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Effects of Mg^{2+} on calcium accumulation by two fractions of sarcoplasmic reticulum from rabbit skeletal muscle

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Calcium accumulation by two fractions of sarcoplasmic reticulum presumably derived from longitudinal tubules (light vesicles) and terminal cisternae (heavy vesicles) was examined radiochemically in the presence of various free Mg^{2+} concentrations. Both fractions of sarcoplasmic reticulum exhibited a Mg^{2+}-dependent increase in phosphate-supported calcium uptake velocity, though half-maximal velocity in heavy vesicles occurred at a much higher free Mg^{2+} concentration than that in light vesicles (i.e., approx. 0.90 mM vs. approx. 0.02 mM Mg^{2+}). Calcium uptake velocity in light vesicles correlated with Ca^{2+}-dependent ATPase activity, suggesting that Mg^{2+} stimulated the calcium pump. Calcium uptake velocity in heavy vesicles did not correlate with Ca^{2+}-dependent ATPase activity, although a Mg^{2+}-dependent increase in calcium influx was observed. Thus, Mg^{2+} may increase the coupling of ATP hydrolysis to calcium transport in heavy vesicles. Analyses of calcium sequestration (in the absence of phosphate) showed a similar trend in that elevation of Mg^{2+} from 0.07 to 5 mM stimulated calcium sequestration in heavy vesicles much more than in light vesicles. This difference between the two fractions of sarcoplasmic reticulum was not explained by phosphoenzyme (EP) level or distribution. Analyses of calcium uptake, Ca^{2+}-dependent ATPase activity, and unidirectional calcium flux in the presence of approx. 0.4 mM Mg^{2+} suggested that ruthenium red (0.5 μM) can also increase the coupling of ATP hydrolysis to calcium transport in heavy vesicles, with no effect in light vesicles. These functional differences between light and heavy vesicles suggest that calcium transport in terminal cisternae is regulated differently from that in longitudinal tubules.

Introduction

Cytoplasmic calcium concentration in skeletal muscle is controlled by the sarcoplasmic reticulum, an intracellular membrane system composed of terminal cisternae (adjacent to the T-tubules) and longitudinal tubules (extending between terminal cisternae in a sarcomere) [1,2]. The terminal cisternae contain an abundance of calcium, which is released into the cytoplasm during excitation-contraction coupling [3]. The longitudinal tubules may then contribute to relaxation of the muscle by sequestering cytoplasmic calcium [4]. Calsequestrin, a calcium binding protein in the terminal cisternae [5–7], may facilitate diffusion of calcium from the longitudinal tubules to a storage site in the terminal cisternae. The preferential release of calcium from the terminal cisternae during excitation-contraction coupling may therefore reflect the abundance of this calcium binding protein, and hence calcium, in this region of the sarcoplasmic reticulum. Recent studies, however, have shown that the calcium release kinetics of terminal cisternae-derived sarcoplasmic reticulum (heavy vesicles) are more rapid than those of longi-

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

tudinal tubule-derived sarcoplasmic reticulum (light vesicles) [8–12]. Moreover, calcium permeability in heavy vesicles appeared to be much more sensitive to the ionic composition of the medium than did that of light vesicles [12]. The terminal cisternae, therefore, appear to be functionally distinct from the longitudinal tubules, at least in terms of calcium release kinetics.

The present study addresses the calcium uptake characteristics of these two regions of the sarcoplasmic reticulum. Louis et al. [13] reported several similarities bteween these two fractions of sarcoplasmic reticulum, including Ca^{2+}-dependence of calcium uptake. Quantitative differences were apparent in that calcium uptake velocity in light vesicles was 3-times faster than that observed in heavy vesicles, presumably because the heavy vesicles contain an abundance of non-pump protein (e.g., calsequestrin). Similar quantitative differences between these two fractions of sarcoplasmic reticulum have been reported by others [14–16]. In the present study, qualitative as well as quantitative differences were observed. The Mg^{2+}-dependence of calcium uptake in light vesicles, for example, was very different from that in heavy vesicles, with half-maximal calcium uptake at approx. 0.02 and approx. 0.90 mM Mg^{2+}, respectively. Moreover, the mechanism(s) involved in these Mg^{2+}-dependent increases in calcium uptake velocity differed between these fractions of sarcoplasmic reticulum in that Mg^{2+} markedly increased the apparent coupling of ATP hydrolysis to calcium transport in heavy vesicles, but had no such effect in light vesicles. Ruthenium red also elucidated functional differences between these two fractions of sarcoplasmic reticulum, affecting heavy vesicles in a manner similar to millimolar Mg^{2+}, with no effect on light vesicles. The present study, therefore, shows that the calcium uptake characteristics of the terminal cisternae are distinct from those in the longitudinal tubules. Moreover, the present study suggests a mechanism for ruthenium red effects on heavy vesicles which differs substantially from that previously reported [16–20].

Methods

Sarcoplasmic reticulum vesicles were isolated from rabbit white skeletal muscle by a combination of differential and zonal centrifugation techniques, as described previously [12]. The zonal centrifugation consisted of centrifugation for 4 h at $91\,000 \times g_{av}$ (Beckman SW 27 rotor) in a discontinuous sucrose gradient containing 3 ml 60%, 10 ml 40%, 10 ml 31%, and 10 ml 28% (w/v) sucrose with 3 ml (40 mg/ml) crude sarcoplasmic reticulum vesicles in 10% (w/v) sucrose. Vesicles at the 28/31% sucrose interface (light sarcoplasmic reticulum vesicles, LSR) and 40/60% sucrose interface (heavy sarcoplasmic reticulum vesicles, HSR), presumably derived from the longitudinal tubules and terminal cisternae (respectively) [5,13,21], were frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the technique of Lowry et al. [22], using bovine serum albumin as a standard.

Calcium uptake by the two fractions of sarcoplasmic reticulum vesicles was determined radiochemically, using $^{45}CaCl_2$. Reaction media contained 120 mM KCl, 5 mM K_2ATP, 40 mM histidine, 50 mM KH_2PO_4 (pH 6.8, 25°C), 200 μM $^{45}CaCl_2$, 6 μg/ml sarcoplasmic reticulum vesicles, and various concentrations of $MgCl_2$ (0.018–35 mM) and EGTA (0.12–0.22 mM) so as to obtain 6 μM free Ca^{2+} and the desired free Mg^{2+} concentration *. Reactions were initiated by addition of sarcoplasmic reticulum vesicles, although similar results were obtained when reactions were initiated by ATP addition. Reactions were terminated by Millipore filtration (0.25 ml aliquot, 0.45 μm HA Millipore filter in Swinney adapter), and filtrates were analyzed for radioactivity by liquid scintillation counting. Filtrates were also analyzed for protein concentration by Coomassie-blue binding [26] to assess retention of sarcoplasmic reticulum vesicles by the Millipore

* A computer routine was used to calculate the amount of EGTA (pH 6 8) and $MgCl_2$ to add to the solution to obtain 10 μM free Ca^{2+} and the desired free Mg^{2+} concentration, assuming the following apparent association constants: 4 10^3 for CaATP [23], $9 \cdot 10^3$ for MgATP [23], 10^6 for CaEGTA [23,24], and 24 for MgEGTA [23] Free Ca^{2+} and Mg^{2+} concentrations were then recalculated by an iterative computer routine [23], using the above apparent association constants plus 3.6 for K-ATP [23], 40 for calcium phosphate [23], 54 for magnesium phosphate [23], and (where appropriate) 5 10^3 for Ca-antipyrylazo III [25] The free ion concentrations calculated from the latter computer routine [23] were used for all figures and tables.

filters. In the latter assay, 0.2 ml of the filtrate was mixed with 0.2 ml H_2O and 0.1 ml dye reagent (Bio-Rad protein assay concentrate), followed by determination of absorbance (595 nm). Unfiltered reaction mixture was assayed for protein concentration similarly, using diluted sarcoplasmic reticulum vesicles (0.3–3 μg) as a standard.

The ATP hydrolysis by sarcoplasmic reticulum vesicles was measured spectrophotometrically. Reaction conditions were as described for calcium uptake, except that $^{40}CaCl_2$ was used. Reactions were terminated by Millipore filtration at minutes 1 and 3, and filtrates analyzed for ADP [27]. For samples with 0.05 and 0.005 mM Mg^{2+}, filtrates were supplemented with $MgCl_2$ (0.4 mM free Mg^{2+} final) before ADP analysis (i.e., prior to addition of pyruvate kinase and phospho*enol*-pyruvate). Basal ATPase activity was measured similarly, except that EGTA (0.42–0.56 mM) was added instead of $CaCl_2$. The EGTA concentration used for basal ATPase activity at a given Mg^{2+} concentration was calculated to reduce free Ca^{2+} concentration to 5 nM (given 4.5 μM contaminating calcium in the reaction medium [28]). The Ca^{2+}-dependent ATPase activity was calculated as the difference between total ATPase and basal ATPase activities.

Calcium influx was determined in the same manner as calcium uptake, except that reactions were initiated in the presence of $^{40}CaCl_2$. Tracer $^{45}CaCl_2$ (20 μl) was added to the reaction medium (2.5 ml) at the desired time (e.g., min 1 or 7), and aliquots filtered periodically, as described above. Calcium efflux was calculated as the difference between calcium influx and calcium uptake velocity [29].

Calcium uptake was also measured by dual wavelength spectrophotometry (Johnson Research Foundation Spectrophotometer), using the calcium-indicator antipyrylazo III (720, 790 nm) [30]. Reaction media contained 120 mM KCl, 5 mM K_2ATP, 100 μM antipyrylazo III, 40 mM histidine, 50 mM KH_2PO_4 (pH 6.8, 25°C), 40 μM $CaCl_2$, 6 μg/ml sarcoplasmic reticulum vesicles, and either 5 mM or 14.9 mM $MgCl_2$. Recordings were calibrated by four serial additions of 0.002 volumes $CaCl_2$, and reactions initiated by addition of 0.005 volumes sarcoplasmic reticulum vesicles. Reactions were also monitored in the absence of antipyrylazo III, and demonstrated no effect of osmotic swelling of the vesicles [31] on the dual wavelength spectrophotometric recordings (data not shown).

Calcium sequestration was also measured by Millipore filtration. Reaction media contained 120 mM KCl, 50 μM $^{45}CaCl_2$, 40 mM histidine (pH 6.8, 25°C), 0.1 mg/ml sarcoplasmic reticulum vesicles, and 0.1 mM MgATP, with or without 5 mM $MgCl_2$. Reactions were initiated by addition of MgATP, and terminated 10 s later by Millipore filtration. For analysis of total ATPase activity, $^{40}CaCl_2$ and [γ-^{32}P]ATP were used, and reactions terminated by perchloric acid quenching [12], followed by benzene/butanol extraction of [^{32}P]phosphate [32]. Basal ATPase activity was determined similarly, except that 1 mM EGTA (pH 6.8) was included in the reaction medium instead of $CaCl_2$. The Ca^{2+}-dependent ATPase activity was calculated as the difference between total ATPase and basal ATPase activities.

Phosphoenzyme (EP) level was determined radiochemically by perchloric acid quenching [12,33]. Reaction media were as described for calcium sequestration, except that [γ-^{32}P]ATP and $^{40}CaCl_2$ were included. Reactions were terminated 10 s after initiation by addition of 1 vol. (0.2 ml) 15% perchloric acid, 1.5 mM H_3PO_4 (4°C). The perchloric acid quenched samples were supplemented with 25 μl 5 mg/ml crude sarcoplasmic reticulum vesicles, and then centrifuged at 2000 $\times$ *g* for 5 min (4°C). 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 M NaOH (1 h, 95°C), and assayed for radioactivity (i.e., total phosphoprotein level) by liquid scintillation counting Nonacyl-phosphoprotein level was determined similarly, except that perchloric acid-washed pellets were incubated in hydroxylamine [33,34] before solubilization in NaOH. Phosphoenzyme (EP) level represents the difference between total phosphoprotein level and nonacyl-phosphoprotein level. Phosphoenzyme distribution was determined by addition of 1 mM ADP 10 s after initiation of the reaction [35], followed 5 s later by perchloric acid quenching and then measurement of total phosphoprotein level. The E_2P (i.e., ADP-insensitive phosphoenzyme) level was calculated as the difference between the ADP-treated phosphoprotein

level and nonacyl-phosphoprotein level. The % E_1P was calculated as $((EP - E_2P)/EP) \times 100$.

Materials

All reagents were analytical grade, and deionized water was glass distilled prior to use. The [γ-^{32}P]ATP was synthesized from [^{32}P]phosphorus [36], and purified by anion-exchange chromatography [12]. The K_2ATP and MgATP were prepared by cation-exchange chromatography of Na_2ATP in protonated Bio-Rad AG 50W-X8, followed by neutralization of the ATP effluent with KOH. For MgATP, this K_2ATP was made equimolar with respect to $MgCl_2$. Ruthenium red, $^{45}CaCl_2$, and [^{32}P]phosphorus were purchased from Sigma, ICN, and NEN, respectively.

Results

Phosphate-supported calcium uptake

The time-course of calcium uptake by sarcoplasmic reticulum vesicles was examined by Millipore filtration (using $^{45}CaCl_2$) in the presence of 0.4 mM free Mg^{2+} (Fig. 1A). This free Mg^{2+} concentration was obtained by inclusion of 4.9 mM $MgCl_2$ with 5 mM K_2ATP, 190 μM EGTA, 200 μM $CaCl_2$ (pCa = 5.2), 40 mM histidine, 50 mM KH_2PO_4 (pH 6.8). As shown in Fig. 1A, light vesicles (LSR) accumulated 5–6 μmol Ca/mg at an initial velocity of 1.1 μmol/mg per min under these conditions; whereas, heavy vesicles (HSR) accumulated much less calcium (2.2 μmol/mg) at a slower velocity (0.4 μmol/mg per min. When the free Mg^{2+} concentration was increased to 3 mM (14.9 mM $MgCl_2$, 211 μM EGTA), heavy vesicles accumulated calcium to a greater extent and at a greater velocity than did light vesicles (Fig. 1B). The change in Mg^{2+} concentration from 0.4 to 3 mM had little effect on calcium content or uptake velocity in light vesicles, but dramatically affected these parameters in heavy vesicles.

To rule out the possibility that this Mg^{2+}-dependent increase in calcium uptake in heavy vesicles was due to incomplete retention of vesicles by Millipore filters at low Mg^{2+} concentrations, filtrates were analyzed for protein concentration using the Coomassie-blue binding technique (see Methods). Reaction media containing 6 μg/ml heavy vesicles and 0.4 mM Mg^{2+} produced an

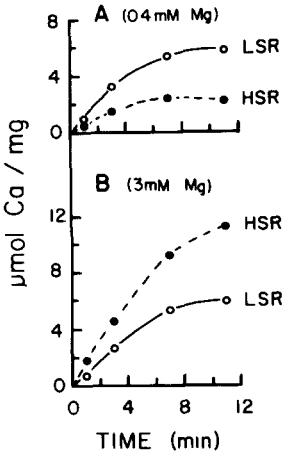


Fig. 1 Time-course of calcium uptake LSR, light sarcoplasmic reticulum vesicles (presumably derived from the longitudinal tubules), HSR, heavy sarcoplasmic reticulum vesicles (presumably derived from the terminal cisternae) Reaction media as described in Methods for analysis of phosphate-supported calcium uptake by Millipore filtration, with either 0.4 mM (A) or 3 mM (B) free Mg^{2+} ○, LSR; ●, HSR. Symbols represent means ± S.E. for $n = 4$, two preparations

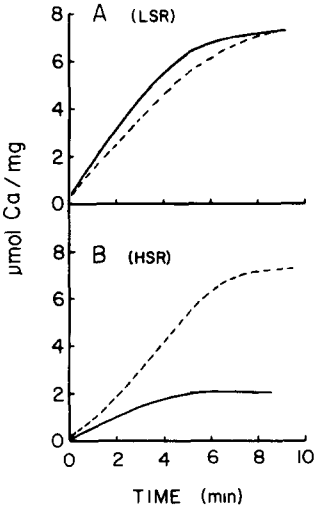


Fig 2. Spectrophotometric analysis of calcium uptake Reaction media as described in Methods for analysis of phosphate-supported calcium uptake by dual-wavelength spectrophotometry in the presence of 0 4 mM (———) or 3 mM (— — —) free Mg^{2+} The total calcium concentration in this analysis is 44.5 μM (i e, 40 μM added $CaCl_2$, 4 5 μM contaminating calcium [28]), so the theoretical maximum calcium content is 7 4 μmol/mg Each curve represents a typical run.

absorbance of 0.130 ± 0.003 ($n = 9$), whereas filtrates contained no detectable protein ($p > 0.05$). To further test this hypothesis, calcium uptake was monitored by dual-wavelength spectrophotometry, using the calcium-indicator antipyrylazo III. As shown in Fig. 2, elevation of free Mg^{2+} from 0.4 to 3 mM had a small inhibitory effect on calcium uptake velocity in light vesicles (1.35 ± 0.08 vs. 1.21 ± 0.03 μmol/mg per min), but markedly increased calcium uptake velocity in heavy vesicles (0.43 ± 0.01 vs. 1.08 ± 0.10 μmol/mg per min), resembling the Mg^{2+} effects seen using Millipore filtration.

To clearly illustrate this functional difference between these two fractions of sarcoplasmic reticulum vesicles, a Mg^{2+} profile of calcium uptake

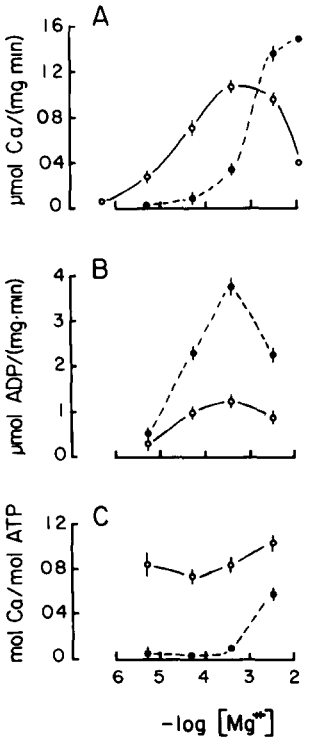


Fig. 3 Mg^{2+}-dependence of calcium uptake and ATP hydrolysis Reaction conditions as in Fig. 1, with free Mg^{2+} concentration varied from 0.5 μM ($pMg = 6\,3$) to 11 mM ($pMg = 1\,95$). (A) Calcium uptake velocity; (B) Ca^{2+}-ATPase activity, (C) coupling ratio (calculated as the ratio of calcium uptake velocity (A) to Ca^{2+}-ATPase activity (B)). O, light sarcoplasmic reticulum vesicles, ●, heavy sarcoplasmic reticulum vesicles. Calcium uptake velocity and ATPase activity were determined between min 1 and 3 Symbols represent means ± S.E. for $8 \leq n \leq 12$, two preparations (or mean for $n = 2$ at $pMg = 1\,95$)

velocity by each fraction of vesicles was determined using Millipore filtration, with free Ca^{2+} concentration buffered at 6 μM *. As shown in Fig. 3, calcium uptake velocity in light vesicles increased when the Mg^{2+} concentration was raised from 0.5 μM to 0.4 mM, with half-maximal velocity at approx. 20 μM Mg^{2+}. As the Mg^{2+} concentration was increased to 11 mM, calcium uptake velocity in light vesicles decreased. Calcium uptake velocity in heavy vesicles showed a very different Mg^{2+} dependence in that half-maximal calcium uptake occurred at approx. 0.9 mM Mg^{2+}. Moreover, calcium uptake velocity in heavy vesicles did not decrease in the presence of 11 mM Mg^{2+}.

To elucidate the mechanism underlying the different Mg^{2+} profiles of calcium uptake velocity, the Mg^{2+} dependence of ATP hydrolysis was examined (Fig. 3B). In light vesicles, the Ca^{2+}-dependent ATPase activity profile resembled that of calcium uptake velocity, yielding a relatively constant Ca/ATP coupling ratio over the Mg^{2+} range 5 μM to 3 mM (Fig. 3C). The coupling ratio for calcium transport in this case was approx. 1, and although this differs from the ideal stoichiometry of 2 [42,47], it is consistent with a report which used similar assay conditions [51]. The Ca^{2+}-dependent ATPase activity in heavy vesicles was higher than that in light vesicles (consistent with a previous report [13]), and showed a similar Mg^{2+} profile, with maximal ATPase activity at approx. 0.4 mM Mg^{2+} (Fig. 3B). The Ca^{2+}-dependent ATPase activity in heavy vesicles, therefore, appears unrelated to calcium uptake velocity; instead, the apparent coupling of ATP hydrolysis to calcium uptake in heavy vesicles increased with increasing Mg^{2+} concentration (Fig. 3C). Technical problems precluded analysis of ATPase activity (and hence coupling ratio) in the presence of 11 mM Mg^{2+}, so it is not clear if coupling ratio in heavy vesicles would increase further at this Mg^{2+} concentration.

If millimolar concentrations of Mg^{2+} are capable of increasing the coupling of ATP hydrolysis to calcium uptake in heavy vesicles, one might expect calcium influx in these vesicles to increase over this Mg^{2+} range. A series of unidirectional calcium flux experiments were undertaken at the

* See footnote on p 334

time of calcium uptake velocity measurements (i.e., min 1–3) (Fig. 4, Table I), and demonstrated that in heavy vesicles, elevation of Mg^{2+} from 0.4 to 3 mM increased calcium influx 3.8-fold ($p < 0.05$), with no change in calcium efflux ($p > 0.05$). A similar effect of Mg^{2+} on calcium influx in heavy vesicles was observed at a later stage of the calcium uptake reaction (i.e., min 7–10), although the influx velocities were lower and the efflux velocities higher than those observed during the initial stage of calcium uptake. Calcium influx in light vesicles, on the other hand, was much less sensitive to Mg^{2+} over the range 0.4 to 3 mM, when assayed at either min 1 or 7 (Table I).

Calcium sequestration

To determine if the observed difference in Mg^{2+}-dependence of calcium uptake by these two fractions of sarcoplasmic reticulum was limited to phosphate-supported calcium uptake, the effects of 0.07 and 5.01 mM free Mg^{2+} on calcium sequestration (i.e., calcium accumulation in the absence of phosphate) were examined (Table II). As a first approximation of calcium sequestration velocity, calcium sequestration reactions were terminated 10 s after initiation, and as shown in Table II, light vesicles accumulated more calcium than did heavy vesicles (52 ± 5 vs. 21 ± 6 nmol/mg, $p < 0.05$) in the presence of equimolar (0.1 mM) concentrations of $MgCl_2$ and ATP (0.07 mM free Mg^{2+}). Elevation of free Mg^{2+} to 5.01 mM (5.1 mM $MgCl_2$, 0.1 mM ATP) increased calcium accumulation in heavy vesicles 3.1-fold ($p < 0.05$), but had no significant effect on calcium accumulation in light vesicles. Thus, stimulation of calcium accumulation in heavy vesicles by millimolar free Mg^{2+} concentrations does not appear to be specific to phosphate-supported calcium uptake.

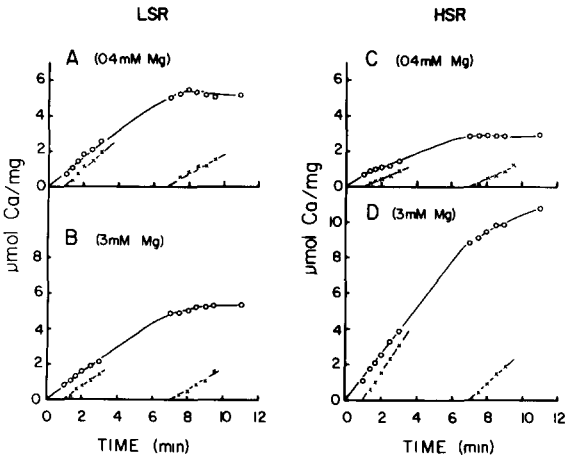


Fig. 4. Analysis of unidirectional calcium flux. Reaction media as in Fig. 1, with either 0.4 mM (A, C) or 3 mM (B, D) free Mg^{2+}. Calcium influx was measured between min 1 and 3 or min 7 and 9, as described in Methods. ○, calcium uptake, ×, calcium influx. (A, B) light sarcoplasmic reticulum vesicles, (C, D) heavy sarcoplasmic reticulum vesicles. Each curve represents a typical run.

In an effort to elucidate the mechanism(s) underlying low calcium sequestration by heavy vesicles in the presence of 0.07 mM Mg^{2+}, ATPase activities and phosphoenzyme levels were measured in the absence of phosphate (Table II). The Ca^{2+}-dependent ATPase activities (V) of light vesicles and heavy vesicles were stimulated $111 \pm 6\%$ and $154 \pm 7\%$ (respectively) by elevation of

TABLE I

EFFECTS OF Mg^{2+} ON UNIDIRECTIONAL CALCIUM FLUX

Calcium influx was measured between min 1 and 3 or min 7 and 9, as specified in the table (and shown in Fig. 3). Calcium efflux was calculated as the difference between calcium influx and calcium uptake velocity, as described in Methods. Calcium fluxes expressed as μmol Ca/mg per min, means ± S.E. ($n = 4$, two preparations). LSR, light sarcoplasmic reticulum vesicles, HSR, heavy sarcoplasmic reticulum vesicles.

Time (min)	$[Mg^{2+}]$ (mM)	LSR		HSR	
		Influx	Efflux	Influx	Efflux
1–3	0.4	1.054 ± 0.049	0.094 ± 0.012	0.389 ± 0.025	0.056 ± 0.023
1–3	3	0.839 ± 0.074	0.072 ± 0.034	1.487 ± 0.019	0.077 ± 0.028
7–9	0.4	0.522 ± 0.014	0.631 ± 0.042	0.360 ± 0.069	0.479 ± 0.047
7–9	3	0.601 ± 0.038	0.456 ± 0.043	0.907 ± 0.040	0.483 ± 0.051

TABLE II

EFFECTS OF Mg^{2+} ON CALCIUM SEQUESTRATION, ATP HYDROLYSIS, AND PHOSPHOENZYME INTERMEDIATES

Reaction media differ substantially from those used in Figs. 1–4, as described in Methods Ca content represents calcium sequestered at $t = 10$ s ($n = 3$) Ca-ATPase is the Ca^{2+}-dependent ATPase activity ($12 \leq n \leq 14$). EP level is total acyl phosphoprotein level at $t = 10$ s ($7 \leq n \leq 10$) EP distribution is percentage of acyl phosphoprotein at $t = 10$ s which is ADP-sensitive ($7 \leq n \leq 10$) All values expressed as means ± S E. for the number of experiments specified above. LSR, light sarcoplasmic reticulum vesicles, HSR, heavy sarcoplasmic reticulum vesicles

$[Mg^{2+}]$ (mM)	LSR		HSR	
	0 07	5 01	0.07	5 01
Ca content (nmol Ca/mg)	52 ±5	52 ±4	21 ± 6	65 ± 4
Ca-ATPase (nmol P_i/mg per min)	125 ±6	264 ±8	323 ±15	820 ±22
EP level (nmol EP/mg)	2 67±0 15	4 79±0 33	1 21± 0 08	2.26± 0.11
EP distribution (%E_1P)	84 ±6	86 ±7	77 ± 7	79 ± 5

Mg^{2+} from 0.07 to 5.01 mM. Total phosphoenzyme (EP) levels in light and heavy vesicles were stimulated 79 ± 12% and 87 ± 9% (respectively) by this increase in free Mg^{2+}, perhaps due to stimulation of EP formation [38,57,58]; whereas phosphoenzyme distribution in neither fraction changed ($P > 0.05$). Thus, elevation of Mg^{2+} concentration resulted in an increased rate of EP decay (V/E_2P), that was slightly, but significantly, greater in heavy vesicles than in light vesicles (51 ± 4% vs. 33 ± 4%, $p < 0.05$). The difference in Mg^{2+}-stimulation of calcium sequestration between fractions (Table II), therefore, cannot be explained on the basis of a Mg^{2+}-dependent change in either EP level or EP distribution. Similarly, the Mg^{2+}-dependent change in EP decay rate in heavy vesicles is only slightly greater than that in light vesicles, suggesting that the Mg^{2+}-dependence of calcium accumulation in heavy vesicles is not related to EP decay rate. Instead, the data are consistent with the previous suggestion that heavy vesicles have a low Ca/ATP coupling ratio in the absence of millimolar Mg^{2+}, though fast kinetic (e.g., quench flow) analyses are needed to accurately determine the coupling ratio during calcium sequestration.

Effects of ruthenium rad

Ruthenium red (0.5 μM) has been reported to increase calcium uptake by terminal cisternae-derived sarcoplasmic reticulum [16,37]. As shown in Fig. 5C, 0.5 μM ruthenium red increased phosphate-supported calcium uptake velocity and maximal calcium content in heavy vesicles in the presence of 0.4 mM free Mg^{2+} ($p < 0.05$); whereas in 3 mM Mg^{2+}, ruthenium red had no effect on the already rapid calcium uptake (Fig. 5D). In contrast, the time-course of calcium uptake in light

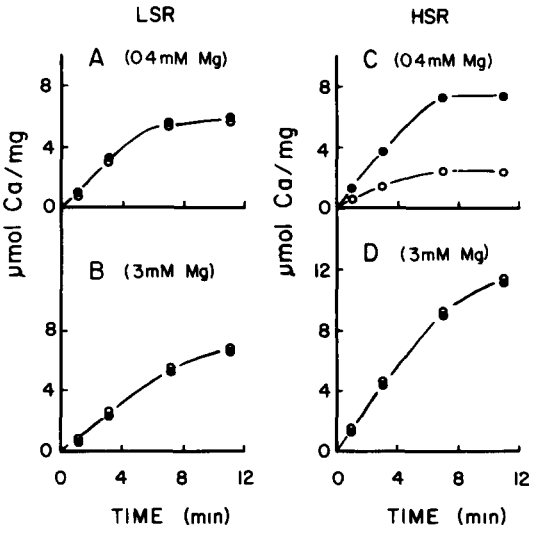


Fig. 5. Time-course of calcium uptake in the presence or absence of ruthenium red. Reaction media as in Fig. 1, with (●) or without (○) 0.5 μM ruthenium red. (A, C) 0 4 mM free Mg^{2+}, (B, D) 3 mM free Mg^{2+}, (A, B) light sarcoplasmic reticulum vesicles, (C, D) heavy sarcoplasmic reticulum vesicles Symbols represent means ± S.E. for $n = 4$, two preparations

vesicles was unaffected by ruthenium red in the presence of either 0.4 or 3 mM Mg^{2-} (Fig. 5). These data are more clearly illustrated in the Mg^{2+} profile (Fig. 6A), which shows that over the Mg^{2+} range 0.5 μM to 3 mM, ruthenium red had no effect on calcium uptake velocity in light vesicles ($p > 0.05$). In the case of heavy vesicles, ruthenium red had no effect on maximal calcium uptake velocity (Fig. 6B, $p > 0.05$), but shifted the Mg^{2+}-dependence of calcium uptake velocity to lower Mg^{2+} concentrations (i.e., half-maximal velocity at approx. 0.08 mM vs. approx. 0.90 mM).

To elucidate the mechanism of the stimulation of calcium uptake in heavy vesicles by ruthenium red, unidirectional calcium fluxes were measured. As shown in Table III, ruthenium red stimulated calcium influx in heavy vesicles 3.8-fold in 0.4 mM Mg^{2+} ($p < 0.05$), consistent with the 3.4-fold increase in calcium uptake velocity at this Mg^{2+} concentration (Fig. 6B). Calcium influx in heavy vesicles in 3 mM Mg^{2+}, and calcium influx in light vesicles in 0.4 or 3 mM Mg^{2+}, on the other hand, were not changed by ruthenium red (Table III), a finding that is consistent with the lack of effect of ruthenium red on calcium uptake velocity under these conditions (Fig. 6). In both fractions of sarcoplasmic reticulum, calcium efflux in the presence of either Mg^{2+} concentration was much slower than calcium influx, and showed no change in the presence of ruthenium red (Table III). There was, however, a trend for slightly slower calcium efflux from heavy vesicles in the presence of ruthenium red (Table III), consistent with previous observations [16–20]. Moreover, Ca^{2+}-dependent

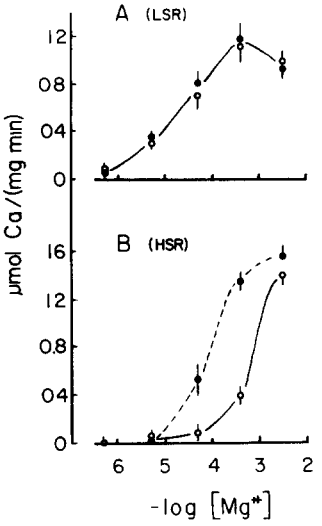


Fig. 6 Effects of ruthenium red on the Mg^{2+}-dependence of calcium uptake Reaction media as in Fig. 5, with free Mg^{2+} varied from 0 5 μM (pMg = 6.3) to 3 mM (pMg = 2 5) (A) Light sarcoplasmic reticulum vesicles, (B) heavy sarcoplasmic reticulum vesicles, ○, no ruthenium red, ●, 0 5 μM ruthenium red. Calcium uptake velocity determined between min 1 and 3, as in Fig 3 Symbols represent means ± S E for $n = 6$, two preparations.

ATPase activity in heavy vesicles in 0.4 mM Mg^{2+} decreased 21% in the presence of ruthenium red ($p < 0.05$), consistent with the observation that Ca^{2+}-dependent ATPase activity is not closely correlated with calcium uptake velocity in heavy vesicles (Fig. 3). Thus, ruthenium red, like millimolar Mg^{2+}, appears to stimulate calcium uptake velocity in heavy vesicles by increasing the coupling of ATP hydrolysis to calcium transport.

TABLE III

EFFECTS OF RUTHENIUM RED ON UNIDIRECTIONAL CALCIUM FLUX

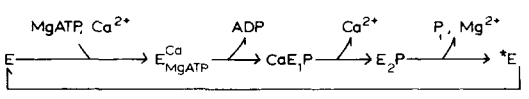
Calcium influx was measured between min 1 and 3 (as shown in Fig. 4) in the presence or absence of 0 5 μM ruthenium red (RR) (0 4 mM or 3 mM free Mg^{2+}). Calcium efflux was calculated as the difference between calcium influx and calcium uptake velocity, as described in Methods. Calcium fluxes expressed as μmol Ca/mg per min, means ± S E ($n = 3$ or 4) LSR, light sarcoplasmic reticulum vesicles, HSR, heavy sarcoplasmic reticulum vesicles

[Mg^{2+}] (mM)	[RR] (μM)	LSR		HSR	
		Influx	Efflux	Influx	Efflux
0.4	0	1 054 ± 0 049	0.094 ± 0.012	0 389 ± 0 025	0.056 ± 0.023
0.4	0 5	1 000 ± 0 024	0.105 ± 0.024	1 472 ± 0 046	0.034 ± 0 046
3	0	0 839 ± 0.074	0.072 ± 0.034	1 487 ± 0 019	0 077 ± 0 028
3	0.5	0 832 ± 0 006	0 091 ± 0.006	1.470 ± 0 014	0 026 ± 0 014

Discussion

In the present study, two fractions of sarcoplasmic reticulum vesicles were shown to differ markedly in terms of the Mg^{2+}-dependence of phosphate-supported calcium uptake. Half-maximal calcium uptake velocity in vesicles presumably derived from longitudinal tubules and terminal cisternae occurred at approx. 0.02 and approx. 0.90 mM Mg^{2+} (respectively). Moreover, elevation of Mg^{2+} from 3 to 11 mM decreased calcium uptake velocity in longitudinal tubule-derived vesicles, perhaps by displacing Ca^{2+} from transport sites [38,50]. In contrast, calcium uptake velocity in terminal cisternae-derived vesicles was unaltered by this change in Mg^{2+}.

In the above experiments, free Ca^{2+} and total ATP concentrations were 6 μM and 5 mM, respectively; thus, MgATP, CaATP, and free ATP concentrations varied with free Mg^{2+} concentration. All of these nucleotide complexes as well as Mg^{2+} and Ca^{2+} have been shown to affect calcium pump activity, though the site of action and/or affinity differ. As shown in model 1 [39–41], the calcium pump protein (M_r 100 000, denoted E) binds Ca^{2+} (1–2 ions) and MgATP in the cytoplasm with high affinity ($K_{Ca} = 1$ μM, $K_{MgATP} = 1$–18 μM). The substrate for the calcium pump appears to be MgATP, the γ-phosphate of which is transfered to the calcium pump to form an ADP-sensitive phosphoenzyme (denoted CaE_1P). A conformational change in the calcium pump then occurs such that the phosphoenzyme releases Ca^{2+} to the inside of the vesicle [42,43], and becomes insensitive to ADP (denoted E_2P). It has been proposed that Mg^{2+} acts allosterically on the calcium pump at this stage of the reaction cycle (e.g., by binding to E_2P) so as to stimulate the subsequent E_2P decay [43–47], resulting in liberation of inorganic phosphate. Alternatively, magnesium bound at the catalytic site may serve this function [39,42,50]. The calcium pump is then thought to undergo a conformational change (*E-E transition) before restarting the cycle, and it has been hypothesized that free ATP acts allosterically to stimulate this transition ($K_m = 100$–500 μM [41,48,49]). Thus, free ATP and MgATP can stimulate calcium pump activity by acting at an allosteric and catalytic site, respectively. CaATP, on the other hand, has been reported to inhibit calcium pump activity by competing with MgATP at the catalytic site ($K_m = 0.3$–2 μM [39,50]).



Model 1

Free ATP concentration in the present study varied inversely with calcium transport velocity; hence, it seems unlikely that changes in this nucleotide concentration contributed to the observed Mg^{2+} profiles of calcium transport velocity. The effect of MgATP on these Mg^{2+} profiles may also be minimal since stimulation of calcium uptake in the heavy vesicles occurred over a Mg^{2+} range (0.4–3 mM) where MgATP concentration (3.5–4.75 mM) greatly exceeded the K_m for MgATP (1–18 μM [39,40,50]). Calcium uptake velocity in light vesicles also increased over a Mg^{2+} range (0.005–0.4 mM) where MgATP concentration (0.156–3.5 mM) was well above the K_m for MgATP. Thus, the observed Mg^{2+} profiles appear to be consistent with the purported allosteric effects of Mg^{2+} on the calcium pump [43–47], and suggest that the affinity of terminal cisternae-derived vesicles for Mg^{2+} is lower than that of longitudinal tubule-derived vesicles. Shigekawa et al. [39], on the other hand, have suggested that competition between MgATP and CaATP for the catalytic site regulates calcium pump activity, and that Mg^{2+} does not act allosterically. The present study cannot completely rule out an effect of CaATP on the calcium pump since CaATP concentration decreased slightly as Mg^{2+} and MgATP concentrations increased. The stimulation of calcium transport activity in both fractions of sarcoplasmic reticulum over a Mg^{2+} concentration range where MgATP concentration greatly exceeded CaATP concentration, however, tends to support the allosteric model. Specifically, calcium transport in heavy vesicles increased approx. 4-fold as MgATP increased from 3.5 to 4.75 mM, and CaATP decreased from 23 to 4 μM (Mg^{2+} range 0.4–3 mM). In light vesicles, calcium transport velocity increased 48% when MgATP increased from 1.2 to 3.5 mM, and CaATP decreased from 59 to 23 μM (Mg^{2+} range 0.05–0.4 mM).

In several reports [13–16], longitudinal tubule-derived vesicles have been shown to accumulate calcium more rapidly than terminal cisternae-derived vesicles when Mg^{2+} concentrations was < 1 mM. Thus, the present study is consistent with previous reports, and suggests that the slow accumulation of calcium by the latter vesicles in the presence of 0.4 mM Mg^{2+} is due to an uncoupling of ATP hydrolysis for calcium transport. Previous reports have shown a Mg^{2+}-dependent decrease in calcium permeability of sarcoplasmic reticulum vesicles [19,20,52,53], which could account for an apparent increase in the coupling ratio. Measurements of unidirectional calcium fluxes (Table I) showed that calcium efflux from both fractions of vesicles was low at the time of calcium uptake velocity determination, which is consistent with a previous report on crude sarcoplasmic reticulum vesicles [51], and was not affected by elevation of Mg^{2+} from 0.4 to 3 mM. In contrast, calcium influx in heavy vesicles increased 3.8-fold over this Mg^{2+} range, which is similar to the effect of Mg^{2+} on calcium uptake velocity. Calcium influx in light vesicles decreased slightly over this Mg^{2+} range, consistent with the Mg^{2+}-effect on calcium uptake velocity. Unidirectional flux analyses, therefore, support the hypothesis that millimolar concentrations of Mg^{2+} increase the Ca/ATP coupling ratio in terminal cisternae-derived vesicles.

A similar increase in Ca/ATP coupling ratio has been reported for a fraction of cardiac sarcoplasmic reticulum vesicles in the presence of ryanodine [54]. This cardiac fraction (which may be terminal cisternae-derived [55]) exhibited a 3-fold stimulation of oxalate-supported calcium uptake velocity in the presence of 0.1 mM ryanodine; whereas, ryanodine had no effect on calcium uptake by a fraction of cardiac sarcoplasmic reticulum possibly derived from longitudinal tubules [54]. Analyses of Ca^{2+}-ATPase activity and unidirectional calcium flux suggested that the ability of ryanodine to stimulate calcium uptake velocity in the terminal cisternae-derived fraction of cardiac sarcoplasmic reticulum was due to an increase in the Ca/ATP coupling ratio [54], although ryanodine may inhibit calcium release from the sarcoplasmic reticulum in situ [56].

The mechanism of calcium transport during calcium sequestration would be expected to be similar to that in the presence of 50 mM phosphate, so that the effects of Mg^{2+} on calcium sequestration and phosphate-supported calcium uptake should be similar. When calcium sequestration reactions were terminated after 10 s, the results substantiated the similarity bteween these two types of calcium accumulation. That is, elevation of Mg^{2+} from 0.07 to 5 mM stimulated calcium sequestration much more in heavy vesicles than in light vesicles. Analyses of Ca^{2+}-ATPase activity were also consistent with those measured in the presence of a calcium precipitating anion in that Ca^{2+}-ATPase activity in heavy vesicles was higher than that in light vesicles, with Ca^{2+}-ATPase activities in both fractions being stimulated by elevation of Mg^{2+} from 0.07 to 5 mM. The similarity of the two fractions of vesicles in terms of Mg^{2+}-dependence of EP level and $\%E_1P$, however, do not support the view that the observed differences in the Mg^{2+}-dependence of calcium sequestration were related to Mg^{2+}-effects on total EP level or EP distribution. Alternatively, the affinity of the ADP-insensitive phosphoenzyme (E_2P) for Ca^{2+} in terminal cisternae-derived vesicles may be regulated allosterically by millimolar Mg^{2+}. The latter hypothesis is not easily tested, although a first approach might involve quantification of calcium bound to the various states of the calcium pump [42].

Ruthenium red, like Mg^{2+}, uncovered a functional difference between longitudinal tubule-derived and terminal cisternae-derived vesicles. This hexavalent cation $[(NH_3)_5Ru–O–(NH_3)_4Ru–O–Ru(NH_3)_5^{6+}]$ [59], inhibits mitochondrial calcium transport [60–62] at concentrations as low as 0.1 μM [62]. Consistent with previous studies using crude sarcoplasmic reticulum vesicles [63,64], ruthenium red (0.5 μM) had no effect on phosphate-supported calcium uptake velocity by light vesicles over the Mg^{2+} range 0.0005–3 mM (Fig. 6). Calcium uptake velocity in heavy vesicles, on the other hand, was markedly stimulated by ruthenium red (0.5 μM), particularly at 0.05–0.4 mM Mg^{2+}, resulting in an apparent shift in the Mg^{2+} dependence of calcium uptake velocity to lower Mg^{2+} concentrations. As ruthenium red (0.5–5 μM) has been suggested to inhibit calcium release from terminal cisternae-derived vesicles

[9,16–19,65], its effects on unidirectional calcium fluxes were measured. Calcium efflux from neither fraction of vesicles was affected by ruthenium red (0.5 μM) in the presence of 0.4–3 mM Mg^{2+}, whereas heavy vesicles exhibited an approx. 4-fold stimulation of calcium influx and calcium uptake by ruthenium red in the presence of 0.4 mM Mg^{2+} (Fig. 6), along with a 21% decrease in Ca^{2+}-ATPase activity. This corresponds to an approx. 5-fold increase in Ca/ATP coupling ratio in heavy vesicles (i.e., 0.09 vs. 0.45). Thus, ruthenium red, like millimolar Mg^{2+}, increases the apparent coupling of calcium transport to ATP hydrolysis in terminal cisternae-derived vesicles, with no effect in longitudinal tubule-derived vesicles.

In summary, the present study has shown that two fractions of sarcoplasmic reticulum vesicles (presumably derived from the longitudinal tubules or terminal cisternae) differ markedly in terms of Mg^{2+} sensitivity of calcium transport and the apparent mechanism by which Mg^{2+} stimulates calcium transport. Ruthenium red also uncovered functional differences between these two fractions of vesicles, and appeared to regulate calcium transport in the terminal cisternae-derived vesicles in the same way as did millimolar Mg^{2+}. These results suggest that calcium transport in the terminal cisternae may be regulated differently from that in the longitudinal tubules.

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