Biochemical Evidence for Functional Heterogeneity of Cardiac Sarcoplasmic Reticulum Vesicles*

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Two subpopulations of cardiac sarcoplasmic reticulum vesicles were resolved functionally, based on their sensitivities to the drug ryanodine. These two subpopulations of sarcoplasmic reticulum vesicles, termed ryanodine-sensitive and ryanodine-insensitive, were separated by preloading crude cardiac microsomes with Ca²⁺ oxalate in the presence of ATP, followed by sucrose density gradient centrifugation. Ryanodine-insensitive vesicles accumulated most of the Ca²⁺ oxalate during the preload, and constituted the densest subfraction recovered from the sucrose gradient. These ryanodine-insensitive vesicles exhibited the highest density of Ca²⁺ pumps, and accounted for 10 to 15% of the total protein in crude cardiac microsomes. Ryanodine-insensitive vesicles continued to transport substantial amounts of Ca²⁺ after isolation.

Ryanodine-sensitive vesicles accumulated negligible Ca²⁺ during the preload, and were recovered from the lower density regions of the sucrose gradient. On a milligrams of protein basis, these vesicles were present in 7-fold excess over ryanodine-insensitive vesicles. Ryanodine-sensitive vesicles transported low amounts of Ca²⁺ under normal incubation conditions, but 3 × 10⁻⁴ M ryanodine strikingly increased their Ca²⁺ uptake 5- to 10-fold. Ca²⁺ uptake by ryanodine-sensitive vesicles was uniquely regulated by Ca²⁺ ion concentration. Elevation of the ionized Ca²⁺ concentration from 2 to 4 µM increased Ca²⁺ uptake by these vesicles greater than 5-fold, but had no effect on their Ca²⁺-dependent ATPase activity. These ryanodine- and Ca²⁺ concentration-dependent effects were apparent for only ryanodine-sensitive vesicles.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed distinct differences in polypeptide staining between ryanodine-sensitive and ryanodine-insensitive vesicles, confirming by an independent method that the two populations of vesicles were different. These data provide the first biochemical evidence for functional and structural heterogeneity of cardiac sarcoplasmic reticulum vesicles.

Sarcoplasmic reticulum vesicles isolated from cardiac and skeletal muscle actively transport Ca²⁺. This Ca²⁺ transport is mediated by a Ca²⁺-dependent ATPase activity, which hydrolyzes ATP in the presence of micromolar concentrations of Ca²⁺ to yield ADP and inorganic phosphate (2-4). In the early studies characterizing Ca²⁺ uptake by these vesicles, it was generally observed that cardiac microsomes accumulated much less Ca²⁺ than skeletal muscle microsomes (4). Although preparative procedures for cardiac sarcoplasmic reticulum vesicles have improved with time (3, 5-8), the Ca²⁺-dependent ATPase activity that these vesicles exhibit is still less than that of fast skeletal muscle vesicles (3, 7).

The low Ca²⁺ transport activities originally observed for cardiac sarcoplasmic reticulum preparations were thought to be due at least in part to the relative impurity of the fractions studied (4, 9). Sarcoplasmic reticulum vesicles actively transporting Ca²⁺ in the presence of oxalic acid generate Ca²⁺ oxalate precipitates, which are readily visualized inside the vesicles with use of the electron microscope (4). Only 5 to 10% of the vesicles formed Ca²⁺ oxalate precipitates in cardiac preparations (2, 9), whereas 20 to 30% of the vesicles developed precipitates in skeletal muscle preparations (2, 9-11). To explain these observations, Baskin and Deamer postulated that contaminating sarcolemmal vesicles devoid of significant Ca²⁺ transport activity might contribute a substantial portion of the total membrane mass in cardiac preparations (9). This idea has been supported by recent studies demonstrating purification of sarcoclemmal vesicles from such crude cardiac microsomal preparations (12, 13). The sarcolemmal content of these crude cardiac preparations, however, was only 15% or less (12, 13), an amount which would not seem sufficient to dilute out appreciably the activity of the sarcoplasmic reticulum vesicles (3).

Carsten and Reddy selectively increased the density of the sarcoplasmic reticulum vesicles in crude cardiac microsomes by loading them to maximum capacity with Ca²⁺ oxalate, thus allowing the separation of the sarcoplasmic reticulum vesicles from the other vesicles by differential centrifugation (6). Ca²⁺ uptake by these purified sarcoplasmic reticulum vesicles, however, could not be further characterized because the isolated vesicles did not continue to transport Ca²⁺ (6). Shortly thereafter, Levitsky et al. successfully used sucrose density gradient centrifugation to purify cardiac sarcoplasmic reticulum vesicles that had been preloaded with much less Ca²⁺ oxalate (7). An advantage of this latter technique was that the purified vesicles were capable of accumulating high levels of additional Ca²⁺ after isolation (7). This was recently confirmed by Misselwitz et al. (13).

In all of the foregoing studies which utilized the technique of Ca²⁺ oxalate loading to facilitate purification of sarcoplasmic reticulum vesicles from cardiac muscle, it was evident that the purified vesicles accounted for only a small percentage of the total membrane content present in the crude preparations (6, 7, 12, 13). Most of the membrane protein was recovered in other subfractions, which were also shown to be

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vesicular, but these subfractions were unable to transport substantial amounts of Ca$^{2+}$. Paradoxically, however, these additional subfractions did exhibit appreciable specific activities of Ca$^{2+}$-dependent ATPase, the enzyme responsible for Ca$^{2+}$-translocation (6, 12, 13). This did not appear to be a unique property of the cardiac preparations, because similar results were also reported in purification studies that used skeletal muscle as the source of the sarcoplasmic reticulum (14-16). The explanation for the inability of these additional sarcoplasmic reticulum subfractions to accumulate Ca$^{2+}$ ions was unclear. It was possible that the vesicles had become leaky or were damaged during their preparation and thus were unable to retain any Ca$^{2+}$ ions that they might have transported (6, 14), or that their membranes were inverted such that any active Ca$^{2+}$ transport by them would have been directed outward into the incubation medium (11, 13, 16).

In the present study, we suggest that these cardiac sarcoplasmic reticulum vesicles thought previously to be deficient in Ca$^{2+}$ uptake are actually capable of transporting substantial amounts of Ca$^{2+}$, and furthermore, that they constitute a distinct subpopulation of sarcoplasmic reticulum vesicles in cardiac microsomes. Subfractions of cardiac sarcoplasmic reticulum vesicles were first isolated by the Ca$^{2+}$-oxalate-loading technique described above, and then, additional Ca$^{2+}$ transport by the subfractions was studied in detail. In agreement with the results of Levitasky et al. (7) and Misselwitz et al. (13), we found that only a fraction of the total sarcoplasmic reticulum vesicles accumulated Ca$^{2+}$ oxalate during the loading step, and that these vesicles continued to transport substantial Ca$^{2+}$ after they were isolated. The lighter sarcoplasmic reticulum vesicles, which did not accumulate significant Ca$^{2+}$ oxalate during the loading step, had only low levels of Ca$^{2+}$ transport after isolation. However, Ca$^{2+}$ transport by the lighter vesicles was markedly and selectively stimulated by ryanodine, a neutral alkaloid shown recently to increase Ca$^{2+}$ uptake into crude cardiac sarcoplasmic reticulum preparations (8). These ryanodine-sensitive vesicles were differentiated from the remainder of the sarcoplasmic reticulum vesicles by several different criteria. The data demonstrate that isolated cardiac sarcoplasmic reticulum vesicles are functionally heterogeneous.

**EXPERIMENTAL PROCEDURES**

**Subfractionation of Crude Cardiac Sarcoplasmic Reticulum Vesicles**—Crude cardiac sarcoplasmic reticulum vesicles were isolated as described previously (12). All operations were conducted at 4°C unless otherwise stated. Briefly, 180 to 200 g of left ventricular tissue was divided into six portions, and each portion was homogenized three times for 30 sec in 100 ml of 10 mM NaHCO$_3$ with a Polytron PT-20 (Brinkman Instruments). Two sequential low speed centrifugations at 14,000 × g$_{max}$ for 20 min were used to remove large particles. The supernatant material from the second centrifugation was next sedimented at 45,000 × g$_{max}$ for 30 min. The pellets were resuspended in 0.6 M KCl and 30 mM histidine, pH 7.0, and sedimented again to yield the crude sarcoplasmic reticulum vesicles.

Ca$^{2+}$-loading of the crude sarcoplasmic reticulum vesicles was modified from our previous report (12). The crude vesicle pellets (75 to 100 mg of protein) were resuspended in 40 ml of an ice-cold medium containing 50 mM histidine, 100 mM KCl, 65 mM MgCl$_2$, 60 mM Na$_2$ATP, 25 mM Tris/EGTA, 20 mM CaCl$_2$, and 3 mM Tris/oxalate (pH 7.1). In experiments in which radioactively labeled Ca$^{2+}$ uptake was measured, 0.3 μCi of $^{45}$CaCl$_2$ was included. The suspension was placed in a waterbath at 37°C to initiate rapid Ca$^{2+}$ uptake, and the incubation was conducted for 10 min with 5 mM additional Tris/oxalate added after the first 5 min of incubation. The suspension was then immediately centrifuged at 4°C for 30 min at 108,000 × g$_{max}$. Consistent with the results of Carsten and Reedy (6), we observed a tan pellet with a white central region or button. The white central region contained the Ca$^{2+}$-filled vesicles. When ATP was omitted from the incubation medium, or when the Ca$^{2+}$ ionophore A23187 (3 μg/ml) was included in the incubation medium, this white part of the pellet was no longer visible.

The Ca$^{2+}$-loaded vesicles were resuspended in 0.25 M sucrose containing 300 mM KCl, 50 mM sodium pyrophosphate, and 100 mM Tris (pH 7.2). This material was layered over a discontinuous sucrose gradient consisting of 7 ml of sucral each of 0.05 M sucrose dissolved in the same buffered solution above. Routinely, a total of 15 ml of crude vesicles was layered over three sucrose gradients. Centrifugation was for 2 h at 27,000 rpm in a Beckman SW 27 rotor. Fraction A was collected at the 0.25 M:0.6 M interface, Fraction B at the 0.6 M:1.0 M interface, and Fraction C at the 1.0 M:1.5 M interface. Fraction E was a white pellet at the bottom of the gradient. The subfractions were diluted with 4 volumes of ice-cold H$_2$O, and then sedimented at 105,000 × g$_{max}$ for 60 min. The pellets were resuspended in 0.25 M sucrose, 10 mM histidine, and stored frozen at -20°C. Protein was determined by the method of Lowry et al. (17).

**Assay of Ca$^{2+}$ Uptake—Ca$^{2+}$ uptake by the vesicles was routinely conducted at 37°C in a medium consisting of 50 mM histidine (free base), 3 mM MgCl$_2$, 3 mM Tris/oxalate, 100 mM KCl, 50 μM CaCl$_2$, and 3 mM Tris/ATP (18). Vesicles were preincubated for 10 min at pH 7.0 prior to initiating Ca$^{2+}$ uptake reactions by adding the ATP. Addition of the ATP lowered the pH to 6.8, which remained stable throughout the incubations. This slight alkaline preincubation was required to demonstrate some of the unique properties of the ryanodine-sensitive vesicles, but it had no effect on Ca$^{2+}$ uptake by the ryanodine-sensitive preparations (19). Ca$^{2+}$ uptake by the fractions was measured simultaneously by a Ca$^{2+}$ electrode connected to a Radiometer PHM 64 pH meter to monitor the extravesicular Ca$^{2+}$ concentration. This was performed as described by Madeira (20), using a Radiometer F21212 Ca$^{2+}$ electrode connected to a Radiometer PHM 64 pH meter to monitor the extravesicular Ca$^{2+}$ concentration.

**Assay of ATPase Activity—Ca$^{2+}$-dependent ATPase activities were measured at 37°C in media identical with those above. Basal ATPase activities were determined in the same media, which had no added Ca$^{2+}$ and 1 mM Tris/EGTA. These basal activities were subtracted from the total ATPase activities, to yield the Ca$^{2+}$-dependent ATPase activities. In experiments in which Ca$^{2+}$ uptake and Ca$^{2+}$-dependent ATPase activities were measured simultaneously, aliquots were taken from the same suspension of vesicles to determine the amount of Ca$^{2+}$ uptake and the total ATPase activity. An additional sample was incubated with EGTA and no added Ca$^{2+}$ to determine the basal ATPase activity. Production of inorganic phosphate from ATP was measured colourimetrically (21).

**Assay of (Na$^+$,K$^+$)-ATPase Activity**—(Na$^+$,K$^+$)-ATPase activities were measured with membranes pre-treated with sodium dodecyl sulfate to expose latent activity (19). The assay was conducted in a medium containing 3 mM Tris/ATP, 50 mM histidine, 3 mM MgCl$_2$, 100 mM NaCl, and 10 mM KCl (pH 6.8). This medium was saturated with 5% CO$_2$ to maintain pCO$_2$ at 25 to 30 mm Hg. Membrane protein (200 μg) was incubated in 1 ml of ice-cold medium containing 50 mM histidine, 3 mM MgCl$_2$, 100 mM NaCl, and 100 mM KCl (pH 6.8), and after 15 min, the reaction was terminated by addition of 0.5 ml of 0.5 M trichloroacetic acid containing 20 mM Na$_2$HPO$_4$ and 10 mM Na$_2$ATP.

1 The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
Subpopulations of Cardiac Sarcoplasmic Reticulum Vesicles

The contents of the tubes were applied to Whatman GF C filters, and the tubes were rinsed two additional times with the same solution, which was also applied to the filters. The filters were then rinsed four times with 10 ml of ice-cold solution containing 5% trichloroacetic acid, 20 mM Na2HPO4, and 2 mM NaATP, followed by one rinse with 5 ml of ice-cold H2O. Radioactivity remaining on the filters was determined by liquid scintillation counting. The incorporation of radioactivity inhibited by EGTA was used to determine the Ca2+-dependent ATPase content of the subfractions (23).

Polyacrylamide Gel Electrophoresis—The subfractions were dissolved in a solution containing 2.5% sodium dodecyl sulfate as previously described (12). Twenty-five μg of protein from each subfraction were applied to a polyacrylamide slab gel, and electrophoresis was conducted according to the method of Laemmli (24). The concentrations of acrylamide in the stacking and resolving gels were 3 and 7%, respectively. Proteins were visualized with the cationic carbocyanine dye Stains-All (25), which best revealed the differences in protein banding between the subfractions.

Materials—[γ-32P]ATP was obtained from ICN Pharmaceuticals. 4CaCl2 was obtained from New England Nuclear. Ryanodine was purchased from the S. P. Penick Co. Stains-All was purchased from the Eastman Kodak Co. A23187 was generously provided by Dr. R. L. Hamill, Eli Lilly and Co. All other reagents were purchased from the Sigma Chemical Co.

RESULTS

Isolation of Subpopulations of Cardiac Sarcoplasmic Reticulum Vesicles—To obtain good separation of different sarcoplasmic reticulum fractions, the crude vesicles were preloaded with Ca2+ plus oxalate in the presence of ATP, and then subjected to sucrose density gradient centrifugation. Virtually all of the Ca2+ accumulated during the preload was recovered in subfraction E, the densest subfraction isolated (Table I). This fraction accounted for about 12% of the total protein recovered (Table I). When the Ca2+ ionophore A23187 was included in the Ca2+-loading medium, or when ATP was omitted from the incubation mixture, the white E vesicles were no longer visible, and no radioactive Ca2+ or protein sediments through the 1.5 M sucrose into the E region of the gradient (data not shown). This demonstrated that the radioactive calcium recovered in the E fraction was accounted for by Ca2+ oxalate which had precipitated inside the vesicles. Loss of radioactive Ca2+ from the Ca2+-loaded vesicles was minimal during the centrifugation procedures, as suggested by the fact that the Ca2+ oxalate recovered in subfraction E accounted for all of the Ca2+ oxalate accumulated by the crude membrane vesicle fraction (Table I). Ca2+ oxalate accumulated by the crude membrane vesicle fraction during the preload was determined by either immediate filtration, or by sedimentation of the membranes with the centrifuge. In both cases, similar results were obtained.

Ca2+-dependent acylphosphoproteins and Ca2+-dependent ATPase activities, which measured the presence of the Ca2+ transport enzyme (21, 23), were present in all the subfractions, although the E vesicles had the highest specific activities of these two parameters (Table I). Basal ATPase activity, measured in the absence of Ca2+, was also present, and it was consistently the lowest in subfraction E (Table I).

To investigate why subfractions A through D exhibited low apparent levels of Ca2+ uptake, but had appreciable Ca2+-dependent ATPase activities, Ca2+ uptake was measured by the subfractions after they had been isolated. Under control incubation conditions, subfraction E continued to accumulate substantial Ca2+, while the other subfractions exhibited only

![Fig. 1. Effect of ryanodine on Ca2+ uptake by sarcoplasmic reticulum subfractions. Ca2+ uptake was determined in a medium containing 50 mM histidine, 3 mM MgCl2, 100 mM KCl, 3 mM Tris/ATP, 5 mM Tris/oxalate, and 50 μM 4CaCl2. Right, 3 × 10-5 M ryanodine was included. The subfractions are designated as follows: A, B, C, D, and E. In this and all other experiments on Ca2+ uptake (except that depicted in Fig. 12), preincubation was at pH 7.4 for 10 min, after which time reactions were initiated by adding ATP, which brought the pH to 6.8.](image)

![Fig. 2. Ca2+-dependent ATPase and (Na+,K+)-ATPase activities of sarcoplasmic reticulum subfractions. MV designates crude membrane vesicles, and fractions A through E are the isolated subfractions. Ca2+-dependent ATPase activities were measured at the optimal Ca2+ concentration of 0.6 mM CaCl2 in the presence of 1.0 mM EGTA. The Ca2+ ionophore A23187 (5 μg/ml) was also included for assay of Ca2+-dependent activities. Results are the means ± S.E. from three separate preparations. With the conditions of assay used presently (under "Experimental Procedures"), purified cardiac sarcolemmal vesicles were previously shown to have a (Na+,K+)-ATPase specific activity of approximately 80 μmol of P/mg of protein/h (Ref. 22).](image)
low levels of Ca\(^{2+}\) uptake (Fig. 1, left). All of this additional Ca\(^{2+}\) uptake was completely prevented by the Ca\(^{2+}\) ionophore A23187 (data not shown). When the drug ryanodine was included in the incubation media, Ca\(^{2+}\) uptake by subfractions B through D was greatly increased (Fig. 1, right). Ca\(^{2+}\) uptake by the E subfraction was unresponsive to ryanodine. Although Ca\(^{2+}\) uptake by the A subfraction was also increased by ryanodine, the absolute level of Ca\(^{2+}\) accumulated remained well below that of the other fractions (Fig. 1, right). These A vesicles were substantially contaminated with sarcolemmal membrane fragments, as evidenced by their relatively high (Na\(^{+},K\(^{+}\))-ATPase activity (Fig. 2), and they were not studied further.

No significant differences have been noted between B, C, and D vesicles with regard to their Ca\(^{2+}\) uptake properties in all of the additional studies to be described. For convenience we will refer to the vesicles in subfractions B, C, and D as the ryanodine-insensitive vesicles. The E vesicles are the ryanodine-sensitive vesicles. We should emphasize that although ryanodine-sensitive and -insensitive vesicles accumulated similar levels of Ca\(^{2+}\) after isolation, ryanodine-insensitive vesicles already contained approximately 8 pmol of Ca\(^{2+}\)/mg of protein at the start of the incubations.

Ryanodine Effects on Ca\(^{2+}\) Uptake by Subpopulations of Cardiac Sarcoplasmic Reticulum Vesicles—Ryanodine stimulated Ca\(^{2+}\) uptake maximally into the ryanodine-sensitive vesicles at a concentration of 300 \(\mu\)M (Fig. 3, right). The concentration effectiveness of ryanodine was the same, when membrane vesicle protein concentrations were between 10 and 100 \(\mu\)g/ml (data not shown). None of the ryanodine concentrations tested had any significant effect on Ca\(^{2+}\) uptake by the ryanodine-insensitive vesicles (Fig. 3, left). Ryanodine stimulation of Ca\(^{2+}\) uptake into the sensitive vesicles was associated with no change or a slight decrease in Ca\(^{2+}\)-dependent ATPase activity (Fig. 4). Ryanodine (300 \(\mu\)M) also had no effect on steady state acylphosphoprotein levels of these vesicles measured as described in Table I (data not shown). Thus, ryanodine did not appear to increase Ca\(^{2+}\) uptake into the sensitive vesicles by increasing the catalytic activities of their Ca\(^{2+}\) pumps.

Ca\(^{2+}\) exchange was measured in ryanodine-sensitive vesicles by adding a trace amount of \(^{40}\)CaCl\(_2\) after active calcium transport had first equilibrated in the presence of \(^{40}\)CaCl\(_2\) (Fig. 5). This rate of Ca\(^{2+}\) exchange was stimulated at least 8-fold by ryanodine. In other experiments, it was determined that ryanodine also stimulated Ca\(^{2+}\) uptake several fold, when the drug was added 20 min after the reactions had been initiated by the addition of ATP (data not shown). Both types of experiments suggested that Ca\(^{2+}\) oxalate already inside cardiac sarcoplasmic reticulum vesicles did not prevent a response to ryanodine.

Ca\(^{2+}\) uptake into ryanodine-sensitive and -insensitive vesicles was measured with use of a Ca\(^{2+}\)-selective electrode, which monitored the extravascular Ca\(^{2+}\) concentration. The ryanodine-insensitive or E vesicles effectively lowered the extravascular Ca\(^{2+}\) concentration, and this process was not affected by ryanodine (Fig. 6, circles). This demonstrated that ryanodine-insensitive vesicles did in fact accumulate additional net Ca\(^{2+}\), in excess of that already transported during the Ca\(^{2+}\)-loading step. With use of the Ca\(^{2+}\)-selective electrode it was further demonstrated that the ryanodine-sensitive vesicles also lowered the extravascular Ca\(^{2+}\) concentration, and that this process, in contrast, was dependent upon the presence of ryanodine (Fig. 6, triangles). We have obtained similar results for both populations of vesicles with use of the dye Arsenazo III to monitor extravascular Ca\(^{2+}\) concentration (data not shown). Comparison of the results in Table I and Figs. 1 and 6 suggests that ryanodine-insensitive vesicles were capable of accumulating approximately 3 times more total Ca\(^{2+}\) oxalate than ryanodine-sensitive vesicles. Ryanodine-insensitive vesicles had been preloaded with approximately 8 pmol of Ca\(^{2+}\)/mg of protein prior to isolation, and they accumulated approximately 4 pmol of additional Ca\(^{2+}\)/mg of protein after isolation. Ryanodine-sensitive vesicles, on the other hand, did not accumulate additional Ca\(^{2+}\), but retained the Ca\(^{2+}\) already present.

**Fig. 3.** Concentration dependence for ryanodine stimulation of Ca\(^{2+}\) uptake into ryanodine-sensitive and -insensitive vesicles. Ca\(^{2+}\) uptake in the left and right was determined for an E and D subfraction of vesicles, respectively, isolated from the same preparation of crude vesicles. For all concentrations of ryanodine tested on the E vesicles (ryanodine-insensitive), Ca\(^{2+}\) uptake determined at each time point was not significantly different from control values, and thus plotted is the average ±S.E. of all the data points. Ca\(^{2+}\) uptake was conducted as described for Fig. 1. Concentrations of ryanodine greater than 3 \(\times\) \(10^{-4}\) M had no further effect on Ca\(^{2+}\) uptake by the ryanodine-sensitive vesicles.

**Fig. 4.** Ryanodine effects on Ca\(^{2+}\) uptake (circles) and Ca\(^{2+}\)-dependent ATPase activities (triangles) of ryanodine-sensitive vesicles. A (D) subfraction was incubated as described in Fig. 1 in the presence (+RY) and absence (−RY) of 3 \(\times\) \(10^{-4}\) M ryanodine. Aliquots were then taken at each time point for measurement of Ca\(^{2+}\) uptake and total ATPase activities. For determination of basal ATPase activities identical reactions were conducted in the presence of 1 mM EGTA and no added Ca\(^{2+}\). The Ca\(^{2+}\)-dependent ATPase activities plotted are the differences between total ATPase and basal ATPase activities. Ryanodine had no effect on the basal ATPase activity.
Fig. 5. Ryanodine effects on Ca\(^{2+}\) exchange by ryanodine-sensitive vesicles. Ca\(^{2+}\) uptake by a (D) subfraction was measured in the presence (●) and absence (□) of 3 × 10\(^{-5}\) M ryanodine. In these two samples (□, ●), \(^{40}\)CaCl\(_2\) was present throughout the entire incubation. Ca\(^{2+}\) exchange was determined by incubating two other samples first with \(^{40}\)CaCl\(_2\), and then adding a trace amount of \(^{45}\)CaCl\(_2\) at the arrow (●). The samples used for determination of Ca\(^{2+}\) exchange were otherwise identical with the samples used for determination of Ca\(^{2+}\) uptake, and were incubated with (●) or without (□) ryanodine. Assays were conducted in a medium identical with that used in Fig. 1, except that a Ca\(^{2+}/\)EGTA buffer (0.8 mM CaCl\(_2\) plus 1 mM EGTA) was included such that the vesicles accumulated only a small fraction of the total Ca\(^{2+}\).

Hand, accumulated approximately 4 μmol of total Ca\(^{2+}\)/mg of protein under optimal conditions in the presence of ryanodine.

Ca\(^{2+}\) Dependence for Ca\(^{2+}\) Uptake by Ryanodine-sensitive and -insensitive Vesicles—Ryanodine-sensitive and -insensitive vesicles were differentiated by their responses to Ca\(^{2+}\) concentration, when active Ca\(^{2+}\) transport was measured (Figs. 7 and 8). For both populations of vesicles, Ca\(^{2+}\)-dependent ATPase activities were maximal at 0.6 mM added Ca\(^{2+}\) in the presence of 1 mM EGTA (or at about 1.5 × 10\(^{-5}\) M ionized Ca\(^{2+}\)) (Figs. 7 and 8, right panels). Ca\(^{2+}\) uptake for the ryanodine-sensitive vesicles, however, was very low at this added Ca\(^{2+}\) concentration, and could barely be detected at the lower Ca\(^{2+}\) concentrations tested (Fig. 7, left). Ryanodine-insensitive vesicles, on the other hand, had already achieved their maximal rates of Ca\(^{2+}\) transport at 0.6 mM added Ca\(^{2+}\), and exhibited considerable levels of Ca\(^{2+}\) transport even at the lower Ca\(^{2+}\) concentrations tested (Fig. 8, left). A spontaneous release of Ca\(^{2+}\) from ryanodine-insensitive vesicles after 10 min of incubation, which occurred at 0.6 mM added Ca\(^{2+}\), was blocked when the Ca\(^{2+}\) concentration was increased to 0.8 mM (Fig. 8, left).

Most striking was the large increase in both the rate and extent of Ca\(^{2+}\) transport for the ryanodine-sensitive vesicles, when the Ca\(^{2+}\) concentration was changed from only 0.8 to 0.8

Fig. 6. Ca\(^{2+}\) uptake by ryanodine-sensitive (triangles) and ryanodine-insensitive (circles) vesicles measured with a Ca\(^{2+}\)-selective electrode. Vesicles (0.4 mg) were preincubated for 10 min in 25 ml of a medium containing 60 mM histidine, 3 mM MgCl\(_2\), 100 mM KCl, 5 mM Tris/ethylenediaminetetraacetic acid (EDTA), and 100 μM CaCl\(_2\) with (filled symbols) or without (open symbols) 1 × 10\(^{-5}\) M ryanodine. Reactions were initiated by adding 3 mM Tris/ATP, which decreased the electrode potential by approximately 16 mV, due to chelation of Ca\(^{2+}\) by ATP. This base-line potential stabilized within 16 s. Ca\(^{2+}\) uptake by the vesicles then caused a further decrease in electrode potential, which is plotted as the change in mV (ΔmV) from the base-line potential on the ordinate. One ΔmV unit corresponds to a change in total extrasolution Ca\(^{2+}\) concentration of approximately 5 μM, as determined from a standard curve made by stepwise addition of CaCl\(_2\) to the above medium containing ATP without membrane vesicles. Maximal Ca\(^{2+}\) accumulation by the vesicles determined with this technique is underestimated, because the amount of Ca\(^{2+}\) chelated by ATP decreased during the course of the incubations, due to ATP hydrolysis. This effect caused ryanodine-sensitive vesicles incubated in the absence of the drug (clear triangles) to exhibit only positive deflections from the base-line potential.

Fig. 7. Ca\(^{2+}\) activation of Ca\(^{2+}\) uptake and Ca\(^{2+}\)-dependent ATPase activity of ryanodine-sensitive vesicles. A (D) subfraction was incubated in the presence of 3 × 10\(^{-5}\) M ryanodine and 1 mM EGTA, and the concentration of \(^{40}\)CaCl\(_2\) was varied between 0.1 and 0.8 mM as follows: 0.1 mM, □; 0.3 mM, ●; 0.6 mM, ▲; 0.8 mM, ●. In addition, a sample was incubated in the presence of only 1 mM EGTA, to determine basal ATPase activity. Otherwise, the incubation mixture was identical with that used in Fig. 1.
mm in the presence of 1 mM EGTA (Fig. 7, left). This increase in Ca\textsuperscript{2+} transport was associated with no increase in Ca\textsuperscript{2+}-dependent ATPase activity (Fig. 7, right).

Ca\textsuperscript{2+} uptake and ATPase rates were obtained at each ionized Ca\textsuperscript{2+} concentration from the data depicted in Figs. 7 and 8 (Table II). The initial rates of Ca\textsuperscript{2+} uptake divided by the initial rates of ATP hydrolysis were used to estimate apparent coupling ratios, or the number of Ca\textsuperscript{2+} ions effectively transported per ATP molecule hydrolyzed during the early, nearly linear phases of Ca\textsuperscript{2+} uptake. Ca\textsuperscript{2+} transport was poorly coupled to ATP hydrolysis for ryanodine-sensitive vesicles at ionized Ca\textsuperscript{2+} concentrations of 1.5 \mu M or below (Table II). However, between 1.5 and 4.0 \mu M ionized Ca\textsuperscript{2+}, a 10-fold increase in apparent Ca\textsuperscript{2+} pumping efficiency occurred. This was primarily accounted for by a large increase or "jump" in the Ca\textsuperscript{2+} uptake rate. In comparison, ATP hydrolysis was fairly well coupled to Ca\textsuperscript{2+} transport at each ionized Ca\textsuperscript{2+} concentration for the ryanodine-insensitive vesicles, and no such jump in the Ca\textsuperscript{2+} uptake rate was evident. We have repeated these experiments many times with different preparations of vesicles and have always obtained similar results. The maximum coupling ratios obtained always approached a value of one for either subpopulation of vesicles, and in no cases were transport ratios greater than one obtained.

Characterization of the Jump in Ca\textsuperscript{2+} Uptake Exhibited by Ryanodine-sensitive Vesicles—The large increase or jump in Ca\textsuperscript{2+} uptake that occurred only for ryanodine-sensitive vesicles was further investigated. Vesicles were incubated under active Ca\textsuperscript{2+} transport conditions for 20 min, which allowed for equilibration of Ca\textsuperscript{2+} uptake (Fig. 7). Ca\textsuperscript{2+} transport and Ca\textsuperscript{2+}-dependent ATP hydrolysis that had occurred during this time were measured at several added Ca\textsuperscript{2+} concentrations, in the presence of 1 mM EGTA (Fig. 9). Between an added Ca\textsuperscript{2+} concentration of 0.7 and 0.8 mM, Ca\textsuperscript{2+} uptake increased sharply in a typical preparation of ryanodine-sensitive vesicles (Fig. 9). This increase in Ca\textsuperscript{2+} uptake was completely prevented by the Ca\textsuperscript{2+} ionophore A23187, which demonstrated that it was not due to Ca\textsuperscript{2+} oxalate precipitating outside the vesicles. Ca\textsuperscript{2+}-dependent ATP hydrolysis, measured with or without A23187, was activated over a much wider range of added Ca\textsuperscript{2+} concentration (0.1 to 0.7 mM), and Ca\textsuperscript{2+} activation of this process was complete before the jump in Ca\textsuperscript{2+} uptake had occurred (Fig. 9). In other experiments it was demonstrated that the jump in Ca\textsuperscript{2+} uptake occurred at a similar added Ca\textsuperscript{2+} concentration when either phosphate or fluoride anions were used as Ca\textsuperscript{2+} precipitants, suggesting that a purely physical interaction between Ca\textsuperscript{2+} and oxalate was...
not responsible for the large increase in Ca\textsuperscript{2+} uptake. Furthermore, the jump in Ca\textsuperscript{2+} uptake was not detected in crude membrane vesicles incubated in the absence of ryanodine, again suggesting that this process was a property of only ryanodine-sensitive vesicles (data not shown).

The jump in Ca\textsuperscript{2+} uptake was measured over a narrower range of added Ca\textsuperscript{2+} concentration, with ryanodine-sensitive vesicles incubated in the presence and absence of the drug (Fig. 10). The increase in Ca\textsuperscript{2+} uptake occurred with or without ryanodine, but the total Ca\textsuperscript{2+} accumulated was much greater when the incubations were conducted in the presence of ryanodine. In the experiment of Fig. 10, the jump in Ca\textsuperscript{2+} uptake was completed between 0.70 and 0.80 mM added CaCl\textsubscript{2}, or between the calculated ionized Ca\textsuperscript{2+} concentrations of about 2 and 4 mM. The steepness of the response was indicated by determining a Hill coefficient of 16 for the effect in a typical preparation of ryanodine-sensitive vesicles (Fig. 11, right). The mean ±S.E. calculated from six such experiments was 17 ± 1.8. In the same preparations, the Hill coefficient for Ca\textsuperscript{2+}-activation of ATPase activity was found to be 1.9 ± 0.13 (shown for a typical preparation in Fig. 11, left), which is consistent with the results of others (26).

In all experiments on Ca\textsuperscript{2+} uptake so far described, the vesicles had been preincubated at pH 7.4 for 10 min, after which time Ca\textsuperscript{2+} transport was initiated by adding an acidic solution of ATP, which lowered the pH of the incubation medium to 6.8. When both the preincubations and incubations were conducted at pH 6.8, the jump in Ca\textsuperscript{2+} uptake noted only for the ryanodine-sensitive vesicles disappeared (Fig. 12, left). Ca\textsuperscript{2+}-activation of Ca\textsuperscript{2+}-ATPase activity, on the other hand, was not affected by the slightly alkaline preincubation (Fig. 12, right). In other experiments, it was demonstrated that Ca\textsuperscript{2+} uptake by the ryanodine-insensitive vesicles was not affected by the pH of the preincubation. Although the jump in Ca\textsuperscript{2+} uptake by ryanodine-sensitive vesicles was not present when the preincubation was conducted at pH 6.8, Ca\textsuperscript{2+} uptake itself was still stimulated 5-fold or greater by ryanodine (data not shown).

**Polyacrylamide Gel Electrophoresis of Cardiac Sarcoplasmic Reticulum Vesicles**—The crude membrane vesicle fraction and the isolated subfractions (A to E) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis...
Subpopulations of Cardiac Sarcoplasmic Reticulum Vesicles

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of sucrose gradient fractions. Twenty-five µg of protein from the crude membrane vesicles and subfractions A through E were submitted to electrophoresis as described under "Experimental Procedures." The M̄ = 55,000 protein band unique to ryanodine-sensitive vesicles is indicated by the double arrow. The protein band characteristic of ryanodine-insensitive vesicles is indicated by the single arrow. The molecular weight of this latter protein could not be accurately estimated, because it migrated behind myosin (M̄ = 200,000), the largest protein standard used.

(Discussion) Differences in protein staining between the subfractions were most clearly revealed by the protein stain, Stains-All. Fractions B, C, and D, the ryanodine-sensitive fractions, had a dark blue, intensely staining protein band of molecular weight 55,000 (double arrow), that was not apparent in the ryanodine-insensitive (E) fraction. We have consistently observed that the intensity of this blue staining protein band increases from fractions B through D. This M̄ = 55,000 protein was also easily visualized in the crude membrane vesicles, confirming by an independent method that most of the protein in the crude microsomes was contributed by ryanodine-sensitive vesicles. Fraction E, on the other hand, characteristically contained a very high molecular weight protein band, which was not prominent in the other subfractions or in the crude membrane vesicles (single arrow). The pink staining protein band of molecular weight 100,000 present in all the subfractions was in large part contributed by the Ca²⁺-dependent ATPase (23, 27). Radioactive Ca²⁺-dependent acylphosphoproteins (Table I) were localized to this region of the polyacrylamide gel for all the subfractions (data not shown).

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Discussion

Sucrose density gradient centrifugation was used in the present study to isolate two biochemically distinct subpopulations of cardiac sarcoplasmic reticulum vesicles. When crude sarcoplasmic reticulum preparations were preloaded to submaximal levels with Ca²⁺ oxalate, only the ryanodine-insensitive vesicles became more dense, and thus they were easily separated from the ryanodine-sensitive vesicles.

Compared to ryanodine-sensitive vesicles, ryanodine-insensitive vesicles had a higher density of Ca²⁺ pumps, a higher Ca²⁺-dependent ATPase activity, and a higher initial velocity and maximal capacity for Ca²⁺ transport. Ryanodine-sensitive vesicles could accumulate substantial levels of Ca²⁺, but only in the presence of the drug, and they exhibited a unique Ca²⁺ concentration-dependent and pH-dependent increase in Ca²⁺ uptake that was not associated with any change in Ca²⁺-dependent ATPase activity. In other experiments, we have observed that 10⁻⁴ M chlorpromazine completely inhibited Ca²⁺ uptake by ryanodine-sensitive vesicles, but it had no effect on Ca²⁺ uptake by ryanodine-insensitive vesicles. Thus, the data above provide evidence for a functional heterogeneity of cardiac sarcoplasmic reticulum vesicles.

Different subpopulations of sarcoplasmic reticulum vesicles were substantiated by examining their poly peptide compositions after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Most apparent was that ryanodine-sensitive vesicles had an intense, blue staining protein band of molecular weight 55,000, which was absent from ryanodine-insensitive vesicles. Ryanodine-sensitive vesicles had an additional protein band of very high molecular weight that was not present in ryanodine-sensitive vesicles. In other recent studies it was demonstrated that, although both populations of vesicles contain a calmodulin-dependent protein kinase activity, ryanodine-sensitive vesicles have several different protein substrates of this kinase that are absent from ryanodine-insensitive vesicles (27). Moreover, although ryanodine-sensitive vesicles have a lower Ca²⁺-dependent ATPase content than ryanodine-insensitive vesicles, they have more of the M̄ = 22,000 protein substrate of cAMP-dependent protein kinase, phospholamban (27).

Some precedent, albeit from a different tissue source, exists for the results described presently. Successful separation of subpopulations of sarcoplasmic reticulum vesicles from fast skeletal muscle have been ongoing for some time. Meissner first isolated skeletal muscle sarcoplasmic reticulum subfractions enriched in either terminal cisterna or longitudinal sarcoplasmic reticulum (28), and this work has since been extended by others (29-31). In different studies, Fairhurst noted that the inhibitory effect of ryanodine on Ca²⁺ transport by skeletal muscle sarcoplasmic reticulum vesicles was specific for only those membrane vesicle fractions that sedimented at relatively low g forces (32). (Ryanodine apparently has opposite effects on Ca²⁺ uptake by skeletal muscle and cardiac microsomes, Ref. 8). Functional heterogeneity of sarcoplasmic reticulum in relatively intact skeletal muscle fibers was recently suggested by the results of Sorenson et al. (33). Using an elegant technique with skinned skeletal muscle fibers, which relied on visualization of Ca²⁺ oxalate crystals deposited during active Ca²⁺ transport, these investigators demonstrated that individual regions of the sarcoplasmic reticulum network within each sarcomere differed in their abilities to transport Ca²⁺ (33). Indeed, Carsten and Reedy originally postulated that functional heterogeneity of cardiac sarcoplasmic reticulum vesicles might explain why only a fraction of the vesicles accumulated Ca²⁺ oxalate (6).

Although our results appear to substantiate functionally different subpopulations of cardiac sarcoplasmic reticulum vesicles, some caution is warranted in interpreting experimental results obtained with ryanodine-insensitive vesicles, which were already partially filled with Ca²⁺ oxalate. Use of a Ca²⁺-selective electrode, however, confirmed that the "Ca²⁺" uptake exhibited by ryanodine-insensitive vesicles reflected a real, net uptake of Ca²⁺ and was not simply a manifestation of Ca²⁺ exchange. This was also supported by the high magnitude of this Ca²⁺ transport, which was 2 to 3 times greater than that observed by us for crude vesicles used in previous studies (8, 18). Ryanodine stimulation of net Ca²⁺ uptake by these vesicles should have been detectable, therefore, if the vesicles

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were capable of responding to the drug. Control experiments conducted with ryanodine-sensitive vesicles also supported this conclusion. Ca\textsuperscript{2+} oxalate precipitates already inside these sensitive vesicles did not prevent a response to ryanodine. Although Carsten and Reedy (6) observed no additional Ca\textsuperscript{2+} uptake by cardiac sarcoplasmic reticulum vesicles isolated by a Ca\textsuperscript{2+}-loading technique, this may have been because the vesicles had already been filled to their maximal levels (5 μmol of Ca\textsuperscript{2+}/mg of protein in the crude cardiac vesicles with 26 μmol of Ca\textsuperscript{2+}/mg of protein recovered in the subsequently purified vesicles). Our results confirm those of Levitisky et al. (7), who observed additional Ca\textsuperscript{2+} transport (11 μmol of Ca\textsuperscript{2+}/mg of protein) by cardiac sarcoplasmic reticulum vesicles isolated by a Ca\textsuperscript{2+}-loading technique similar to that used in this study.

Since the cellular localization of ryanodine-sensitive and -insensitive vesicles was not established in the present study, it is relevant to ask if in fact both populations of vesicles originated from sarcoplasmic reticulum. This can best be addressed by stating what the vesicles did not appear to be. They did not appear to be sarcolemmal vesicles because they contained very low levels of all of the different sarcolemmal markers including (Na\textsuperscript{+},K\textsuperscript{+})-ATPase, adenylate cyclase, sialic acid, β-adrenergic receptors (12), and regulatory and catalytic subunits of type II cAMP-dependent protein kinase (27). Although several investigators have recently suggested that sarcolemmal vesicles can actively transport Ca\textsuperscript{2+}, the levels of Ca\textsuperscript{2+} accumulated in these studies were only about 1% of those presently reported, and the Ca\textsuperscript{2+} uptake was not stimulated by oxalate (34, 36). The vesicles described presently did not appear to be mitochondria because their Ca\textsuperscript{2+} uptake was insensitive to sodium azide, and their ATPase activities were only marginally inhibited by this agent. Moreover, purified cardiac sarcolemmal membranes and mitochondria had none of the blue staining protein bands observed in the sarcoplasmic reticulum fractions after polyacrylamide gel electrophoresis and staining with Stains-All. Thus it seems most likely that the Ca\textsuperscript{2+} uptake reported presently was accounted for by different subpopulations of sarcoplasmic reticulum vesicles, although it cannot be definitely excluded that a subpopulation of sarcolemmal vesicles containing none of the markers above contributed to some of the Ca\textsuperscript{2+} uptake.

Ratios for Ca\textsuperscript{2+} ions transported per ATP molecule hydrolyzed were substantially less than two for both populations of vesicles. Since the accepted stoichiometry is two for fast skeletal muscle preparations (26), one could argue that the cardiac vesicles were damaged during the Ca\textsuperscript{2+}-loading step. However, we have obtained similar coupling ratios for crude vesicles not preloaded with Ca\textsuperscript{2+} (8, 18). Although Tada et al. have reported Ca\textsuperscript{2+} transport stoichiometries of two for cardiac preparations (36), most other investigators have obtained coupling ratios of less than one (37-40). Thus, the coupling ratios reported presently were compatible with those obtained by most of the investigators who have used cardiac preparations.

For ryanodine-sensitive vesicles it was apparent that calculated coupling ratios could be increased dramatically by either ryanodine itself (Fig. 4), or Ca\textsuperscript{2+} concentration (Fig. 7). Ryanodine, an alkaloid known to cause relaxation in intact heart (41), was previously postulated to increase net Ca\textsuperscript{2+} uptake into crude preparations of cardiac microsomes by blocking passive efflux of Ca\textsuperscript{2+} from these vesicles (8). However, such an increase in net Ca\textsuperscript{2+} uptake was also compatible with an increased number of Ca\textsuperscript{2+} ions transported per ATP molecule hydrolysed (3). The data of Fig. 5 clearly showed that ryanodine stimulated Ca\textsuperscript{2+} exchange in ryanodine-sensitive vesicles after net Ca\textsuperscript{2+} uptake had reached a steady state.
Mueller is gratefully acknowledged. The technical skills of Ann Yoshia are equally appreciated.

Note Added in Proof—Campbell, Jorgensen, and MacLennan (personal communication) have isolated a protein of $M_r$ = 55,000 from cardiac membranes which stains blue with Stains-All, and they have shown that it is physically similar to calsequestrin, a protein localized inside terminal cisternae in fast skeletal muscle sarcoplasmic reticulum. By analogy, the presence of this blue-staining protein in the ryanodine-sensitive cardiac vesicles suggests that they originate in part from functional sarcoplasmic reticulum in intact myocardium.

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