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Functional Characterization of Junctional Terminal Cisternae from Mammalian Fast Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: Junctional terminal cisternae are a recently isolated sarcoplasmic reticulum fraction containing two types of membranes, the junctional face membrane with morphologically intact "feet" structures and the calcium pump membrane [Saito, A., Seiler, S., Chu, A., & Fleischer, S. (1984) *J. Cell Biol. 99*, 875–885]. In this study, the Ca^{2+} fluxes of junctional terminal cisternae are characterized and compared with three other well-defined fractions derived from the sarcotubular system of fast-twitch skeletal muscle, including light and heavy sarcoplasmic reticulum, corresponding to longitudinal and terminal cisternae regions of the sarcoplasmic reticulum, and isolated triads. Functionally, junctional terminal cisternae have low net energized Ca^{2+} transport measured in the presence or absence of a Ca^{2+}-trapping anion, as compared to light and heavy sarcoplasmic reticulum and triads. Ca^{2+} transport and Ca^{2+} pumping efficiency can be restored to values similar to those of light sarcoplasmic reticulum with ruthenium red or high $[Mg^{2+}]$. In contrast to junctional terminal cisternae, heavy sarcoplasmic reticulum and triads have higher Ca^{2+} transport and are stimulated less by ruthenium red. Heavy sarcoplasmic reticulum appears to be derived from the nonjunctional portion of the terminal cisternae. Our studies indicate that the decreased Ca^{2+} transport is referable to the enhanced permeability to Ca^{2+}, reflecting the predominant localization of Ca^{2+} release channels in junctional terminal cisternae. This conclusion is based on the following observations: (1) The Ca^{2+},-Mg^{2+}-dependent ATPase activity of junctional terminal cisternae in the presence of a Ca^{2+} ionophore is comparable to that of light sarcoplasmic reticulum when normalized for the calcium pump protein content; i.e., the enhanced Ca^{2+} transport cannot be explained by a faster turnover of the pump. (2) Ruthenium red or elevated $[Mg^{2+}]$ enhances energized Ca^{2+} transport and Ca^{2+} pumping efficiency in junctional terminal cisternae so that values approaching those of light sarcoplasmic reticulum are obtained. (3) Rapid Ca^{2+} efflux in junctional terminal cisternae can be directly measured and is blocked by ruthenium red or high $[Mg^{2+}]$. (4) Ryanodine at pharmacologically significant concentrations blocks the ruthenium red stimulation of Ca^{2+} loading. Ryanodine binding in junctional terminal cisternae, which appears to titrate Ca^{2+} release channels, is 2 orders of magnitude lower than the concentration of the calcium pump protein. (5) By contrast, light sarcoplasmic reticulum has a high Ca^{2+} loading rate and slow Ca^{2+} efflux that are not modulated by ruthenium red, ryanodine, or Mg^{2+}. These studies lead to the conclusion that Ca^{2+} release channels, likely the ones important in excitation–contraction coupling, are localized predominantly in the junctional terminal cisternae. The possible coupling of Ca^{2+} pumping and its relationship to Ca^{2+} release channels are discussed.

The sarcoplasmic reticulum (SR)[1] of muscle is an internal membranous network controlling Ca^{2+} release and uptake, which, in turn, brings about contraction and relaxation [reviews by Endo (1977), Berman (1982), Martonosi (1984), and Inesi (1985)]. The skeletal SR membrane is composed mainly of two morphologically distinct regions, longitudinal tubules and terminal cisternae; the latter are junctionally associated with the transverse tubule via bridging "feet" structures at the triads (Franzini-Armstrong, 1980). The uptake of Ca^{2+} into the lumen of the SR, mediated by the calcium pump protein, enables the muscle to relax. Muscle contraction is triggered by the release of Ca^{2+} from the terminal cisternae via the

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[1] Abbreviations: SR, sarcoplasmic reticulum; RR, ruthenium red; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CaATPase, Ca^{2+},Mg^{2+}-dependent ATPase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; $[Ca^{2+}]_f$ and $[Mg^{2+}]_f$, free (not chelated) Ca^{2+} and Mg^{2+} concentrations, respectively; Tris, tris(hydroxymethyl)aminomethane.

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transverse tubule in response to an action potential at the sarcolemma. However, the nature of the signal transmitted across the triad junction and the molecular events underlying Ca^{2+} release from the SR in excitation–contraction coupling are poorly understood (Endo, 1977; Martonosi, 1984).

Fractions referable to terminal cisternae and longitudinal tubules can be isolated by using density gradient centrifugation, and the two types of vesicles express distinct functional characteristics reflecting their origin in the intact muscle. In general, light SR is obtained at about 28–33% sucrose and heavy SR at about 38–43% sucrose (Meissner, 1975; Louis et al., 1980; Campbell et al., 1980; Caswell & Brandt, 1981; Miyamoto & Racker, 1982; Watras & Katz, 1984). Heavy SR contains electron-dense material within the lumen (Meissner, 1975), consisting mainly of Ca^{2+}-binding protein, also referred to as calsequestrin (MacLennan et al., 1983). Such electron-dense material is diagnostic of the terminal cisternae region in situ (Fleischer et al., 1979; Jorgensen et al., 1983). Recently, we have isolated a junctional terminal cisternae preparation with morphologically intact "feet" structures, corresponding to the terminal cisternae in situ (Saito et al., 1984). The "feet" structures occupy 15–20% of the vesicular membrane surface area. This distinct portion of the membrane is referred to as the junctional face membrane, in contrast to the other nonjunctional portion of the membrane referred to as the calcium pump membrane (Fleischer, 1985).

In this study, the junctional terminal cisternae preparation has been characterized with regard to net Ca^{2+} transport and efflux and compared with other well-defined SR fractions, heavy SR, triads, and light SR. The results are consistent with the hypothesis that a unique Ca^{2+} efflux pathway is localized in the terminal cisternae, in contrast with the longitudinal region of the sarcotubular system. A preliminary report of this paper has been presented (Chu et al., 1984).

Materials and Methods

Isolation of Sarcoplasmic Reticulum. All SR fractions were prepared from fast skeletal leg muscles of female New Zealand White rabbits. Junctional terminal cisternae and a light SR fraction from the same gradient (R2) were isolated as described by Saito et al. (1984). Mitochondrial contamination of junctional terminal cisternae was 2–3%, as estimated by succinate-cytochrome *c* reductase activity (Fleischer & Fleischer, 1967). Isolated triads were prepared as previously described (Mitchell et al., 1983); the pyrophosphate variant of a stage II procedure (band 4) was used. Light and heavy SR vesicles were prepared according to Meissner (1975), with modification, in which a 0.6 M KCl step was included in the first zonal centrifugation in addition to the regular salt-extraction step before the final centrifugation.

Ca^{2+} Transport Assays. Ca^{2+} uptake (in the absence of a Ca^{2+}-trapping anion) and loading (in the presence of phosphate) were measured by several methods. A metallochromic indicator (Scarpa, 1979), antipyrylazo III (Sigma Chemical Co., St. Louis, MO), was used to continuously monitor Ca^{2+} flux at the wavelength pair of 710 and 790 nm in a Hewlett-Packard UV/vis spectrophotometer, Model 8450A (Atlanta, GA). For Ca^{2+} loading, the reaction medium contained 200 μM antipyrylazo III, varying amounts of phosphate (phosphoric acid–KOH, pH 7.0), 5 mM NaN_3, various concentrations of $MgCl_2$, 1 mM Na_2ATP, and 50 μM $CaCl_2$.

A filtration assay with $^{45}CaCl_2$ was also used to measure Ca^{2+} flux. For $^{45}Ca^{2+}$ uptake, the reaction medium at 25 °C contained 25 or 50 μM $^{45}CaCl_2$ (specific activity of 6000–11 000 cpm/nmol, ICN Pharmaceuticals, Inc., Irvine, CA), 100 mM KCl, varying amounts of $MgCl_2$, 10 mM K-HEPES (pH 7.0), 5 mM NaN_3, and 150 μg of SR protein/mL. The reaction was initiated by the addition of 1 mM Na_2ATP. At appropriate time intervals, 200-μL aliquots of reaction mixture were passed through Millipore filters (0.22 μm, type GSWP; Bedford, MA), and the filters were washed immediately with 2 mL of ice-cold 100 mM KCl/10 mM K-HEPES (pH 7.0).

For $^{45}Ca^{2+}$ loading assay, under constant $[Ca^{2+}]_f$, $[Mg^{2+}]_f$, and MgATP levels described in the figure legends, the standard reaction medium contained 5 mM Tris–EGTA (pH 7.0), 5 mM NaN_3, different amounts of $MgCl_2$ and $CaCl_2$, and about 100 μg of SR protein/mL. Each reaction also contained $^{45}CaCl_2$ (2 μL of a 4 mCi/mL stock solution, the specific activity varied for each pCa condition). Prior to the initiation of the reaction at 25 °C, phosphate (phosphoric acid–KOH, pH 7.0) was added to give a final concentration of 100 mM. Various amounts of Na_2ATP were used to start the reaction in order to maintain a constant level of MgATP. At appropriate time intervals, aliquots were filtered and washed with 2 mL of ice-cold distilled water.

All filters were subsequently dissolved in Bray's solution, and the radioactivity was measured by liquid scintillation counting. Nonspecific binding of $^{45}Ca^{2+}$ to filters and to SR protein was subtracted. The rates of $^{45}Ca^{2+}$ loading were calculated by taking the initial linear phases of time courses during $^{45}Ca^{2+}$ loading and analyzing the slopes by linear regression.

$^{45}Ca^{2+}$ Influx Assay. $^{40}Ca^{2+}$ loading was initiated by 1 mM Na_2ATP in a reaction mixture containing 100 mM phosphate (pH 7.0), 5 mM NaN_3, 80–100 μg of SR protein/mL, and 3 μM $[Ca^{2+}]_f$ and 0.1 mM $[Mg^{2+}]_f$ maintained by 5 mM Tris–EGTA, as described above. At the desired time interval, pulses of tracer $^{45}CaCl_2$ (2 μL of 4 mCi/mL) were added to ongoing $^{40}Ca^{2+}$ loading reactions and 200-μL aliquots were rapidly filtered and washed (Katz et al., 1980). The filters were then processed for liquid scintillation counting. A control $^{45}Ca^{2+}$ loading reaction was carried out separately for comparison. According to the procedure, the "influx" rate is obtained by measuring the uptake of the pulsed $^{45}Ca^{2+}$.

$^{45}Ca^{2+}$ Efflux after Passive Loading. SR (8–10 mg of protein/mL) was loaded with 5 mM $^{45}CaCl_2$ overnight at 4 °C in a medium containing 100 mM KCl and 10 mM K-HEPES (pH 7.0). The SR vesicles were then diluted about 100-fold into 12-mL isoosmolar solutions of various constant $[Ca^{2+}]_f$ and $[Mg^{2+}]_f$, which were preadjusted in the presence of 5 mM K-EGTA and 20 mM K-HEPES (pH 7.0) at 25 °C. Immediately, 950-μL aliquots were passed through Millipore filters, and the filters were washed with 2 mL of ice-cold 20 mM $MgCl_2$/10 mM $LaCl_3$ (Kim et al., 1983) and counted by liquid scintillation. For time zero data points, the 950-μL aliquots were delivered into test tubes containing the Mg^{2+}/La^{3+} quench solutions before filtering. The half-time ($t_{1/2}$) of $^{45}Ca^{2+}$ efflux was obtained from a semilog plot of the efflux time course with regression analysis of the linear slope. The equation used was based on first-order kinetics (Segel, 1975): $\log s = kt/2.3 + \log s_0$, where s = substrate ($^{45}Ca^{2+}$ retained in SR), t = time, k = rate constant, and s_0 = $^{45}Ca^{2+}$ in SR at zero time.

Rapid Kinetics of Passive $^{45}Ca^{2+}$ Efflux. A Biologic rapid filtration system (distributed by CosmoLogic, Pullman, WA) was used. Junctional terminal cisternae (1 mg of protein/mL) were preincubated in 80 mM KCl, 10 mM MOPS (pH 6.8), and 1 mM $^{45}CaCl_2$ (~8000 cpm/nmol) at room temperature for at least 2.5 h. An 80-μL aliquot containing 80 μg of protein was added to 1 mL of a "nonrelease" medium [5 mM $MgCl_2$, 10 mM MOPS (pH 6.8), 1 mM EGTA, 80 mM KCl], and

then the mixture was transferred onto a 0.45-μm Millipore filter (type HAWP). After 10 s in this medium, a filtration buffer was forced through the sample at preset rates (3 mL/s at 100 ms to 1 mL/s at 2 s). The filtration buffer contained 80 mM KCl, 10 mM MOPS (pH 6.8), and 3.5 μM $[Ca^{2+}]_f$ (using 0.5 mM K-EGTA buffer) in the absence or presence of 10 μM ruthenium red. The radioactivity on the filters was measured by liquid scintillation counting.

Calculations of Free and Bound Ligands. $[Ca^{2+}]_f$, $[Mg^{2+}]_f$, and [MgATP] were calculated by computer programs using association constants of various ligands provided by Fabiato and Fabiato (1979). For example, the K_{app}'s of Ca^{2+}, Mg^{2+}, K^+, and Na^+ complexes of ATP (5.09×10^3, 1.14×10^4, 4.46, 6.53), phosphate (33.13, 50.14, 2.04, 2.64), and EGTA (2.51×10^6, 40.48, 0, 0), respectively, were determined for pH 7.0. The constants at pH 6.8 were derived similarly. The K_{app}'s of Ca^{2+} and Mg^{2+} complexes of antipyrylazo III (8.33×10^3, 3×10^3) were derived from the literature (Scarpa et al., 1978; Scarpa, 1979; Durham & Walton, 1983). For $^{45}Ca^{2+}$ loading, the standard reaction medium contained 100 mM phosphate (pH 7.0), 5 mM Tris–EGTA (pH 7.0), and 5 mM NaN_3. For $^{45}Ca^{2+}$ efflux, the standard reaction medium contained 5 mM K-EGTA and 20 mM K-HEPES (pH 7.0).

CaATPase Activity. A coupled-enzyme assay monitoring NADH oxidation at 340 nm was used to measure ATPase activity at 25 °C (Schwartz et al., 1969) in a reaction medium containing 0.1 or 5 mM $[Mg^{2+}]_f$, respectively, 5 mM Tris–EGTA, 100 mM phosphate (phosphoric acid–KOH, pH 7.0), and 5–10 μg of SR protein/mL. The reaction was started by 1 mM Na_2ATP to give a final [MgATP] of 0.45 or 1 mM, respectively. The CaATPase rate was the difference between the rate obtained from the linear portion of total ATPase activity curve (in the presence of 3 μM $[Ca^{2+}]_f$) and the rate obtained from the linear portion of basal ATPase activity curve (in the absence of Ca^{2+} and in the presence of 5 mM Tris–EGTA). The coupled-enzyme system of pyruvate kinase and lactic dehydrogenase used was obtained in lyophilized powder form (salt free; Sigma), and was reconstituted in 100 mM KCl and 20 mM Tris–MOPS (pH 7.0).

[^{3}H]Ryanodine Binding. The binding of [^{3}H]ryanodine to junctional terminal cisternae was carried out as described by Fleischer et al. (1985).

Protein was estimated according to the method of Lowry et al. (1951) with bovine serum albumin as standard. All experiments were repeated at least 3 times, and when appropriate, the values are expressed as the mean ± SE (standard error of the mean) unless indicated otherwise. The Ca^{2+} ionophore A23187 (Calbiochem-Behring, La Jolla, CA) was dissolved in 95% ethanol. Ruthenium red was used as purchased (Sigma). Its molecular weight was taken as 786.35 (Stimers & Byerly, 1982), and the concentration was adjusted for the purity as indicated on the Sigma label. Ruthenium red was also purified according to Luft (1971). There was no difference in the Ca^{2+} loading rates using purified vs. nonpurified ruthenium red; i.e., the ratio (+RR/−RR) using purified vs. nonpurified ruthenium red was 0.99 ± 0.05, n = 4.

Results

Ca^{2+} Transport by Junctional Terminal Cisternae and Other SR Fractions. In the absence of Ca^{2+}-precipitating anions, the preparation of junctional terminal cisternae accumulated a small amount of Ca^{2+} at 1 mM $MgCl_2$, but the uptake of Ca^{2+} could be enhanced severalfold by adding ruthenium red (Figure 1). The accumulated Ca^{2+} was released by adding the Ca^{2+} ionophore A23187 in the presence or absence of ruthenium red, indicating that the accumulated Ca^{2+} had been translocated into the SR compartment.

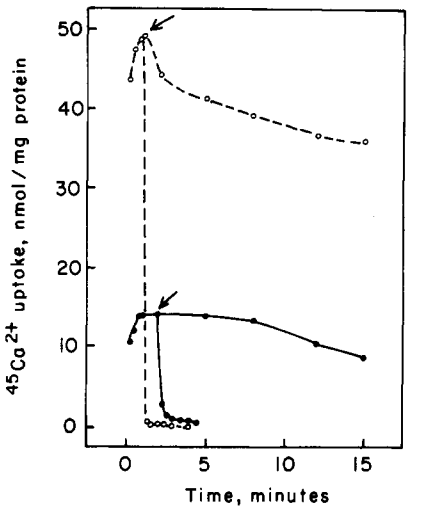

FIGURE 1: Ca^{2+} uptake by junctional terminal cisternae vesicles. $^{45}Ca^{2+}$ uptake was measured by the filtration assay at 25 °C (Materials and Methods) under the conditions of 1 mM $MgCl_2$, 5 mM NaN_3, 10 mM K-HEPES (pH 7.0), 100 mM KCl, and 50 μM $^{45}CaCl_2$. Immediately after 150 μg of SR protein was added, Na_2ATP was used to start the reaction mixture in the presence (○) and absence (●) of 20 μM ruthenium red. The Ca^{2+} ionophore, 1.5 μg of A23187/mL (2.9 μM), was added (arrow) to release accumulated $^{45}Ca^{2+}$. Ruthenium red stimulated peak $^{45}Ca^{2+}$ accumulation by 3.7- (±0.6) fold (n = 3) compared to the control of 14 ± 4 nmol of $^{45}Ca^{2+}$/mg of protein, n = 3. Peak Ca^{2+} accumulation is defined as the maximal Ca^{2+} uptake. The $[Mg^{2+}]_f$, $[Ca^{2+}]_f$, and [MgATP] were estimated to be 0.3 mM, 25 μM, and 0.7 mM, respectively.

Table I: Ca^{2+} Uptake of Vesicles Derived from Different Regions of SR[a]

SR fraction	$[MgCl_2]$ (mM)	peak Ca^{2+} accumulation (nmol/mg of protein) no RR	with RR	ratio (+RR/−RR)	n
junctional terminal cisternae	1	7 ± 1	37 ± 5	5.0 ± 0.2	4
	10	18 ± 2	39 ± 6	1.8 ± 0.3	4
triads	1	16	31	1.9	1
	10	36	44	1.2	1
heavy SR	1	17 ± 4	33 + 5	2.0 ± 0.3	3
	10	41 ± 2	47 ± 8	1.1 ± 0.1	3
light SR	1	42 ± 6	44 ± 6	1.1 ± 0.1	3
	10	56 ± 2	66 ± 11	1.2 ± 0.1	3

[a] Ca^{2+} uptake was measured by $^{45}Ca^{2+}$ with a filtration procedure (Materials and Methods). The reaction mixture contained 25 μM $^{45}CaCl_2$, 100 mM KCl, 10 mM K-HEPES (pH 7.0), 5 mM NaN_3, 1 mM Na_2ATP, 1 or 10 mM $MgCl_2$, and 150 μg of SR protein/mL.

On the other hand, Ca^{2+} uptake by other SR preparations derived from terminal cisternae (heavy SR and triads) was higher as compared with the junctional terminal cisternae vesicles, particularly at the lower 1 mM $MgCl_2$ (Table I). Ruthenium red stimulated the Ca^{2+} uptake of both heavy SR and triads 2-fold, whereas junctional terminal cisternae were enhanced 5-fold. In contrast, Ca^{2+} uptake by light SR was not enhanced by ruthenium red. At the higher $MgCl_2$ concentration (10 mM), the uptake by each terminal cisternae derived SR preparation appeared to approach equivalent uptake capacity in the presence of ruthenium red and the stimulation by the polyvalent cation was less. Light SR accumulated somewhat higher amounts of Ca^{2+} than the preparations derived from terminal cisternae at both 1 and 10 mM $MgCl_2$.

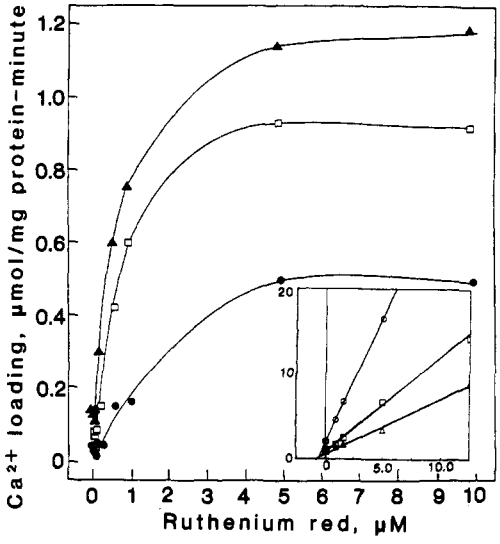

FIGURE 2: Effect of Mg^{2+} on ruthenium red stimulation of Ca^{2+} loading of junctional terminal cisternae. Phosphate-supported Ca^{2+} loading was measured in the presence of the metallochromic dye, antipyrylazo III, as described under Materials and Methods. The reaction mixture contained 100 mM phosphate (phosphoric acid–KOH, pH 7.0), 5 mM NaN_3, 200 μM antipyrylazo III, 1 mM Na_2ATP, and 50 μg of SR protein/mL, at 25 °C, and was started by 50 μM $CaCl_2$. $MgCl_2$ concentration was varied as follows: (●, ○) 1, (□) 3, and (▲, △) 5 mM. The $[Mg^{2+}]_f$, $[Ca^{2+}]_f$, and [MgATP] were estimated to be 93, 7, and 0.44 μM at 1 mM $MgCl_2$; 360, 9, and 0.8 μM at 3 mM $MgCl_2$; and 690, 11, and 0.9 μM at 5 mM $MgCl_2$, respectively. The inset represents a double-reciprocal plot of the data. The K_m for ruthenium red varies from 0.6 to 1.0 μM and is not significantly different (linear correlation coefficients are 0.998 or better).

We further examined Ca^{2+} loading of junctional terminal cisternae, as compared to that of heavy SR and triads, in the presence of phosphate, a Ca^{2+}-trapping agent, in order to reduce the Ca^{2+} leakage rate (Table II). Ruthenium red and elevated concentrations of Mg^{2+} and phosphate were found to enhance the Ca^{2+} loading rates. For each of the terminal cisternae derived SR fractions, in the presence of ruthenium red, the Ca^{2+} loading rates at 1 mM $MgCl_2$ were equivalent to or approached those at higher $MgCl_2$ in the absence of ruthenium red. The enhanced Ca^{2+} loading rate at 10 mM $MgCl_2$ was already optimal, and ruthenium red did not further increase the rate. Both heavy SR and triads were stimulated 2-fold by ruthenium red at 1 and 5 mM $MgCl_2$. In contrast, junctional terminal cisternae were more sensitive to ruthenium red stimulation, especially at the lower Mg^{2+} concentration (see also Figure 3 below). In general, junctional terminal cisternae behave similarly to other heavy SR preparations with regard to the enhancement of Ca^{2+} transport by ruthenium red and/or Mg^{2+} (Kirino & Shimizu, 1982; Seiler et al., 1984; Morii et al., 1985; Meszaros & Ikemoto, 1985a), except that the ruthenium red enhancement of both Ca^{2+} uptake and loading is appreciably higher in junctional terminal cisternae.

The influence of Mg^{2+} and ruthenium red concentrations on the stimulation of Ca^{2+} loading of junctional terminal cisternae was further examined (Figure 2). Ruthenium red was found to be noncompetitive with respect to Mg^{2+} and MgATP. The apparent K_m for ruthenium red was about 0.8 μM.

Ca^{2+} Dependence of $^{45}Ca^{2+}$ Loading Rates by SR Fractions. Since increasing the amount of phosphate and Mg^{2+} at a constant $CaCl_2$ concentration caused a change in $[Ca^{2+}]_f$, $[Mg^{2+}]_f$, and MgATP, the Ca^{2+} dependence of the Ca^{2+} loading rate in junctional terminal cisternae was measured and compared with other terminal cisternae derived fractions

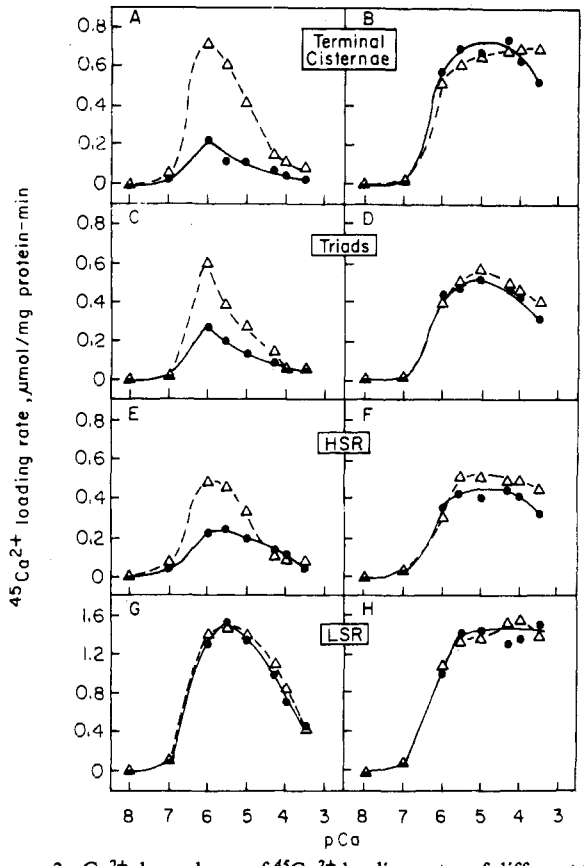

FIGURE 3: Ca^{2+} dependence of $^{45}Ca^{2+}$ loading rates of different SR fractions. $^{45}Ca^{2+}$ uptake in the presence of 100 mM phosphate (phosphoric acid–KOH, pH 7.0) was measured by the filtration procedure at 25 °C (Materials and Methods). Each reaction tube of 2-mL total volume contained about 100 μg of SR protein/mL and different pCa, with either constant $[Mg^{2+}]_f$ = 0.1 mM and [MgATP] = 0.45 mM (panels A, C, E, G) or constant $[Mg^{2+}]_f$ = 5 mM and [MgATP] = 1 mM (panels B, D, F, H). Phosphate was added just prior to initiation of the reaction with Na_2ATP. (●) Without ruthenium red. (△) With 20 μM ruthenium red. (▲) Overlapping data points in the presence and absence of ruthenium red.

(triads and heavy SR) and with longitudinal tubules (light SR), at two constant $[Mg^{2+}]_f$ levels of 0.1 (MgATP = 0.45 mM) and 5 mM (MgATP = 1 mM), designated low and high $[Mg^{2+}]_f$ conditions, respectively (Figure 3).

Each of the SR fractions displayed Ca^{2+} dependence on the $^{45}Ca^{2+}$ loading rates (Figure 3). At the low $[Mg^{2+}]_f$ condition, the rates for all the fractions were maximal at 1–3 μM $[Ca^{2+}]_f$, with apparent half-maximal $[Ca^{2+}]_f$ of 0.25–0.3 μM. At the high $[Mg^{2+}]_f$ condition, the rates were maximal at 3 μM $[Ca^{2+}]_f$ and above, with apparent half-maxima at 0.5 μM $[Ca^{2+}]_f$. This is within the range of $[Ca^{2+}]_f$ for half-maximal Ca^{2+} loading rates reported by others (Louis et al., 1980; Inesi, 1985).

Only at the lower $[Mg^{2+}]_f$ condition were the $^{45}Ca^{2+}$ loading rates of junctional terminal cisternae, triads, and heavy SR stimulated by ruthenium red, over a pCa range of 5–7. Under the conditions of the assay, the maximal enhancement by ruthenium red was approximately 5-, 2-, and 2-fold for terminal cisternae, triads, and heavy SR, respectively. The higher $[Mg^{2+}]_f$ enhanced net Ca^{2+} transport, but there was no additional ruthenium red stimulation over the entire pCa range examined. In contrast, the $^{45}Ca^{2+}$ loading rate of light SR was optimal without the addition of ruthenium red or high Mg^{2+} concentration.

CaATPase Rates of SR Fractions. In order to evaluate the basis for the enhanced Ca^{2+} transport, the influence of ru-

Table II: Effects of Ruthenium Red, Mg^{2+}, and Phosphate on Ca^{2+} Loading of Different SR Fractions[a]

								Ca^{2+} loading of								
								junctional terminal cisternae			triads			heavy SR		
		estimated ion concentrations						rate [nmol/(mg of protein·min)]		ratio	rate [nmol/(mg of protein·min)]		ratio	rate [nmol/(mg of protein·min)]		ratio
$[MgCl_2]$ (mM)	[potassium phosphate] (mM)	$[Ca^{2+}]_f$ (μM)	$[Mg^{2+}]_f$ (μM)	$[P_i]_f$ (μM)	[MgATP] (μM)	[CaATP] (μM)	$[ATP^{2-}]_f$ (μM)	−RR	+RR	+RR/−RR	−RR	+RR	+RR/−RR	−RR	+RR	+RR/−RR
1	25	12	177	21	570	17	284	23 ± 7	164 ± 91	7.2	62 ± 16	138 ± 9	2.2	193 ± 53	402 ± 21	2.1
5		24	1986	19	940	5	41	94 ± 29	298 ± 111	3.2	171 ± 30	250 ± 42	1.5	350 ± 108	818 ± 21	2.3
10		28	4729	17	970	3	18	353 ± 56	516 ± 67	1.5	272 ± 44	332 ± 52	1.2	641 ± 159	916 ± 255	1.4
1	50	10	138	43	520	16	328	64 ± 31	425 ± 204	6.7	181 ± 31	337 ± 53	1.9	457 ± 142	777 ± 185	1.7
5		17	1317	40	910	5	61	142 ± 43	615 ± 191	4.3	391 ± 60	561 ± 113	1.4	685 ± 80	1217 ± 196	1.8
10		20	3104	37	960	3	27	647 ± 113	750 ± 89	1.2	474 ± 86	490 ± 119	1.0	884 ± 182	1364 ± 173	1.5
1	100	7	99	85	440	14	392	154 ± 39	1025 ± 216	6.7	591 ± 133	1017 ± 260	1.7	669 ± 107	1270 ± 100	1.9
5		11	783	82	860	5	96	350 ± 62	1541 ± 219	4.4	918 ± 152	1144 ± 166	1.2	804 ± 130	1420 ± 102	1.8
10		12	1800	79	930	3	46	1247 ± 268	1671 ± 350	1.3	993 ± 196	1094 ± 286	1.1	930 ± 107	1460 ± 280	1.6

[a] Ca^{2+} loading was measured in the presence of the metallochromic indicator antipyrylazo III (Material and Methods). Varying amounts of KCl were added to maintain total K^+ concentration at 100 mM in reaction mixtures that contain 5 mM NaN_3, 50 μg of SR protein/mL, and 1 mM Na_2ATP at 25 °C. The reaction was started by the addition of 50 μM $CaCl_2$. The concentrations of $MgCl_2$ and phosphate (phosphoric acid–KOH, pH 7.0) were varied. Values of estimated free and bound ligand concentrations under these conditions are included in the table. There was considerable variability in the Ca^{2+} loading rates of different preparations, as indicated by the standard error.

Table III: CaATPase Activities of Different SR Fractions[a]

SR fraction	RR present	A23187	CaATPase activity $[Mg^{2+}]_f$ = 0.1 mM rate	ratio (+RR/−RR)	$[Mg^{2+}]_f$ = 5 mM rate	ratio (+RR/−RR)
junctional terminal cisternae	−	−	1.76 ± 0.17		1.03 ± 0.13	
	+	−	1.34 + 0.14	0.8	0.87 ± 0.13	0.8
	−	+	2.06 ± 0.27		1.12 + 0.27	
	+	+	1.88 ± 0.28	0.9	0.96 ± 0.15	0.9
triads	−	−	1.86 ± 0.23		1.03 + 0.08	
	+	−	1.26 ± 0.16	0.7	0.87 ± 0.08	0.8
	−	+	1.98 ± 0.04		1.01 ± 0.11	
	+	+	1.85 ± 0.19	0.9	0.93 ± 0.10	0.9
heavy SR	−	−	1.85 ± 0.22		1.29 + 0.23	
	+	−	1.50 + 0.09	0.8	1.10 + 0.13	0.9
	−	+	2.18 ± 0.29		1.11 ± 0.21	
	+	+	2.06 ± 0.19	1.0	1.02 + 0.21	0.9
light SR	−	−	2.61 ± 0.44		1.26 ± 0.16	
	+	−	2.52 + 0.34	1.0	1.43 + 0.04	1.1
	−	+	5.81 ± 0.52		2.74 + 0.23	
	+	+	5.76 ± 0.40	0.9	2.60 + 0.37	1.0

[a] CaATPase activity was measured spectrophotometrically by monitoring continuously the oxidation of NADH in the presence of a coupled-enzyme assay with pyruvate kinase and lactic dehydrogenase (9 and 12 units/mL, respectively), 400 μM NADH, and 2 mM phosphoenolpyruvate at 25 °C. The $[Ca^{2+}]_f$ was held constant at 3 μM, and $[Mg^{2+}]_f$ was held constant at 0.1 ([MgATP] = 0.45 mM) and 5 mM ([MgATP] = 1 mM), under similar conditions as for Figure 3, in the presence of 5 mM Tris–EGTA (pH 7.0) and 100 mM phosphoric acid–KOH (pH 7.0) (Materials and Methods). The assay was carried out in the presence and absence of 1.5 μg/mL (2.9 μM) A23187, with 5 and 10 μg of SR protein/mL, respectively. The final concentration of 0.29% ethanol as a solvent for A23187 did not significantly alter the CaATPase activities. Ruthenium red, when present, was 20 μM. CaATPase activity, measured by the release of inorganic phosphate, gave similar results (data not shown). The basal ATPase rate (in the absence of Ca^{2+} and in the presence of 5 mM Tris–EGTA) of all SR fractions was not affected by ruthenium red. The rate is in μmol/(mg of protein·min), n = 3. The calcium pump accounts for 35 and 90% protein of the junctional terminal cisternae and light SR, respectively (Costello et al., 1986). Therefore, when normalized for the calcium pump protein content, the average CaATPase activities in the presence of the ionophore were 5.63 and 6.42 μmol/(mg of protein·min) at $[Mg^{2+}]_f$ = 0.1 mM and 2.97 and 2.96 μmol/(mg of protein·min) at $[Mg^{2+}]_f$ = 5 mM for junctional terminal cisternae and light SR, respectively.

thenium red on CaATPase activity was examined (Table III). Ruthenium red did not enhance the CaATPase activities of any SR fraction but actually had some inhibitory effect between 3 and 32%. Under Ca^{2+} loading conditions similar to those in Figure 3, at 3 μM $[Ca^{2+}]_f$ and 0.1 or 5 mM $[Mg^{2+}]_f$, ruthenium red decreased the CaATPase activities of junctional terminal cisternae, triads, and heavy SR by 24, 32, and 19%, respectively. The CaATPase of light SR was not significantly affected by ruthenium red (3%). In the presence of A23187, which dissipates the Ca^{2+} gradient across the SR membrane, ruthenium red also did not appreciably decrease the CaATPase rates (<9%). Our results are consistent with previous reports that ruthenium red slightly inhibited the CaATPase activities of SR microsomes (Vale & Carvalho, 1973; Howell, 1982). Under our conditions, the CaATPase activities at 5 mM $[Mg^{2+}]_f$ were lower than those at 0.1 mM $[Mg^{2+}]_f$, consistent with other reports using microsomes (Vianna, 1975) or light and heavy SR (Louis et al., 1980). Likewise, there were no significant changes when ruthenium red was present at the high Mg^{2+} condition. Our MgATP concentration range (0.4–1 mM) was well above the K_m for MgATP (1–18 μM) (Vianna, 1975; Shigekawa et al., 1983), so the decreased CaATPase rates are not due to limiting MgATP. Ca^{2+} ionophores have been observed to stimulate CaATPase activities (Neet & Green, 1977), reflecting tight coupling of Ca^{2+} transport to ATP hydrolysis and low Ca^{2+} permeability of the SR membrane. In this regard, the CaATPase activity of the light SR fraction was enhanced about 2-fold by A23187. At the low Mg^{2+} condition, ruthenium red decreased the CaATPase activities of terminal cisternae derived fractions, and then the ionophore enhanced the activities in the presence of ruthenium red.

The CaATPase rates of junctional terminal cisternae and light SR in the presence of ionophore at both low and high $[Mg^{2+}]$ were approximately the same when normalized for the content of the calcium pump protein, although the rates at the high $[Mg^{2+}]$ condition were about 2-fold lower. This means that the turnover number for CaATPase activity is the same for the calcium pump protein in junctional terminal cisternae and light SR.

Table IV: Ca^{2+} Pumping Efficiency of SR Fractions[a]

SR fraction	$[Mg^{2+}]_f$ (mM)	pumping efficiency (Ca^{2+}/ATP) no RR	with RR
junctional terminal cisternae	0.1	0.21 ± 0.03	1.45 ± 0.31
	5	1.69 ± 0.28	1.56 ± 0.26
light SR	0.1	1.62 ± 0.06	1.69 ± 0.20
	5	1.92 ± 0.13	1.70 ± 0.09

[a] The Ca^{2+} pumping efficiency (Ca^{2+}/ATP) is the ratio of $^{45}Ca^{2+}$ loading rate to CaATPase activity determined under similar conditions. Initial rates of $^{45}Ca^{2+}$ loading and CaATPase activity at 25 °C were measured as described in Figure 3 and Table III (Materials and Methods), at 3 μM $[Ca^{2+}]_f$. Ruthenium red was 20 μM. n = 3.

Ca^{2+} Pumping Efficiency of Terminal Cisternae and Light SR. The junctional terminal cisternae exhibited low Ca^{2+} transport activities (uptake and loading), albeit appreciable CaATPase rates. The Ca^{2+} pumping efficiency, i.e., the molar ratio of Ca^{2+} transported to ATP hydrolyzed (Ca^{2+}/ATP), was only 0.2 at the lower $[Mg^{2+}]_f$ (Table IV). Ruthenium red or high Mg^{2+} concentration increased the efficiency to 1.6, approaching the level found with light SR. The Ca^{2+} transport efficiency of light SR was not significantly enhanced by either ruthenium red or elevated Mg^{2+} concentration.

Thus, the enhanced Ca^{2+} loading by ruthenium red in the fractions derived from terminal cisternae could not be correlated with an increase in ATP hydrolytic activity of the Ca^{2+} pump protein. This suggests that the stimulation of net Ca^{2+} transport is related to the inhibition of Ca^{2+} efflux by ruthenium red or increased Mg^{2+} concentration.

Passive $^{45}Ca^{2+}$ Efflux from SR. $^{45}Ca^{2+}$ efflux was measured over the same range of pCa as for $^{45}Ca^{2+}$ loading, at both the low and high $[Mg^{2+}]_f$ conditions (Figure 4). SR fractions

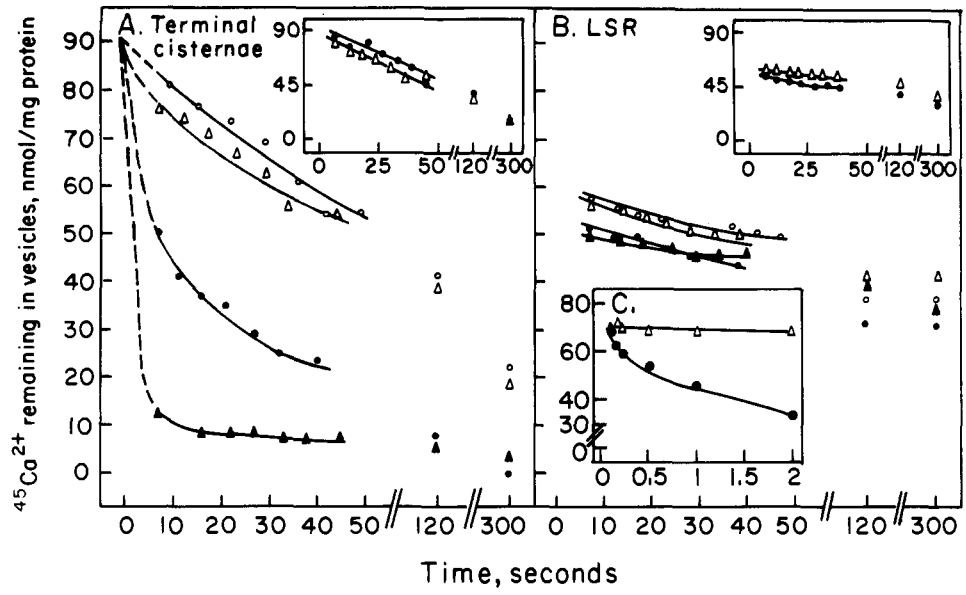

FIGURE 4: Ca^{2+} dependence of $^{45}Ca^{2+}$ efflux of different SR fractions after passive loading. SR vesicles (junctional terminal cisternae, panel A; light SR, panel B) were equilibrated with 5 mM $^{45}CaCl_2$ overnight at 4 °C and diluted into various pCa solutions at 25 °C with either 0.1 (panels A and B) or 5 mM (insets) $[Mg^{2+}]_f$. The $^{45}Ca^{2+}$ remaining in the vesicles was measured by the filtration assay (Materials and Methods). Closed symbols, without ruthenium red; open symbols, with 20 μM ruthenium red; (○, ●) $[Ca^{2+}]_f$ = 0.11 μM, (△, ▲) $[Ca^{2+}]_f$ = 3.3 μM. Panel C shows a rapid $^{45}Ca^{2+}$ efflux study of junctional terminal cisternae (a different sample from that used in panel A) at 3.5 μM $[Ca^{2+}]_f$ (●, no ruthenium red; △, with ruthenium red) using a rapid filtration procedure (Dupont, 1984). The half-time of $^{45}Ca^{2+}$ efflux was 0.66 s.

derived from terminal cisternae exhibited Ca^{2+} stimulation of $^{45}Ca^{2+}$ efflux at the low $[Mg^{2+}]_f$ condition, whereas ruthenium red or high $[Mg^{2+}]_f$ inhibited the Ca^{2+}-dependent component of the $^{45}Ca^{2+}$ efflux. In contrast, the $^{45}Ca^{2+}$ efflux from light SR was slower and insensitive to Ca^{2+}, ruthenium red, and Mg^{2+}. We compared the efflux patterns of the SR fractions and used the half-time of efflux as an index. In order to measure efflux without specialized rapid kinetics equipment, the slower Ca^{2+} efflux patterns at pCa 8 were examined. The half-times ($t_{1/2}$) of $^{45}Ca^{2+}$ efflux of heavy SR, triads, and light SR were 2, 3, and 5 times slower than that of junctional terminal cisternae ($t_{1/2} \sim 22$ s).

A rapid filtration apparatus was used to measure Ca^{2+} efflux from junctional terminal cisternae (Figure 4C). The measured rapid $^{45}Ca^{2+}$ efflux was inhibited with ruthenium red and was more than 2 orders of magnitude faster than the passive release in the presence of ruthenium red, indicating the existence of Ca^{2+} release channels in junctional terminal cisternae in contrast with light SR.

$^{45}Ca^{2+}$ Influx in SR Fractions. There have been suggestions that ruthenium red and Mg^{2+} regulate Ca^{2+} influx by directly influencing the calcium pump protein (Watras, 1985). In such a study, Ca^{2+} loading is measured with $^{45}Ca^{2+}$. "Unidirectional influx" is measured by pulsing in $^{45}Ca^{2+}$ at different time intervals to SR vesicles carrying out $^{40}Ca^{2+}$ loading. The $^{45}Ca^{2+}$ loading rate measured during the same time interval is subtracted from the $^{45}Ca^{2+}$ influx rate to give an "efflux" rate (Katz et al., 1980). We investigated Ca^{2+} "influx" of the SR fractions under similar Ca^{2+} loading conditions at 3 μM $[Ca^{2+}]_f$ and 0.1 mM $[Mg^{2+}]_f$ (Figure 5). The apparent $^{45}Ca^{2+}$ influx rates (between 0.25 and 2 min) were enhanced by ruthenium red to similar extents as the $^{45}Ca^{2+}$ loading rates for junctional terminal cisternae, heavy SR, and triads, consistent with the results reported by Watras (1985).

The effect of ryanodine on Ca^{2+} loading and influx in junctional terminal cisternae were also examined (Figure 6) with the idea that the action of ryanodine would discriminate between the direct action of the ruthenium red on Ca^{2+} release

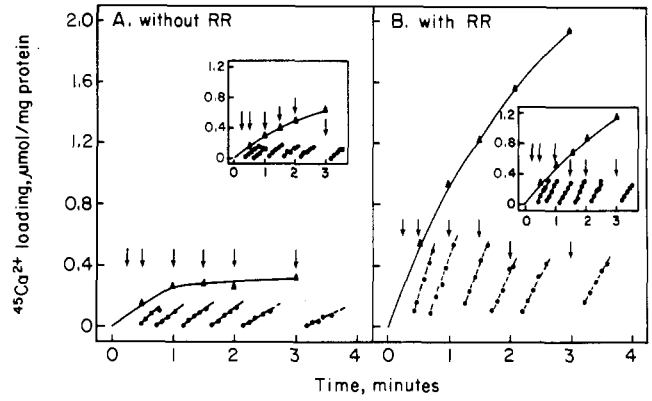

FIGURE 5: $^{45}Ca^{2+}$ loading and "influx" of SR fractions. $^{45}Ca^{2+}$ loading (▲) was measured as in Figure 3, with 0.1 mM $[Mg^{2+}]_f$, 3 μM $[Ca^{2+}]_f$, and 0.45 mM MgATP, in the absence (panel A) and presence (panel B) of 20 μM ruthenium red. The total $CaCl_2$, $MgCl_2$, and Na_2ATP concentrations were 4.43, 0.972, and 1.02 mM, respectively. $^{45}Ca^{2+}$ "influx" (●) was measured by pulsing $^{45}Ca^{2+}$ into an ongoing reaction of $^{40}Ca^{2+}$ loading at the time intervals of 0.25, 0.5, 1, 1.5, 2, and 3 min as indicated by the arrows (see Materials and Methods). Junctional terminal cisternae and triads are represented in the panels and insets, respectively. Heavy SR exhibited a similar transport pattern (data not shown). At 0.5 min, the amount of Ca^{2+} taken up by junctional terminal cisternae in the presence of ruthenium red (panel B) was 1% of the total $CaCl_2$ in the medium; at 3 min it was 5%.

channels vs. the calcium pump protein. At low concentrations (2 μM or less), ryanodine specifically blocks the action of ruthenium red in stimulating Ca^{2+} loading in junctional terminal cisternae (Fleischer et al., 1985; also see legend, Figure 6). The concentration of specific ryanodine binding sites was in the range of 20 pmol/mg of protein, approximately 2 orders of magnitude less than the concentration of calcium pump protein (in nmol/mg of protein range). In the absence of ruthenium red, ryanodine had essentially no effect on either apparent $^{45}Ca^{2+}$ influx or $^{45}Ca^{2+}$ loading. Ryanodine largely blocked the action of ruthenium red in enhancing both the apparent $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ loading rates to similar extents. In contrast, the Ca^{2+} influx and loading of light SR

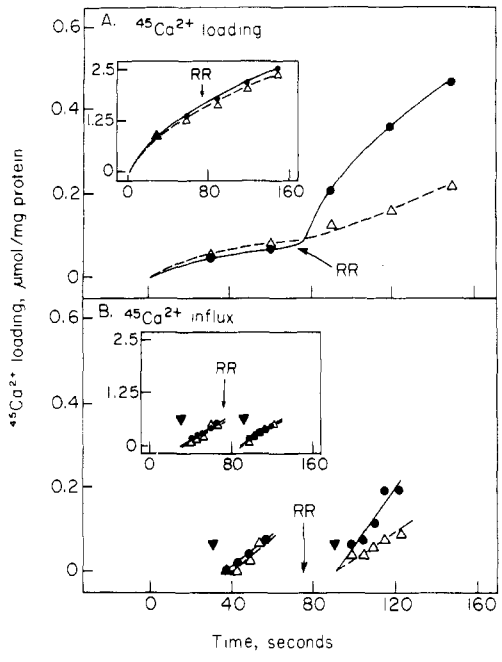

FIGURE 6: Effect of ryanodine on $^{45}Ca^{2+}$ loading and "influx" of junctional terminal cisternae. Reaction media are similar to those in Figure 5, except that 7 μM ruthenium red was added to an ongoing Ca^{2+} loading reaction at 75 s as indicated at the arrows. Panel A shows $^{45}Ca^{2+}$ loading, and panel B shows $^{45}Ca^{2+}$ "influx", in which $^{45}Ca^{2+}$ was pulsed at 30 and 90 s, as indicated by the arrowheads (▼). (●) Without ryanodine. (△) With 2 μM ryanodine, preincubated for 15 min at 25 °C prior to the addition of Na_2ATP to start the reaction (zero time). Junctional terminal cisternae and light SR (prepared from the same gradient) are represented in the panels and insets, respectively. Ruthenium red stimulated the Ca^{2+} loading rates of two different junctional terminal cisternae preparations 5-fold over control, and ryanodine inhibited the ruthenium red enhanced loading rate approximately 60%. Similarly, ryanodine inhibited the ruthenium red stimulated influx rate about 67%. Similar experimental results were obtained when 1 μM ryanodine was used to block the action of ruthenium red in the experiment (data not shown). In separate studies, [^{3}H]ryanodine binding was measured in junctional terminal cisternae at 270 nM, 1 μM, and 2 μM. The specific ryanodine binding was 18.5, 19.7, and 22.6 pmol/mg of protein, respectively, and nonspecific binding was 3.7, 12.6, and 25.8 pmol/mg of protein. Specific binding for light SR bound was 1.7, 3.2, and 4.0 pmol/mg of protein, respectively.

were not affected by ruthenium red or ryanodine. The results indicate that the primary action of ryanodine and ruthenium red is on the Ca^{2+} release channels.

DISCUSSION

The approach of our laboratory to study muscle contraction and relaxation from fast skeletal muscle has been to isolate and characterize defined fractions of the sarcotubular system (Fleischer, 1985). In this report, four such preparations developed in our laboratory have been studied with regard to Ca^{2+} transport and efflux. The most recently available preparation is junctional terminal cisternae (Saito et al., 1984), which differs from heavy SR (Meissner, 1975) in that it contains about 15–20% junctional face membrane with morphologically intact "feet" structures; the remainder of the membrane, about 80–85%, consists of calcium pump membrane. The heavy SR has very little junctional face membrane (<3%). Isolated triads (Mitchell et al., 1983) differ from junctional terminal cisternae in that junctional face membrane of the terminal cisternae is junctionally associated with transverse tubule via the "feet" structures. Light SR (Meissner, 1975), derived from longitudinal tubules, is essentially a calcium pump membrane, containing mainly the Ca^{2+} pump protein (~90% of the membrane protein).

Energized Ca^{2+} uptake by the calcium pump membrane of SR enables muscle to relax. Since light SR and terminal cisternae consist mainly of calcium pump membrane, Ca^{2+} uptake takes place in both longitudinal and terminal cisternae regions of SR. Terminal cisternae, in situ, and both junctional and nonjunctional (heavy) SR vesicles contain, within their compartment, electron-opaque contents, mainly the Ca^{2+}-binding protein. This protein has a high capacity to bind Ca^{2+} (MacLennan et al., 1983) and appears to serve as a reservoir for Ca^{2+} after it has been translocated into the SR lumen. Thus, transported Ca^{2+} becomes localized within the terminal cisternae, and the release of Ca^{2+} would be expected to occur from this region of the SR. This study provides evidence for the localization of a unique Ca^{2+} release system in terminal cisternae of SR, thereby supporting the concept of Ca^{2+} release from this locus.

The junctional terminal cisternae are distinct compared with the other SR fractions with regard to energized net Ca^{2+} transport. There is very little Ca^{2+} uptake at low Mg^{2+} concentration. Likewise, the Ca^{2+} loading rate, measured in the presence of phosphate, a Ca^{2+}-trapping anion, is also low. The basis for the impaired Ca^{2+} transport by the junctional terminal cisternae appears to be mainly due to the presence of Ca^{2+} release channels in the "open state". This conclusion is based on the following observations: (1) The CaATPase activity of terminal cisternae fractions in the presence of Ca^{2+} ionophore is comparable to light SR when normalized for calcium pump protein (Table III). (2) Elevation of Mg^{2+} concentration or addition of ruthenium red restores Ca^{2+} uptake and loading (Tables I and II). (3) The enhanced Ca^{2+} transport by ruthenium red cannot be explained by a faster turnover of the pump [see CaATPase activities in Table III; also see Meszaros and Ikemoto (1985a) and Watras (1985)]. (4) The Ca^{2+} pumping efficiency is enhanced from 0.2 to nearly 2 with either ruthenium red or elevated Mg^{2+} concentration (Table IV). (5) Passively loaded junctional terminal cisternae exhibit rapid Ca^{2+} efflux, which can be blocked by ruthenium red (Figure 4). (6) Ryanodine at low concentrations (2 μM or less) blocks the ruthenium red enhancement of Ca^{2+} loading (Figure 6). The number of [^{3}H]ryanodine binding sites (in pmol/mg of protein) in junctional terminal cisternae is 2 orders of magnitude lower than that of the calcium pump protein (in the nmol/mg of protein range), and the binding can be inhibited by ruthenium red (Fleischer et al., 1985; Pessah et al., 1985). Thus, the ryanodine binding appears to titrate the Ca^{2+} release channels rather than the calcium pump protein. (7) In contrast, light SR, which is derived from longitudinal tubules, has a high Ca^{2+} loading rate and slow Ca^{2+} efflux, which are not modulated by ruthenium red, ryanodine, or Mg^{2+} concentration. Junctional terminal cisternae are distinct from light SR in that they have a high permeability to Ca^{2+}. The permeability can be modulated by Mg^{2+} or ruthenium red. The action of ruthenium red in turn can be blocked by ryanodine at pharmacologically significant levels. Therefore, these studies provide strong evidence that Ca^{2+} release channels, likely the ones important in excitation–contraction coupling, are localized in the junctional terminal cisternae.

It is worth noting that the heavy SR and triads, which are also derived from terminal cisternae, are less leaky to Ca^{2+} than the junctional terminal cisternae fraction. Both heavy SR and triads exhibit intermediate sensitivity to ruthenium red with respect to Ca^{2+} transport, the maximal stimulation being 2-fold. The Ca^{2+} efflux for both heavy SR and triads

also appears to be slower as compared to junctional terminal cisternae. Heavy SR contains few "feet" structures, suggesting that this fraction is derived from the nonjunctional terminal cisternae. The isolated triad fraction, containing terminal cisternae in junctional association with the transverse tubule by way of the junctional face membrane, is also relatively impermeable to Ca^{2+}, compared with isolated junctional terminal cisternae. These observations suggest that the transverse tubule is involved in maintaining Ca^{2+} channels in the "closed state". A recent report by Ikemoto et al. (1984) comes to a similar conclusion using a somewhat different approach.

There is now a diversity of evidence in the literature for the existence of Ca^{2+} release channels [review by Endo (1977); Ohnishi, 1979; Miyamoto & Racker, 1982; Kirino & Shimizu, 1982; Kim et al., 1983; Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Meissner 1984, 1986; Meissner et al., 1986]. Some of these studies make use of rapid kinetics to measure Ca^{2+} fluxes. A recent study makes use of electrical measurements by incorporating a heavy SR fraction into a black lipid film and recording a large Ca^{2+} conductance of 170 pS (Smith et al., 1985). Smaller Ca^{2+} conductances of 5–10 pS have been reported from unfractionated SR and appear to be different from the large conductance (Orozco et al., 1985; Cukierman et al., 1986). Ca^{2+} efflux in isolated skeletal muscle SR vesicles is generally favored in the absence of Mg^{2+} or at reduced Mg^{2+} concentrations [Meissner, 1984, 1986; see also reviews by Endo (1977), Stephenson (1981), and Martonosi (1984)]. In the context of our studies, the differential Mg^{2+} dependence for Ca^{2+} loading, CaATPase activity, and coupling efficiency of heavy vs. light SR (Watras, 1985) reflects the localization of Ca^{2+} release channels in junctional terminal cisternae.

Ryanodine can have different effects on sarcoplasmic reticulum depending on the system and how it is used. The unique application of ryanodine at low concentration in this study is based on its locking the channels in the open state, obviating the action of ruthenium red, which closes the channel. The action of ryanodine can be correlated with a small number of specific binding equivalents, ~20 pmol/mg of protein (Fleischer et al., 1985). Our study is not to be confused with the use of ryanodine in different experimental conditions that can close or open the Ca^{2+} release channels (Sutko et al., 1985; Meissner, 1986).

There is the suggestion by Watras (1985) that the action of ruthenium red on a heavy SR fraction is to increase the coupling of ATP hydrolysis to transport. We have repeated these studies with our fractions and obtain similar experimental results (Figure 5). However, we find that ryanodine blocks the enhanced influx by ruthenium red, indicating that the primary effect is on a small number of equivalents (a factor of 10^2 smaller than the calcium pump protein). Thus, the primary effect of ruthenium red and ryanodine is better explained by their direct action on the Ca^{2+} release channels rather than on the calcium pump protein. The action of ruthenium red to enhance Ca^{2+} "influx" might then be explained as secondary to the closing of the channels, via a global membrane phenomenon, transmitted through the membrane to the calcium pump protein. The secondary effect would then result in an increase in the coupling efficiency of the calcium pump protein itself. The "slippage" of the pump has already been considered by Berman (1982). The primary effect would be too fast to be detected by the unidirectional flux type of experiments, which are in the time scale of seconds or longer. An example of a well-studied global membrane phenomenon is the generation of protonmotive force by one transducer, which is expressed via the membrane, to energize a different transducer (Mitchell, 1979). In this context, Meszaros and Ikemoto find that ruthenium red induces a small increase in the steady-state level of phosphoenzyme (Meszaros & Ikemoto, 1985a), and in a separate study, they suggest that conformational changes of the calcium pump protein attend early events during Ca^{2+} release (Meszaros & Ikemoto, 1985b).

The uniqueness of our study is the comparison, under comparable conditions of Ca^{2+} transport and efflux, of four SR fractions corresponding to *defined* segments of the internal membrane system that regulates Ca^{2+} uptake and release in skeletal muscle. Junctional terminal cisternae containing junctional face membrane are distinct morphologically and functionally from the other SR fractions. The Ca^{2+} efflux characteristics of junctional terminal cisternae reflect the existence of Ca^{2+} release channels in the open state. In vitro, these channels can be chemically gated. In situ, the terminal cisternae are junctionally associated with the transverse tubule to form the triad. The state of polarization of the transverse tubule maintains those channels in the closed state. The channels can be transiently opened, in response to an action potential that triggers Ca^{2+} release.

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Registry No. ATPase, 9000-83-3; Ca, 7440-70-2; Mg, 7439-95-4; PO_4^{3-}, 14265-44-2; ruthenium red, 11103-72-3; ryanodine, 15662-33-6.

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