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CALCIUM RELEASE FROM TWO FRACTIONS OF SARCOPLASMIC RETICULUM FROM RABBIT SKELETAL MUSCLE

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Calcium release from sarcoplasmic reticulum vesicles presumably derived from longitudinal tubules (LSR) and terminal cisternae (HSR) of rabbit skeletal muscle was investigated by dual wavelength spectrophotometry using the calcium-indicator antipyrilazo III. In 120 mM KCl, 5 mM $MgCl_2$, 30 μM $CaCl_2$, 50 μM MgATP, 100 μM antipyrilazo III, 40 mM histidine (pH 6.8, 25°C), LSR and HSR sequestered approx. 115 nmol calcium/mg, and then spontaneously released calcium. Analysis of ATP hydrolysis and phosphoenzyme level during LSR and HSR calcium sequestration indicated that this calcium release process was passive, occurring in the virtual absence of ATP and phosphoenzyme. Moreover, subsequent addition of ATP reinitiated the calcium sequestration-release sequence. Calcium release by HSR was more than 4-times faster than that by LSR. Analysis of the calcium release phase demonstrated a biexponential decay for both LSR (0.10 and 0.63 min^{-1}) and HSR (0.26 and 1.65 min^{-1}), suggestive of heterogeneity within each fraction. Replacement of 120 mM KCl with either 120 mM choline chloride, 240 mM sucrose, or H_2O reduced maximal calcium sequestration by LSR, but had less effect on LSR calcium release rate constants. In the case of HSR, these changes in the ionic composition of the medium drastically reduced calcium release rate constants with little effect on calcium content. These marked differences between LSR and HSR are consistent with the hypothesis that the calcium permeability of the terminal cisternae is greater and more sensitive to the ionic environment than is that of the longitudinal tubules of sarcoplasmic reticulum.

Introduction

The sarcoplasmic reticulum of skeletal muscle is an internal membranous network consisting of terminal cisternae, located adjacent to the T-tubules, and longitudinal tubules, which extend the length of the sarcomere [1,2]. The terminal cisternae are characterized by an abundance of electron

dense material (calsequestrin) [3], which appears to bind calcium with low affinity [4,5], and electron probe analyses suggest that the terminal cisternae contain most of the calcium in the muscle fiber [6,7]. It therefore seems likely that calcium released during excitation-contraction coupling is derived primarily from the terminal cisternae [6,7]. The longitudinal tubules may participate in the relaxation phase by sequestering cytoplasmic calcium, which then diffuses to the terminal cisternae where it binds to calsequestrin [3,7].

In an effort to determine if the terminal cisternae represent a specialized region of the sarcoplasmic reticulum for calcium release, several inves-

Abbreviations: bisTris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N' -tetraacetic acid; HSR and LSR, sarcoplasmic reticulum derived from terminal cisternae and longitudinal tubules, respectively.

tigators have examined EGTA-induced calcium release from terminal cisternae-derived (HSR) and longitudinal tubule-driven (LSR) sarcoplasmic reticulum vesicles after calcium loading. The results, however, are inconsistent in that calcium release from HSR was shown to be faster [3,8] or slower [9] than that from LSR. The calcium content of HSR relative to that of LSR varied widely in the above studies, hence it would have been more appropriate to express the calcium release data in terms of rate constants (which are independent of concentration). It would also seem preferable not to initiate calcium release by addition of EGTA, since the effects of EGTA on membrane permeability are not clear.

The present study was undertaken to reconcile the above discrepancy involving calcium release kinetics of HSR and LSR and thus clarify functional differences between terminal cisternae and longitudinal tubules. Experimental conditions were selected to optimize calcium accumulation by both LSR and HSR [10] in the presence of a low concentration of ATP, with calcium release occurring after depletion of ATP and phosphoenzyme. Kinetic analysis of the calcium release phase (monitored continuously by dual wavelength spectrophotometry) suggested heterogeneity within each fraction of vesicles, with a much higher rate constant for calcium release by HSR than LSR. Comparison of calcium release kinetics in various media also suggested a greater ionic dependence of the rate constant for calcium release from HSR than LSR, consistent with the hypothesis that these two regions of the sarcoplasmic reticulum are functionally distinct in terms of calcium release characteristics.

Methods

Crude sarcoplasmic reticulum vesicles were isolated from rabbit white skeletal muscle as described previously [11], with 4-morpholinopropane sulfonate instead of Tris as buffer. The vesicles were then suspended in 10% (w/v) sucrose and centrifuged in a discontinuous sucrose gradient for 4 h at $91\,000 \times g_{av}$ in a Beckman SW 27 rotor. The discontinuous gradient was composed of 3 ml 60%, 10 ml 40%, 10 ml 31%, 10 ml 28% (w/v) sucrose with 3 ml 40 mg/ml crude sarcoplasmic reticulum

(in 10% sucrose) at the top. The vesicles at the 28/31% sucrose interface (LSR) and 40/60% sucrose interface (HSR), presumably derived from the longitudinal tubules and terminal cisternae, respectively [3,9,12], were frozen in liquid nitrogen and stored at -80°C . Freezing was shown not to affect calcium sequestration or calcium release kinetics by LSR or HSR (data not shown), and no loss of enzymic activity or calcium transport was observed for up to 5 weeks. Protein concentration was measured by the technique of Lowry et al. [13], using bovine serum albumin as a standard.

Calcium accumulation and release were measured by dual wavelength spectrophotometry (Johnson Research Foundation Multichannel Spectrophotometer) using the calcium indicator antipyrilazo III (720, 790 nm) [14]. Unless specified otherwise, reaction media contained 5 mM MgCl_2 , 120 mM KCl, 30 μM CaCl_2 , 100 μM antipyrilazo III, 50 μM MgATP, 0.2 mg/ml HSR or LSR, 40 mM histidine (pH 6.8, 25°C). Recordings were calibrated by four serial additions of 0.006 vol. CaCl_2 , and reactions initiated by addition of 0.006 vol. MgATP. The small baseline shift caused by MgATP addition was subtracted from all recordings. The amount of 'releaseable calcium' within the sarcoplasmic reticulum was determined by addition of 5 μM A23187 after ATP depletion.

Calcium fluxes, ATP hydrolysis, and phosphoenzyme level were monitored by a combination of spectrophotometric and radiochemical analysis. Reaction conditions were as described above, except for the inclusion of 50 μM [γ - ^{32}P]ATP and 20 μM CaCl_2 . Reactions were initiated with 0.008 vol. [γ - ^{32}P]MgATP, and calcium flux monitored by dual wavelength spectrophotometry as described above. At appropriate intervals, 0.1-ml aliquots were transferred from the cuvette to 0.2 ml 15% perchloric acid, 1.5 mM H_3PO_4 (4°C). Within 10 min, each of these quenched samples was supplemented with 25 μl 5 mg/ml crude sarcoplasmic reticulum and then centrifuged at $2000 \times g$ for 5 min (4°C). A 0.1-ml aliquot of the supernatant fluid was assayed for liberated [^{32}P]phosphate after benzene-butanol extraction [15] followed by liquid scintillation counting [16]. Pellets were washed three times with 0.6 ml 10% perchloric acid, 1 mM H_3PO_4 , 0.5 mM ATP (4°C), solubilized in 1 N NaOH (1 h, 95°C), and assayed

for radioactivity (i.e., phosphoenzyme level) by liquid scintillation counting [16]. In all experiments, contaminating levels of ^{32}P in the phosphate and phosphoenzyme analyses (determined by reversing the order to perchloric acid and ATP addition) were subtracted. The hydroxylamine-insensitive phosphoprotein level (i.e., non-acyl phosphate) was also determined [16,17], and subtracted from all phosphoenzyme measurements.

Analysis of LSR and HSR calcium release was performed graphically by plotting $\ln[(C_i - C_f)/(C_o - C_f)]$ versus time, where C_i = calcium content at any time t , C_o = calcium content at the onset of calcium release, and C_f = final calcium content after addition of $5\text{ }\mu\text{M}$ A23187. The plot was non-linear, consistent with the previous suggestion of heterogeneity within sarcoplasmic reticulum vesicles [18,19]; hence, a parallel model (shown below) was tested.

$$C_i - C_f = (C_o - C_f)[(A) \exp(-k_1 t) + (1 - A) \exp(-k_2 t)]$$

where k_1 and k_2 are apparent rate constants for calcium release from the two suspected populations of vesicles, and A = fraction of vesicles with rate constant k_1 . Estimates of $[(A)k_1 + (1 - A)k_2]$ and k_2 were obtained from regression analysis of the initial and terminal linear regions, respectively, of the graph $\ln[(C_i - C_f)/(C_o - C_f)]$ versus time, with the Y-intercept of the terminal linear region used to calculate $(1 - A)$ and hence k_1 . Similar results were obtained when a computer routine was employed to calculate A and k_1 (given k_2) which best fit the experimental data (using least-squares analysis).

Electrophoresis of HSR and LSR was performed by the method of Laemmli [20], using a 7.5% acrylamide separating gel, and a 3% acrylamide stacking gel. Molecular weight standards included myosin (220 000), phosphorylase *b* (92 000), albumin (66 000), ovalbumin (45 000) and trypsinogen (24 000).

Cytochrome oxidase activity of HSR and LSR was analyzed at 25°C as described previously [21]. To estimate mitochondrial contamination of these sarcoplasmic reticulum fractions, cytochrome oxidase activity of mitochondria isolated from canine ventricular myocardium was also determined. The mitochondrial pellet, obtained by

differential centrifugation ($300\text{--}15\,000 \times g$ pellet) in 10 mM NaHCO_3 buffer (4°C), was washed twice in buffer ($15\,000 \times g$), suspended in 10% sucrose, frozen in liquid nitrogen, and stored at -80°C .

Ouabain binding was analyzed using $[^3\text{H}]$ ouabain in the presence of 50 mM Tris (pH 7.4), 4 mM MgCl_2 , 4 mM Tris-phosphate, 500 nM $[^3\text{H}]$ ouabain, $20\text{ }\mu\text{g/ml}$ sarcoplasmic reticulum (37°C) [22]. Reactions were terminated by filtration (GFb filters, Brandel Cell Harvester, 40 cm Hg) 90 min after initiation. Filters were washed three times with 50 mM Tris (pH 7.4) and then assayed for radioactivity by liquid scintillation counting. To estimate sarcolemmal contamination in these sarcoplasmic reticulum fractions, ouabain binding to sarcolemmal vesicles isolated from canine ventricular myocardium [23] was also determined. Nonspecific binding, determined by addition of $200\text{ }\mu\text{M}$ nonradioactive ouabain to the incubation solution, was subtracted from all measurements of ouabain binding.

All reagents were analytical grade, and deionized H_2O was glass distilled prior to use. The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was synthesized as described previously [24], and applied to an anion exchange column (Bio-Rad AG 1-X8). The column was washed with H_2O and 30 mM HCl , and then the ATP eluted with 300 mM HCl . The effluent was neutralized with bisTris propane, brought to $0.5\text{ }\mu\text{M}$ ATP, and stored at -20°C . The MgATP was prepared by cation exchange chromatography of

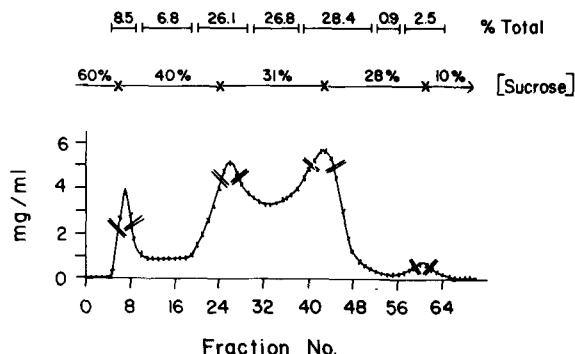


Fig. 1. Fractionation profile of sarcoplasmic reticulum following zonal centrifugation in a discontinuous sucrose gradient. Protein recovery was approx. 95%. Fractions between cross-bars on each peak were pooled for subsequent enzymic analyses. Sucrose concentration expressed as % (w/v).

Na_2ATP in protonated Bio-Rad AG 50W-X8. The ATP effluent was neutralized with KOH, and made equimolar with respect to MgCl_2 .

Results

Fractionation / characterization

Zonal centrifugation of crude sarcoplasmic re-

ticulum in the discontinuous sucrose gradient described above yielded four protein peaks (Fig. 1). The peak at the 28/31% sucrose interface ('light' SR, or LSR) contained approx. 28% of the total protein. Slightly less protein (approx. 26%) appeared at the 31/40% sucrose interface ('intermediate' SR, or ISR), with approx. 27% of the

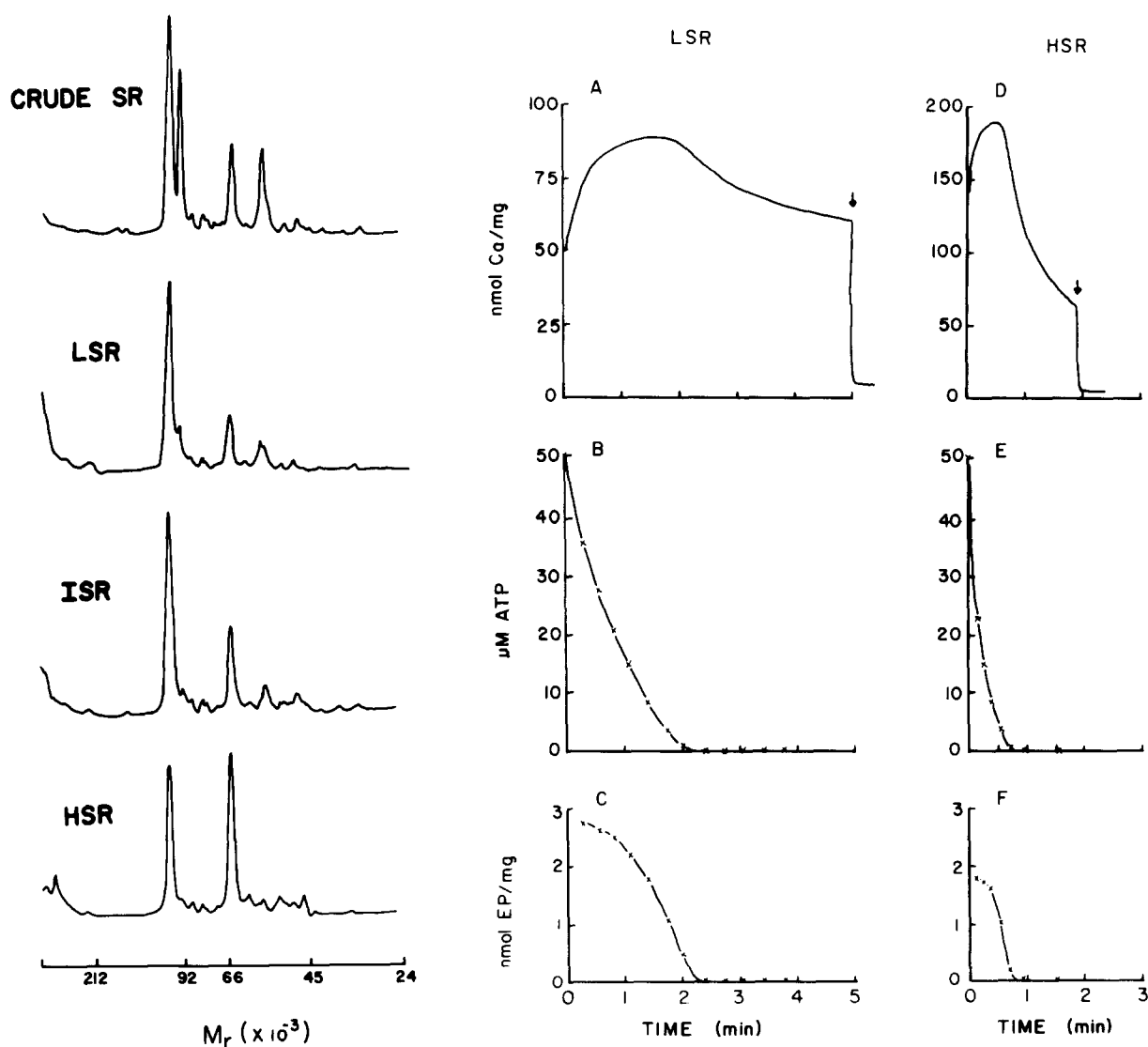


Fig. 2. Electrophoretograms of various sarcoplasmic reticulum fractions. Samples ($50 \mu\text{g}$) were electrophoresed in the presence of SDS, as described in Methods, stained with Coomassie blue, and scanned at 550 nm in a Gilford spectrophotometer.

Fig. 3. Simultaneous monitoring of sarcoplasmic reticulum calcium content, ATP hydrolysis, and phosphoenzyme (EP) level. A, B and C: LSR; D, E and F: HSR; A and D: calcium flux; B and E: ATP hydrolysis; C and F: EP level; $5 \mu\text{M}$ A23187 added at arrow. Reaction conditions as described in text, with 0.2 mg/ml LSR or HSR. Depletion of ATP under these conditions corresponds to the liberation of $250 \text{ nmol P}_i/\text{mg}$.

total protein distributed between these peaks. Thus, LSR and ISR (representing 81% of the protein) constituted the predominant species in this study, although the distribution of LSR and ISR was very sensitive to slight changes in sucrose concentration. The 'heavy' fraction (i.e., HSR) at the 40/60% sucrose interface usually represented less than 10% of the total protein, with an additional 5–8% between ISR and HSR. A small peak containing approx. 3% of the protein was evident at the 10/28% sucrose interface.

Electrophoretic analysis of crude sarcoplasmic reticulum demonstrated a predominance of calcium pump protein M_r approx. 110 000 [4,25], with secondary bands at 90 000, 65 000, and 52 000 (Fig. 2). The LSR exhibited a similar electrophoretic profile, except that the 90 000 band was markedly attenuated (Fig. 2). The ISR and HSR exhibited electrophoretic profiles similar to that of LSR, except that the relative amount of calsequestrin (M_r 65 000) [25] increased progressively with increasing buoyant density of the vesicles, whereas the protein band of M_r 52 000 (which contains high affinity calcium binding protein and an intrinsic glycoprotein) [26] exhibited the reverse trend (Fig. 2). Vesicles from the 10/28% sucrose interface exhibited an intense band of M_r 90 000, which did not comigrate with the calcium-ATPase when LSR was coelectrophoresed with the latter (data not shown). Two secondary proteins (M_r 110 000 and 150 000) were also observed in this very low buoyant density fraction.

Purity of the various sarcoplasmic reticulum fractions was also assessed by enzyme marker analyses. Mitochondrial cytochrome *c* oxidase activities were 1.45 ± 0.05 , 0.004 ± 0.003 , and 0.028 ± 0.004 $\mu\text{mol/mg per min}$ for cardiac mitochondria, LSR, and HSR, respectively; suggesting less than 3% mitochondrial contamination in HSR. Similarly, [^3H]ouabain binding to LSR and HSR was 6 and 10 pmol/mg, respectively, which is less than 5% that seen in canine cardiac sarcolemmal vesicles (237 pmol/mg).

Calcium sequestration and release

Analysis of calcium sequestration demonstrated that both LSR and HSR accumulated calcium rapidly (Figs. 3–5). The ISR also accumulated approx. 100 nmol calcium/mg rapidly, whereas vesicles from the 10/28% sucrose interface accumulated approx. 25 nmol calcium/mg. In the presence of limiting ATP, spontaneous calcium release by LSR and HSR was observed (Figs. 3–5). As shown in Fig. 3A, LSR sequestered 90 nmol calcium/mg (i.e., 18 μM calcium) over the course of 1.6 min, and at minute 2 began to release calcium at a maximal rate of 17 nmol calcium/mg per min. Simultaneous monitoring of ATP hydrolysis demonstrated that at the onset of calcium release, more than 99% of the ATP had been hydrolyzed. Within the next 25 s, ATP level fell below the level of detection, and did not increase during the course of calcium release (Fig. 3B). Similarly, phosphoenzyme level decreased during

TABLE I

SARCOPLASMIC RETICULUM CALCIUM CONTENT AND RELEASE RATES IN VARIOUS MEDIA

Data obtained from experiments like those shown in Fig. 5. Reaction media as described in text, with 120 mM KCl in A, 120 mM choline chloride in B, neither KCl nor choline chloride in C, and 240 mM sucrose in D. (mean \pm S.E., $5 \leq n \leq 7$, three preparations).

Condition	Calcium content ^a (nmol/mg)		Calcium release ^b (nmol/mg per min)	
	LSR	HSR	LSR	HSR
A	108 \pm 2	122 \pm 4	32 \pm 3	143 \pm 14
B	91 \pm 2	109 \pm 3	20 \pm 1	70 \pm 6
C	64 \pm 2	105 \pm 4	13 \pm 2	33 \pm 2
D	62 \pm 4	112 \pm 4	10 \pm 1	34 \pm 4

^a Calcium content at the time of calcium release.

^b Maximal calcium release rate.

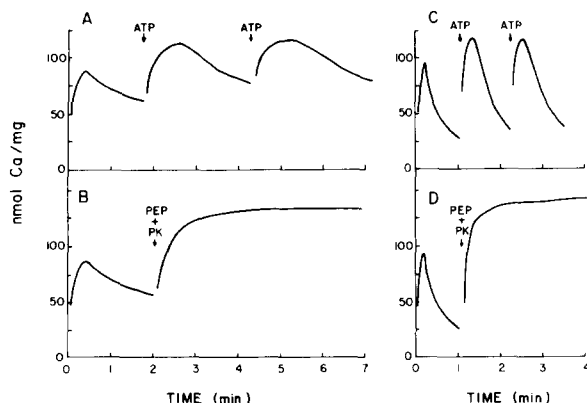


Fig. 4. Reversibility of calcium release. A and B: LSR; C and D: HSR. A and C: Reactions initiated with $10\ \mu\text{M}$ MgATP, with two subsequent additions of $20\ \mu\text{M}$ MgATP at arrows. B and D: Same as A, except that $500\ \mu\text{M}$ phosphoenolpyruvate (PEP) and $20\ \mu\text{g/ml}$ pyruvate kinase (PK) were added after calcium release.

calcium sequestration, although approx. 18% of the phosphoenzyme remained at the onset of calcium release. The phosphoenzyme level decreased to the hydroxylamine insensitive level of $0.05\ \text{nmol/mg}$ within the next 25 s (Fig. 3C); whereas calcium release continued (exhibiting maximal calcium release between minutes 2.2 and 2.5) (Fig. 3A). Analysis of calcium flux, ATP hydrolysis, and phosphoenzyme level by HSR demonstrated similar phenomena, although the rates of ATP hydrolysis and calcium release were much greater than those of LSR (Figs. 3D–F). In this preparation, HSR sequestered more calcium (i.e., $188\ \text{nmol/mg}$) than the three preparations shown in Table I, although the trends in Fig. 3 and Table I are very similar.

The reversibility of the calcium release phase was examined under standard reaction conditions except that $10\ \mu\text{M}$ CaCl_2 , $10\ \mu\text{M}$ MgATP, and $0.1\ \text{mg/ml}$ LSR or HSR were employed. With this low MgATP concentration, calcium release from LSR began within 25 s after initiation of the reaction (Fig. 4A). Subsequent addition of MgATP ($20\ \mu\text{M}$) reinitiated calcium sequestration, with calcium release occurring approx. 54 s later (Fig. 4A). Addition of an ATP-regenerating system (phosphoenolpyruvate and pyruvate kinase) stimulated calcium sequestration with no evidence of calcium release (Fig. 4B). Similar results were

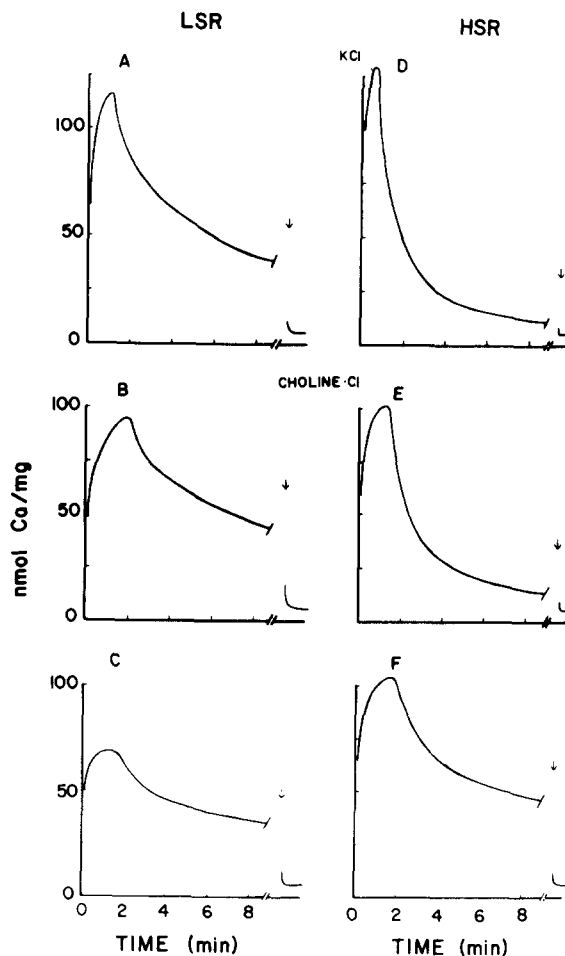


Fig. 5. Ionic dependence of calcium sequestration and release. Reaction media as described in text, with $120\ \text{mM}$ KCl in A and D, $120\ \text{mM}$ choline chloride in B and E, and neither KCl nor choline chloride in C and F. A, B and C: LSR; D, E and F: HSR. $5\ \mu\text{M}$ A23187 added at arrow.

obtained in the presence of HSR in that ATP reinitiated the calcium sequestration-release process (Fig. 4C), whereas the ATP regenerating system initiated calcium sequestration with no evidence of calcium release (Fig. 4D).

The ionic dependence of calcium sequestration and release by LSR and HSR was examined with various KCl, choline-chloride, and sucrose concentrations. In the presence of $120\ \text{mM}$ KCl, LSR sequestered $108 \pm 2\ \text{nmol}$ calcium/mg within 1.2 min (Fig. 5A and Table I), and then released calcium at a rate of $32 \pm 3\ \text{nmol/mg per min}$ (Table I). HSR, on the other hand, sequestered

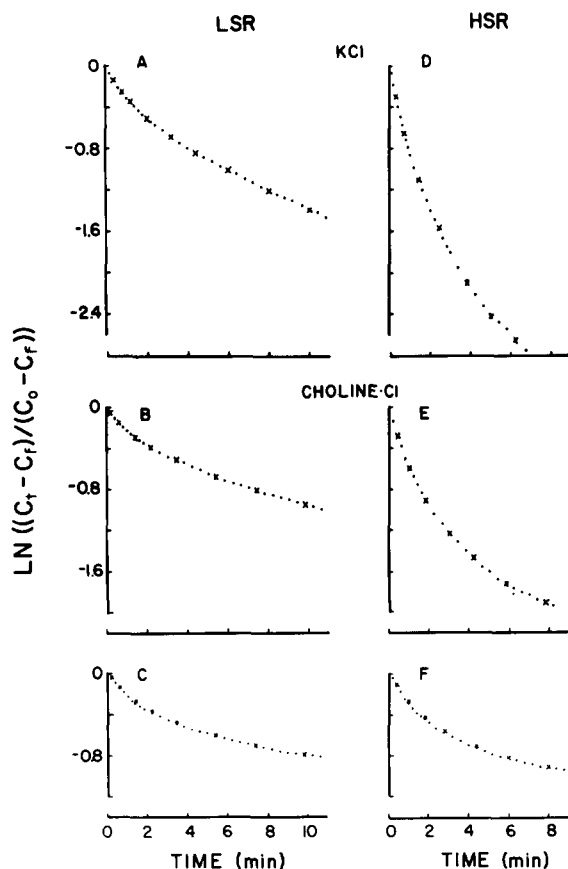


Fig. 6. Rate constant analyses of calcium release in various media. Data obtained from experiments like those shown in Fig. 5. A and D: 120 mM KCl; B and E: 120 mM choline chloride; C and F: neither KCl nor choline chloride; A, B and C: LSR; D, E and F: HSR; ●, Experimental points; ×, predicted points using rate constants shown in Table II.

122 ± 4 nmol calcium/mg within 30 s, and released calcium at a rate of 143 ± 14 nmol/mg per min (Fig. 5D and Table I).

Replacement of the KCl with 120 mM choline-chloride prolonged the time to onset of calcium release and reduced both calcium content and calcium release rate by LSR and HSR (Fig. 5 and Table I). The reduction in LSR calcium content, however, was greater than that of HSR ($16 \pm 2\%$ vs. $11 \pm 2\%$ inhibition), whereas HSR calcium release rate was inhibited to a greater extent than that of LSR ($51 \pm 4\%$ vs. $38 \pm 3\%$ inhibition, $p < 0.05$). Reduction of ionic strength by omission of KCl and choline chloride also prolonged the time to onset of calcium release by LSR and HSR (Figs. 5C and 5F), and further reduced LSR calcium content, but caused a much smaller decrease in HSR calcium content ($41 \pm 1\%$ vs. $14 \pm 3\%$ inhibition, $p < 0.05$) (Table I). The HSR calcium release rate, however, decreased more than that of LSR when ionic strength was reduced ($77 \pm 1\%$ vs. $60 \pm 6\%$ inhibition, $p < 0.05$) (Table I). Inclusion of 240 mM sucrose in the absence of KCl and choline chloride had little effect on the above trend (Table I).

Rate constant analyses of the calcium release phase in the various media did not fit a mono-exponential relationship, but instead were consistent with a bi-exponential model (Fig. 6). In the presence of 120 mM KCl, rate constants for calcium release by LSR were $0.63 \pm 0.05 \text{ min}^{-1}$ and $0.10 \pm 0.005 \text{ min}^{-1}$, with the rapidly releasing fraction representing $38 \pm 2\%$ of the total, and an overall

TABLE II

IONIC DEPENDENCE OF SARCOPLASMIC RETICULUM CALCIUM RELEASE RATE CONSTANTS

Data obtained from graphs like those shown in Fig. 7. $(A)k_1 + (1 - A)k_2$ represents the initial (weighted average) calcium release rate constant. k_1 and k_2 are the calculated rate constants for calcium release from the fast and slow releasing fractions, respectively. Reaction conditions as in Table I, with 120 mM KCl in A, 120 mM choline chloride in B, neither KCl nor choline chloride in C, and 240 mM sucrose in D. All rate constants expressed as min^{-1} (mean \pm S.E., $5 \leq n \leq 7$, three preparations).

Condition	$(A)k_1 + (1 - A)k_2$		k_1		k_2	
	LSR	HSR	LSR	HSR	LSR	HSR
A	0.30 ± 0.02	1.27 ± 0.13	0.63 ± 0.05	1.65 ± 0.16	0.10 ± 0.01	0.26 ± 0.04
B	0.24 ± 0.01	0.77 ± 0.07	0.64 ± 0.05	1.10 ± 0.10	0.07 ± 0.01	0.15 ± 0.02
C	0.26 ± 0.03	0.33 ± 0.03	0.58 ± 0.04	0.63 ± 0.03	0.03 ± 0.01	0.06 ± 0.01
D	0.19 ± 0.02	0.31 ± 0.03	0.59 ± 0.04	0.60 ± 0.03	0.06 ± 0.01	0.06 ± 0.01

TABLE III

EFFECTS OF IONOPHORES ON LSR CALCIUM CONTENT AND RELEASE

Reaction media contained 120 mM KCl, with or without 10 μ M ionophore. (mean \pm S.E., $n = 4$, four preparations).

Ionophore	Calcium content ^a (nmol/mg)	Calcium release ^b (nmol/mg per min)
None	98 \pm 6	27 \pm 2
Gramicidin	99 \pm 6	26 \pm 1
Valinomycin	104 \pm 6	49 \pm 3

^a Calcium content at time of calcium release.

^b Maximal calcium release rate.

initial rate constant of $0.30 \pm 0.02 \text{ min}^{-1}$ (Table II). Rate constants for calcium release by HSR were $1.65 \pm 0.16 \text{ min}^{-1}$ and $0.26 \pm 0.04 \text{ min}^{-1}$, with the rapidly releasing fraction representing $74 \pm 1\%$ of the total, and an overall initial rate constant of $1.27 \pm 0.13 \text{ min}^{-1}$ (Table II). As in the case of the calcium release rate data, replacement of KCl with choline chloride reduced the overall initial rate constant of HSR calcium release more than that of LSR ($40 \pm 5\%$ vs. $20 \pm 3\%$ inhibition, $p < 0.05$) (Table II). Comparison of the component rate constants (k_1 and k_2) showed that replacement of KCl with choline chloride inhibited both HSR rate constants 40%, whereas only the slow rate constant (k_2) for LSR calcium release was altered by this ionic substitution (Table II). Reduction of ionic strength by omission of KCl and choline chloride reduced the overall rate constant for HSR still further (74% inhibition), with little effect on that of LSR (14% inhibition) (Table II). Comparison of the component rate constants showed that this reduction of ionic strength inhibited both HSR release rate constants (k_1 and k_2) 70%, whereas in the case of LSR, only the slow rate constant (k_2) was affected (Table II). Inclusion of 240 mM sucrose in place of KCl and choline chloride had little effect on these differences between LSR and HSR (Table II).

As the observed differences in the ionic dependences of LSR and HSR calcium release may be due in part to a greater potassium permeability of HSR (see Discussion), the effect of the potassium ionophore valinomycin on LSR calcium release was examined under standard conditions. Valinomycin (10 μ M), present from the beginning

of the reaction, had no effect on LSR calcium content (Table III), but increased calcium release rate from 27 ± 2 to $49 \pm 3 \text{ nmol/mg per min}$ ($p < 0.05$) (Table III). The overall rate constant for the initial phase of calcium release increased from 0.36 ± 0.04 to $0.51 \pm 0.03 \text{ min}^{-1}$ ($p < 0.05$) in the presence of 10 μ M valinomycin. Analysis of the calcium release data on the assumption of a bi-exponential curve indicated that valinomycin increased the rate constant of the fast releasing fraction (0.98 ± 0.10 vs. $0.74 \pm 0.07 \text{ min}^{-1}$, $p < 0.05$) more than that of the slow releasing fraction (0.16 ± 0.04 vs. $0.16 \pm 0.05 \text{ min}^{-1}$, $p > 0.05$). To determine if these effects were specific for potassium, the effects of the proton ionophore gramicidin D on LSR calcium release were also examined. As shown in Table III, neither LSR calcium content nor calcium release rate were altered by gramicidin D.

Discussion

The present study compared calcium release kinetics following ATP depletion in two fractions of sarcoplasmic reticulum vesicles from rabbit skeletal muscle. Calcium release began at the time of ATP depletion, and was not accompanied by a detectable increase in ATP level; hence, this calcium release appears to be passive, and not due to complete reversal of the calcium pump reaction (which would be expected to be accompanied by ATP synthesis and phosphoenzyme formation). Moreover, the residual phosphoenzyme observed at the onset of calcium release decayed shortly thereafter, whereas calcium release continued and reached a maximum when phosphoenzyme was absent. Feher and Briggs [27] also found no evidence for ATP resynthesis during calcium release from sarcoplasmic reticulum vesicles following hexokinase-induced ATP depletion. Thus, it appears that calcium release began when ATP concentration fell to a level where active calcium transport velocity was less than that of passive calcium efflux. This would result in a gradual calcium release while some phosphoenzyme still remained, followed by full expression of passive calcium efflux when ATP and phosphoenzyme levels fell essentially to zero. An initial (gradual) phase of calcium release was evident from the

spectrophotometric recordings, but only lasted a few seconds and was not included in the rate constant analyses.

Rate constant analysis of calcium release from LSR was inconsistent with a mono-exponential function, but instead fit a bi-exponential (0.63 and 0.10 min^{-1}). The fast releasing fraction of vesicles was calculated to represent 38% of the total, with an initial (weighted average) release rate constant of 0.30 min^{-1} . The latter rate constant agrees with that obtained by Takenaka et al. [28] for the initial phase of EGTA-induced calcium release from a similar sarcoplasmic reticulum fraction in the presence of acetylphosphate, where pump reversal does not occur. Moreover, similar rate constants (i.e., 0.5 min^{-1} and 0.06 min^{-1} for 30% and 70% of the releasable calcium, respectively) have been observed for EGTA-induced calcium release from passively loaded vesicles [19]. These similarities in rate constants lend further support for the hypothesis that calcium release observed in the present study is not due to calcium pump reversal. Feher and Briggs [27] who studied calcium release following hexokinase-induced ATP depletion, obtained a calcium release constant of 0.7 min^{-1} . These investigators argued that the calcium release kinetics followed a mono-exponential function when calcium binding to internal sites (e.g., calsequestrin) was taken into consideration. Measurement of total 'releasable calcium' by addition of $5 \mu\text{M}$ A23187, however, demonstrated an immediate, rapid calcium release (Fig. 3), indicating that the second (slow) phase of calcium release cannot be attributed to a slow dissociation of calcium from internal binding sites. Instead, the data are more readily explained by heterogeneity within the LSR fraction.

Analysis of HSR calcium release also suggested a bi-exponential function (1.65 and 0.26 min^{-1}), with the fast releasing fraction representing 74% of the total. As the calsequestrin content of HSR was approx. 7-times greater than that in the LSR (Fig. 2), calcium binding to this protein would decrease the proportion of 'fast releasing' vesicles in HSR, compared to LSR, if binding of calcium to internal sites was responsible for the slow phase of the bi-exponential release curve. The opposite was observed, however.

Another possible explanation for the observed

bi-exponential release of calcium is that this process is calcium dependent, so that the permeability of the sarcoplasmic reticulum membrane changes as a function of either extravesicular calcium concentration or the calcium concentration gradient across the sarcoplasmic reticulum membrane, as previously suggested for calcium-oxalate and calcium-phosphate loaded vesicles [29]. Nagasaki and Kasai [18], who reported calcium-dependent calcium release from passively loaded sarcoplasmic reticulum vesicles in the absence of MgCl_2 , found that passive calcium efflux appeared to be calcium-independent in the presence of 5 mM MgCl_2 . Thus, it is likely that in the present experiments, carried out in 5 mM MgCl_2 , calcium release is calcium-independent. A time-dependent change in calcium permeability of the sarcoplasmic reticulum [30] is another possible explanation of the observed bi-exponential release of calcium. The reproducibility of the calcium release phase upon subsequent addition of ATP (Fig. 4), however, argues against such a mechanism. Furthermore, calcium release rate did not change when the time to onset of calcium release was increased by reducing the protein:ATP ratio (data not shown), which is also inconsistent with a time-dependent change in calcium permeability.

Direct comparison of calcium release kinetics in LSR and HSR demonstrated that, starting with similar calcium contents, HSR released calcium 4.4-fold faster than that of LSR (Table I). The HSR, however, has a smaller intravesicular volume [8,31] and contains more calsequestrin [3,9 and Fig. 2] than LSR; consequently, comparisons are more appropriately expressed by rate constant analyses, which are independent of concentration gradients. This method gave similar differences, with HSR and LSR exhibiting an initial (weighted average) release rate constants of 1.27 and 0.30 min^{-1} , respectively. Analysis of the data assuming a bi-exponential function also maintained this trend, with 74% of the HSR vesicles exhibiting a rate constant of 1.65 min^{-1} , as opposed to 62% of the LSR vesicles releasing calcium with a rate constant of 0.10 min^{-1} . Using vesicles loaded with calcium in the presence of acetylphosphate, Meissner [3] observed that EGTA-induced calcium release from HSR was faster than from LSR, though the time required for 50% calcium release (which is

inversely related to the rate constant for calcium release) was similar for the two fractions of vesicles. Louis et al. [9] reported the reverse trend in the presence of ATP or acetylphosphate in that HSR exhibited a slower EGTA-induced calcium release than LSR, though it is not clear if the rate constants for calcium release differ. Campbell et al. [8], on the other hand, observed a 2-fold higher calcium release rate constant for EGTA-induced calcium release from HSR than LSR after passive equilibration in 1 mM CaCl_2 , though the rate constants for LSR (0.20 min^{-1}) and HSR (0.35 min^{-1}) are lower than those observed in the present study (i.e., 0.30 and 1.27 min^{-1}). Analyses of calcium-induced, caffeine-induced, and anion substitution-induced calcium release by HSR and LSR have also shown inconsistencies [32–37], although calcium release by HSR was generally found to exceed that by LSR. Comparison of initial (weighted average) rate constants for LSR and HSR in the present study are therefore consistent with the majority of the above reports in that the rate constant for HSR was 4-times that of LSR. Examination of the rate constants from the predominant fractions LSR and HSR, however, suggests an even greater (i.e., 15-fold) difference in calcium permeability between terminal cisternae and longitudinal tubules.

Reduction of ionic strength or replacement of KCl with choline chloride decreased maximal calcium content and calcium release rate for both LSR and HSR. These interventions reduced LSR calcium content at the time of calcium release to a greater extent than that of HSR, whereas rate constants for HSR calcium release were consistently inhibited more than those of LSR. This observation implies a greater dependence of HSR calcium permeability on the ionic environment, although the mechanism for this cation-specific change in calcium permeability is not clear.

Analysis of the cation-dependence of the calcium transport mechanism of sarcoplasmic reticulum has demonstrated that potassium affects several reaction steps [38,39], and that replacement of potassium by various monovalent cations (e.g., sodium or lithium) reduced calcium uptake velocity [40]. It has been proposed that approximately 67% of the sarcoplasmic reticulum vesicles are potassium permeable [41], and that calcium uptake

is couple to potassium efflux [42]. Thus, the observation that LSR calcium content was reduced by replacement of KCl with choline chloride, and/or by reduction of ionic strength is in accord with these earlier studies. The less dramatic changes in HSR calcium content upon ionic substitution or reduction of ionic strength suggests a difference between LSR and HSR in terms of ionic regulation of calcium transport. Further study of these differences, using both stopped flow and quench flow analyses to define the affinity for potassium as an allosteric regulator of the calcium transport reaction in the two fractions of sarcoplasmic reticulum is needed.

Electron probe analysis of skeletal muscle has shown that calcium release from the terminal cisternae is accompanied by accumulation of potassium and magnesium [6], so that the potassium-dependence of HSR calcium release may reflect a mechanism in which calcium efflux is balanced by potassium influx so as to maintain electroneutrality. The higher calcium release rate constants and greater potassium-dependence of calcium release by HSR may, therefore, reflect a higher potassium influx rate in HSR. Inclusion of valinomycin with LSR should maximize potassium influx and thereby augment calcium release rate constants in this fraction. In the present study, valinomycin was found to increase LSR calcium release rate, although the rate constant remained much less than that for HSR. The low rate constant for LSR calcium release, therefore, can be explained only partially by slow potassium influx. Furthermore, the relative insensitivity of the predominant fraction of LSR to valinomycin is consistent with previous reports of a high potassium permeability in 67% of the vesicles (Type I) in a similar preparation [41].

In summary, both LSR and HSR appear to be heterogeneous, with HSR exhibiting a weighted average calcium release rate constant 4-fold higher than that of LSR. The weighted average rate constant for calcium release from HSR was also much more sensitive to the ionic composition of the medium than that of LSR. Thus, it is hypothesized that the calcium permeability of the terminal cisternae is greater and more sensitive to the ionic environment than that of the longitudinal tubules.

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