

BBA 78938

FURTHER CHARACTERIZATION OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES

IDENTIFICATION OF THE 'SARCOPLASMIC RETICULUM FEET' ASSOCIATED WITH HEAVY SARCOPLASMIC RETICULUM VESICLES *

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(Received December 20th, 1979)

(Revised manuscript received May 7th, 1980)

Key words: Light vesicle; Heavy vesicle; Sarcoplasmic reticulum feet; Terminal cisterna; (Rabbit muscle)

Summary

Light and heavy sarcoplasmic reticulum vesicles were isolated from rabbit leg muscle using a combination of differential centrifugation and isopycnic zonal ultracentrifugation. Light sarcoplasmic reticulum vesicles obtained from the 30–32.5% and heavy sarcoplasmic reticulum vesicles obtained from the 38.5–42% sucrose regions of the linear sucrose gradient were determined to be free of surface and mitochondrial membrane contamination by marker enzyme analysis and electron microscopy. Thin sections of the light vesicles revealed empty vesicles of various sizes and shapes. Freeze-fracture replicas of the light vesicles showed an asymmetric distribution of intramembranous particles with the same orientation and distribution as the longitudinal sarcoplasmic reticulum *in vivo*. Heavy vesicles appeared as rounded vesicles of uniform size filled with electron dense material, similar to that seen in the terminal cisternae of the sarcoplasmic reticulum. The cytoplasmic surface of the membrane was

* This work was submitted (by K.P.C.) to the Graduate School of the University of Rochester in partial fulfillment of the requirements for a Ph.D. degree.

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Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol bis (β -aminoethyl ether)-*N,N'*-tetraacetic acid.

decorated by membrane projections, closely resembling the 'feet' which join the sarcoplasmic reticulum to the transverse tubules in the intact muscle fiber. Freeze-fracture replicas of the heavy vesicles revealed an asymmetric distribution of particles which in some areas of the vesicle's surface are larger and less densely aggregated than those of the light vesicles. In the best quality replicas, some regions of the luminal leaflet were not smooth but showed evidence of pits. These structural details are characteristic of the area of sarcoplasmic reticulum membrane which is covered by the 'feet' in the intact muscle.

Heavy vesicles contained greater than six times the calcium content of light vesicles, 54 vs. 9 nmol $\text{Ca}^{2+}/\mu\text{l}$ of water space. After KCl washing both contained less than 4 nmol $\text{Ca}^{2+}/\mu\text{l}$ of water space. Although they transported at the same rate and the same total amount of calcium, the rate of passive Ca^{2+} efflux from the heavy vesicles was double that of light vesicles. The higher rate of calcium efflux from the heavy vesicles was inhibited by dantrolene, an inhibitor of Ca^{2+} release. High resolution sodium dodecyl sulfate gel electrophoresis showed that the light vesicles contained predominantly Ca^{2+} -ATPase along with several approx. 55 000-dalton proteins and a 5000-dalton proteolipid, while the heavy vesicles contained Ca^{2+} -ATPase and calsequestrin along with several approx. 55 000-dalton proteins, extrinsic 34 000- and 38 000-dalton proteins, intrinsic 30 000- and 33 000-dalton proteins and two proteolipids of 5 000 and 9 000 daltons. KCl washing of the heavy vesicles removed both the approx. 34 000- and 38 000-dalton proteins, and the 'sarcoplasmic reticulum feet' were no longer seen on the heavy vesicles. The KCl supernatant was enriched in the 34 000- and 38 000-dalton proteins, indicating that these proteins are possible components of the sarcoplasmic reticulum feet. The biochemical and morphological data strongly support the view that the light vesicles are derived from the longitudinal sarcoplasmic reticulum and that the heavy vesicles are derived from the terminal cisternae containing junctional sarcoplasmic reticulum membrane with the intact 'sarcoplasmic reticulum feet'.

Introduction

It is generally accepted that the depolarization of the transverse tubular system of skeletal muscle initiates the release of calcium from the terminal cisternae of the sarcoplasmic reticulum [1-4]. In recent years there have been several mechanisms proposed for the link between the depolarization of the walls of the T-tubule and the release of calcium from the sarcoplasmic reticulum [1-4] but it still remains one of the least understood processes in the muscle contraction. The skeletal muscle membranes directly involved in excitation-contraction coupling are the transverse tubular membrane and the junctional sarcoplasmic reticulum membrane [5]. The morphology of the transverse tubular system and the sarcoplasmic reticulum has been extensively studied [6-10]. In the region of the triad the sarcoplasmic reticulum membrane has several distinctive features: (1) the internal space of the terminal cisternae is filled with electron dense material; (2) at periodic intervals of about 30 nm the terminal cisternae membrane facing the T-tubules forms small projections ('sarcoplasmic

reticulum feet') which apparently join the sarcoplasmic reticulum to the transverse tubular membrane. This portion of the sarcoplasmic reticulum is called junctional sarcoplasmic reticulum membrane [3] and it has large intramembraneous particles mostly on the cytoplasmic leaflet and pits on the luminal leaflet. The terminal cisternae is also known to have a high calcium content [11]. The longitudinal sarcoplasmic reticulum, in contrast, has no internal material and has an asymmetric distribution of intramembraneous particles of 8 nm on the cytoplasmic leaflet [9,10], which represent the calcium pump protein [12]. The pump particles do not leave a distinct pit on the luminal leaflet.

Meissner [13] developed preparations of light and heavy sarcoplasmic reticulum vesicles in which he found that calsequestrin and a 55 000-dalton protein were localized in the heavy vesicles and that the heavy vesicles contained an electron dense content similar to that seen in the terminal cisternae of the sarcoplasmic reticulum. He suggested that the electron dense material within the terminal cisternae was calsequestrin and/or the 55 000-dalton protein. Caswell and his collaborators [14,15] have isolated terminal cisternae vesicles from French press treatment of isolated dyads and triads. They have also found that the terminal cisternae is the primary location for calsequestrin. In contrast to Meissner's results, they have found that the 55 000-dalton protein was enriched in the light vesicles. Caswell et al. [16,17] have also characterized isolated transverse tubules and studied the recombination of transverse tubules and terminal cisternae to form dyads. Jorgensen et al. [18] used immunofluorescent staining to show that while the ATPase was uniformly distributed throughout sarcoplasmic reticulum, calsequestrin was localized in regions which correspond to the terminal cisternae.

The purpose of our investigation was three-fold: (1) to isolate heavy sarcoplasmic reticulum vesicles which have retained 'sarcoplasmic reticulum feet'; (2) to show that the heavy vesicles contain junctional sarcoplasmic reticulum membrane; and (3) to carry out a detailed study of the protein composition of the light and heavy vesicles in order to identify the proteins of the junctional sarcoplasmic reticulum membrane. Preliminary reports of this work have appeared [19,20].

Experimental procedures

Materials

Ultra pure sucrose was obtained from Schwarz/Mann. Electrophoresis reagents were obtained from Bio-Rad. Adenosine 5'-[γ - ^{32}P]triphosphate (20 mCi/mmol); inulin [^{14}C]carboxylic acid (5 mCi/mmol) and $^{45}\text{CaCl}$ were obtained from Amersham/Searle. Tritiated water was obtained from New England Nuclear. All solutions were made with deionized distilled water.

Protein was determined by the method of Lowry et al. [21] using bovine serum albumin as a standard. Percent sucrose (w/w) was measured at 25°C using an Abbe refractometer. Calcium and magnesium were determined by atomic absorption spectroscopy. Phosphate was determined by the method of Chen et al. [22].

Isolation of light and heavy sarcoplasmic reticulum vesicles

The isolation procedure for the light and heavy sarcoplasmic reticulum vesi-

cles was similar to the method of Meissner [13] with the major difference being that the vesicles were not washed with 0.6 M KCl. Male albino rabbits were stunned by a blow on the head and then exsanguinated. The white, proximal hind leg muscles were excised and placed on ice. All subsequent steps were carried out at 0–4°C. The muscle was cleaned and then ground in a meat grinder. It was then homogenized in 0.25 M sucrose in a Waring Blendor for 30 s. The homogenate of approx. 1000 g of muscle was then centrifuged for 10 min at $1600 \times g$ (R_{max}). The supernatant was then passed through six layers of cheesecloth and centrifuged for 30 min at $9000 \times g$. Sarcoplasmic reticulum membranes were pelleted using a Beckman 45-Ti rotor at 40 000 rev./min ($127\,000 \times g$) for 60 min. The resulting pellets were gently resuspended using a Teflon homogenizer into 0.25 M sucrose, 1 mM Hepes (pH 7.4) to approx. 10 mg/ml. Sarcoplasmic reticulum vesicles were placed on a linear sucrose gradient 28–45% sucrose (w/v) of 1200 ml. After 14–16 h at 32 000 rev./min ($102\,000 \times g$) the rotor was allowed to slow down without any braking to 3000 rev./min and 55% sucrose (w/w) was pumped in at the edge to unload the gradient. The effluent was continuously monitored for protein by measuring the 280 nm absorbance with an ISCO zonal flow cell attached to an ISCO UV monitor. The percent sucrose of every other fraction was subsequently measured using an Abbe refractometer.

Fractions from 30–32.5% sucrose (Light), 33.5–38% sucrose (Intermediate) and 38.5–42% sucrose (Heavy) were combined and then diluted with an equal volume of 0.25 M sucrose, 1 mM Hepes (pH 7.4). The purified Sarcoplasmic reticulum vesicles were pelleted using a Beckman 45 Ti rotor at 45 000 rev./min ($160\,000 \times g$) for 2h. The pellets were gently resuspended using a Teflon homogenizer into 0.25 M sucrose, 1 mM Hepes (pH 7.4) quickly frozen and stored in liquid nitrogen (–70°C). These fractions are referred to as sucrose-diluted vesicles. Light and heavy vesicles were also prepared with 0.6 M KCl wash as described by Meissner [13]. These fractions are referred to as KCl-diluted vesicles. Proteolipid extraction from light and heavy vesicles was carried out according to MacLennan et al. [23].

Enzymatic assays

($Ca^{2+} + Mg^{2+}$ -ATPase activity was measured in 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM EGTA, 10 mM Hepes (pH 7.4) and 5 mM ATP ($[\gamma\text{-}^{32}P]$ -ATP, 500 cpm/nmol ATP) at a protein concentration of 50–80 $\mu\text{g/ml}$ at 37°C. ($Na^+ + K^+$ -ATPase activity was measured by the method of Takachuk and Boldyrev [24] which employs the optimal conditions for skeletal sarcolemma ($Na^+ + K^+$ -ATPase. ($Na^+ + K^+$ -ATPase activity was calculated from the difference between the total and the Mg^{2+} -ATPase activity. Succinate dehydrogenase was measured by the method of King [25] following the absorbance of 2,6-dichlorophenol indophenol at 600 nm in the presence of phenazine methosulfate. Controls were run in the absence of succinate. Monoamine oxidase activity was measured by the method of Schnaitman et al. [26] by following the formation of benzaldehyde spectrophotometrically at 250 nm and 37°C in a medium containing 2.5 mM benzylamine and 50 mM potassium phosphate (pH 7.6).

Ca^{2+} uptake was measured by the Millipore filtration method of Martonosi

and Feretos [27] or with arsenazo III [28]. Calcium efflux from sarcoplasmic reticulum vesicles was measured by the Millipore filtration method [27] using 0.45 micron filters. Vesicles were passively loaded by incubating them at 0°C in 100 mM KCl and 5 mM MgCl₂, 10 mM Hepes (pH 7.1) and 1 mM ⁴⁵Ca²⁺ at a protein concentration of 20 mg/ml. The vesicles were diluted 40-fold into the same medium with 1 mM EGTA replacing the ⁴⁵CaCl₂. Aliquots (0.5 ml) were removed at timed intervals, filtered, and washed immediately with 4 ml of dilution buffer. The filters were dried and counted for radioactive calcium.

Volume measurements

The intravesicular water content of the sarcoplasmic reticulum vesicles was measured by the radiochemical technique of Johnson and Scarpa [29], using tritiated water (0.8 μCi/ml) and [¹⁴C]inulin (0.125 μCi/ml) in the presence of 0.25 M sucrose, 1 mM Hepes (pH 7.4).

Electron microscopy

Samples of sarcoplasmic reticulum vesicles were fixed for 2 h at 0°C in 3% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.2) after they had been pelleted by centrifugation at 25 000 × *g* for 1 h. An alternate procedure of fixation used less frequently, was to fix the vesicles in solution (3% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.2) and 0.25 M sucrose) at a protein concentration of 2.5 mg/ml for 30 min and then pellet the vesicles using a Beckman JA-10 rotor at 20 000 rev. /min (10 000 × *g* for 1 h). In either case fixed samples were stored in 0.1 M sodium cacodylate (pH 7.2) and were subsequently fixed in 1% OsO₄ for 1–2 h. They were dehydrated in ethanol, stained with 1% uranyl acetate in 95% ethanol and embedded in epon. Pellets were sectioned perpendicular to the surface of the pellet and stained with uranyl acetate and lead. Representative micrograms were taken at regular intervals from top to bottom of the pellet. Samples for freeze fracture were fixed with glutaraldehyde and then gradually infiltrated in glycerol up to 30%. They were frozen in liquid Freon and fractured in Denton DFE-3 freeze-fracture apparatus. Pictures were taken in AE1 100B and JEOL 100B and S microscopes. Samples of sarcoplasmic reticulum vesicles were also fixed in the presence of 1% tannic acid according to the method of Saito et al. [30].

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [31] using a 10% acrylamide running gel with a 3% acrylamide stacking gel. The samples to be analyzed by electrophoresis were solubilized in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue at a protein concentration of 1 mg/ml for 2 min at 100°C followed by 2 h at 37°C. Polyacrylamide gel electrophoresis was also carried out according to the method of Weber and Osborn [32] using 7.5% acrylamide gels. In all cases 40 μg of protein were run per gel. Cylindrical gels were scanned at 560 nm with a Gilford spectrophotometer equipped with a linear scanner. Slab gels were scanned using a Beckman densitometer. Apparent molecular weights were calculated from a graph of relative mobilities versus log of the molecular weight. Molecular weight standards used were spectrin

(220 000 and 240 000), phosphorylase α (94600), bovine serum albumin (68 000), catalase (57 500), actin (43 000), lactate dehydrogenase (35 000), carbonic anhydrase (30 000), trypsin inhibitor (21 000) and lysozyme (14 400).

Low molecular weight proteins (3000–20000 daltons) were resolved on Swank and Munkres [33] gel system. Cylindrical gels (5.5×125 mm) consisted of 10% acrylamide, 1% bis-acrylamide, 8 M urea, 0.1 phosphoric acid (pH 7.0) and 0.1% SDS. Samples were solubilized in 1% SDS, 8 M urea, 1% mercapto-ethanol, 0.01 M phosphoric acid (pH 6.8). Molecular weight standards used were carbonic anhydrase (30 000), trypsin inhibitor (21 000), cytochrome c (12 600), parathyroid hormone (9800) and glucagon (3480).

Detergent treatment of light and heavy sarcoplasmic reticulum vesicles

Light and heavy vesicles were treated with deoxycholate according to the method of MacLennan [34] to remove the extrinsic membrane proteins. Heavy vesicles were treated with Triton X-100 in the following manner: a suspension of heavy sucrose-diluted vesicles at 2 mg/ml in 0.25 M sucrose, 1 mM Hopes (pH 7.4) were solubilized with 2% Triton X-100 on ice for 1 h. The solubilized membranes were then centrifuged for 1 h at $200\,000 \times g$. The resulting pellets were fixed for electron microscopy or homogenized on 0.25 M sucrose, 1 mM Hepes (pH 7.4).

Results

Isolation of light and heavy sarcoplasmic reticulum vesicles

Two distinct protein peaks, determined by 280 nm absorbance with an ISCO UV monitor, appeared at sucrose densities 1.0854 and 1.1663 g/cm³ (21% and 38%) following zonal ultracentrifugation of the sarcoplasmic reticulum vesicles isolated according to the detailed method in Experimental procedures. Peak I (15–26% sucrose) which lay in the non-linear region of the gradient contained 14% of the protein that was applied to the gradient. After dilution with sucrose and centrifugation at $160\,000 \times g$ for 2 h only a very small amount of protein was recovered as particulate material. Therefore much of the protein in Peak I appeared to be soluble proteins, such as phosphorylase α . Peak II (30–43%

TABLE I

ENDOGENOUS CALCIUM AND WATER VOLUMES OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES

The values given are the mean of the number of preparations \pm S.E.M. The number of preparations measured is given in parenthesis. Ca²⁺ was determined using atomic absorption spectroscopy. Water volumes were measured according to the method of Johnson and Scarpa [22].

| Fraction of sarcoplasmic reticulum vesicles (buoyant density) | Ca ²⁺ content (nmol Ca ²⁺ /mg protein) | | Water volumes (μ l/mg protein) | |
|---|--|----------------------|-------------------------------------|----------------------|
| | Sucrose-diluted vesicles | KCl-diluted vesicles | Sucrose-diluted vesicles | KCl-diluted vesicles |
| Light (30–32.5% sucrose) | 34 \pm 5 (3) | 16 \pm 2 (4) | 3.6 \pm 0.2 (3) | 3.2 \pm 0.5 (3) |
| Intermediate (33.5–38% sucrose) | 58 \pm 9 (3) | 15 \pm 2 (5) | 2.9 \pm 0.4 (3) | 2.5 \pm 0.6 (3) |
| Heavy (38.5–42% sucrose) | 114 \pm 12 (3) | 15 \pm 1 (3) | 2.1 \pm 0.3 (3) | 2.4 \pm 0.4 (3) |

sucrose) consisted of highly purified sarcoplasmic reticulum vesicles and contained 85% of the applied protein. The calcium content of the light and heavy vesicles is given in Table I. There was a striking difference in the amount of calcium per mg of protein in the sucrose-diluted vesicles. The heavy vesicles have over three times the amount of calcium content as the light vesicles (114 vs. 34 nmol Ca^{2+} /mg). The KCl dilution lowered the calcium content in all three fractions to 15 nmol Ca^{2+} /mg of protein. In contrast to the calcium content, the magnesium content was approximately the same in all three sucrose diluted fractions (40 nmol Mg^{2+} /mg). It was also reduced to less than 10 nmol Mg^{2+} /mg by the KCl dilution.

Biochemical characterization of light and heavy vesicles

The maximum (Na^+ + K^+ -ATPase activity occurred in Peak I membranes (15–26% sucrose) whereas the sarcoplasmic reticulum fractions contained little (Na^+ + K^+ -ATPase activity. Peak I had an activity of 5 $\mu\text{mol P}_i$ per mg per h, which would indicate that it was approx. 25% surface or T-tubule membrane. The amount of surface or T-tubule membrane contamination in the sarcoplasmic reticulum fractions determined from the (Na^+ + K^+ -ATPase activity was less than 1%.

The activity of monamine oxidase ranged from 0.05 to 0.45 nmol of benzylamine formed per min per mg of protein. These values were insignificant when compared to 35 nmol/min per mg in isolated mitochondrial membrane in the vesicles. Succinate dehydrogenase activity ranged from 0.01 to 0.02 $\mu\text{mol/min}$ per mg at 37°C in the vesicles. Values obtained for purified inner mitochondrial membrane were in the range of 1.0 $\mu\text{mol/min}$ per mg for succinate dehydrogenase activity. Therefore, the light and heavy vesicles had low specific activities of marker enzymes for the inner mitochondrial membrane. Electron microscopy studies showed that all sarcoplasmic reticulum vesicles were free of mitochondrial contamination.

The sarcoplasmic reticulum vesicles contained (Ca^{2+} + Mg^{2+})-ATPase activity, with the highest activity occurring in the light vesicles. The ATPase activity was higher in the KCl-washed vesicles than in the sucrose-diluted vesicles. The internal water volumes of the light and heavy vesicles (Table I) were 3.6 and 2.1 μl /mg of protein, respectively. Ca^{2+} uptake in all light and heavy vesicles was around 100 nmol Ca^{2+} /mg of protein. Similar results were found by Meissner [13].

Ca^{2+} efflux from the light and heavy vesicles

Ca^{2+} efflux was studied from the vesicles which had been passively loaded by equilibration for 24–36 h in 100 mM KCl, 5 mM MgCl_2 , 10 mM Hepes (pH 7.1) and 1 mM $^{45}\text{Ca}^{2+}$ on ice. Passive efflux is biphasic in the vesicles (Fig. 1). The first order rate constant for the initial fast phase of calcium efflux shows that the efflux rate from the heavy vesicles (0.43 min^{-1}) is approximately twice the efflux rate from the light vesicles (0.17 min^{-1}) (Table II). The heavy vesicles have a 30% faster rate constant for efflux than the heavy vesicles which were washed with KCl. Sodium dantrolene is a drug which selectively inhibits the Ca^{2+} release from the sarcoplasmic reticulum in resting and activated muscle [38]. When the vesicles were incubated for 5 min in 20 μM sodium dantrolene,

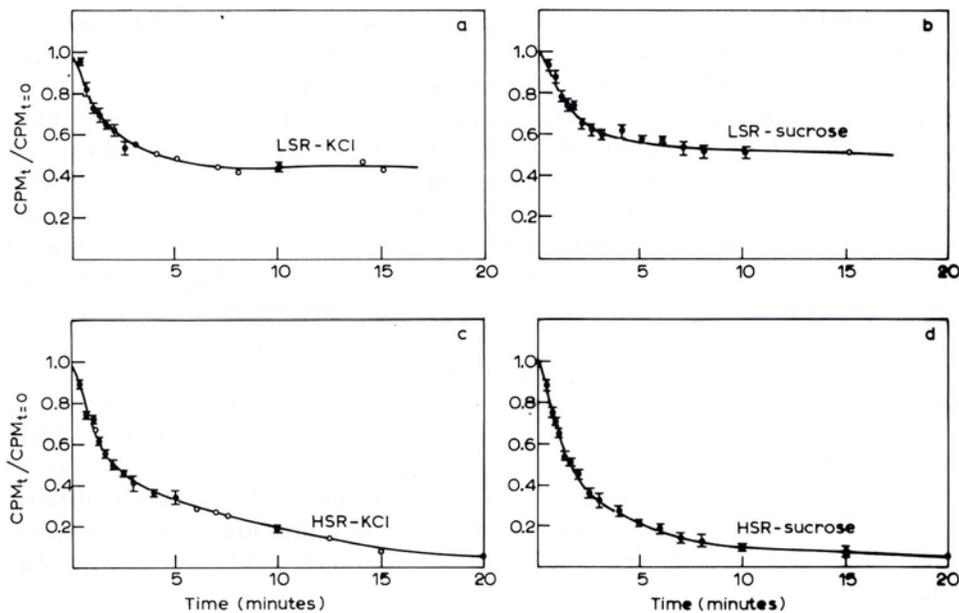


Fig. 1. Passive calcium efflux from light and heavy sarcoplasmic reticulum vesicles. The results from at least three efflux experiments were averaged and the percent calcium remaining is plotted vs. time (see Experimental procedures), (a) KCl-washed light vesicles, (b) light vesicles, (c) KCl-washed heavy vesicles, (d) heavy vesicles. Open circles are used for time points where only one measurement was made. The first-order rate of calcium efflux was 0.17 and 0.20 min^{-1} for light and KCl-light vesicles, respectively, and 0.43 and 0.35 min^{-1} for heavy and KCl-heavy vesicles, respectively.

the rate of calcium efflux from the heavy vesicles was greatly reduced (from 0.43 to 0.34 min^{-1} , Table II), whereas the rate of calcium efflux from the light vesicles was not affected.

Morphological studies of light and heavy vesicles

An electron micrograph of light vesicles is shown in Fig. 2a. Light fractions contain empty vesicles of various shapes and sizes. Light vesicles often formed

TABLE II

Ca²⁺ EFFLUX FROM LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES

Calcium efflux was measured from passively loaded sarcoplasmic reticulum vesicles. The raw data (cpm) was plotted on semi-log paper, and the cpm at time equals 0.0 was found by extrapolating back the least squares fit line of the initial efflux phase (up to 3 min). The results from at least three efflux experiments were averaged and plotted on linear and semi-log graph paper. The first order rate constants of the initial rapid phase were calculated by taking the least squares fit of the linear portion of each phase on the semi-log paper.

| | First-order rate constant (min^{-1}) | |
|----------------|---|-------------------------------------|
| | Control | +20 μM sodium dantrolene |
| Light vesicles | 0.17 | 0.15 |
| Heavy vesicles | 0.43 | 0.34 |

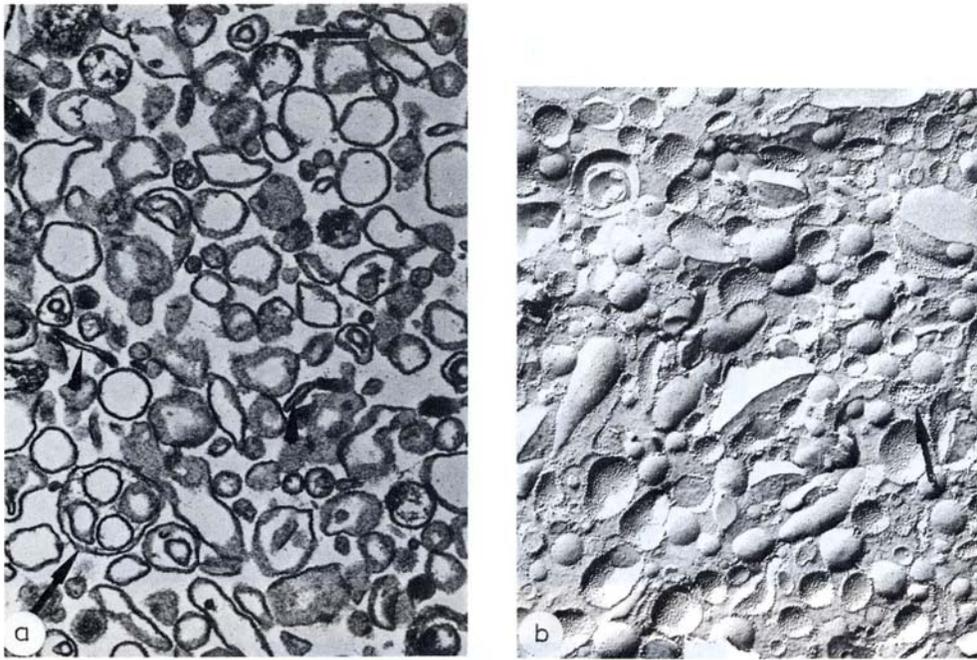


Fig. 2. (a) Thin section of light sarcoplasmic reticulum vesicles. The vesicles have variable size and shape and no visible content. Centrifugation of the fraction before fixation may result in flattening of the vesicles. Light vesicles often form invaginations, producing the appearance of vesicles with a double wall (arrows). Small, flat profiles probably derive from the transverse tubular system (arrow heads). A small number of profiles of probable T-tubular origin are present in most fractions. Magnification: $\times 39\ 400$. (b) Freeze-fracture replica of light sarcoplasmic reticulum vesicles. In most vesicles the cytoplasmic leaflet (concave) is occupied by particles and the luminal leaflet (convex) is smooth. In invaginated vesicles, the appropriate polarity of the membrane is maintained (i.e. the cytoplasmic leaflet remains in contact with the external fluid). Profiles with fewer, larger particles on the cytoplasmic leaflet may belong to heavy vesicles (arrow). A double-walled vesicle is indicated (arrowhead). Magnification: $\times 39\ 400$.

invaginations producing the appearance of vesicles with a double wall (see Fig. 2). Small flat vesicles (arrowheads) in gently centrifuged samples were probably derived from the transverse tubular system since vesicles of T-tubule origin have a flat shape even when fixed before centrifugation. A small number of profiles, of probably T-tubular origin, were present in most fractions. A freeze-fracture replica of the light vesicles is given in Fig. 2b. The luminal membrane face (convex) of the vesicles appeared as smooth surfaces whereas the cytoplasmic face (concave) was filled with particles which are known to represent the calcium pump protein [12].

A thin section of heavy vesicles is seen in Fig. 3b. The vesicles appeared as rounded vesicles of uniform size and shape, filled with a visible content which was denser on one side of the vesicle. The cytoplasmic (outer) surface of the membrane in correspondence to the regions of denser content, were decorated by small projections, resembling the feet which join the transverse tubules to the sarcoplasmic reticulum in the intact muscle fiber. The separation between adjacent dense projections was approximately $350\ \text{\AA}$, which is close to the value for the spacing between the feet in the intact triads. Fig. 3a shows a thin

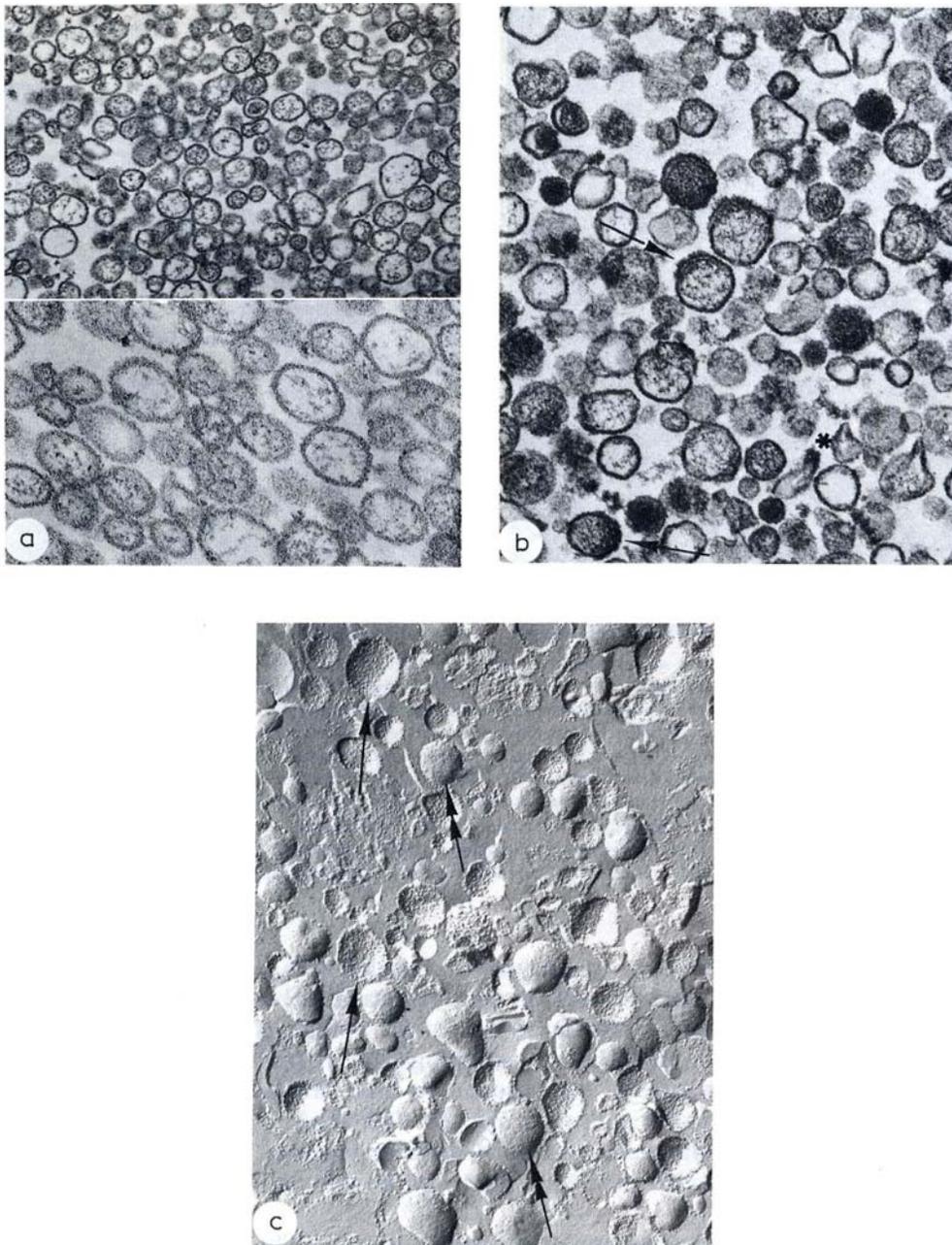


Fig. 3. (a) Thin section of KCl-washed heavy sarcoplasmic reticulum vesicles. The vesicles are round, of fairly uniform size, and with a visible content (probably calsequestrin, see text). This particular fraction is extremely pure. The cytoplasmic side of the membrane is smooth. Magnification: upper, $\times 24\ 750$; bottom, $\times 47\ 250$. (b) Thin section of heavy sarcoplasmic reticulum vesicles. The content of the vesicles is denser on one side of the vesicle. The cytoplasmic surface of the membrane, in correspondence to the regions of denser content, is decorated by small projections (arrows), closely resembling the 'feet' which join transverse tubules to sarcoplasmic reticulum in the intact muscle fiber. Asterisk indicates T-tubule profiles. Magnification: $\times 19\ 500$. (c) Freeze-fracture replica of heavy sarcoplasmic reticulum vesicles. The cytoplasmic leaflet contains particles, which in many cases (arrows) are larger and less densely aggregated than those typical of light vesicles. Some areas of the luminal leaflet (double arrows) are not smooth, but show evidence of pits. These structural details are characteristic of the area of sarcoplasmic reticulum membrane which are covered by 'feet' in the intact muscle. Compare with Fig. 2b. Magnification: $\times 39\ 750$.

section of KCl washed heavy vesicles under two different magnifications. The major difference between the heavy vesicles before and after KCl washing is that the small projections from the membrane of the heavy vesicles were missing in the KCl washed heavy vesicles. KCl treatment also removed several proteins from the heavy vesicles which are possibly the components of the 'sarco-plasmic reticulum feet' (see Fig. 7).

A freeze-fracture replica of heavy vesicles is shown in Fig. 3c. The cytoplasmic leaflet contained particles which in many cases were larger and less densely aggregated than those seen in the light vesicles. The luminal leaflet which was smooth in the light vesicles showed evidence of pits in the heavy vesicles (arrows). The area in the intact muscle which was covered by feet showed similar structural details [10]. It should be noted that pits in the feet-covered sarco-plasmic reticulum are difficult to see in both heavy vesicles and in intact muscle. Not all replicas showed them.

The results of tannic acid treatment of the vesicles is shown in Fig. 4. The outer leaflet of the light vesicles was surrounded by a uniform dense band (Fig. 4a). This has been shown to represent extensions of the Ca^{2+} -pump protein into the cytoplasm [30]. In the heavy vesicles (Fig. 4b) some portion of the vesicle surface was covered by the uniform dense band. The other portion was covered

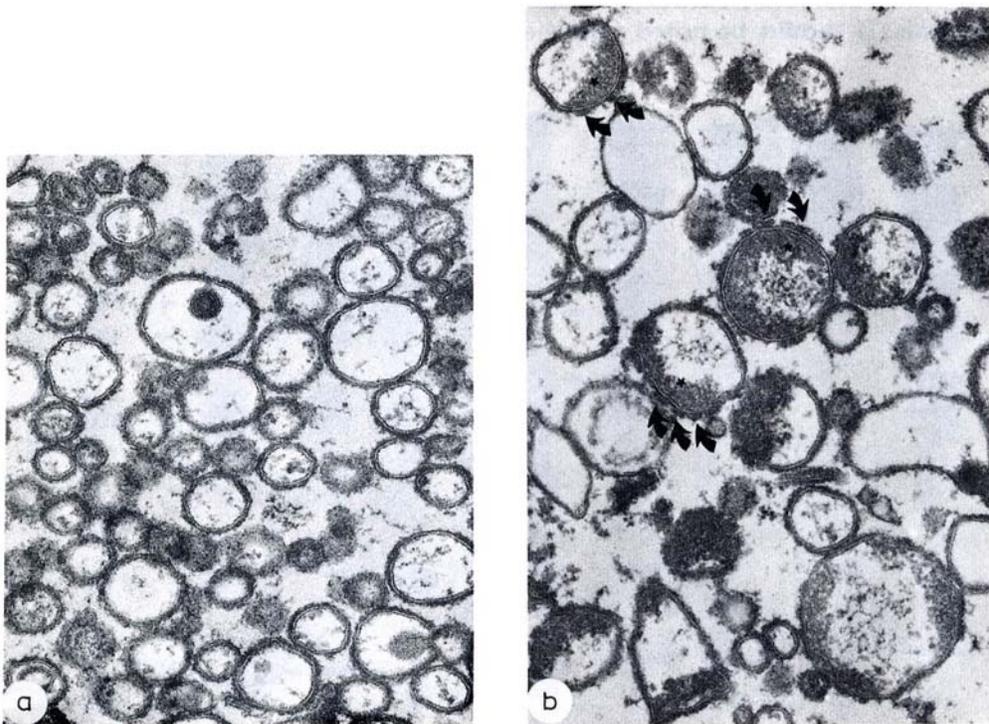


Fig. 4. Thin sections of sarcoplasmic vesicles treated with 1% tannic acid. In the light vesicles (a) the outer leaflet of the membrane is surrounded by a uniform dense band. This was shown to represent extensions of the calcium-pump proteins into the cytoplasm (Saito et al. [30]). In the heavy vesicles (b), the 'feet' (between arrows) are obviously discontinuous. Notice that the presence of feet on the cytoplasmic leaflet always coincides with a dense aggregations of the vesicles' content (probably calsequestrin, asterisk, see text). Magnification: $\times 60\ 000$.

by the 'feet', which are obviously discontinuous (i.e., the heavy vesicles contained regions of membrane covered by feet as well as regions occupied by the Ca^{2+} pump).

Protein composition of light and heavy vesicles

Fig. 5 illustrates SDS-gels according to the method of Weber and Osbom [32] of light, intermediate and heavy vesicles. Several proteins have been identified as calcium ATPase (105 000), 55 000-dalton protein and calsequestrin (44 000) [35]. Light vesicles (Fig. 5, sets 2 and 6) contained Ca^{2+} -ATPase and 55 000-dalton protein. Heavy vesicles (Fig. 5, sets 4 and 8) contained Ca^{2+} -ATPase and were enriched in calsequestrin. The major difference in the heavy vesicles (Fig. 5, set 4) and KCl (Fig. 5, set 8) washed heavy vesicles was in the 30 000-dalton proteins. The heavy vesicles contained approximately equal amounts of 30 000-dalton protein and 34 000-dalton proteins whereas the KCl heavy vesicles had little 34 000-dalton protein. The vesicles were relatively free of higher molecular weight proteins although they did contain a substantial amount of minor protein components. An example of this is a 20 000-dalton protein which was consistently seen in all fractions but it was less than 1% of the total protein.

The results of Laemmli slab gel electrophoresis [31] can be seen in Fig. 6. The resolution and sensitivity of this gel system is much better than the previous one. It should be noted that the same samples were run on both gel sys-

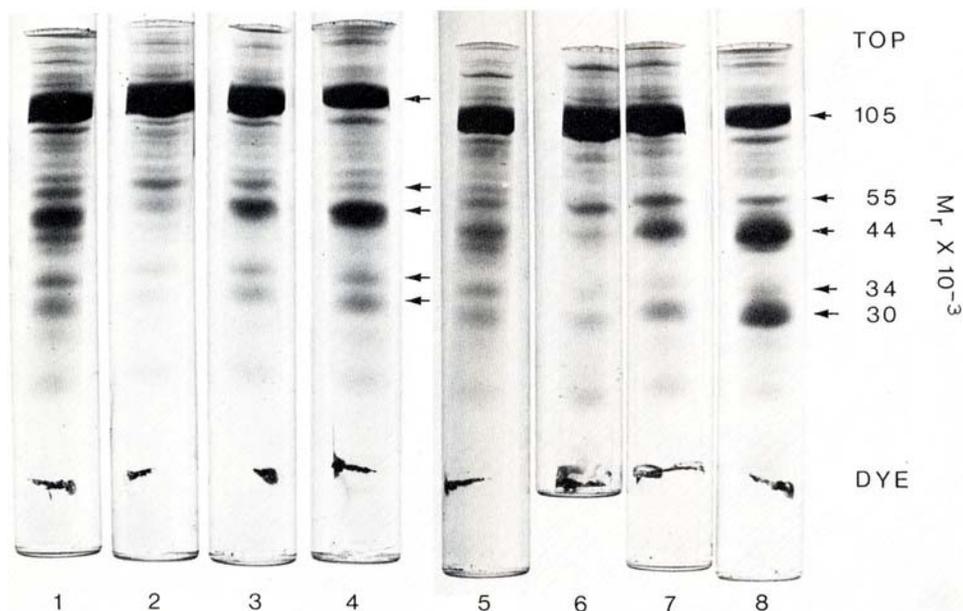


Fig. 5. Gel electrophoresis (7.5% acrylamide gels) of the sarcoplasmic reticulum vesicles according to Weber and Osbom [24]. The protein composition of the sarcoplasmic reticulum vesicles and the KCl-washed sarcoplasmic reticulum vesicles is shown in the first (1-4) and second (5-8) sets of gels, respectively. Crude sarcoplasmic reticulum vesicles (1,5), light vesicles (2,6), Intermediate sarcoplasmic reticulum vesicles (3,7), and heavy sarcoplasmic reticulum vesicles (4,8). Molecular weight determination and gel electrophoresis procedures are described under Experimental procedures.

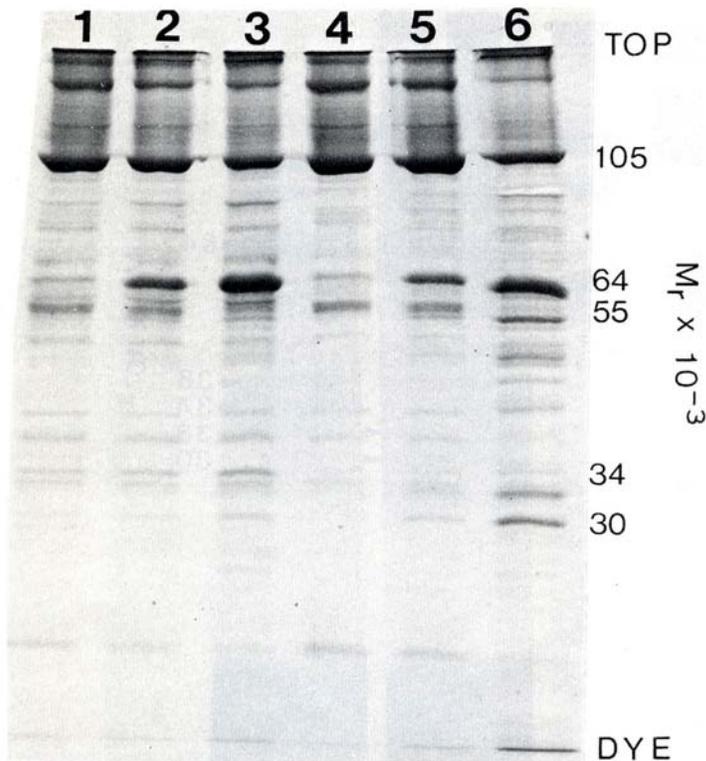


Fig. 6. Slab gel electrophoresis of sarcoplasmic reticulum vesicles according to Laemmli [23]. The running gel and stacking gel consisted of a 10% and 3% acrylamide gel, respectively. Sarcoplasmic reticulum fractions contain 40 μg of protein per sample. Well numbers 1-3 contain sucrose-washed vesicles and 4-6 contain KCl-washed vesicles. Light vesicles (1,4), intermediate vesicles (2,5) and heavy vesicles (3,6). Molecular weight determination and gel electrophoresis procedures are described under Experimental procedures. Note that the sarcoplasmic reticulum samples run on this gel are the same ones run on the gels in Fig. 5.

terns. Light vesicles (Fig. 6, set 1) consisted of predominantly Ca^{2+} -ATPase, 56 000-dalton protein, 55 000-dalton protein (which stains diffusely) and a small amount of 64 000 and 34 000-38 000-dalton proteins. KCl washed light vesicles had the same composition. Heavy vesicles (Fig. 6, set 3) contained roughly equal amounts of ATPase and calsequestrin all three 55 000-dalton proteins (53 000, 55 000, and 56 000) and four 30 000-dalton proteins (38000, 34 000, 33 000, and 30 000). KCl washed heavy vesicles (Fig. 6, set 6) contained a similar protein composition except the 34 000- and 38 000-dalton proteins are greatly reduced.

The results of comparing the protein composition of sarcoplasmic reticulum vesicles on Weber and Osborn [32] and Laemmli [31] gels has shown that the following bands, which are seen on Weber and Osborn gels, consist of at least two or three proteins when run on Laemmli gels: (1) 30 000-dalton band consists of the 30 000- and 33 000-dalton proteins; (2) 34 000-dalton band consists of 34000- and 38 000-dalton proteins; and (3) 55 000-dalton band consists of 53 000-, 55 000-, and 56 000-dalton proteins.

In order to investigate further what proteins were removed during KCl wash-

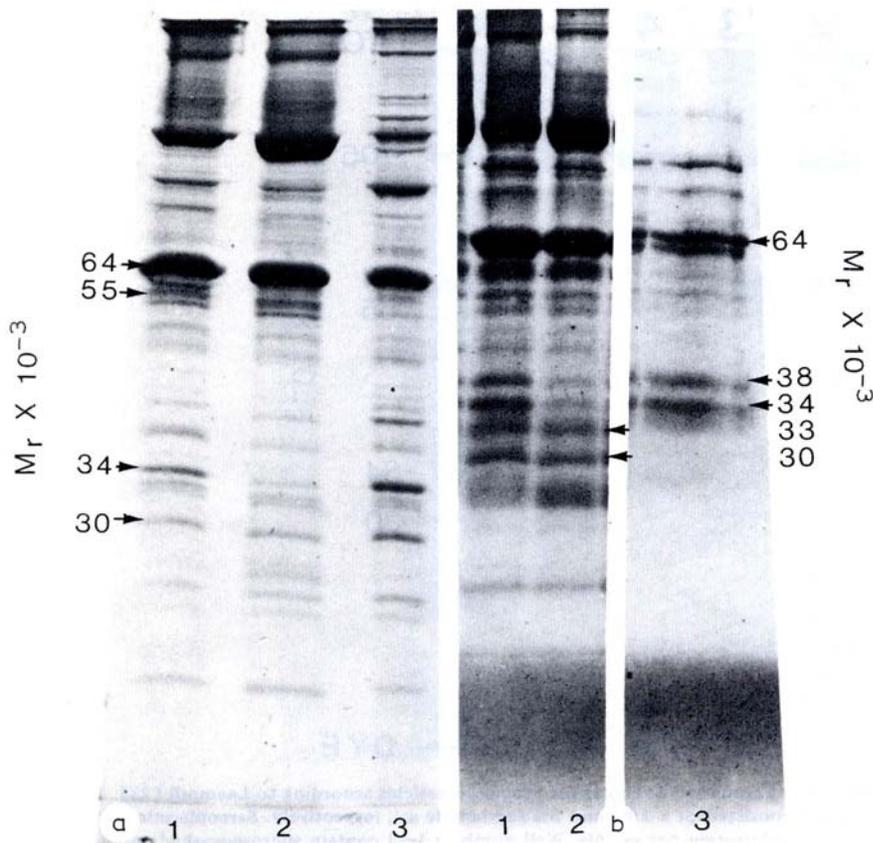


Fig. 7. (a) Slab gel electrophoresis according to Laemmli [23] is described under Experimental procedures. Heavy vesicles (1) showing $M_r = 64\ 000$ (calsequestrin) $M_r = 55\ 000$ along with $M_r = 53\ 000$ and $M_r = 56\ 000$, $M_r = 34\ 000$ and $M_r = 30\ 000$. Heavy vesicles which were subsequently washed with 0.6 M KCl (2) note the removal of the 34 000- and 38 000-dalton proteins. Triton X-100 insoluble pellet from the heavy vesicles (3). (b) Heavy vesicles (1). Heavy vesicles which were subsequently washed with 0.6 M KCl (2) and the 0.6 M KCl supernatant (3) from the KCl-washed heavy vesicles.

ing of the heavy vesicles we have analyzed the resulting KCl supernatant. In Fig. 7 the results of KCl washing of heavy vesicles are shown. The heavy vesicles contained all four 30 000-dalton proteins (Fig. 7a, set 1 and 7b, set 1). In the KCl washed vesicles the 34 000- and 38 000-dalton proteins were greatly reduced (Fig. 7a, set 2 and 7b, set 2). The KCl supernatant (Fig. 7b, set 3) contained approx. 5% of the protein and both the 34 000- and 38 000-dalton proteins along with some calsequestrin (64 000 daltons). After KCl washing the sarcoplasmic reticulum feet were no longer seen on the vesicles and a small number of vesicles had lost their content. These results suggest that the 34 000- and 38 000-dalton proteins were possible protein components of the 'sarcoplasmic reticulum feet'. It is also possible that non-protein material contributed to the structure of the feet and is removed by KCl washing.

Solubilization studies

Sarcoplasmic reticulum vesicles have been reported to contain intrinsic and

extrinsic membrane proteins [35]. Mild deoxycholate and high salt have been used to remove the extrinsic proteins and leave the intrinsic proteins membrane bound [34]. We have used this technique to look at both the intrinsic and extrinsic proteins of light and heavy vesicles. In the presence of 0.1 mg deoxycholate per mg of protein and 1 M NaCl almost all of the calsequestrin from the heavy vesicles was solubilized. The remaining proteins are the Ca^{2+} -ATPase, some 55 000-dalton protein and the 30 000- and 33 000-dalton proteins (Fig. 8a, set 4). Deoxycholate treatments of the light vesicles left just the Ca^{2+} -ATPase. The 30 000- and 33 000-dalton proteins were therefore intrinsic membrane proteins, and were not related to the low molecular weight acid proteins. Triton X-100 (2%) could also produce the same effects of KCl washed heavy vesicles, leaving the ATPase, 30 000- and 33 000-dalton proteins in the membrane. It should be noted that our experience in trying to purify the 30 000- or 33 000-dalton proteins shows that they are very insoluble in deoxycholate, cholate or Triton X-100. It should be noted that the gels in Fig. 8 are Laemmli disc gels and that the light vesicles (Fig. 8a, set 1) appeared as almost completely pure Ca^{2+} -ATPase. We consistently found that the disc gels revealed less of the minor proteins than did the slab gels.

The results of treating heavy vesicles with 2% Triton X-100 in the presence

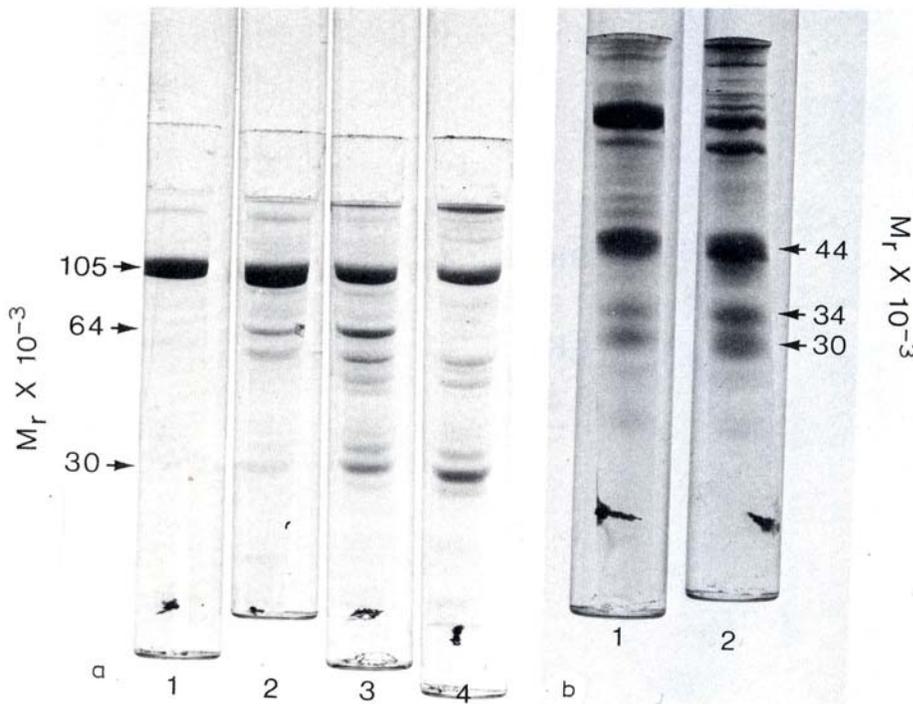


Fig. 8. (a) Disc gel electrophoresis according to Laemmli is described under Experimental procedures. KCl-washed light vesicles (1), intermediate (2), and heavy (3). Deoxycholate-treated heavy vesicles (4) showing the intrinsic membrane proteins, $M_r = 100\ 000$ (ATPase) and $M_r = 30\ 000$. Note that the KCl-washed light vesicles show almost pure ATPase on disc gels. (b) Disc gel electrophoresis according to Weber and Osborn is described under Experimental procedures. Heavy vesicles (1) and the Triton X-100 insoluble pellet from the heavy vesicles (2).

of 0.25 M sucrose are shown in Figs. 7 and 8. On Laemmli slab gels (Fig. 7) one can see the Triton X-100 extract (Fig. 7a, set 3) was enriched in 110 000-, 90 000-, 64 000 (calsequestrin), 34 000-, and 30 000-dalton proteins. The results of Weber and Osborn gel electrophoresis on the Triton X-100 extract (Fig. 8b) showed the same proteins as the Laemmli slab gels but calsequestrin's molecular weight was 44 000. An electron micrograph (not shown) of the Triton X-100 extract shows that it consists predominantly of electron dense material which is similar to that seen in the heavy vesicles. It seems to contain very little membrane material which is consistent with the low amount of phosphate ($0.07 \mu\text{mol P}_i/\text{mg}$ of protein). Whether the Triton X-100 insoluble fraction contains the sarcoplasmic reticulum feet remains to be seen. A possible interpretation of the data on solubilization and the morphology of the heavy vesicles and Triton X-100 insoluble complex is that the intrinsic 30 000- and 33 000-dalton proteins are localized in the junctional region of the terminal cisternae

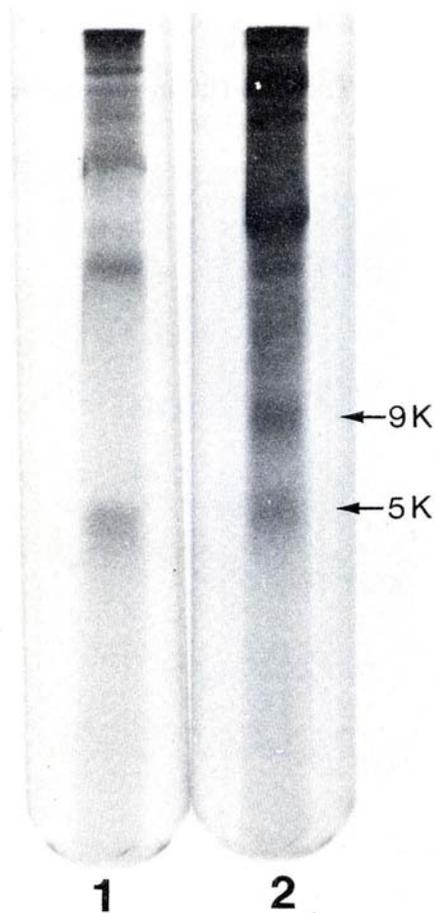


Fig. 9. SDS-gel electrophoresis according to Swank and Munkres is described under Experimental procedures. Light vesicle proteolipid extract (1) and the heavy vesicle proteolipid extract (2). The 5000- and 9000-dalton proteolipids are indicated. Proteolipid extraction from light and heavy vesicles is described under Experimental procedures.

and that calsequestrin and several other proteins are physically associated with them.

When light and heavy vesicles were analyzed for low molecular weight proteins using the gel system of Swank and Munkres [25], a 5000-dalton protein was seen in both fractions and a 9000-dalton protein was seen in the heavy sarcoplasmic reticulum (gels not shown). The results of a proteolipid extraction from light and heavy vesicles is given in Fig. 9. It can be seen that the light (Fig. 9, set 1) had a 5000-dalton proteolipid and the heavy (Fig. 9, set 2) had 5000- and 9000-dalton proteolipids.

Discussion

It has been shown that sarcoplasmic reticulum vesicles derived from longitudinal reticulum and terminal cisternae can be isolated in a very pure form with a combination of differential centrifugation and isopycnic sucrose gradient ultracentrifugation [13,15]. In our studies the calcium contents reveal major differences in the vesicles types: light and heavy vesicles contain 34 and 114 nmol Ca^{2+} /mg protein, respectively. The localization of the highest amount of calcium in the heavy sarcoplasmic reticulum vesicles was consistent with the electron probe studies of calcium in terminal cisternae of the sarcoplasmic reticulum [II], and with the higher content of Ca^{2+} -binding proteins, i.e. calsequestrin (see below). The KCl wash removes most of the calcium from all the vesicles. The larger volume of the light vesicles compared to the heavy vesicles was consistent with the morphology of the vesicles and with the fact that the heavy vesicles contain a large amount of extrinsic protein. If one assumes that in the KCl-washed vesicles the Ca^{2+} and Mg^{2+} content is at background level, (probably binding to the phospholipids), and knowing the water volumes of the vesicles, it is possible to calculate the total calcium and magnesium concentrations within vesicles. The resulting concentrations are 5 mM and 50 mM Ca^{2+} for light and heavy vesicles, respectively, and 10 mM and 14 mM Mg^{2+} for the light and heavy vesicles, respectively.

The morphological data was the basis for concluding that the light vesicles are derived from the longitudinal reticulum and the heavy vesicles from the terminal cisternae of the sarcoplasmic reticulum. The light vesicles, which are empty vesicles, are heterogeneous in size and shape. The freeze-fracture replicas of the light vesicles confirmed the biochemical data, that the vesicle's membrane is in the same orientation as in the longitudinal reticulum *in vivo* and the major membrane protein is the calcium pump protein.

Heavy vesicles are rounded vesicles of a more homogeneous size. The visible content was identified as calsequestrin (Ref. 13, and below). It is our contention that the very dense content of the heavy vesicles is calsequestrin with a large amount of bound calcium. This seems likely, since the content becomes more diffuse following KCl extraction, concomitant to calcium lost. The dense appearance is probably due to the aggregation of calsequestrin by calcium. Calsequestrin has been shown to have a large conformational charge upon binding of calcium [36,37].

The material on the cytoplasmic surface of the heavy vesicles was identified as forming the sarcoplasmic reticulum feet [7] on the basis of size and period-

icity. The appearance of the sarcoplasmic reticulum feet on the heavy vesicles is definite proof that they contain the junctional sarcoplasmic reticulum membrane, the portion of sarcoplasmic reticulum facing T-tubules in intact triads and covered by feet. Heavy vesicles also contain non-junctional sarcoplasmic reticulum, with Ca^{2+} ATPase. The observed localization of the electron dense content, presumed calsequestrin, in the region of the junctional sarcoplasmic reticulum membrane might indicate attachment of calsequestrin, to the junctional sarcoplasmic reticulum membrane of the terminal cisternae.

The freeze-fracture replica of the heavy vesicles shows basically the same asymmetric pattern as the light vesicles indicating that the orientation of the heavy vesicles was right side out. Identification by this technique of junctional membrane in the heavy vesicles was possible only in few fractions, due to the difficulty of detecting the pits on the cytoplasmic leaflet. Where present, these pits confirmed derivation of heavy vesicles from lateral sacs of the triad.

We have employed two criteria for the purity of our vesicles in addition to marker enzymatic assays. First, all samples used for electron microscopy were also analyzed by gel electrophoresis. Each electron micrograph presented is from a sample which was analyzed by SDS-gel electrophoresis and is shown in one of the figures. Second, we have always cut the section perpendicular to the pellet and taken sample pictures at approximately 'regular intervals across the pellet. An estimate of the purity of each fraction was made from the overall pellet.

Passive calcium efflux rates from the vesicles indicate that the heavy vesicles have a higher rate of passive calcium efflux and are more sensitive to dantrolene. We speculate that this extra efflux may be located in the area of the membrane covered by 'sarcoplasmic reticulum feet'. This would be consistent with the much smaller effect of dantrolene on the light vesicles which lack feet. These results agree with the work of Desmedt and Hainaut [38] on barnacle muscle fibers injected with aequorin. They have shown that dantrolene inhibits rather selectively the resting calcium efflux from the sarcoplasmic reticulum. In addition, it inhibits the Ca^{2+} -release mechanism.

The results of analyzing vesicles by Weber and Osborn [32] and Laemmli [31] SDS-gel electrophoresis enable us to tentatively localize the various proteins to the longitudinal or terminal regions of the sarcoplasmic reticulum. The light vesicles consist predominantly of the 105 000-dalton calcium-ATPase along with a 55 000-dalton protein. Although the 55 000-dalton band appears as a single band on Weber and Osborn [32] gels it consists of several proteins (53 000, 55 000, 56 000) when analyzed on Laemmli SDS gels [31]. It is our contention that the longitudinal reticulum consists predominantly of (Ca^{2+} + Mg^{2+} -ATPase along with the 55 000-dalton protein, since these proteins are enriched in the light vesicles. The heavy vesicles have in addition to the Ca^{2+} -ATPase and 55 000-dalton protein, several unique proteins: (1) calsequestrin, (2) extrinsic 34 000- and 38 000-dalton proteins, (3) intrinsic 30 000- and 33 000-dalton proteins and (4) a 9000-dalton proteolipid. Meissner [13] had identified terminal cisternae vesicles by their electron dense content. He has shown that the electron dense content of the terminal cisternae is due to calsequestrin and/or 55 000-dalton protein. We have confirmed Meissner's results on the heavy vesicles and also on identifying the terminal cisternae vesicles from

their sarcoplasmic reticulum feet and their freeze-fracture pattern, which resembles the junction sarcoplasmic reticulum membrane. Our studies of the protein composition of the heavy vesicles indicate that the dense content is due to calsequestrin and the 55 000-dalton protein is in the longitudinal reticulum and the terminal cisternae. The heavy vesicles preparation of Lau et al. [15] contains a high percentage of T-tubule in the form of dyads and triads. After French press treatment they are able to recover heavy vesicles which do not have T-tubules but have lost their ability to transport calcium [39]. Their analysis of the protein composition of light and heavy vesicles agrees with ours in that they find the light vesicles contain the 55 000-dalton protein and that the heavy vesicles contain calsequestrin. It should be noted that our analysis of the protein composition of the vesicles shows a more complicated protein composition than previously shown by other investigators [13,15].

Ikemoto et al. [40] have preliminary data indicating that a 30 000-dalton protein might be a component of the sarcoplasmic reticulum feet. Hidalgo and Ikemoto [41] have shown that about half of this protein is located on the cytoplasmic surface of the sarcoplasmic reticulum vesicles. Since the 30 000-dalton protein was identified using only the gel electrophoresis method of Weber and Osborn [32] it is probably a mixture of proteins as we have shown here when analyzed by gel electrophoresis according to Laemmli [31]. Our results have indicated that the extrinsic 34 000- and 38 000-dalton proteins are most likely components of the sarcoplasmic reticulum feet and that the intrinsic 30 000- and 33 000-dalton proteins are possible membrane components of the feet.

In conclusion, we feel that the biochemical and morphological data strongly indicate that the light vesicles are derived from the longitudinal reticulum and the heavy vesicles are from the terminal cisternae and contain junctional sarcoplasmic reticulum membrane. In addition, the heavy vesicles which have intact 'sarcoplasmic reticulum feet' and a high calcium content, will be an excellent preparation to study the mechanism of calcium release.

Acknowledgments

This paper is based on work performed under contract with the U.S. Department of Energy at the University of Rochester Department of Radiation Biology and Biophysics and has been assigned Report No. UR-3490-1616. This paper was also supported by a grant 1R01 18892 from the National Institute of Health, Program Project ES-10248 from N.I.E.H.S. and a grant from Muscular Dystrophy Association of America. K.P.C. van an Elon Huntinton Hooker Graduate Fellow. A.E.S. is an Established Investigator of American Heart Association, Inc.

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