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Depolarization-Induced Calcium Release from Isolated Triads Measured with Impermeant Fura-2

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Summary. Depolarization-induced Ca^{2+} release was studied in a mixture of triads and terminal cisternae isolated from rabbit skeletal muscle. The vesicles were actively loaded with known amounts of Ca^{2+} in the absence of precipitating anions in a solution containing 100 mM K propionate buffer. Changes in extravesicular Ca^{2+} were monitored with 10 μM Fura-2 (membrane impermeant form). Ca^{2+} release was initiated by diluting an aliquot of the loaded vesicles into a TEACl release solution designed to maintain a constant $[K^+] \cdot [Cl^-]$ product. Fast release, defined as the percentage of total Ca^{2+} loaded which released in less than 10 sec, occurred when extravesicular free Ca^{2+} was in the submicromolar range and was unaffected by 5 mM caffeine under depolarizing conditions, change in external pH to 6.5, and an increase in external Mg^{2+} concentration from 0.1 to 0.2 mM. Thus, the Ca^{2+} release measured in these studies is distinct from Ca^{2+}-induced Ca^{2+} release. The fast release more than doubled when a greater dilution (1 : 20 *versus* 1 : 10) of the loaded vesicles into the release solution, which would produce a larger depolarization, was used. The percentage of loaded Ca^{2+} which released rapidly in a particular triad preparation was similar to the percentage of vesicles structurally coupled as visualized by electron microscopy.

Key Words triads · skeletal muscle · excitation-contraction coupling · sarcoplasmic reticulum · fura

Introduction

Isolated triadic vesicles have been used by a number of investigators (Caswell, Lau & Brunschwig, 1976; Mitchell, Palade & Fleischer, 1983; Ikemoto, Antoniu & Kim, 1984) to probe the mechanism of excitation-contraction coupling in skeletal muscle. The isolated vesicles offer a certain advantage over cut or skinned skeletal muscle fibers, in that rapid diffusion of substances to the triadic junction is attained while complete control of extravesicular components is maintained. Much work has been presented that demonstrates the rapid release of calcium from heavy sarcoplasmic reticulum vesicles induced by micromolar free calcium addition (Ikemoto, Antoniu & Mészános, 1985; Meissner, 1984, 1986; Meissner, Darling & Eveleth, 1986). Calcium-induced calcium release, which is believed to be a direct stimulation of the sarcoplasmic reticulum, requires micromolar free Ca^{2+} as a trigger (Meissner, 1984; Meissner et al., 1986; Ikemoto et al., 1985), is enhanced by ATP and is inhibited by Mg^{2+} (Meissner, 1984; Meissner et al., 1986; Ikemoto et al., 1985) and an extravesicular pH of 6.5 (Meissner & Henderson, 1987; Rousseau & Pinkos, 1990). Calcium release induced by some drugs, such as caffeine and quercetin, is believed to act through the same mechanism as calcium-induced calcium release (Endo, 1975; Miyamoto & Racker, 1982; Kim, Ohnishi & Ikemoto, 1983; MacLennan, Shoshan & Wood, 1983; Palade, 1987). However, to date, only limited evidence has been obtained that supports the mechanism of depolarization-induced calcium release in triadic vesicles (Ikemoto et al., 1984; Ohkusa, Smilowitz & Ikemoto, 1990; Ohkusa et al., 1991). This is particularly disturbing since the leading hypothesis for excitation-contraction coupling in skeletal muscle involves the opening of the Ca^{2+}-release channel (the ryanodine receptor), in response to a signal transmitted in some unknown manner from the voltage-sensor in T-tubule (the dihydropyridine receptor) upon T-tubule membrane depolarization.

"Depolarization" is induced in isolated vesicles and in membrane-enclosed compartments in skinned fibers by the ionic replacement of impermeant anions with permeant anions and permeant cations with impermeant cations. If, during ionic replacement, the $[K^+] \cdot [Cl^-]$ in the bathing solution increases, an additional stimulus would be provided by osmotic swelling, which has been shown to enhance Ca^{2+} release (Mobley, 1979; Stephenson, 1985*a*). In skinned fibers, where care was taken to ensure that the ionic replacement used to depolarize internal membranes would not induce osmotic swelling

(Donaldson, 1985; Volpe & Stephenson, 1986), it was determined that depolarization-induced release required polarized transverse-tubules, whereas caffeine-induced release did not. Work by Stephenson (1985*a,b*) indicated that in skinned fibers step depolarization by diffusion potentials elicited at constant $[K^+] \cdot [Cl^-]$ product produce a small Ca^{2+} insensitive calcium release, which is not inhibited by EGTA, that potentiates a much larger Ca^{2+}-dependent efflux. The presence of ATP was critical for this Ca^{2+}-insensitive release, whether through repolarization of the transverse tubules or requirement of some phosphorylation to initiate this release.

Earlier work (Kasai & Miyamoto, 1976*a,b*) that explored depolarization-induced calcium release in vesicular preparations had focused on the effect of depolarization on isolated sarcoplasmic reticulum (heavy terminal cisternae), although no precautions were generally made to ensure that triadic vesicles were absent. Calcium release under these conditions was slow, and, in general, was believed to be caused by osmotic shock (Beeler, Russell & Martonosi, 1979; Meissner & McKinley, 1976). Other studies that have focused on depolarization-induced calcium release in triads (Ikemoto et al., 1984; Ohkusa et al., 1990) have observed a small initial phase of calcium release, which appears to require the transverse tubule, followed by a large slower calcium release. These studies used a 1 : 1 dilution of the loaded vesicles into an altered ionic environment, which might be expected to produce a relatively small depolarization, in order to obtain a signal that can be measured. A constant $[K^+] \cdot [Cl^-]$ product was not maintained upon dilution, so osmotic changes might have contributed to the release measured in these studies. The low percentage of functional triads in a preparation composed of a mixture of heavy sarcoplasmic reticulum (terminal cisternae), longitudinal reticulum, isolated transverse tubules and triads, as well as the insensitivity of previous calcium indicators were responsible for many of the problems related to signal resolution in these studies. The Ca^{2+} indicator used in part of these studies, arsenazo III, with a K_D of 20 μM, would not be able to ensure that Ca^{2+} levels in the extravesicular medium were in the nanomolar range prior to depolarization in order to eliminate the possibility that Ca^{2+}-induced Ca^{2+} release played some role in the observed calcium release. Additionally, the large amount of material (0.8 mg) which was used in the optical measurements caused light scattering artifacts which had to be subtracted from the original records in certain conditions. Parallel experiments with equal concentrations of unloaded vesicles would have ensured that the optical changes measured under these conditions were not due to the vesicular light scattering. In a later paper (Ohkusa et al., 1991), the phase of release which was reduced by dihydropyridine antagonists, demonstrated by Rios and Brum (1987) to inhibit the charge movement in the DHP[1] receptor/voltage sensor that acts as a trigger for depolarization-induced Ca^{2+} release, was not the same phase which was originally claimed to be dependent on the presence of transverse tubules. Hence, some question exists as to the nature of the fast release phase of depolarization-induced release in these studies.

In previous work on depolarization-induced calcium release using vesicular preparations the following problems hindered unambiguous interpretation of the results: (i) release was not demonstrated in the absence of micromolar calcium; in many cases, no attempt was made to control calcium levels; (ii) "depolarization" involved possible osmotic shock through alteration of the $[K^+] \cdot [Cl^-]$ product, which has been shown to cause calcium release; (iii) no effort was made to ensure that the calcium levels outside of the loaded vesicles was similar to that of the release solution prior to dilution; (iv) no effort was made to eliminate spontaneous calcium release from the loaded vesicles; (v) no morphological correlation between the number of coupled vesicles and the percentage of vesicles capable of functional release was established; (vi) no voltage dependence of depolarization-induced release was established (i.e., no attempt was made to demonstrate that some dilution which would generate a small depolarization was subthreshold or produced a less than maximal response whereas greater depolarizations produce a maximal response).

The purpose of the present study was to characterize "depolarization-induced" Ca^{2+} release in a preparation of isolated triads/terminal cisternae under conditions that would differentiate it from Ca^{2+}-induced Ca^{2+} release and enable one to obtain reasonable signals in fluorescence measurements with samples of 40–50 μg. Care was taken to ensure that ionic replacement did not induce osmotic changes in the vesicles, that the loaded vesicles did not spontaneously release calcium, and that the vesicles were able to actively load Ca^{2+} in the absence of precipitating anions. Dilution of loaded triadic vesicles into a solution of similar ionic composition, as well as dilution of longitudinal reticulum into a "depolarizing" medium, served as controls for the depolarization-induced release. Additionally, if the process studied was in fact depolarization-induced calcium release, then some voltage dependence of the re-

[1] The abbreviations used: EDTA (ethylenediaminetetraacetic acid); TEACl (tetraethylammonium chloride); NMG (N-methyl-D-glucamine); DHP (dihydropyridine).

sponse should be evident. This was examined by testing different dilutions which would be expected to produce different degrees of depolarization. Electron micrographs were used to provide some estimate of the number of coupled vesicles in a given preparation in order to see if any correlation existed with the percentage of total calcium loaded that released during the relatively rapid ("fast" phase of release) in that triad preparation.

Materials and Methods

Preparation of Isolated Triads/Terminal Cisternae

Triads/terminal cisternae were prepared from the fast-twitch muscle in both legs and back of 5–7 lb New Zealand White rabbits using a modification of the procedure of Caswell et al. (1976). The animals were sacrificed through either carbon dioxide inhalation or cervical dislocation and subsequent exsanguination. The muscles were removed and minced into 500 ml of ice cold sucrose buffer (250 mM sucrose, 2 mM EDTA) containing protease inhibitors (1 mM 1,10-phenanthroline and 1 μM leupeptin). This mixture was homogenized in a Waring Blender for a total of 1.5 min using three 30-sec rests to prevent heating of the sample. Occasionally, an additional 100–200 ml of sucrose buffer was added during homogenization, for larger net muscle mass. The remainder of the protocol for triad preparation was similar to that used by Caswell et al. (1976). The homogenate was subjected to a 10,000 × g spin in a Sorvall GSA rotor for 20 min. The supernatant from this spin was poured through three layers of cheesecloth to remove any floating material, the pH adjusted to 7.0 with NaOH, and centrifuged at 42,000 rpm in a Beckman Ti45 rotor for 1 hr. The resulting supernatant was discarded; the pellets were rehomogenized in 280–420 ml sucrose buffer (70 ml per centrifuge tube) and subjected to an additional spin at 42,000 rpm for 1 hr. Again the supernatant was discarded; the pellets were homogenized in a small volume (25–30 ml) of sucrose buffer.

The separation of microsomal fractions on continuous sucrose gradients was similar to that used by Caswell et al. (1976). Continuous exponential sucrose gradients were prepared using 15 and 65% sucrose (w/w) and chilled to 5°C. Approximately 5–6 ml of the rehomogenized microsomes were loaded onto the top of each gradient and then spun at 27,000 rpm overnight (12–16 hr). ^{3}H-nitrendipine was used as a marker for the transverse tubules, and ^{3}H-ryanodine was used as a marker for terminal cisternae. The sucrose gradients were fractionated into 2 ml fractions which were assayed for both ^{3}H-nitrendipine and ^{3}H-ryanodine binding, protein content (*see below*) and for % sucrose using a refractometer. A typical radioligand binding pattern from a fractionated sucrose gradient is shown in Fig. 1, with protein peaks and % sucrose shown. The fractions at 35–45% sucrose which display ^{3}H-nitrendipine and ^{3}H-ryanodine binding activities, contained a mixture of triads and terminal cisternae while those fractions at 25–30% sucrose contained longitudinal reticulum (Lau et al., 1977). Fractions of interest were pooled and diluted slowly (over a 30 min period) with 8% sucrose buffer (250 mM sucrose, 2 mM histidine), and concentrated through centrifugation (pelleting) and rehomogenization. The concentrated samples (25–30 mg protein/ml) were stored in 8% sucrose buffer at −70°C in small aliquots.

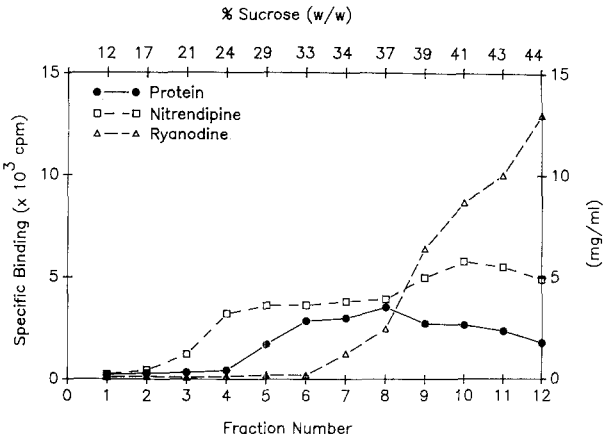

Fig. 1. Radioligand binding of fractionated microsomes on a sucrose gradient. The sucrose gradients were fractionated into 2 ml fractions, from which small aliquots were taken for protein and radioligand assays and for measurement of percent sucrose (wt/wt). Protein measurements were an average of values obtained at several dilutions using the Bradford (1976) protein assay (shown on right axis). ^{3}H-nitrendipine binding was performed as described in Materials and Methods. ^{3}H-ryanodine binding was performed according to the method of Imagawa et al. (1987) with minor modifications.

^{3}H-Nitrendipine Binding

Samples were diluted in 50 mM Tris HCl, pH 7.0, and incubated with 3 nM ^{3}H-nitrendipine (New England Nuclear) at 30°C in the dark for 30 min. Nonspecific binding was determined through the addition of 5 μM cold nitrendipine. Following incubation, the samples were filtered through glass fiber filters and washed three times with 4.5 ml of 140 mM Choline chloride. The filters were counted by liquid scintillation.

Protein Assay

Protein was determined by the methods of Bradford (1976) and Lowry et al. (1951) using bovine serum albumin as the standard.

Measurement of Ca^{2+} Loading and Release in Isolated Triads

Three different solution stocks were used routinely in these experiments: Solution A (100 mM K propionate, 2 mM $MgCl_2$, 36 mM imidazole); Solution B (44 mM TEACl, 60 mM NMG propionate, 36 mM imidazole); and Solution C (84 mM TEACl, 20 mM NMG propionate, 36 mM imidazole). Vials containing an ATP regenerating system (sufficient to produce 2 mM NaATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase in final solution for a single series of experiments) were stored dessicated at −20°C until the time of the experiment. At this time, a 10X stock solution was made with the addition of 0.5 ml of the appropriate buffer (solution A for the load solution; solution B for the 1 : 10 release solution, solution C for the 1 : 20 release).

Assay of Ca^{2+} Loading

Solution A (100 mM K propionate, 2 mM $MgCl_2$, 36 mM imidazole) was the base solution used to load triads with Ca^{2+}. Isolated triads (20–40 μl of a 30 mg/ml stock) were slowly diluted into the 0.84–0.86 ml Solution A with 10 μl of a 1 mM Fura-2 stock (pentapotassium salt: impermeable; final concentration 10 μM) and 10 μl of a 100 mM Na azide stock (1 mM final concentration). The ATP regenerating system (100 μl of a 10X stock) was added following a 15-min incubation, giving the reaction mixture a final total volume of 1 ml.

Ca^{2+} uptake and release, measured as changes in extravesicular Ca^{2+} content by the impermeable Fura-2, were monitored in various quartz cuvettes in a spectrofluorometer. Two different spectrofluorometers were used in this study: the Aminco-Bowman Model J4-8966 spectrofluorometer and the SLM Model 8000 spectrofluorometer. Using a constant emission wavelength (510 nm), three excitation wavelengths (340, 358 and 380 nm) were monitored in succession on the Aminco spectrofluorometer by manual adjustment of the excitation wavelength setting. During Ca^{2+} loading of the triads, only the signal at 380 nm was monitored during actual uptake, with the other wavelengths monitored at the completion of uptake. Those experiments performed on the SLM Model 8000 spectrofluorometer utilized a 100 Hz chopper to continuously provide measurements at two excitation wavelengths (340 and 380 nm), again at a constant emission wavelength (505 nm).

In order to monitor the loading of the vesicles with Ca^{2+}, 0.9 ml of the mixture containing load solution (solution A), vesicles and ATP regenerating system was placed in a 1-ml quartz cuvette in a spectrofluorometer. Vesicular concentration in this solution generally ranged from 0.8 to 1.2 mg protein/ml. Aliquots (3.6 μl) of a known 10 mM Ca^{2+} standard were added to the vesicle preparation and Ca^{2+} uptake was monitored through changes in the 380 nm signal or the 340/380 nm ratio. Vesicles typically received 5–10 loads, each containing 36 nmol Ca^{2+} (40 μM additions).

Assay of Ca^{2+} Release

Release solutions were designed to depolarize the transverse tubules through dilution without altering the $[K^+][Cl^-]$ product of the solutions. The osmolarity of the load and release solutions were matched through the addition of NMG propionate. Two different dilutions were examined in this study: 1 : 10 dilution into 44 mM TEACl, 60 mM NMG propionate, 36 mM imidazole (Solution B) and 1 : 20 dilution into 84 mM TEACl, 20 mM NMG propionate, 36 mM imidazole (Solution C). The release solution components were designed to parallel those of the load solution: 0.84–0.86 ml of solution B or C, 10 μl of a 1 mM Fura stock, 10 μl of a 100 mM Na azide stock, 20–40 μl sucrose buffer (vehicle used to store triadic vesicles), 100 μl of a 10X stock of ATP regenerating system. Based on the changes in the cytoplasmic K^+ concentrations upon dilution (Nernst equation), the anticipated depolarizations were estimated to be 58 mV using the 1 : 10 dilution and 75 mV using the 1 : 20 dilution. Releases from several aliquots of the same loaded vesicle suspension were monitored through the use of multiple cuvettes containing identical release solutions. Four releases (two using Ca^{2+}-loaded vesicles and two using unloaded vesicles) were typically performed in less than 5 min, then the remaining loaded vesicles were rechecked to ensure that spontaneous Ca^{2+} release had not occurred.

Calibration of Release Solution

Calibration of the fluorescence response observed upon dilution of loaded vesicles into the release solution was performed with unloaded vesicles (vesicles in a load solution mixture which received aliquots of water rather than Ca^{2+}). Following vesicle addition to the release solution, additions of 1 and 5 μM Ca^{2+} aliquots were made to obtain stepwise changes in fluorescence until R_{max} was obtained; EGTA additions were made to subsequently obtain R_{min}. Fura fluorescence was expressed as percent saturation using the following equation:

$$\% \text{ saturation} = k \cdot (R - R_{min})/[R_{max} - (k \cdot R_{min}) - (R \cdot (1 - k)] \quad (1)$$

where k is the ratio of the fluorescence of free Fura-2 to the fluorescence of the complexed Fura-2 at the reference wavelength (380 or 358 nm), R is the ratio of the fluorescence at two wavelengths (either 340/380 or $-380/358$) and R_{max} and R_{min} are the values of R for calcium-saturated and calcium-free Fura-2 (Grynkiewicz, Poenie & Tsien, 1985). The percent saturation was plotted against the amount of Ca^{2+} added to the calibration sample and fit with the equation

$$y = ((x - A) \cdot B)/((x - A) + M) \quad (2)$$

where y is the percent saturation, x is the added Ca^{2+}, A is the contaminating Ca^{2+} prior to Ca^{2+} additions, B is the maximum saturation, and M is the K_D of the dye. Values of A, B and M were obtained using a nonlinear least squares curve fitting program (Nfit: Island Products). This curve fit was then used to calculate changes in the amount of extravesicular Ca^{2+} from fluorescence changes in the release solutions. As an added test, known amounts of Ca^{2+} were added to separate release solutions in which unloaded vesicles had been diluted. If the calculated change in extravesicular Ca^{2+} varied more than 20% from the known addition in this test, the experiment was not used unless some common factor was able to correct the calibration in the entire experimental series (total of six releases used this factor).

Ultrastructural Evaluation of Membrane Vesicles

The incidence of coupled vesicles in isolated triad preparations was evaluated using thin-resin methodology. Briefly, vesicles were loaded and diluted into release solution (Solution B or C), as above. Vesicles diluted to 20–40 μg protein/ml were applied to glass coverslips freshly coated with 1 mg/ml poly-L-lysine (70,000–150,000 mol wt, Sigma). After allowing 5 min for attachment, excess vesicles were washed away and attached vesicles were fixed for 30 min in 2% glutaraldehyde buffered in 100 mM Na phosphate, 50 mM KCl, 5 mM $MgCl_2$, pH 7.0, and then for 1 hr in 1% glutaraldehyde with 0.2% tannic acid in the same buffer. Following three buffer washes, coverslips were post-fixed on ice for 1 hr in 1% OsO_4 buffered to pH 6.0. Samples were dehydrated in a graded ethanol series (from 70–100% ethanol) and infiltrated with two changes of LR White (Polysciences). Excess resin was blotted from the coverslips using Whatman #50 filter paper to produce a thin-resin layer on the coverslips. Resin was polymerized overnight at 60°C in a N_2 atmosphere. After scoring the resin side with a razor blade to produce squares about the size of an electron microscope grid, thin-resin samples were

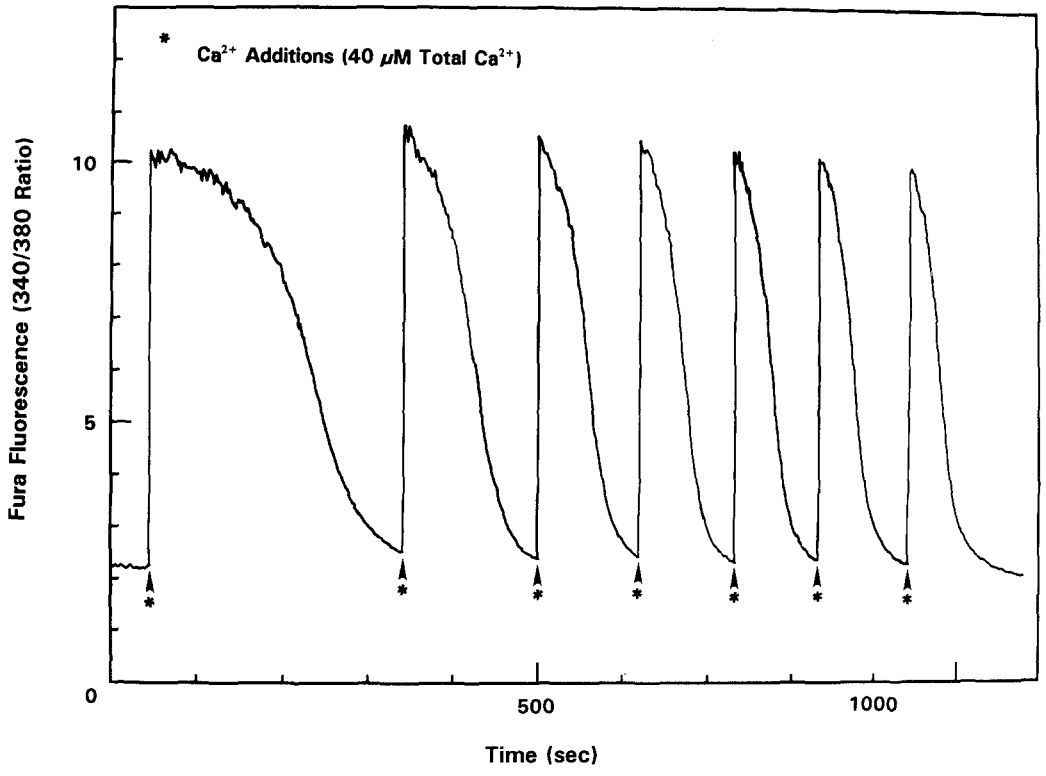

Fig. 2. Active Ca^{2+} loading of triads/terminal cisternae. Approximately 0.5–1 mg/ml vesicles were incubated in a loading solution (100 mM K propionate, 2 mM $MgCl_2$, 36 mM imidazole, 10 μM Fura, 1 mM Na azide, pH 7.0) for 15–30 min before addition of an ATP regenerating system (2 mM NaATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase). The reaction mixture (0.9 ml) was placed in a quartz cuvette and fluorescence monitored at 340 and 380 nm excitation wavelengths (using a 100-Hz chopper) at a constant emission wavelength, 510 nm. At the arrowheads, 36 nanomoles of a Ca^{2+} standard were manually added to the reaction mixture (40 μM total concentration). Increases in the fluorescence ratio (340/380 nm) of Fura indicated an increase in extravesicular Ca^{2+}, while a decrease in the fluorescence ratio was indicative of a decrease. In general, further Ca^{2+} additions were made when the fluorescence level was similar to the baseline obtained prior to loading (a ratio less than 3.0). Vesicles were generally loaded in 15–20 min.

released from the coverslips by slowly inserting the coverslips into 5% hydrofluoric acid. The floating resin samples were transferred to distilled water, picked up on formvar coated grids, stained with uranyl acetate and Reynolds lead citrate and examined with a Zeiss 10 CA electron microscope.

Results

In Fig. 2, an aliquot of triads/terminal cisternae was diluted into a solution for Ca^{2+} loading (100 mM K propionate, 2 mM $MgCl_2$, 36 mM imidazole, 10 μM Fura, 1 mM Na azide, pH 7.0) and equilibrated for 15–30 min at room temperature. Immediately prior to insertion of this reaction mix into a spectrofluorometer cuvette, an ATP regenerating system (2 mM NaATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase final concentration, pH 7.0) was added to the equilibrated vesicles. An impermeant form of Fura-2 (pentapotassium salt) was chosen for these studies so that changes in extravesicular Ca^{2+} only are monitored. For Fig. 2, Fura fluorescence was measured at two excitation wavelengths (340 and 380 nm), through the use of a 100-Hz chopper, at a constant emission wavelength of 510 nm. In order to correct for any abnormalities in fluorescence induced by distortions in ultraviolet lamp emission, the ratio of two excitation wavelengths was used to monitor changes in extravesicular calcium (340/380 ratio only is shown in figures, while an average value obtained from the 340/380 ratio and the −380/358 ratio is used in some Tables).

Known amounts of Ca^{2+} were added to the vesicles in loading solution at the arrows and manually mixed. Upon addition, rapid increases in Fura fluorescence, indicative of high extravesicular Ca^{2+}, were observed, followed by a steady lowering of fluorescence to a level slightly higher than the initial baseline over a period of 75–300 seconds. Lowering of the Fura 340/380 fluorescence ratio is indicative of Ca^{2+} sequestration into some of the vesicles, or removal from the extravesicular space. This lowering of the 340/380 ratio was not seen when the ATP

regenerating system was omitted (*data not shown*) or when Mg^{2+} was omitted, indicating that the Ca^{2+} uptake was dependent on Mg^{2+} and ATP. These observations are consistent with the uptake of Ca^{2+} into vesicles of sarcoplasmic reticulum origin via the Ca-ATPase. Although transverse tubules also contain a Ca-ATPase, the rate of Ca^{2+} uptake is substantially lower than that of the sarcoplasmic reticulum (SR) (Hildalgo, Gonzalez & Lagos, 1983) and, thus, are not thought to play a major role in Ca^{2+} uptake seen under these conditions. As a control, an identical preparation of triadic vesicles received aliquots of distilled water rather than calcium (referred to in subsequent figures as unloaded vesicles).

Depolarization-induced Ca^{2+} release was initiated by diluting the loaded triadic vesicles into various TEA chloride buffers (called release solutions) which were designed to maintain a constant $[K^+] \cdot [Cl^-]$ product, thus reducing the possibility that any Ca^{2+} release might result from osmotic shock in the vesicles. Replacement of both cations/anions with a impermeant/permeant species upon dilution was found by other investigators to be more effective than replacement of a single species in the production of depolarization (Ikemoto et al. 1984). Replicates were obtained by using identical release solutions in 3–4 different quartz cuvettes. The calcium content of the release solution was sometimes adjusted with small calcium additions prior to the addition of loaded vesicles in order to match that of the loaded vesicles.

In Fig. 3*A*, a typical Ca^{2+}-release experiment with the triad/terminal cisternae preparation is depicted. The loaded vesicles were monitored both prior to release and following all releases in order to ensure that spontaneous Ca^{2+} release had not occurred at any time during the depolarization-induced releases. (Note that the fluorescence level of the "loaded vesicles" before release and of an aliquot of the same loaded vesicles which was measured after completion of release experiments were approximately equal.) To monitor release, a cuvette containing the release solution was placed into the spectrofluorometer and the calcium content was checked. A steady fluorescence level had to be obtained in the release solution before any dilutions were performed. In Fig. 3, a 1 : 20 dilution of loaded vesicles was performed; the addition of vesicles to the release solution and subsequent mixing is indicated by the arrowheads. A rise in the 340/380 fluorescence ratio is seen following mixing, indicating a rise in extravesicular calcium due to vesicular calcium release. Calcium release was only measured in this phase for 60–100 sec. The next cuvette containing an identical release solution was inserted at the period marked *Release 2*, and unloaded vesicles rather than loaded vesicles were added to the solution at the arrowhead. Note that a very slight increase in the fluorescence ratio was observed which did not increase with time. A replicate of the first release was performed under *Release 3*. Note that in both *Release 1* and *Release 3*, the rise in fluorescence ratio following addition of vesicles can easily be separated into two components: a more rapid phase which is complete in approximately 10 sec, and a slower phase which releases calcium at a rate approximately 1/3 that of the rapid phase. The rapid phase of release was routinely determined in all releases as that portion which occurred in 10 sec. In all experiments for which a continuous monitoring of the 340/380 ratio was achieved, the contribution of the slow phase to the release measured in the fast phase was calculated by fitting the curves with double exponentials. An average of 6.93% ($n = 51$) of the amount reported in the fast phase was actually contributed by the slow phase (obtained by extrapolation), which was generally within the SEM for the release from the fast phase.

The relatively rapid phase of release appeared to be associated with depolarization-induced calcium release in triads. The "fast" phase of release observed with triads/terminal cisternae occurred within 10 sec of diluting the loaded vesicles into the depolarizing solution. The exact time course of release during the 10 sec was not resolved due to the time required for manual mixing of solutions. In this sense the present observations on depolarization-induced calcium release in triad/terminals cisternae vesicles are analogous to previous studies on depolarization-induced calcium release in skinned skeletal muscle fibers, where the speed of solution change also limited the time resolution of the release measurement to the range of seconds (Donaldson, 1985; Stephenson, 1985*a*,*b*).

As a control, the same release protocol was used with a preparation of longitudinal reticulum (LR), which should not display any depolarization-induced release (Fig. 3*B*). Note that the "fast" phase of release was eliminated in these trials with LR (Fig. 3*B*: *Release 1* and *3*; Table 6), but the slow phase of release remained. With LR, the contribution of the slow phase to the release measured in the fast phase (release measured in 10 sec) was 53.7% ($n = 10$), substantially greater than the 6.9% observed with triadic vesicles.

The release solutions did not contain any added magnesium (the only Mg^{2+} present came from the dilution of the loading solution), so one might expect that the Ca-ATPases would be relatively ineffective under the release conditions. In the face of de-

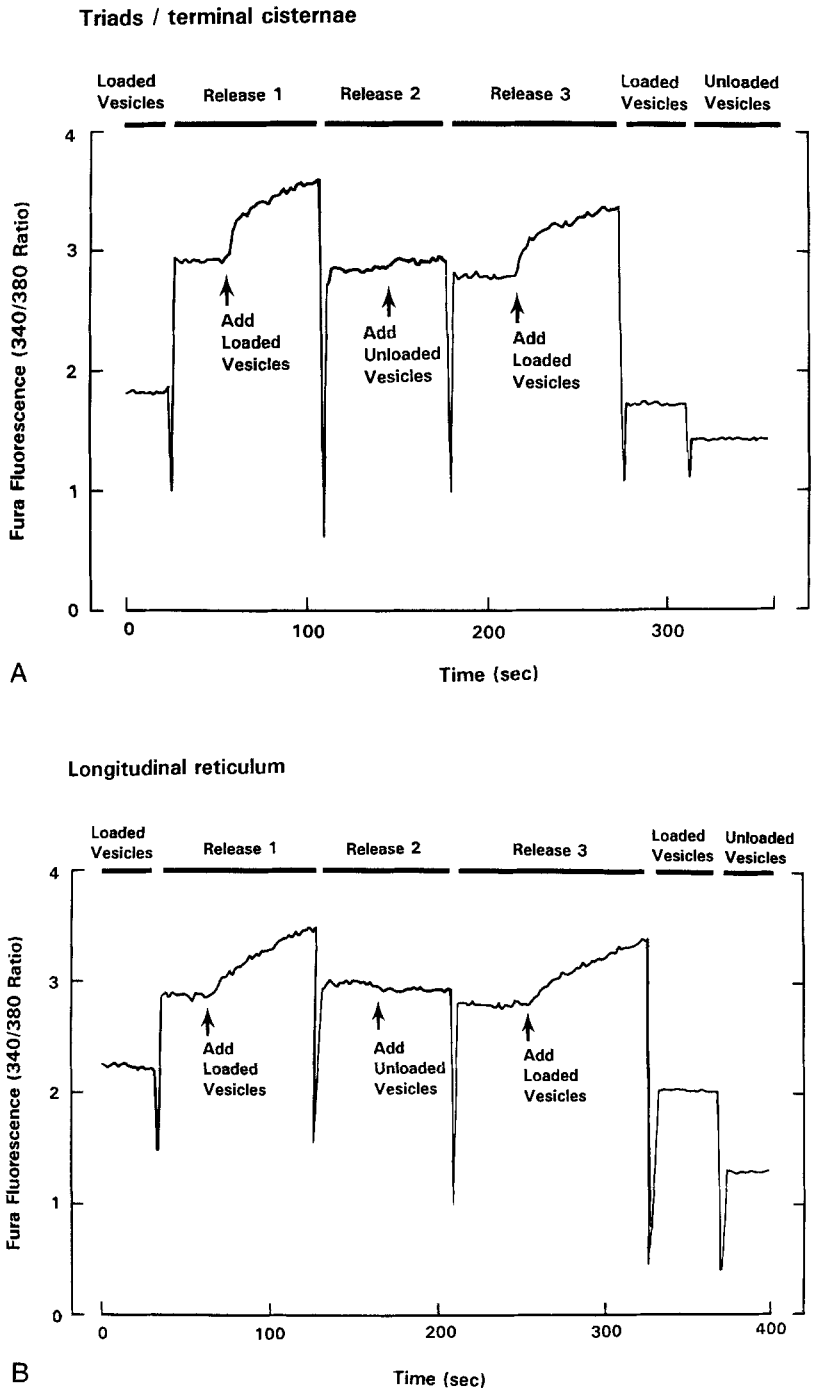

Fig. 3. Calcium release experiments with: (*A*) triads/terminal cisternae and (*B*) longitudinal reticulum. Fura fluorescence was monitored at 340 and 380 nm excitation wavelengths using a 100-Hz chopper in a series of cuvettes (the sharp transient downward deflections in the fluorescence ratio indicate a cuvette change). Changes in the ratio of the two wavelengths varied with changes in extravesicular Ca^{2+} (an increase in the ratio reflected an increase in extravesicular Ca^{2+}, etc.). Loaded vesicles were monitored before and the remaining vesicles monitored immediately after a series of three Ca^{2+} release trials. *Release 1* and *3* depict Ca^{2+} release of actively loaded vesicles: at the arrowhead, 40 μl of loaded vesicles were diluted into 0.76 ml of release solution (84 mM TEACl, 20 mM NMG propionate, 36 mM imidazole, 10 μM Fura, 1 mM Na azide) complete with ATP regenerating system. *Release 2* depicts the release observed when an equal amount of unloaded vesicles are diluted (at the arrowhead) into the release solution. Unloaded vesicles were monitored after the series of releases were complete.

pressed pump activity, the second slow phase of the calcium release seen in *Release 1* and *Release 3* probably corresponds to a slow leak of accumulated calcium. Approximately 10 min after the initial dilution of loaded triads or longitudinal reticulum into each of the release solutions, the fluorescence of the release solutions were re-evaluated: the change in extravesicular Ca^{2+} measured at this time was equivalent to approximately 80–100% of the total amount of Ca^{2+} loaded.

The fluorescence response of loaded vesicles diluted into various release solutions was calibrated using unloaded triadic vesicles which had been diluted into the corresponding release solution. As shown in Fig. 4*A*, additions of small aliquots of Ca^{2+} (1 μM additions) to this preparation produced step-

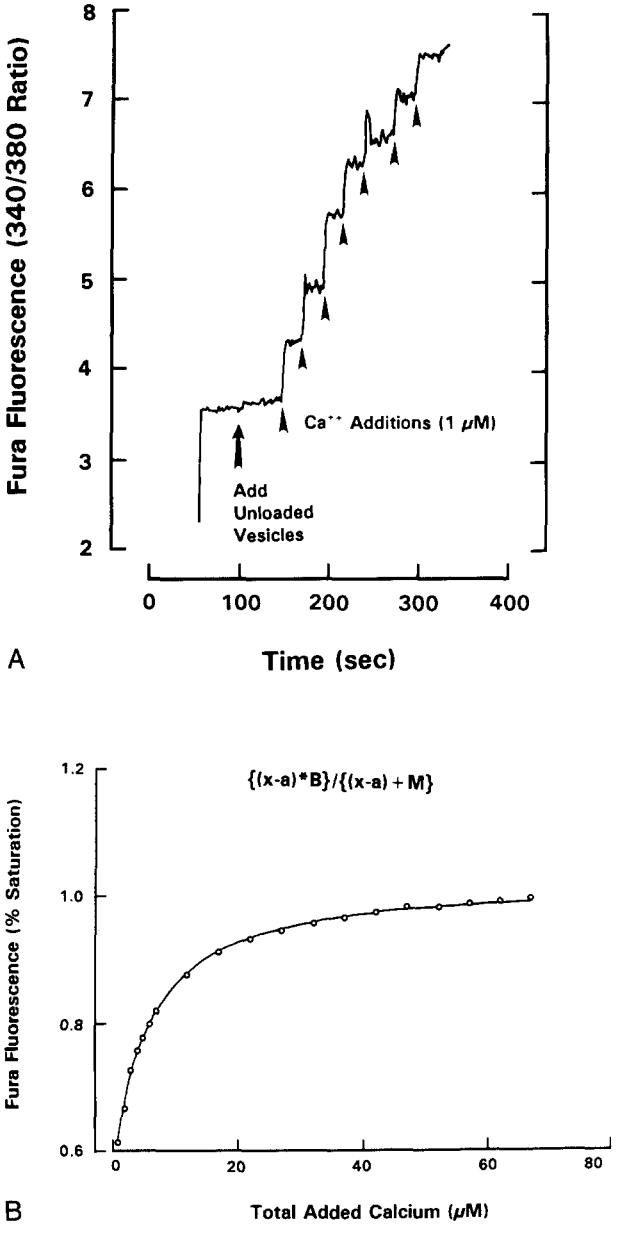

Fig. 4. Calibration of Ca^{2+} release. (*A*) Fluorescence changes with calibrating Ca^{2+} additions. In a separate cuvette, unloaded vesicles are diluted into Ca^{2+} release solution (depolarizing solution) at the arrow. Sequential Ca^{2+} additions are made at two different concentrations, 1 and 5 μM (only 1 μM additions shown at the arrowheads) until Fura saturation is obtained. The Fura fluorescence at the final Ca^{2+} addition (approximately 67 μM total added calcium) is taken to be R_{max}. Aliquots of 100 mM EGTA are added until a stable minimum fluorescence ratio is obtained (R_{min}). (*B*) Calibration curve. Using R_{max} and R_{min} values determined during calibration, Fura fluorescence at 340/380 ratio is converted into % Fura saturation (*see* Materials and Methods) and plotted against total added calcium. Using the equation denoted in the figure, a nonlinear least squares fitting program (Nfit, Island Products) was used to obtain values for a (contaminating Ca^{2+}), B (B_{max}), and M (K_D). These values were then used to convert changes in Fura saturation obtained in release experiments to changes in extravesicular calcium concentration.

wise changes in the Fura fluorescence ratio. Using equations described in Materials and Methods, these fluorescence ratios were converted to percent Fura saturation and plotted against total *added* calcium (Fig. 4*B*). The fit to this curve was then used to correlate fluorescence changes with changes in extravesicular calcium. The change in calcium associated with the rapid phase of release was expressed as a percentage of the total calcium loaded into the vesicles (percent release).

Various conditions were tested to ensure that the dilution of loaded vesicles into release solutions produced a response different from that of either osmotic shock or Ca^{2+}-induced Ca^{2+} release. Following this, this response should display some characteristics of depolarization-induced calcium release, such as voltage dependence of the response.

In the initial test condition, loaded triads/terminal cisternae were diluted into either a modified load solution or into a 1 : 20 dilution release solution. In all of the release solutions, Mg^{2+} was absent except for the small amount that results from dilution of the load solution so that the Ca^{2+} release could be visualized with minimal interference from Ca^{2+} uptake. Thus, in order to appropriately compare the K propionate release solution with a 1 : 20 release solution, the Mg^{2+} had to be eliminated from the modified load solution used for release, without altering the rest of the ionic composition. In these experiments, the individual components of the loading solution were altered slightly while the total ionic composition remained essentially the same (96 mM K propionate, 4 mM KCl, 36 mM imidazole, 2 mM MgATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase, 10 μM Fura, 1 mM Na azide) so that only one substitution (use of NaATP rather than MgATP) was necessary to modify the loading solution into a release solution. The "fast release" component (percent release) was eliminated except for a small baseline response (approximately 1% of total loaded calcium) when loaded triads were diluted into the modified load solution (Table 1, Load), although the slow release component remained. In contrast, 6% of the loaded calcium was released in less than 10 sec (Table 1, Release) upon 1 : 20 dilution into TEACl buffer.

The next series of experiments examined the effect of two release solutions on the fast phase of Ca^{2+} release. The two release solutions were designed to maintain constant $[K^+] \cdot [Cl^-]$ product upon either a 1 : 10 dilution (44 mM TEACl, 60 mM NMG propionate, 36 mM imidazole) or a 1 : 20 dilution (84 mM TEACl, 20 mM NMG propionate, 36 mM imidazole) of loaded vesicles. Based on the rapid change in K^+, the 1 : 20 dilution should produce a slightly larger depolarization (75 mV) than that asso-

Table 1. 1 : 20 Dilution into load solution *versus* release solution

Prep	Test	Ca^{2+} load (nmoles/mg)	Percent release	
			Load	Release
6/26	713RELF	248	1.91	5.51
			0.02	5.17
6/26	630RELF	248	−0.73	2.20
			1.77	4.59
6/26	628RELF	248	−1.06	12.39
			1.09	8.69
6/26	710RELF	248	5.10	6.54
			−0.48	8.01
10/25	7XXRELF	216	1.27	6.07
			1.77	5.49
		Mean ± SEM	1.06 ± 0.4	6.46 ± 0.6

Triads/terminal cisternae were loaded with calcium in a normal loading solution (solution A: 100 mM K propionate, 2 mM $MgCl_2$, 36 mM imidazole, 2 mM NaATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase, 1 mM Na azide, 10 μM Fura; *see* Materials and Methods) and diluted (1 part into 20) into either a modified load solution (96 mM K propionate, 4 mM KCl, 36 mM imidazole, 2 mM Na ATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase, 10 μM Fura, 1 mM Na azide) or a normal 1 : 20 release solution (solution C: 84 mM TEA chloride, 20 mM NMG propionate, 36 mM imidazole, 2 mM NaATP, 15 mM Na creatine phosphate, 15 units/ml creatine phosphokinase, 1 mM Na azide, 10 μM Fura; *see* Materials and Methods). Two different fluorescence ratios (340/380 and −380/358) were used to calculate the percent release (figure shown is the mean value). The mean release obtained in 10 sec differed significantly ($P > 0.001$; Student's *t*-test) in the two solutions.

Table 2. 1 : 10 Dilution *versus* 1 : 20 dilution (0.2 mM final total Mg^{2+})

Prep	Test	Ca^{2+} load (nmol/mg)	Percent release	
			1 : 10	1 : 20
6/26	712RELF	216	0.95	1.87
			1.70	1.56
6/26	718RELF	360	2.23	2.76
			1.01	3.95
6/26	721RELF	287	2.73	6.64
			2.60	6.09
6/26	728RELF	217	1.92	5.32
			1.84	6.94
10/25	725RELF	180	0.93	4.18
			2.70	2.78
		Mean ± SEM	1.86 ± 0.2	4.21 ± 0.4

Triads/terminal cisternae were loaded to the level indicated (Ca^{2+} load) in a K propionate buffer (Solution A: Table 1 or Materials and Methods). The loaded vesicles were either diluted one part in 10 (using Solution B: *see* Materials and Methods) or one part in 20 (using Solution C to which an additional 0.1 mM $MgCl_2$ was added: Materials and Methods) into TEACl release solutions. Two different Fura fluorescence ratios (340/380 and −380/358) were used to determine amount of Ca^{2+} release (number shown is the mean of the two determinations). The average amount of the total Ca^{2+} load released in 10 sec in each of the two different test conditions was significantly different at $P > 0.01$ (Student's *t*-test).

ciated with a 1 : 10 dilution (58 mV) according to the Nernst equation. Note that the final Mg^{2+} content would be 0.2 mM following the 1 : 10 dilution and only 0.1 mM following the 1 : 20 dilution. As mentioned previously, we intentionally removed Mg^{2+} from the release solution to minimize any antagonistic effect of Ca^{2+} uptake while we are attempting to measure Ca^{2+} release. There was a fourfold difference between the release measured in the 1 : 10 dilution *versus* the 1 : 20 dilution under these conditions (2.6% release at 1 : 10 dilution; 10.1% release at 1 : 20 dilution, *data not shown*). The possibility exists that the release at the 1 : 10 dilution was partially masked by a higher degree of Ca^{2+} uptake induced by the correspondingly higher Mg^{2+} concentration. In order to compensate for any effect that the increased Mg^{2+} might have on the individual releases, 0.1 mM Mg^{2+} was added to the 1 : 20 release solution, so that the total final Mg^{2+} content in both solutions was 0.2 mM. As shown in Table 2, the 1 : 20 dilution produced a larger percent release than that of the 1 : 10 dilution, either in the presence (4.2% *versus* 1.8% release) or absence of added Mg^{2+} in the 1 : 20 release solution.

In the experiments shown above, the measured "percent release" was considerably smaller in the 1 : 20 dilution where the Mg^{2+} concentration had effectively been doubled; however, this might simply reflect a preparation-to-preparation variation in the percentage of functional triads. To determine whether the presence of higher concentration of Mg^{2+} acted to effectively reduce Ca^{2+} release, comparisons were made using the same triad preparation and a 1 : 20 dilution of vesicles to initiate release (Table 3). In the preparations tested, no significant difference in percent release was observed when the Mg^{2+} content of the release solution was doubled to 0.2 mM final concentration. The contribution of the slow phase to the release measured in the fast phase was essentially the same for both conditions tested.

As mentioned in the Introduction, two additional pathways for Ca^{2+} release from SR have been described: Ca^{2+}-induced Ca^{2+} release and drug-induced Ca^{2+} release. The following experiments were designed to determine if either of these pathways contribute to the "depolarization-induced" release measured in these studies.

In general, the experimental conditions selected to elicit Ca^{2+} release were carefully designed to minimize any contribution by Ca^{2+}-induced Ca^{2+} release: free Ca^{2+} concentrations were in the submicromolar range and Mg^{2+} was present in at least a

Table 3. Effect of $[Mg^{2+}]$ on release

Prep	Test	Ca^{2+} load (nmol/mg)	Percent release Final total Mg^{2+} concentration	
			0.1 mM	0.2 mM
6/28	713RELF	613	4.16	4.59
			4.92	3.94
6/28	717RELF	532	5.84	7.07
			5.93	6.19
6/28	726ARELF	532	4.91	6.32
			3.05	3.78
6/28	726BRELF	532	2.74	0.85
			4.59	8.68
6/28	727RELF	532	4.58	2.44
			2.61	2.37
	Mean ± SEM		4.33 ± 0.4	4.62 ± 0.9

Triads/terminal cisternae were actively loaded with Ca^{2+} in Solution A (Materials and Methods) and diluted one part in 20 into a depolarizing TEACl buffer (Solution C; shown under 0.1 mM Mg^{2+} column) or into the same TEACl buffer plus 0.1 mM $MgCl_2$ (shown under 0.2 mM Mg^{2+} column). The percent of total Ca^{2+} loaded which was released in 10 sec is indicated under "Percent release" (determined using 340/380 Fura fluorescence ratio). The average slow phase contribution to the fast phase measurement was 5.46% (n = 10) at 0.1 mM Mg^{2+} and 4.58% (n = 10) at 0.2 mM Mg^{2+}, or within the SEM for these measurements. The average percent "fast" release obtained under these two test conditions was not significantly different (Student's t-test; P = 0.5).

Table 4. Release at pH 6.5 *versus* pH 7.0

Prep	Test	Ca^{2+} load (nmol/mg)	Percent release	
			pH 6.5	pH 7.0
5/25	717RELF	180	3.29	3.81
			7.91	7.47
10/25	55RELF	144	7.18	11.70
	420RELF	144	7.58	6.04
6/28	89RELF	532	9.48*	4.01*
			7.19*	7.01*
6/28	814RELF	456	14.04*	7.43*
			6.08*	4.15*
6/28	814CRELF	532	4.42	6.07
			2.98	3.78
6/28	821RELF	532	4.23	2.12
			5.76	4.26
6/28	926RELF	608	4.25*	7.20*
			8.43*	4.69*
6/28	926CRELF	532	4.85*	8.71*
			3.56*	3.60*
6/28	927RELF	684	5.29*	5.10*
			5.31*	4.91*
	Mean ± SEM		6.21 ± 0.6	5.67 ± 0.5

Triads/terminal cisternae were loaded with Ca^{2+} in Solution A at pH 7.0 (Materials and Methods) to the extent indicated (Ca^{2+} load) and diluted one part in 20 into Solution C (Materials and Methods) at either pH 6.5 or pH 7.0. Ca^{2+} release was determined using the 380/340 Fura fluorescence ratio for those experiments using the 6/28 triad prep, and both the 380/340 and −380/358 Fura ratios for the experiments using other triad preps (5/25 and 10/25). In those experiments where two Fura ratios were used, the number shown under percent release is the mean value obtained from these two ratios. Those numbers marked with an asterisk (*) had the final calculated Ca^{2+} release adjusted by a factor in order to obtain the correct calcium calibration in the release solution. The slow phase contribution to the fast phase measurement was estimated to be 8.87% at pH 6.5 (n = 18) and 3.02% at pH 7.0 (n = 18), both within the 10% SEM tabulated. There was no significant difference in the average "fast" release obtained at pH 6.5 *versus* pH 7.0 (Student's t-test; P = 0.5).

0.1 mM concentration. Recent work (Rousseau & Pinkos, 1990) has suggested that Ca^{2+}-induced Ca^{2+} release is pH dependent, showing complete inhibiton at pH 6.5 and maximum stimulation at pH 7.5. Under normal conditions, our release experiments were performed at pH 7.0. In Table 4, vesicles were loaded and diluted into release solution at two different pHs: 6.5 and 7.0. The lower pH had no effect on the rapid phase of release, as shown in the Table. However, if the vesicles were particularly leaky and subject to spontaneous release after few loads, the lower pH appeared to decrease the rate of the slow phase of release. If the vesicles were very "tight" and not susceptible to spontaneous release, the lower pH had a negligible effect on either of the release phases (*data not shown*).

Caffeine has been shown to enhance Ca^{2+} release from SR in the absence of T-tubule depolarization in skinned fibers (Donaldson, 1985), as well as in isolated heavy SR vesicles (Kim et al., 1983; MacLennan et al., 1983; Rousseau et al., 1988). This seems to imply that caffeine has some direct effect on the Ca^{2+} release channel which might be independent of T-tubular control. In the next series of experiments (Table 5), loaded triadic vesicles and terminal cisternae were diluted into a 1 : 20 release solution (84 mM TEACl, 20 mM NMG propionate, 36 mM imidazole, etc.) which was compared to the same solution with an additional 5 mM caffeine. There was no effect of caffeine at this concentration on the fast phase of release, although an enhanced slow phase of release was observed (percent slow phase contribution). A small effect of 5 mM caffeine on the fast phase of release was observed when loaded vesicles were diluted into a nondepolarizing solution (*see* legend to Table 5).

Morphological estimates of the number of coupled vesicles in various preparations used in these studies were made using a new preparative technique as described in Materials and Methods. Vesicles from the different preparations were subjected to the same loading and release protocols which

Table 5. Effect of 5 mM caffeine on release

Prep	Test	Ca^{2+} load (nmol/mg)	Percent release	
			+Caffeine	−Caffeine
10/25	724RELF	180	7.37**	7.83**
			6.83**	7.61**
6/26	727RELF	239	4.62**	5.22**
				5.60**
6/26	3RC19RELF	504	6.22	5.42
			7.65	9.74
6/28	821RELF	532	4.78	5.34
			4.23	4.45
6/28	823RELF	608	3.50	3.94
			3.86	3.39
6/28	827RELF	608	2.81	3.69
			4.79	3.60
	Mean ± SEM		5.15 ± 0.5	5.48 ± 0.6

Triads/terminal cisternae were actively loaded with calcium to the extent noted under "Ca^{2+} load" in Solution A (Materials and Methods). Loaded vesicles were then diluted one part in 20 into Solution C (Materials and Methods) with and without additional 5 mM caffeine ("+Caffeine" and "−Caffeine" columns). Calcium release was determined from two Fura fluorescence ratios in two experiments (340/380 and −380/358; number shown represents mean of the two determinations: marked by **) and by the 340/380 fluorescence ratio in the remainder. The slow phase contribution to the fast phase measurement was slightly greater in the presence of caffeine (14.6%; $n = 11$) than that measured without caffeine (8.4%; $n = 12$). The average percent "fast" calcium release in each of the two treatments was not significantly different (Student's t-test; $P = 0.5$). In additional experiments not tabulated above, 5 mM caffeine was tested using a nondepolarizing solution. In these experiments, vesicles diluted into a nondepolarizing solution without caffeine produced a 0.63 ± 0.1% fast release ($n = 3$, ±SEM), and a 1.15 ± 0.4% fast release in the presence of 5 mM caffeine.

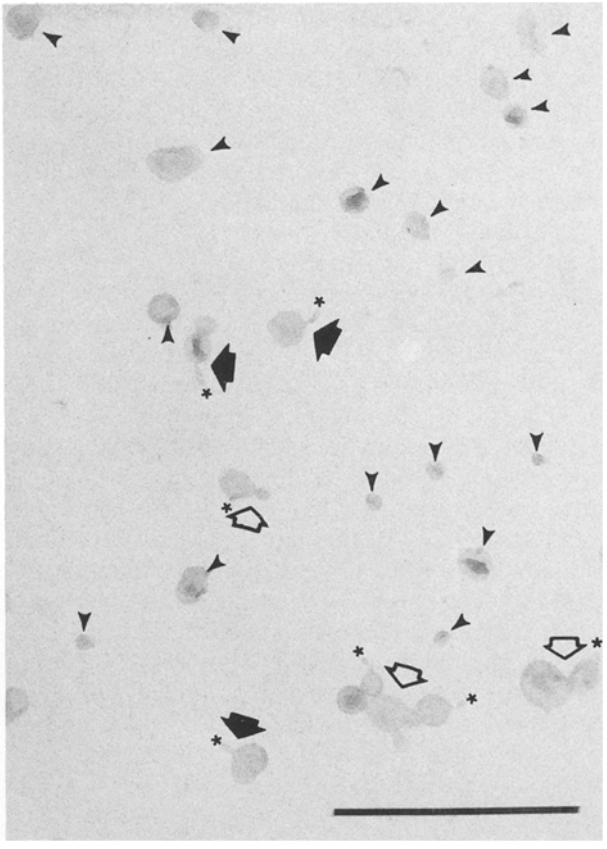

Fig. 5. Electron micrograph of the thin-resin embedded triad/terminal cisternae preparation. Along with coupled tubule-vesicle structures (solid arrows) and irregular or spherical attached vesicles (open arrows), there are many vesicles which are not attached to adjacent vesicles (arrowheads). Transverse tubules which have retained their original elongated morphology are indicated with an asterisk (*). Bar = 0.5 μm, Magnification: 65,000×.

were used in the above experiments and generally diluted to 40 μg/ml in order to obtain sufficiently dispersed vesicles after addition to coverslips. Samples were prepared for electron microscopy by applying a layer of LR white resin to the dehydrated coverslip and allowing the resin to harden. After the coverslip was etched away with hydrofluoric acid, the thin layer of vesicles embedded in resin were floated onto a grid and stained for transmission electron microscopy. This preparation allows entire vesicles to be visualized along with attached tubules (solid arrows, Fig. 5). There are also cases where irregular or spherical vesicles are attached (open arrows, Fig. 5) and many examples of vesicles not attached to any adjacent vesicles (arrowheads, Fig. 5). Transverse tubules, which retain their original elongated morphology, are indicated by asterisks (*); round vesicles are considered to be primarily terminal cisternae. Since in some instances, a swollen transverse tubule might appear to be round, those cases in which two rounded vesicles are attached are also indicated as possible triadic or diadic structures (open arrow). It is clear that most of the vesicles in this preparation are not attached to other vesicles. Quantitative analysis of four such preparations indicates that the triad preparations examined may have as few as 11–23% of the vesicles structurally coupled in a manner suggestive of triads or diads. This number would provide a high estimate of the number of functional triads or diads which are capable of depolarization-induced release.

Discussion

Before one is able to demonstrate Ca^{2+} release from a vesicular prep, one must first develop a method whereby the vesicles are loaded with Ca^{2+} and are able to maintain that load with a minimum of leak.

Traditionally, vesicles prepared from skeletal muscle have been somewhat leaky, so that the rate of calcium loading or total amount of Ca^{2+} loaded have been low. Various methods had been used in the past to minimize these problems. It was discovered that precipitating anions inside the vesicle could increase the rate of Ca^{2+} uptake and the amount of Ca^{2+} loaded (Martonosi & Feretos, 1964). However, once a critical level of calcium loading had been achieved in the presence of high phosphate, spontaneous Ca^{2+} release was observed after some time lag when extravesicular free Ca^{2+} had been reduced to the micromolar range (Palade, Mitchell & Fleischer, 1983). A number of recent papers (Meissner, 1984, 1986; Meissner et al., 1986) which examine Ca^{2+} release using triadic vesicles or heavy terminal cisternae employ passive Ca^{2+} loading rather than active or ATP-stimulated loading. The extent of loading is rather low for these vesicles (60–90 nmol Ca^{2+}/mg protein), requiring the use of radiolabeled Ca^{2+} for detection of release.

In order to ensure that the transverse tubules in our triadic preparation had repolarized before release was attempted, it was necessary for us to provide the vesicles with ATP, an ATP regenerating system, Na and K, so that the Na-K ATPase in the T-tubules might re-establish the normal ionic gradient and therefore the normal resting potential. This requirement and its low level of loading eliminated the passive loading regime for our studies. We had also observed that although we could load large amounts of Ca^{2+} into the vesicles using precipitating anions (Na pyrophosphate, Na oxalate), we were unable to observe any Ca^{2+} release upon dilution of the vesicles into a depolarizing solution. As we also preferred to conduct our studies in a system which was as close to physiological conditions as possible, we decided to use ATP-stimulated Ca^{2+} uptake into vesicles in this study. Taking care to prevent any osmotic damage to membrane vesicles during their preparation which might result in "leaky" vesicles, a preparation of triads and terminal cisternae were prepared which displayed reasonable Ca^{2+} uptake rates (Fig. 2) and minimum Ca^{2+} leak (Fig. 3, loaded vesicles generally displayed no Ca^{2+} leak for a period greater than 30 min following loading). If vesicles displayed a tendency to leak with time, the slow phase of their release (Fig. 3) generally had a steeper slope, an effect which could be reduced by a pH of 6.5. Also, loading "leaky" vesicles at pH 6.5 allowed one to load to a greater extent than possible at pH 7.0 without inducing spontaneous release. In general, unloaded vesicles had very little stored Ca^{2+} which remained after the extensive membrane preparation protocol as indicated by negligible release upon dilution into depolarizing solution (Fig. 3*A* and *B*, *Release 2*). Therefore, we were able to express release as a percentage of total amount of Ca^{2+} which was actively loaded into the preparation by the addition of known amounts of Ca^{2+} standard, without overestimating due to unaccounted stored Ca^{2+}.

It was essential that we attempt to differentiate the Ca^{2+} release which was observed upon dilution of vesicles into a depolarizing solution from: (i) osmotically induced release from vesicular swelling or breakage, (ii) Ca^{2+}-induced Ca^{2+} release, and (iii) a release induced by a direct effect on sarcoplasmic reticulum vesicles (e.g. drug-induced release). By designing the loading solution and release solution so that a constant $[K^+] \cdot [Cl^-]$ product was maintained at a given dilution, as well as balancing the two solutions so that the load and release solution had the same osmolarity (determined independently by an osmometer), we believe that we eliminated any possibility of release due to osmotic shock. To ensure this, we tested the dilution of loaded vesicles into release solution which contained 2 mM Mg^{2+} and a full ATP regenerating system. If the vesicles released any calcium due to breakage or swelling, there should be a lower rate of calcium uptake than that seen previously, as the vesicles should become "leaky." First, we found that there was no detectible fast or slow Ca^{2+} release, possibly due to the large number of vesicles capable of taking up Ca^{2+} (over 80%, Fig. 5) and the small number of vesicles capable of releasing it (less than 20%, Fig. 5 and Tables). We found that Ca^{2+} uptake was only limited by the concentration of vesicles following dilution into the release solution, greatly reducing the possibility that osmotically induced Ca^{2+} release had occurred. Finally, osmotically induced release would effect vesicles capable of loading calcium indiscriminately. Under normally used conditions of release (no Mg^{2+} in release solution to minimize Ca^{2+} uptake), the loaded vesicles (primarily SR in the triadic preparation and control longitudinal reticulum vesicles) would be expected to produce a large rapid release when compared to the total amount of Ca^{2+} loaded if osmotic shock had occurred. Although both vesicle types displayed slow Ca^{2+} release, we only observed a rapid release phase with triadic vesicles, not with loaded longitudinal reticulum. Additionally, this rapid release accounted for only a small portion (4–10%) of the total loaded calcium, which argues against the possibility that the fast release observed in these studies was produced by osmotic shock.

The use of 10 μM Fura as a calcium indicator allowed us to monitor calcium release at submicromolar free Ca^{2+} concentration, a condition which in itself would severely inhibit any Ca^{2+}-induced Ca^{2+}

release. A Mg^{2+} concentration of 1.5 mM has been shown to inhibit Ca^{2+}induced Ca^{2+} release in the presence of 1 mM adenine nucleotide and 10 μM Fura (Meissner, 1984). When releases at two different Mg^{2+} concentrations (0.1 and 0.2 mM) were compared, no significant difference in "fast" release was found using the 1 : 20 dilution protocol. It is not clear if the small amount of total Mg^{2+} (0.2 or 0.1 mM) used in our release studies would be expected to have any large inhibitory effect on Ca^{2+}-induced Ca^{2+} release in the presence of 2 mM Na ATP, but doubling the Mg^{2+} concentration might be expected to have some small effect. The lack of an effect, therefore, might be used to distinguish our "fast" release from Ca^{2+}-induced Ca^{2+} release. Finally, Ca^{2+} release channels have been shown to be very pH dependent: at low pH (6.5) the channel activation is low, whereas at physiological pH (7.0–7.4) the probability of the channel being open is high (Rousseau & Pinkos, 1990). This pH effect is also evident in Ca^{2+}-induced Ca^{2+} release (Meissner & Henderson, 1987). However, the "fast" release in our studies was not affected by an extravesicular pH of 6.5, whereas Ca^{2+}-induced Ca^{2+} release would have been inhibited. Thus, based on these three criteria, we believe that the "fast" release observed in our studies is distinct from Ca^{2+}-induced Ca^{2+} release.

Various agents, such as caffeine and quercetin, have been shown to act directly on SR to stimulate Ca^{2+} release (Miyamoto & Racker, 1982; MacLennan et al., 1983). Ca^{2+} release was tested in the presence or absence of 5 mM caffeine, and no effect on "fast" calcium release was observed under depolarizing conditions, although a slightly enhanced release rate in the second slow phase of release was observed. In similar studies by Rousseau et al. (1988), only a small stimulation of Ca^{2+} release (rates of approximately 0.5–1/sec) by 5 mM caffeine was observed at nanomolar Ca^{2+} levels. We observed a small increase in the fast release when loaded vesicles were diluted into a nondepolarizing solution containing 5 mM caffeine *versus* control (minus caffeine). With the limited number of experiments performed, it is not clear if this difference is significant. In any event, our "fast" calcium release was not affected by caffeine under depolarizing condition and, thus, appears to be different from "drug" induced effects on the sarcoplasmic reticulum.

One would expect that depolarization-induced release would display some voltage-dependence (i.e., a small dilution which would produce little depolarization might not produce any fast release). We found that a 1 : 10 dilution (expected to produce a 58 mV depolarization) of loaded vesicles produced very little fast release (Table 2: approximately 2% of the total load), comparable to that of control longitudinal reticulum (Table 6) and dilution of loaded triadic vesicles into modified load solution (Table 1), whereas a 1 : 20 dilution (expected to produce a 75 mV depolarization) more than doubled the percentage of Ca^{2+} released rapidly. Using single muscle fibers, it was determined that Ca^{2+} release displays a steep voltage dependence once the threshold potential is reached but levels off for somewhat larger depolarizations (Melzer et al., 1986). Thus, once a dilution that produces a reasonably large response is obtained, it is unlikely that larger dilution (hence a larger depolarization) would produce a much greater response. When dilutions larger than 1 : 20 were attempted, it was found that little increase in the percent fast release was observed (*data not shown*) and, due to the small response, the system noise made more of a contribution to error in the measurements. For this reason, various conditions were tested at a single dilution of 1 : 20 in order to produce a depolarization-induced release. The time course of the "fast" release reported here is relatively slow when compared with physiological depolarization-induced release. However, the release of Ca^{2+} in the present studies was performed by manually mixing a small aliquot of loaded vesicles into its release solution at the proper dilution. Hence, the rate of the response seen in these studies is simply a reflection of the speed with which the vesicles were mixed into the altered ionic environment. Even additions of large amounts of calcium to the loading solution required seconds to obtain the full fluorescent response due to manual mixing. Although we would

Table 6. 1 : 20 Release using longitudinal reticulum

Prep	Test	Ca^{2+} load (nmol/mg)	Percent release
10/25	LR1RELF	604	3.45
			2.30
10/25	LR2RELF	704	2.54
			1.68
10/25	LR3RELF	626	2.11
			1.22
10/25	LR4RELF	704	1.61
			1.33
10/25	LR5RELF	704	2.12
			1.75
	Mean ± SEM		2.01 ± 0.21

Longitudinal reticulum were actively loaded with Ca^{2+} using Solution A (Materials and Methods), and then diluted one part in 20 into Solution C (Materials and Methods). Virtually no "fast" release phase was observed with this vesicle preparation (*see* Fig. 2*B*). The contribution of the slow phase to this measured release is 53.7%, which would reduce the fast phase measurement to 0.93%: an amount similar to that observed for a dilution of triadic vesicles into a modified load solution (Table 1).

have preferred to use a stopped-flow apparatus for these studies in order to obtain the true time course of the response, the largest dilution obtainable in standard stopped-flow devices at the time of this study was 1 : 10, a dilution which was incapable of producing a maximal depolarization-induced response in our studies.

Various attempts were made to assess the role of the transverse tubule in the depolarization-induced release observed in these studies. In one series of experiments, we attempted to poison the transverse tubule Na-K ATPase with a lipid soluble form of ouabain (digitoxin), but found that we were unable to complete vesicle loading before spontaneous release occurred. In an alternate series of experiments, we passed a triad mixture through a french press to mechanically separate transverse tubule from sarcoplasmic reticulum, then spun the mixture on a continuous sucrose gradient to assess the separation achieved. ^{3}H-nitrendipine binding, used to localize transverse tubules in the gradient, indicated that full separation of transverse tubules was not achieved in two different trials. Furthermore, the reisolated vesicles which had been exposed to the french press were incapable of loading Ca^{2+}, whereas untreated vesicles had demonstrated Ca^{2+} loading capability and acceptable stability following loading. It was postulated that some aspect of the french press treatment (either exposure to mechanical sheer force and/or additional exposure to sucrose gradients) made the vesicles too leaky for further use in our studies.

Electron microscopy was used in an attempt to correlate the number of coupled vesicles (triadic or diadic) with the "fast" release observed in our fluorescent assays. In normal thin-section procedures, it would be difficult to count the number of coupled vesicles since the plane of the cut determines what one is able to see. A triad with a vertical orientation might appear to be a single vesicle when the thin section is cut on a horizontal plane. In order to effectively count all coupled vesicles, we had to use a method by which we could unambiguously see all of a sample of vesicles in one plane, yet thin enough to use transmission electron microscopy and stain so that structures might be clear. The coverslip technique employed in this paper (*see* Materials and Methods; Fig. 5) met these criteria. We were careful to use vesicles that had been loaded in a normal manner and diluted into release solution for visualization of coupled vesicles, so that any component of the loading or release solutions that might affect the coupling between transverse tubules and terminal cisternae would be controlled for. Basically, less than 20% of all vesicles in any field were found to be coupled to either one or two vesicles (this number was obtained for a preparation which gave us 10–12% fast release in fluorescence assays). This number would be expected to provide a high estimate for the number of functional triads or diads, since some vesicles might be nonspecifically coupled or some triads or diads might be functionally damaged so that depolarization-induced release is not possible.

In order to correlate the data from the fluorescence studies with the estimates of coupled vesicles obtained through electron microscopy, one must make certain assumptions about the nature of vesicular loading and release. If, for example, one assumes that triadic or diadic terminal cisternae load and release calcium at the same rate as terminal cisternae that are uncoupled to transverse tubules, and that all of the vesicular calcium is released in these stimulated vesicles, then the percent of the total calcium loaded which is released in the "fast" phase should correlate exactly with the number of functional triads or diads. If, however, alternate situations exist (the stimulated vesicles do not release **all** of their stored calcium rapidly or the terminal cisternae which are coupled to transverse tubules load less calcium than isolated terminal cisternae), then the percent of "fast" release would be a low estimate of the number of functional coupled vesicles. Given all of these possibilities, we can say that at least 4–10% of the loaded vesicles are functionally coupled so that they can respond to a "depolarizing" stimulus, implying that the presence of transverse tubules are essential for the depolarization-induced response.

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