2.0 mg/mL) was incubated in a solution containing 0.15 M KCl, 5.0 mM $MgCl_2$, 5.0 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM TMX, and 20 mM MES, pH 6.8 (solution A). After a period during which the maximal level of $[Ca^{2+}]_i$ and TMX loading was reached (5–15 min after the addition of ATP to solution A), $Ca^{2+}$ release was induced by two alternative methods described below.

(*a*) *Single-Step Mixing.* Solution A was mixed with an equal volume of solution B containing 0.15 M KCl, various concentrations of release triggers (e.g., polylysine and caffeine), 20 mM EGTA, various concentrations of $CaCl_2$, and 20 mM MES (pH 6.8), in a stopped-flow system (BioLogic SFM-3) using two syringes. Then the changes in the difference in the absorbance of TMX at 510 and 550 nm ($A_d = A_{510} - A_{550}$) were monitored in a dual-wavelength spectrophotometer system (Ikemoto et al., 1988). As control, solution A was mixed with an equal volume of solution $B_0$, which was identical with solution B except that no release trigger was added.

(*b*) *Two-Step Mixing.* To allow a direct comparison of the $[Ca^{2+}]_i$ levels before and after the induction of $Ca^{2+}$ release, we devised the two-step mixing technique to perform a sequential recording first of the control trace (mixing of solution A with solution $B_0$; see above) and then of the release trace (mixing of solution A with solution B; see above). Figure 2 illustrates the schedule of sequential driving of the three syringes ($S_1$, $S_2$, and $S_3$) to perform such experiments. First, 40 $\mu$L of solution A (in $S_1$) was driven to fill the delay line (DL). After 0.1 s, 40 $\mu$L of solution A ($S_1$) and 40 $\mu$L of solution $B_0$ ($S_3$) were mixed to fill the cuvette with a 1:1 mixture of solution A + solution $B_0$. After 0.6 s, 50 $\mu$L of solution A ($S_1$) and 50 $\mu$L of solution B ($S_2$) were mixed in a 1:1 ratio to replace the content of the whole system including the cuvette with a 1:1 mixture of solution A + solution B.

Recording of the TMX absorbance signal was initiated immediately after mixing of solution A and solution $B_0$, and continued for 2 s. As control, the same type of multiple-step mixing was carried out by replacing solution B in $S_2$ with solution $B_0$.

There was a transient (≤50 ms) artifactual signal immediately after each mixing both without and also with release triggers. This was removed by subtracting the trace without release triggers (viz., solution A + solution $B_0$ mixing) from the trace with release triggers (viz., solution A + solution B mixing).

*$^{45}Ca^{2+}$ Release Assays.* Loading of SR with $^{45}Ca^{2+}$ and TMX was carried out as in the above spectroscopic measurements of the $[Ca^{2+}]_i$ transient, but solution A contained 1.0 μCi of $^{45}CaCl_2$/mL. Between 6 and 11 min after mixing, during which period the amount of the total calcium in SR remained constant (95 nmol/mg), a portion of the reaction mixture was diluted with 9 volumes of a solution containing 0.15 M KCl, 2.0 mM EGTA, 4.0 mM $MgCl_2$, and 20 mM MES (pH 6.8). A 0.5-mL sample of the mixture was placed on a Millipore filter (0.65 μm) and filtered through with a releasing solution containing 0.15 M KCl, 20.0 mM EGTA, 12.87 mM $CaCl_2$, various concentrations of polylysine, 0.1 mM TMX, and 20 mM MES, pH 6.8, for various durations (40 ms–1.4 s) using a rapid filtration apparatus (BioLogic). The filter was air-dried, and the radioactivity retained on the filter was counted.

*Measurements of Release Trigger Induced $Ca^{2+}$ Dissociation from the Isolated JFM–Calsequestrin Complex and Its Components.* Portions (0.12 mL) of the various fractions described above (the isolated JFM–calsequestrin complex, the calsequestrin-depleted JFM, and the reconstituted JFM–calsequestrin complex) were diluted to 3.0 mL (final concentrations: 0.15 M KCl, various concentrations of $CaCl_2$ as indicated, 0.1 mM TMX, and 20 mM MES, pH 6.8) and incubated for 5–15 min. For the assay with the dissociated calsequestrin, the calsequestrin sample was diluted to 0.5–0.7 mg $mL^{-1}$ (the concentration approximately equivalent to that present in the above solution of the JFM–calsequestrin complex) with the same solution as above. Then, 1 part of the protein solution was mixed with a reaction solution containing 0.15 M KCl, various concentrations of $CaCl_2$ (equivalent to those in the protein solution), 0.1 mM TMX, release triggers (10 μg $mL^{-1}$ polylysine or 8 mM caffeine), and 20 mM MES, pH 6.8, in the stopped-flow apparatus. As control, the same type of mixing was carried out but with release triggers omitted. As another type of control, reactions with or without release triggers were carried out in the absence of TMX. The time course of the changes in the absorbance of TMX ($A_d = A_{510} - A_{550}$) was recorded. To determine the $A_d/[Ca^{2+}]$ ratio to be used for calculation of the amounts of $Ca^{2+}$ dissociated from the complex, the isolated JFM–calsequestrin complex was mixed with various concentrations of $Ca^{2+}$ in the presence of TMX, and the traces were recorded as described above. The values of $A_d$ at $t = 0$ at different $Ca^{2+}$ concentrations were plotted as a function of $[Ca^{2+}]$. In the range of $0 < [Ca^{2+}] < 2$ mM, $A_d$ (output in millivolts) = $360[Ca^{2+}]$ (in millimolar).

## Results

*Conditions That Permit Selective Monitoring of the Changes in the Intravesicular $[Ca^{2+}]$ ($[Ca^{2+}]_i$ Transient) of SR during $Ca^{2+}$ Release. (A) Loading of SR Vesicles with $Ca^{2+}$ and TMX.* As shown in Figure 3A (curve a), the absorbance ($A_d = A_{515} - A_{550}$) of TMX increased when SR vesicles were incubated with 0.1 mM TMX in a solution containing an EGTA–Ca buffer (free $[Ca^{2+}] = 1$ μM) and

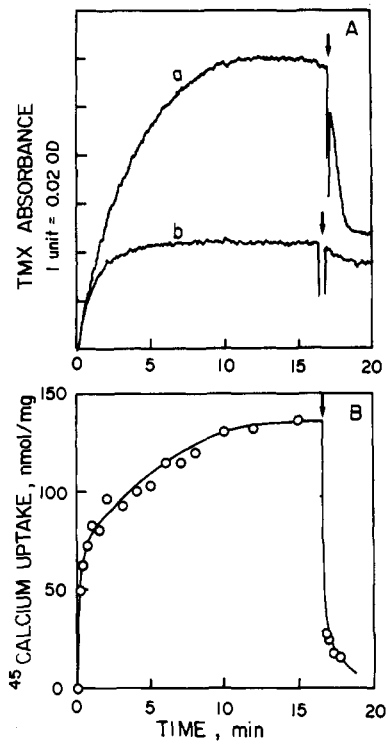


FIGURE 3: (A) Changes in the absorbance of TMX during $Ca^{2+}$ uptake by SR vesicles and subsequent addition of the $Ca^{2+}$ ionophore A23187, in the presence of 1 μM $Ca^{2+}$ (curve a) and in the virtual absence of $Ca^{2+}$ (curve b). (Curve a) SR (2 mg $mL^{-1}$) was added to a solution containing 0.15 M KCl, 1.0 mM EGTA, 0.645 mM $CaCl_2$, 5 mM MgATP, 0.1 mM TMX, and 20 mM MES (pH 6.8) to start the $Ca^{2+}$ uptake reaction, and changes of the TMX absorbance ($A_{515}$–$A_{550}$) were recorded. At the time when the TMX absorbance reached a plateau (17 min), 10 μM ionophore A23187 was added. (Curve b) The same reactions as above were carried out in a solution containing 1.0 mM EGTA but no added $CaCl_2$. (B) Time course of $^{45}Ca^{2+}$ uptake and the ionophore-induced discharge of the accumulated $^{45}Ca^{2+}$. The same reactions as above (curve a) were carried out in a reaction solution containing 1 μCi of $^{45}CaCl_2$/mL of the solution. The reaction was quenched by mixing 1 volume of the reaction solution with 2 volumes of a quenching solution containing 0.15 M KCl, 7.5 mM $MgCl_2$, 15.0 mM EGTA, 7.5 μM ruthenium red, and 20 mM MES (pH 6.8). A 0.5-mL portion of the quenched reaction mixture was filtered through a Millipore filter (0.45 μm) and washed by filtering 2 × 2.5 mL of a diluted (2/3) quenching solution. The filter was air-dried, and the radioactivity retained on the filter was counted.

MgATP. The time course of the increase of the TMX absorbance was approximately parallel to that of $^{45}Ca^{2+}$ uptake determined under equivalent conditions (Figure 3B). Since the extravesicular $[Ca^{2+}]$ remained constant under these conditions (because of the presence of an EGTA–calcium buffer), the increase of the TMX signal seems to reflect (a) accumulation of TMX in the SR lumen during $Ca^{2+}$ uptake and (b) increase of $[Ca^{2+}]_i$ that is reported by the internally loaded TMX. In support of this view, upon addition of 4 μM $Ca^{2+}$ ionophore A23187 after the maximum level of $Ca^{2+}$ loading and TMX had been achieved, the TMX signal decreased considerably (Figure 3A, curve a). However, the extent of the ionophore-induced decrease in the TMX signal (~60% in 1 min) was much smaller than that in the $^{45}Ca$ content (~90% in 1 min, cf. Figure 3B). If SR vesicles were incubated with TMX and MgATP in the presence of EGTA alone (curve b of Figure 3A), there was a small but rapid increase of the TMX absorbance followed by a plateau, whose level was approximately identical with that of curve a after the addition of the $Ca^{2+}$ ionophore. The addition of the $Ca^{2+}$ ionophore under these conditions produced virtually no change (Figure 3A, curve b). Thus, it appears that the TMX signal

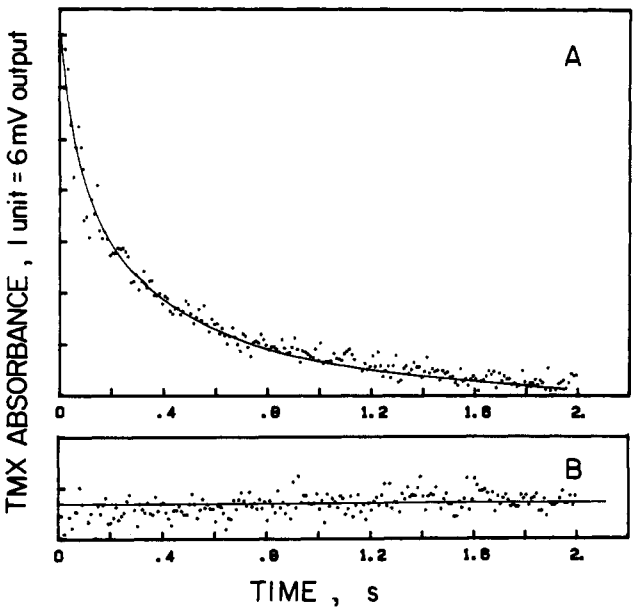


FIGURE 4: Changes in the absorbance of TMX during polylysine-induced $Ca^{2+}$ release from SR at $[Ca^{2+}]_o$ = 1.0 μM. (A) To SR that had been loaded with $Ca^{2+}$ and TMX as shown in Figure 3A (curve a) was added polylysine, and (B) to SR that had been loaded with $Ca^{2+}$ but not with TMX were added polylysine and TMX. SR (2 mg $mL^{-1}$) was loaded with $Ca^{2+}$ by incubating in solution A containing 0.15 M KCl, 5 mM MgATP, 0.1 mM $CaCl_2$, and 20 mM MES, pH 6.8, in the presence of 0.1 mM TMX (A) or in the absence of TMX (B). Then, solution A was mixed with an equal volume of solution B containing 20 mM EGTA, 12.87 mM $CaCl_2$, 10 μg $mL^{-1}$ polylysine, and 0.1 mM TMX (A) or 0.2 mM TMX (B), and the time course of the changes in the TMX absorbance was recorded. The trace was obtained by signal-averaging a total of ~80 traces.

in the steady state of $Ca^{2+}$ uptake consists of two components: (i) a portion (~60%) of the total TMX absorbance which is sensitive to the $Ca^{2+}{}_i$, (ii) the rest which is insensitive to $Ca^{2+}{}_i$.

*(B) Monitoring of the $[Ca^{2+}]_i$ Transient.* The intravesicular volume of SR vesicles is not more than 0.84% of the total volume of the reaction solution at 2 mg/mL SR, since the internal volume of our HSR vesicles is 4.2 ± 0.27 μL/mg of SR (Ikemoto et al., 1989b). Therefore, a large portion of the added TMX remains in the extravesicular space under the above conditions, and the extravesicular TMX would contribute to the TMX signal, unless changes in the extravesicular $[Ca^{2+}]$ during $Ca^{2+}$ release are prevented. This can be done by using high concentrations of EGTA–calcium buffer (e.g., 20 mM EGTA and 12.87 mM calcium, free $[Ca^{2+}]$ = 1 μM), as shown in Figure 4. In the experiments shown in Figure 4A, SR vesicles were loaded with TMX and $Ca^{2+}$, and then $Ca^{2+}$ release was induced by polylysine in the presence of the above EGTA–calcium buffer. On the other hand, in the experiments shown in Figure 4B, the vesicles were loaded with $Ca^{2+}$ but without TMX, and $Ca^{2+}$ release was induced by polylysine in the same EGTA–calcium buffer containing TMX. As shown in Figure 4A, in the vesicles loaded with TMX the TMX absorbance decreased, reflecting the decrease of $[Ca^{2+}]_i$ due to release. However, if no time was allowed for TMX to enter the SR lumen as shown in Figure 4B, there was no change in the TMX absorbance on the time scale of release. Therefore, for most of the $[Ca^{2+}]_i$ transient measurements described below, we induced $Ca^{2+}$ release in the presence of the 20 mM EGTA/12.87 mM calcium buffer.

*Biphasic Nature of the Intravesicular $Ca^{2+}$ Transient during $Ca^{2+}$ Release from SR.* We have noticed in preliminary experiments that if $Ca^{2+}$ release was induced by release triggers at the concentration that led to the maximum rate of $Ca^{2+}$ release (see Table I) the TMX absorbance decreased monotonically, but if lower concentrations of release triggers were used the TMX absorbance first increased and subsequently decreased. In order to investigate how upon the induction of $Ca^{2+}$ release the $[Ca^{2+}]_i$ changes relative to the resting level of $[Ca^{2+}]_i$, the two-step mixing experiments (see Experimental Procedures) were carried out using polylysine ($M_r$ 20600) as a release trigger. As shown in Figure 5A (upper panel), upon addition of 5 μg $mL^{-1}$ ($2.43 \times 10^{-7}$ M) polylysine (see arrow) after having recorded the resting level of $[Ca^{2+}]_i$ for 0.6 s, the $[Ca^{2+}]_i$ showed a rapid monotonic decrease. However, if $Ca^{2+}$ release was induced with lower concentrations of polylysine (e.g., 2.5 μg/mL or $1.21 \times 10^{-7}$ M, Figure 5B, upper panel; 1.0 μg/mL or $4.85 \times 10^{-8}$ M, Figure 5C, upper panel), the $[Ca^{2+}]_i$ first increased to a higher level than the resting level and then decreased below the resting level. The period during which the $[Ca^{2+}]_i$ was higher than the resting level (overshoot period) became longer by lowering the concentration of polylysine.

Table I: Amplitude ($A$) and Rate Constant ($k$) of the Decrease in Intravesicular $[Ca^{2+}]$ Release Induced by Various Release Triggers at Concentrations That Led to the Maximum Rate of Release[a]

| release trigger | concn | $A_d$ output (mV) | $k$ ($s^{-1}$) |
|---|---|---|---|
| caffeine | 4.0 mM | 9.3 ± 1.4 | 1.2 ± 0.6 |
| polylysine ($M_r$ 3800) | 1.84 μM (7 μg $mL^{-1}$) | 12.1 ± 1.3 | 2.2 ± 0.5 |
| polylysine ($M_r$ 20600) | 0.25 μM (5 μg $mL^{-1}$) | 41.8 ± 5.5 | 6.3 ± 0.8 |

[a] Data are average ± standard deviation ($n$ = 5).

Lower panels of Figure 5A,B,C depict the time courses of $Ca^{2+}$ release determined by rapid filtration using $^{45}Ca^{2+}$ under conditions corresponding to those of the TMX experiments. With the maximally activating concentration of polylysine (Figure 5A), there was a sharp decrease in the intravesicular content of $^{45}Ca$. At lower concentrations of polylysine, however, there was an appreciable lag period before the decrease of intravesicular $^{45}Ca$. Interestingly, the lag periods in the $^{45}Ca$ traces (~100 ms in Figure 5B and ~400 ms in Figure 5C, lower panels) are on the same order of magnitude as the overshoot periods of the TMX traces (160 ms in Figure 5B and 660 ms in Figure 5C, upper panels), respectively. This suggests that the total amount of the intravesicular calcium remained unchanged during the period when the overshoot in the $[Ca^{2+}]_i$ was occurring. These results suggest that upon the addition of the $Ca^{2+}$ release trigger the internally bound calcium is dissociated, leading to the transient increase in $[Ca^{2+}]_i$; subsequently, the dissociated $Ca^{2+}{}_i$ is released to the extravesicular space.

*Release Trigger Induced Dissociation of Calsequestrin-Bound Calcium Is Mediated by the Junctional Face Membrane.* We found that the addition of the release triggers—5 μg $mL^{-1}$ polylysine (Figure 6) and 4 mM caffeine (Figure 7)—to the isolated JFM–calsequestrin complex (Figures 6A and 7A) preincubated in 2 mM $Ca^{2+}$ and 0.1 mM TMX produced a large increase of the TMX signal (see curve +RT). On the other hand, the control mixing experiment with a solution containing no release triggers produced a different type of signal (curve −RT); this represented the changes in light scattering rather than the changes in $Ca^{2+}$, as evidenced by that fact that mixing in the absence of TMX yielded the same signal (data not shown). Therefore, the TMX signal attributable to the release trigger induced $Ca^{2+}$ dissociation proper (curve ΔCa) was calculated by subtraction of curve −RT from curve +RT. The amplitude of the curve ΔCa corresponds to approximately 80 nmol of $Ca^{2+}$ dissociated from 1 mg of protein of the JFM–calsequestrin complex, determined

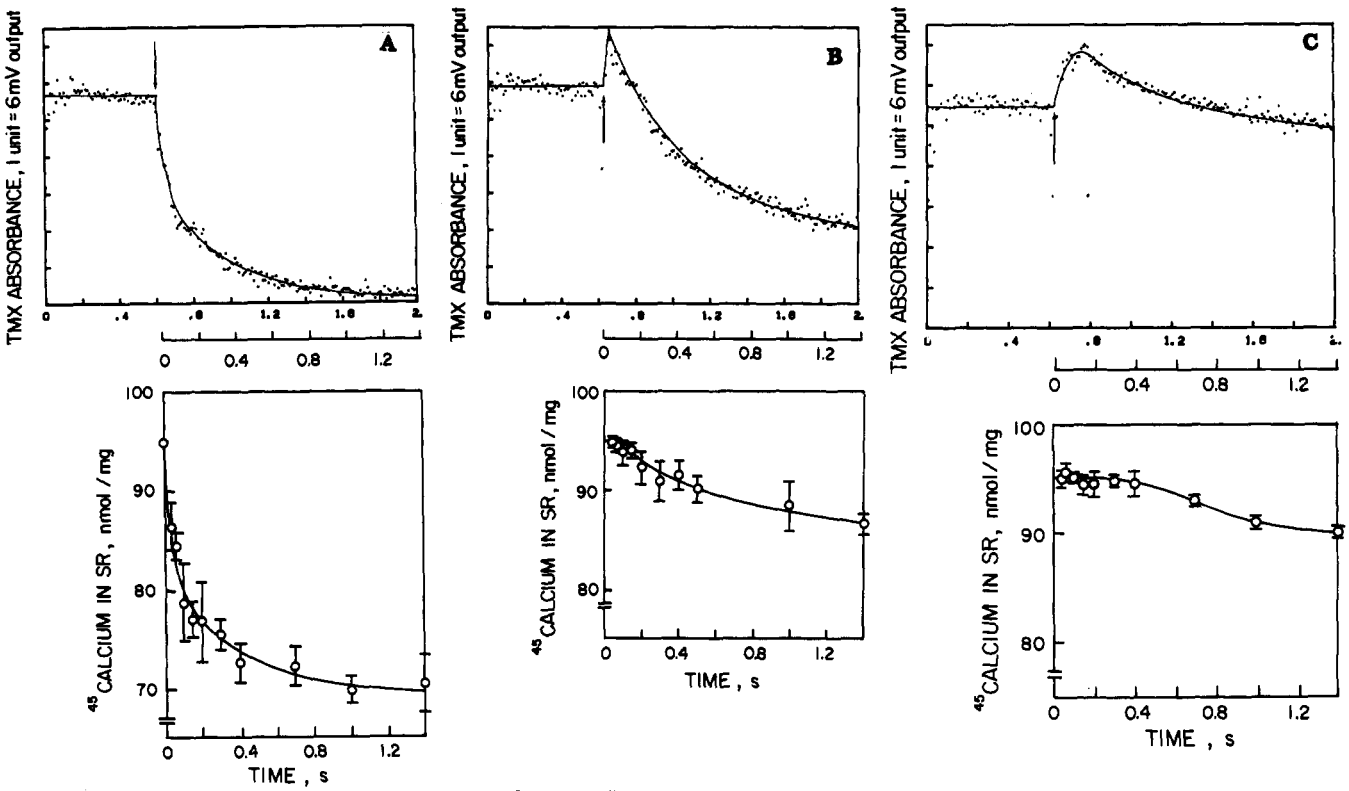


FIGURE 5: (Upper traces) Sequential monitoring of the intravesicular $[Ca^{2+}]$ by TMX before and after the induction of $Ca^{2+}$ release from SR with different concentrations of polylysine: (A) 5 $\mu g\ mL^{-1}$; (B) 2.5 $\mu g\ mL^{-1}$; (C) 1.0 $\mu g\ mL^{-1}$. The arrow indicates the time of addition of polylysine. In first mixing, SR that had been incubated with MgATP, $Ca^{2+}$, and TMX (solution A; see Experimental Procedures) was mixed with an equal volume of solution $B_0$ which was the same as the triggering solution (solution B) except that the release trigger polylysine was excluded. At 0.6 s after the first mixing, the content of the reaction cuvette was replaced by the mixture of equal volumes of solution A and solution B containing various concentrations of polylysine (the concentrations after mixing are as indicated above), and the recording of the TMX absorbance was continued for further 1.4 s. As a control, mixing of solution A and solution $B_0$ was repeated in the second step. There was no change in the TMX absorbance during the sequential mixing. An artificial signal that occurred immediately after each mixing was removed by the subtraction procedure described under Experimental Procedures. The trace was obtained by signal-averaging a total of ~120 traces. (Lower traces) Time courses of the changes in the intravesicular $^{45}Ca$ content after the addition of various concentrations of polylysine as above. SR was actively loaded with $^{45}Ca^{2+}$ in solution A containing $^{45}Ca^{2+}$ in the same way as done in the above stopped-flow experiments. $Ca^{2+}$ release was induced by solution B in a rapid filtration apparatus, and the time course of the changes in the $^{45}Ca$ content was determined as described under Experimental Procedures. Each datum point: average ± standard deviation ($n$ = 3–4).

as described under Experimental Procedures. This suggests that a large amount of the bound calcium was dissociated from the JFM–calsequestrin complex upon the addition of the release triggers.

Mixing of the calsequestrin-free JFM with solutions containing, or devoid of, release triggers (curve +RT and curve −RT, respectively) in the presence of TMX (see Figures 6B and 7B) produced practically identical signals. Therefore, the signal attributable to the release trigger induced $Ca^{2+}$ dissociation (curve +RT minus curve −RT = curve ΔCa) was virtually zero in the calsequestrin-depleted JFM.

Mixing of the dissociated calsequestrin with solutions containing, or devoid of, release triggers (curve +RT and curve −RT, respectively; see Figures 6C and 7C) produced a relatively large signal of about the same magnitude. However, the same type of signal was obtained even in the absence of added TMX, suggesting that the dissociated calsequestrin underwent much larger and different type of light-scattering changes compared with those of the JFM-attached calsequestrin. Thus, the signal attributable to the release trigger induced $Ca^{2+}$ dissociation (viz., curve +RT minus curve −RT) was again virtually zero also in the dissociated calsequestrin (curve ΔCa, Figures 6C and 7C).

As shown in Figures 6D and 7D, upon reassociation of the dissociated calsequestrin with the calsequestrin-depleted JFM, the release trigger induced $Ca^{2+}$ dissociation was restored almost completely. These results suggest that in order to dissociate the bound calcium from the JFM–calsequestrin complex the JFM and calsequestrin moieties have to be linked with each other.

Figures 8 and 9 illustrate that the amplitude as well as the shape of the traces of the caffeine-induced $Ca^{2+}$ dissociation from the JFM–calsequestrin complex varies with the concentration of $Ca^{2+}$ in the reaction solution. The amount of the dissociated $Ca^{2+}$ increased sharply as the $[Ca^{2+}]$ increased from the submillimolar range to the millimolar range in parallel with the amount of calcium bound to the internal binding sites (Ikemoto et al., 1989a,b), and leveled off at 2 mM (Figure 8). The $[Ca^{2+}]$ dependence shown in Figure 9 is essentilly identical with the recently reported $[Ca^{2+}]_i$ dependence of caffeine-induced $Ca^{2+}$ release from the SR vesicles (Ikemoto et al., 1989b), suggesting that the $[Ca^{2+}]$ of the reaction solution in the experiment with the nonvesicular JFM–calsequestrin system is equivalent to the $[Ca^{2+}]_i$ in the $Ca^{2+}$ release experiments with intact SR vesicles.

## Discussion

As shown in our recent study (Ikemoto et al., 1989b), most of the $Ca^{2+}$ transported into the SR vesicles (e.g., more than 90% of the transported calcium) is bound to the intravesicular $Ca^{2+}$ binding sites, while upon stimulation of the SR vesicles by release-inducing reagents (release triggers) a significant portion (e.g., 50%) of the accumulated calcium is liberated from the vesicles. Therefore, the major portion of the $Ca^{2+}$

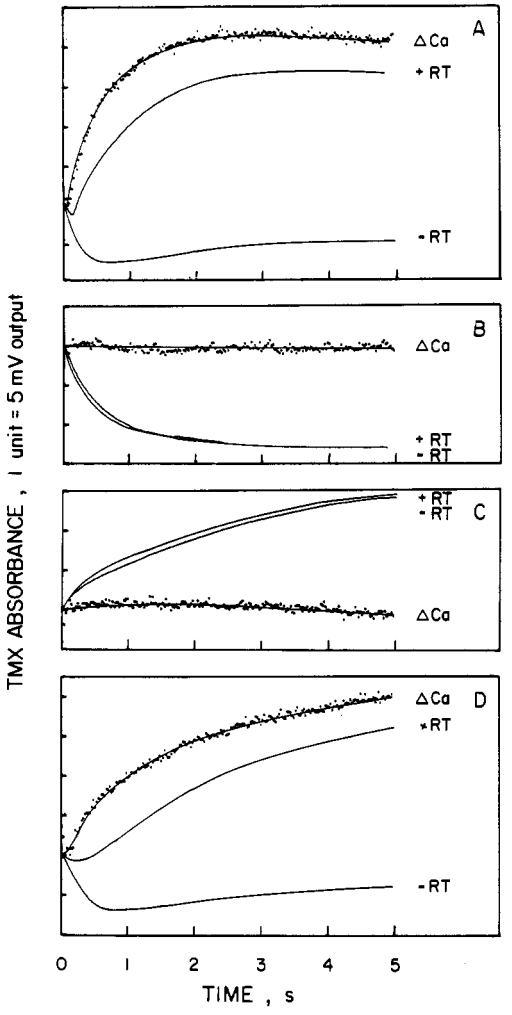


FIGURE 6: Polylysine dissociates the bound calcium from the isolated JFM–calsequestrin complex (A) and the reconstituted JFM–calsequestrin complex (D), but not from the calsequestrin-free JFM (B) nor from the dissociated calsequestrin (C). These fractions (the amounts added were as described under Experimental Procedures) were suspended in a solution containing 0.15 M KCl, 2.0 mM $CaCl_2$, 0.1 mM TMX, and 20 mM MES, pH 6.8. The protein suspension was mixed with an equal volume of reaction solution containing the release trigger polylysine (5 $\mu$g $mL^{-1}$ after mixing; see curve +RT, trace of the original plot) or the same solution devoid of polylysine (see curve -RT, trace of the original plot). The changes in the TMX absorbance were monitored in a stopped-flow spectrophotometer system (Experimental Procedures). Each trace was obtained by signal-averaging a total of 80–140 traces originated from 2–3 preparations. The traces attributable to the changes of $[Ca^{2+}]$ proper, viz., curve ΔCa (the original plot and its trace), were obtained by subtracting curve -RT from curve +RT (for the rationale for the procedure, see the text).

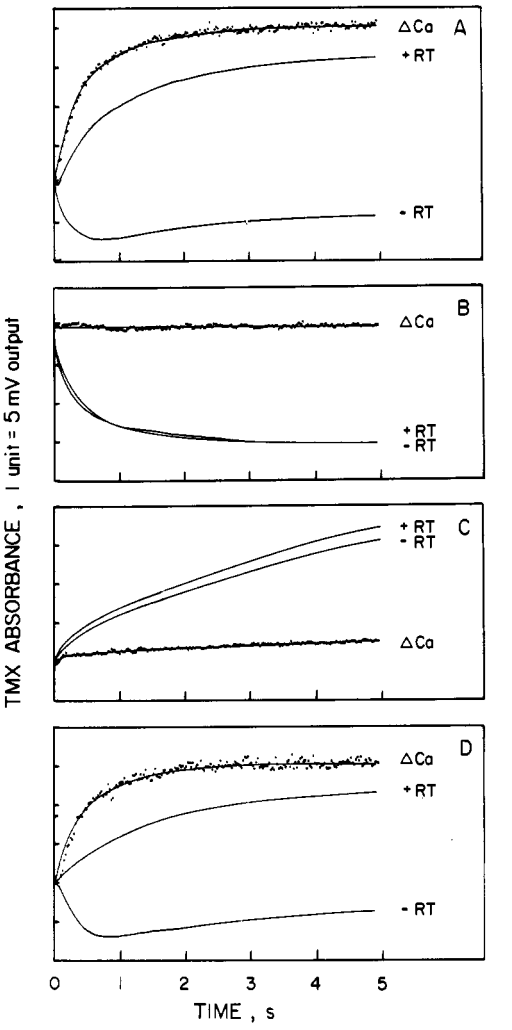


FIGURE 7: Caffeine dissociates the bound calcium from the isolated JFM–calsequestrin complex (A) and from the reconstituted JFM–calsequestrin complex (D), but not from the calsequestrin-free JFM (B) nor from the dissociated calsequestrin (C). Stopped-flow reactions were carried out as described in the legend to Figure 6, except that caffeine (4 mM after mixing) was used as a release trigger. Each trace was obtained by signal averaging a total of 80–220 traces originated from 2–6 preparations.

released must have originated from the internal binding sites. Thus, it is anticipated that the release trigger dissociates the internally bound calcium during $Ca^{2+}$ release.

The main finding in this study is that upon induction of $Ca^{2+}$ release by lower concentrations of release trigger there is an increase of the $[Ca^{2+}]_i$ prior to $Ca^{2+}$ release. Thus, the release triggers dissociate the internally bound calcium by an active, rather than a passive, process. As shown in Figure 5, upon increase of the concentration of polylysine (from 1.0 to 2.5 $\mu$g $mL^{-1}$), both the initial increase and the subsequent decrease of $[Ca^{2+}]_i$ become faster, and at the maximally activating concentration of polylysine (5 $\mu$g $mL^{-1}$), the initial increase of $[Ca^{2+}]_i$ becomes unresolvable as a result of further increase of the $Ca^{2+}$ release rate. These results suggest that both phases of $[Ca^{2+}]_i$ transient (viz., active $Ca^{2+}$ dissociation and subsequent release across the membrane) are regulated by a common mechanism. As shown in our recent studies (Ohkusa et al., 1990), upon the binding of release trigger the foot protein undergoes rapid conformational changes (e.g., $k = 70\ s^{-1}$ with polylysine), leading to the activation of $Ca^{2+}$ release. Thus, it appears that the dissociation of internally bound calcium is also mediated by the trigger-induced conformational change of the foot protein.

Since calsequestrin is localized in the terminal cisternal SR (Meissner, 1975; Volpe et al., 1987) at a high concentration and has an extraordinarily large capacity of $Ca^{2+}$ binding (about 40 mol of Ca/mol) in the $[Ca^{2+}]$ range expected to prevail within the vesicles during $Ca^{2+}$ transport (MacLennan & Wong, 1971; Ikemoto et al., 1974, MacLennan et al., 1983; Campbell, 1986), the main source from which the internally bound calcium is dissociated would be calsequestrin. The present experiments with a nonvesicular membrane complex consisting of the JFM and the attached calsequestrin have provided direct evidence for this hypothesis. As shown here, the addition of the $Ca^{2+}$ release triggers (polylysine or caffeine) to the JFM–calsequestrin complex resulted in the dissociation of the bound calcium. The release trigger dependent $Ca^{2+}$ dissociation did not occur from the calsequestrin-free JFM nor

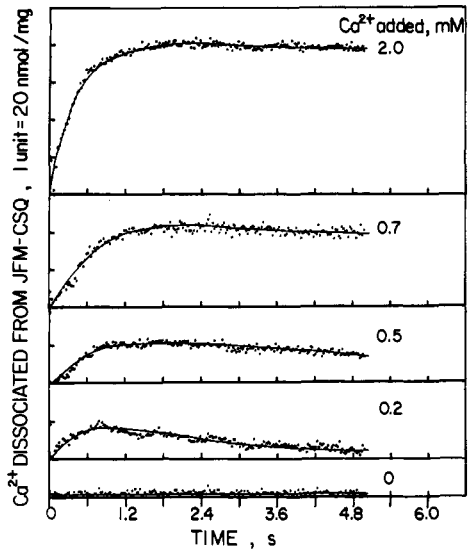


FIGURE 8: Caffeine-induced $Ca^{2+}$ dissociation of the isolated JFM–calsequestrin complex at different $Ca^{2+}$ concentrations as indicated. The isolated JFM–calsequestrin complex (0.86 mg $mL^{-1}$) was suspended in 0.15 M KCl, 0.1 mM TMX, various concentrations of $CaCl_2$ as indicated, and 20 mM MES, pH 6.8. The protein suspension was mixed with an equal volume of reaction solution [0.15 M KCl, 0.1 mM TMX, various concentrations of $CaCl_2$ (equivalent to that of the protein suspension), and 20 mM MES, pH 6.8] containing 8 mM caffeine or the same solution containing no caffeine. About 40–60 traces were signal-averaged, and the signal-averaged trace without caffeine was subtraced from that with caffeine. The amount of $Ca^{2+}$ dissociated from the isolated JFM–calsequestrin complex was calculated from the TMX absorbance as described under Experimental Procedures.

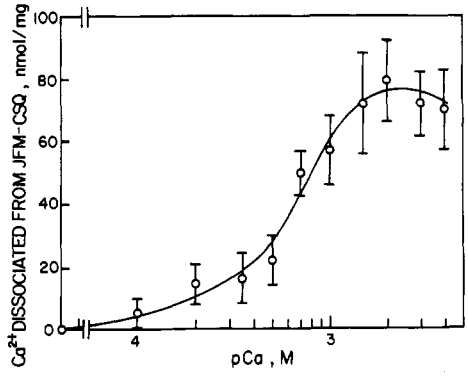


FIGURE 9: $[Ca^{2+}]$ dependence of the amount of $Ca^{2+}$ dissociated from the isolated JFM–calsequestrin complex by caffeine. $Ca^{2+}$ dissociation from the isolated JFM–calsequestrin complex was induced and monitored as described in the legend to Figure 8. The amounts of $Ca^{2+}$ dissociated from the complex were plotted as a function of $[Ca^{2+}]$ during the reaction. Each datum point: average (±) standard deviation ($n$ = 3).

from the dissociated calsequestrin. However, $Ca^{2+}$ dissociation activity was restored when the complex was reconstituted from the calsequestrin-free JFM and the dissociated calsequestrin. The dissociated $Ca^{2+}$ must have originated from calsequestrin for the following reasons. First, no $Ca^{2+}$ dissociation occurred from the calsequestrin-depleted JFM. Second, a large amount of $Ca^{2+}$ dissociated (e.g., 80 nmol/mg of JFM–calsequestrin complex) at higher $[Ca^{2+}]$, at which a large-capacity $Ca^{2+}$ binding occurs in calsequestrin, suggests the involvement of low-affinity and large-capacity $Ca^{2+}$ binding proteins. Calsequestrin is the only such protein present in the JFM–calsequestrin complex in an appreciable quantity. On the other hand, dissociation of the calsequestrin-bound calcium must have been mediated via the JFM, since no release trigger dependent $Ca^{2+}$ dissociation occurred from the dissociated calsequestrin. Thus, the process of $Ca^{2+}$ dissociation in the JFM–calsequestrin system appears to be mediated by some kind of communication between the JFM and calsequestrin, probably by communication between the foot protein and calsequestrin.

Since the dissociated calsequestrin fraction used in the reconstitution experiments contained a few non-calsequestrin proteins (e.g., a ~40-kDa protein, cf. Figure 1, lane 4) as minor contaminants, we have carried out the same type of reconstitution experiment with an exhaustively purified preparation of calsequestrin. Our preliminary experiments suggest that the purified calsequestrin can be effectively reassociated with the JFM, but the extent of restoration of the trigger-induced $Ca^{2+}$ dissociation activity is very partial (10–20% of the activity in the isolated JFM–calsequestrin complex). Thus, we cannot exclude the possibility that the JFM to calsequestrin communication is catalyzed by some of these contaminants present in the dissociated calsequestrin preparation; in fact, the possible involvement of several proteins with low molecular weight in the interaction of calsequestrin with the JFM was suggested (a ~27K protein; Mitchell et al., 1988; several proteins in the molecular weight range of 25–47K; Damiani & Margreth, 1990).

Appreciable $Ca^{2+}$ dissociation from the JFM–calsequestrin complex occurred on preincubation in high concentrations of $Ca^{2+}$ (e.g., >0.5 mM), when a large amount of calcium is bound to calsequestrin. The optimum $[Ca^{2+}]$ range for the caffeine-induced $Ca^{2+}$ dissociation from the nonvesicular JFM–calsequestrin complex was essentially identical with the optimum range of the intravesicular $[Ca^{2+}]$ for caffeine-induced $Ca^{2+}$ release from the SR vesicles (Ikemoto et al., 1989b); this suggests that the JFM–calsequestrin complex serves as a suitable model for studies of the intravesicular events during $Ca^{2+}$ release. However, the $Ca^{2+}$ concentrations required to produce sufficient binding of dissociable $Ca^{2+}$ from the JFM–calsequestrin complex are within the range of the extravesicular $[Ca^{2+}]$ in which $Ca^{2+}$ release from the SR vesicles is suppressed [the optimal concentrations of the $[Ca^{2+}]_o$ for $Ca^{2+}$ release from the SR vesicles being 1–10 $\mu$M (Kim et al., 1983; Kirino et al., 1983; Meissner et al., 1986)]. As a matter of fact, the rate of $Ca^{2+}$ dissociation from the JFM–calsequestrin complex (e.g., $k$ = 1.8 $s^{-1}$ with 5 $\mu$g $mL^{-1}$ polylysine at 2 mM $Ca^{2+}$; Figure 6A) is much slower that that of the transient increase of the intravesicular $Ca^{2+}$ concentration even with lower concentrations of polylysine (e.g., $k \geq 19.5$ $s^{-1}$ at 2.5 $\mu$g $mL^{-1}$; Figure 5B).

On the basis of the present results, we propose that several important events occur in a sequential fashion in the following order: (i) the binding of release triggers to the foot protein; (ii) conformational changes of the foot protein; (iii) dissociation of the calcium bound to calsequestrin; and (iv) release of the dissociated $Ca^{2+}_i$ to the extravesicular space. The active dissociation of the internally bound calcium described here would have a 2-fold physiological significance. First, the rapid $Ca^{2+}$ dissociation would elevate the $[Ca^{2+}]_i$ from several millimolar (the $[Ca^{2+}]_i$ level in the steady state of $Ca^{2+}$ uptake) to a much higher level. Higher levels of the luminal or trans $[Ca^{2+}]$ (as high as ≥50 mM) have been used for most conductance measurements of the foot protein incoporated in lipid bilayers unless holding potential was imposed (Smith et al., 1987; Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988; Rousseau & Meissner, 1989), suggesting that such a high level of $[Ca^{2+}]_i$ would in fact be required for the activation of the $Ca^{2+}$ channel. Second, the resultant creation of the high $Ca^{2+}$ concentration gradient across the SR membrane would

facilitate the $Ca^{2+}$ efflux through the activated channel.

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**Registry No.** Ca, 7440-70-2; caffeine, 58-08-2; polylysine (homopolymer), 25104-18-1; polylysine (SRU), 38000-06-5.

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