Characterization of the Junctional Face Membrane from Terminal Cisternae of Sarcoplasmic Reticulum

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Abstract. We have recently described a preparation of junctional terminal cisternae (JTC) from fast skeletal muscle of rabbit hind leg. The fraction differs from other heavy sarcoplasmic reticulum (SR) fractions in that it contains a substantial amount of junctional face membrane (JFM) (15–20% of the membrane) with morphologically well-defined junctional feet structures. In common with other heavy SR preparations, it contains predominantly the calcium pump membrane (80–85% of the membrane) and compartmental contents (CC), consisting mainly of calcium-binding protein (calsequestrin). In this study, a modified procedure for the preparation of JTC from frozen rabbit back muscle is described. The yield is substantially greater (threefold per weight of muscle), yet retaining characteristics similar to JTC from fresh hind leg muscles.

Methodology has been developed for the disassembly of the JTC. This is achieved by selectively extracting the calcium pump membrane with 0.5% Triton X-100 in the presence of 1 mM $CaCl_2$ to yield a complex of JFM with CC. The CC are then solubilized in the presence of EDTA to yield JFM. This fraction contains unidirectionally aligned junctional feet structures protruding from the cytoplasmic face of the membrane with repeat spacings comparable to that observed in JTC. The JFM contains 0.16 μmol phosphorus (lipid) per milligram protein. Characteristic proteins include 340 and 79-kD bands, a doublet at 28 kD, and a component that migrates somewhat slower than or equivalent to the calcium pump protein. Approximately 10% of the calcium-binding protein remains bound to the JFM after EDTA extraction, indicating the presence of a specific binding component in the JFM. The JFM, which is involved in junctional association with transverse tubule and likely in the Ca^{2+} release process in excitation–contraction coupling, is now available in the test tube.

MUSCLE contraction and relaxation are regulated by the sarcoplasmic reticulum (SR)[1] by way of controlling the myoplasmic-free calcium concentration. The calcium pump protein, the major constituent of the Ca^{2+} pump membrane of SR, transports calcium from the myoplasm into the SR lumen where it is stored during relaxation. The calcium pump membrane is the major membrane of SR comprising most of the terminal cisternae membrane and essentially all of the longitudinal cisternae. In excitation–contraction coupling, the signal that triggers calcium release is transmitted from the transverse tubule across the triad junction to the junctional face membrane (JFM) of the terminal cisternae. The detailed mechanism of the calcium release process is not understood and is one of the important frontiers in muscle cell and molecular biology (12, 23).

The isolation and characterization of enriched junctional terminal cisternae (JTC) has recently been achieved in this laboratory (33). JTC consist of two distinct types of membranes, i.e., the JFM and the calcium pump membrane. JTC also contain compartmental contents (CC) consisting mainly of calcium-binding protein (CBP) (25), which is also referred to as calsequestrin (22). The JTC fraction is unique because it contains a substantial amount of JFM (15–20% of the surface area) as compared with previously described heavy SR fractions. In this paper, we describe the isolation and characterization of JFM containing unidirectionally aligned junctional feet structures. A preliminary report of this work has appeared (7).

Materials and Methods

Isolation of JTC

JTC were isolated via a modification of the procedure of Saito et al. (33) using frozen back muscle from female New Zealand White rabbits (~3 kg).

1. *Abbreviations used in this paper*: CBP, calcium-binding protein; CC, compartmental contents; JFM, junctional face membrane; JFM-CC, junctional face membrane-compartmental contents; JTC, junctional terminal cisternae; SR, sarcoplasmic reticulum.

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Dissected muscle was sealed in a plastic bag and submerged in an ice-water bath to rapidly cool the muscle to 0°C. The bag was then placed in a −70°C freezer for freezing and storage of the muscle. Just before use, muscle was removed from the bag and submerged in distilled water at room temperature; the muscle was agitated to achieve rapid thawing. The thawed muscle was trimmed of fat and connective tissue and processed according to Saito et al. (33) with the following modifications. Homogenization buffers were 0.3 M sucrose, 0.5 mM EDTA, 20 mM imidazole (pH 7.4) for the first homogenization and 0.3 M sucrose, 0.5 mM MgATP, 20 mM imidazole (pH 7.4) for the second homogenization. MgATP was added to decrease the viscosity in the second homogenate. The yield of JTC using frozen back muscle was 17 mg/50 g ground muscle, which is about three times greater than from fresh leg or back muscle. A typical JTC preparation uses 300 g of back muscle (the two strips obtained from a 3 kg rabbit provide ~200 g) and yields ~100 mg JTC. JTC from frozen and fresh back muscle or fresh hind leg are similar with respect to protein composition, morphology observed by electron microscopy, detergent solubilization behavior, and enzyme activities. The latter include Ca^{2+}-ATPase in the presence or absence of the ionophore A23187, and phosphate-facilitated calcium loading and its enhancement with ruthenium red. We note in particular that the SDS PAGE band patterns of JTC from fresh and frozen muscle are indistinguishable, indicating that proteolysis is not a problem with the frozen muscle.

Detergent Solubilization of JTC

JTC were incubated for 10 min at 0°C in buffered sucrose (0.3 M sucrose, 5 mM Hepes, pH 7.4) with a number of additions as indicated in the text. A one-tenth volume aliquot of an appropriate stock solution of Triton X-100 was added to achieve a final concentration as indicated. This solubilization mixture was maintained at 0°C for 20 min and then either (*a*) pelleted at 110,000 g_{max} for 60 min in a Beckman L-8 Ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) using a type 75Ti rotor or in a Beckman airfuge, or (*b*) loaded onto a continuous 14–50% sucrose gradient (containing 5 mM Hepes, pH 7.4) and centrifuged for 3 h at 97,000 g_{max} in a Beckman SW41 or SW28 rotor (see legend to Fig. 7). The supernatant was recovered and treated to remove detergent as outlined below. The pellet was rinsed with buffered sucrose to remove adhering supernatant and resuspended in buffered sucrose with a Dounce homogenizer.

Protocol for Isolation of Junctional Face Membrane with Compartmental Complex (JFM-CC) and JFM

All operations were in the cold (0–4°C). JTC in buffered sucrose were adjusted to 3.3 mg protein/ml and 1 mM $CaCl_2$ and maintained for 10 min. Triton X-100 was added from a 10% (vol/vol) stock solution to a final concentration of 0.5% and mixed thoroughly using a Vortex mixer. After 20 min, the sample was centrifuged in a type 75 Ti rotor at 35,000 rpm (110,000 g_{max}) for 60 min in a Beckman L-8 Ultracentrifuge or in a Beckman Airfuge for 30 min at 29 pounds per square inch air pressure (160,000 g_{max}) in an A95 or A110 rotor. The supernatant, consisting mainly of solubilized calcium pump membrane, was decanted. The pellet, which is JFM-CC, was resuspended in buffered sucrose to 1.0 mg protein/ml. To obtain JFM the suspension was adjusted to 2.0 mM EDTA using a stock solution of 100 mM EDTA, pH 7.4. After 10 min, the mixture was centrifuged as before and the supernatant containing CC was decanted. The pellet, consisting of JFM, was rinsed with and then resuspended in buffered sucrose. The fractions were stored at −80°C.

Removal of Detergent

Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) were used to remove Triton X-100 via the rapid column centrifugation procedure of Horigome and Sugano (17). Briefly, the resin was washed (16) and the columns were prepared from 1.5-ml plastic sample storage tubes with a pinhole in the bottom, or from disposable hyperdermic syringe barrels. A plug of silanized glass wool was placed in the bottom to retain the packed Bio-Beads. The column was equilibrated with buffered sucrose for 5 min, placed into a glass tube (the flanges around the top of the column prevented it from falling into the tube), and centrifuged to remove free liquid. Supernatants containing Triton X-100 at concentrations up to 1.0% were then loaded onto the column at a ratio of 0.15-ml supernatant to 0.5-ml packed beads. After 5 min at 0°C, the columns were centrifuged for 3 min at 100 *g* in a Beckman model TJ-R refrigerated table top centrifuge. The supernatants were recovered from the glass tubes.

SDS PAGE

Slab gels (0.75-mm thick) were run using the discontinuous buffer system of Laemmli for stacking and separating gels (20). All gels used 5% acrylamide for stacking gels and 5–20% linear acrylamide gradients for separating gels unless otherwise indicated. Fractions in Laemmli sample buffer were heated to 95°C for 5 min before loading on the gels. Loading was 10 μg per lane unless otherwise noted. After electrophoresis, gels were fixed for 2 h in 10% acetic acid, 50% methanol, stained for 1 h in 0.25% Coomassie Brilliant Blue R-250 in 10% acetic acid and 50% methanol, and destained in several washes containing 7% acetic acid and 10% methanol. Gels were scanned using an LKB Ultroscan XL Laser Densitometer or a model EC910 scanning densitometer (E-C Apparatus Corp., St. Petersburg, FL). The percentage of the total protein of a particular band in a sample is the area under the peak divided by the total area of the complete scan. For such quantitation, variations in band width were taken into account by integrating the areas of several scans across the width of the gel lanes. Molecular weights were determined by reference to a linear calibration curve constructed by plotting the log of the molecular weight of standards versus log percentage acrylamide. The molecular weight standards used were α_2-macroglobulin (340,000, nonreduced; 170,000, reduced), myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), glutamate dehydrogenase (55,400), ovalbumin (45,000), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Peptide Analysis

The CBP was subjected to peptide analysis in SDS PAGE performed according to Cleveland (6). In this way, proteolytic fragments of CBP, both extractable and non-extractable from the JFM-CC with EDTA, could be compared. Preparative gels were used, consisting of 5–20% linear gradient slabs, 0.75-mm thick, with 5% stacking gels using the Laemmli (20) buffer system. Total loading of the preparative gels was adjusted so that approximately equal amounts of CBP were loaded for both fractions. After brief staining of the preparative gels with Coomassie Brilliant Blue, the CBP bands from the two fractions were cut out. The bands were processed as described by Cleveland for hydrated gel slices and loaded into the wells of the stacking gel (5%) of the 1.5-mm thick 10% analytical slab gels (20). Either *Staphylococcus aureus* V8 protease (Miles Scientific Div., Miles Laboratories Inc., Naperville, IL), or α-chymotrypsin (Cooper Biomedical, Inc., Malvern, PA) was loaded together with the preparative gel slices. The amounts of protease loaded, and the length of time in the stacking gel of the mixture of protease and CBP was adjusted to obtain an appropriate range of proteolysis. Gels were fixed, stained with Coomassie Brilliant Blue, and destained as outlined above under SDS PAGE.

Electron Microscopy

Samples were fixed for thin-section electron microscopy according to either of two procedures. The first is a modification of the tannic acid enhancement method of Saito et al. (34). Membranes (~40 μg protein) in buffered sucrose were fixed in suspension by dilution into 5–10 vol 0.1 M sodium cacodylate (pH 7.4), 2% glutaraldehyde, 1% electron microscopic grade tannic acid (Polysciences, Inc., Warrington, PA), for 2 h at 0°C. Alternatively, the samples were fixed for 30 min by addition of one-tenth volume of 20% glutaraldehyde followed by addition of ~5–10 vol of the above glutaraldehyde/tannic acid solution and further incubation for 2 h. After exposure to tannic acid for 2 h, samples were pelleted in a Beckman airfuge at 19 psi for 15 min. The pellets were postfixed in 1% OsO_4 in Michaelis buffer (pH 7.2) then block stained with 0.5% uranyl acetate in Michaelis buffer (pH 6.0) (10) for 2 h at 0°C. The samples were dehydrated in a series of increasing ethanol concentrations followed by propylene oxide, and embedded in Epon 812 as described by Fleischer et al. (11). Thin sections were cut on an LKB Ultratome (LKB Instruments, Inc., Bromma, Sweden), poststained sequentially with 1% uranyl acetate and lead citrate (35) for 3 min each, and examined in a JEOL 100S electron microscope (Jeol Ltd., Tokyo, Japan).

The second fixation method was used in Fig. 1, *a–c*, and is a modification of the fixation/filtration procedure of Palade et al. (31). The sample, ~70 μg protein in buffered sucrose, was fixed for 2 h at 0°C by addition of one-tenth volume of 20% glutaraldehyde. This fixation mixture was then mixed with 200 μl of 0.1% Ficoll 70 (Pharmacia Fine Chemicals, Piscataway, NJ) before filtration onto 0.1-μM pore size filter disks (No. VCWP01300; Millipore Corp., Bedford, MA). Filters were postfixed with OsO_4 as outlined above for pellets and subjected to tannic acid mordanting using low molecu-

lar weight galloylglucoses (Mallinckrodt No. 1764 tannic acid) as described by Simeonescu and Simeonescu (38). They were then processed for thin section preparation, as for the pellets.

Biochemical Assays

Ca^{2+}-ATPase activity was measured at 25°C by following the change in optical density at 340 nm resulting from oxidation of NADH in a coupled enzyme assay system (37, 40). The reaction medium contained 50 mM KCl, 50 mM potassium phosphate, 5 mM $MgSO_4$, 50 μM $CaCl_2$, 5 mM NaN_3, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 8.75 U/ml pyruvate kinase, 12.5 U/ml lactate dehydrogenase, 1 mM Na_2ATP, 1.5 μg/ml A23187, and 10–20 μg/ml sample protein (pH 7.0). The basal ATPase rate was determined in the presence of 2 mM EGTA. The Ca^{2+}-ATPase rate was calculated as the difference of the rates in the absence and presence of EGTA.

Calcium loading was measured at 25°C by following the decrease in absorbance (A_{710}–A_{790}) of the metallochromic indicator dye antipyrylazo III (36). The reaction medium contained 50 mM potassium phosphate, 50 mM KCl, 5 mM $MgSO_4$, 0.2 mM antipyrylazo III, 50 μM $CaCl_2$, 1 mM Na_2ATP, and 20–50 μg/ml sample protein (pH 7.0). For determination of ruthenium red stimulation of loading, a low Mg^{2+} reaction medium was used. It contained 100 mM potassium phosphate, 1 mM $MgCl_2$, 0.2 mM antipyrylazo III, 50 μM $CaCl_2$, 1 mM Na_2ATP, 4 mM NaN_3, with and without 20 μM ruthenium red, and 20–50 μg/ml sample protein (pH 7.0).

Phosphorus was determined as described by Rouser and Fleischer (32), and converted to milligram lipid by dividing by 40 μg P/mg phospholipid. Protein was measured by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Results

This study is primarily concerned with the stepwise disas-

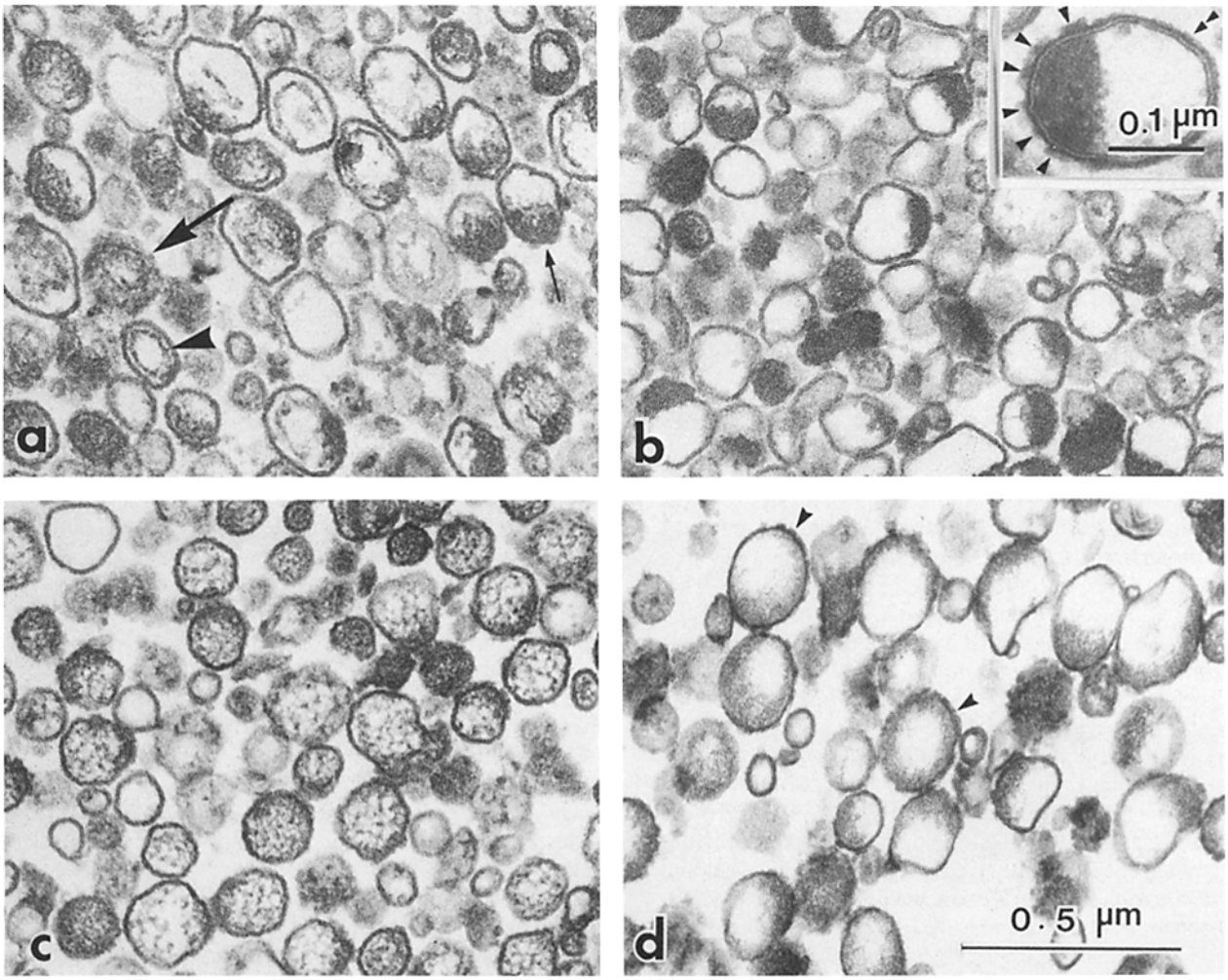


Figure 1. Modification of the appearance of the CC of the JTC. *a–c* show vesicles that were fixed after a 30-min preincubation in buffered sucrose (0.3M sucrose, 5 mM Hepes, pH 7.4) containing the following additional components: (*a*) none (control), (*b*) 1 mM $CaCl_2$, and (*c*) 100 mM KCl. In the control (*a*), the CC within the JTC exhibit varying degrees of aggregation, appearing either as a condensed mass (*small arrow*), strands (*arrowhead*), or more evenly dispersed (*large arrow*). In calcium-treated vesicles (*b*) the CC appear as a condensed mass bound to the luminal surface of the JFM. The inset shows a vesicle at higher magnification. Arrowheads indicate feet structures that are present on the JFM opposite the bound CC. The double arrow denotes the asymmetric calcium pump membrane. (*c*) KCl, by contrast, causes the CC to appear dispersed. The same appearance results from EDTA treatment of JTC vesicles (not shown). (*d*) JTC vesicles were subjected to two sequential preincubations before fixation. The first preincubation was in 150 mM KCl, 5 mM Hepes (pH 7.4) for 30 min. The appearance of the vesicles at this stage (not shown) was similar to that seen after treatment with 100 mM KCl (*c*). The vesicles were then pelleted and resuspended in physiological cationic mixture (125 mM KCl, 10 mM NaCl, 5 mM $CaCl_2$; 0.7 mM $MgCl_2$, 5 mM K-Hepes, pH 7.4), designed to mimic the cationic milieu inside the SR in situ when the muscle is at rest. The vesicles were fixed after incubation in this mixture for 70 min. Exposure to the physiological cationic mixture caused the CC, which had been dispersed by KCl, to become condensed and associated with JFM (arrowheads indicate junctional feet). The sample in *d*, processed by both tannic acid enhancement procedures (see Materials and Methods), displayed essentially the same morphology.

sembly of JTC to prepare JFM-CC and JFM. The JTC used in these studies were obtained from frozen rabbit back muscle by a modification of the procedure previously described by Saito et al. (33) (see Materials and Methods). The preparation is comparable with regard to morphology, protein profile in SDS PAGE, Ca^{2+}-ATPase activity with and without ionophore, and enhancement of Ca^{2+} loading rates with ruthenium red (about fivefold) (5). The yield is considerably greater than the preparation from rabbit hind leg described by Saito et al., approximately threefold per weight of muscle.

JTC consist mainly of two different types of membranes and CC. We found that the state of aggregation of the CC was an important variable for the preparation of intact JFM, and that it was influenced by the ionic environment. In buffered sucrose (Fig. 1 *a*), the CC exhibit a variable degree of aggregation which appears to reflect the endogenous calcium and magnesium concentration within the vesicles after subcellular fractionation. After preincubating in the presence of 1 mM $CaCl_2$ (Fig. 1 *b*), the CC become highly condensed and are bound to the luminal surface of the JFM. A similar appearance of the CC is obtained by exposure of JTC to 1 mM $MgCl_2$ (not shown). Although not all JTC vesicles exhibit feet structures in a thin section, those that do and have been exposed to divalent cations always have their CC associated with the JFM rather than with calcium pump membrane. By contrast, the CC appear more evenly dispersed after incubation in buffer containing 100 mM KCl (Fig. 1 *c*) which decreases binding of divalent cations to CBP (18, 24, 30). A similar appearance is obtained by treatment with 2.0 mM EDTA (not shown).

We next attempted to mimic the internal cationic environment of the JTC in situ, during muscle relaxation, in which divalent cations and K^+ are both present in a physiological cationic mixture. JTC vesicles were incubated in 150 mM KCl to disperse the CC, and then resuspended in a medium approximating physiological cationic composition. This medium reinitiated aggregation of CC and binding to the JFM (Fig. 1 *d*), similar to that observed in buffered sucrose supplemented with divalent cations (Fig. 1 *b*).

A procedure was developed to prepare JFM based on the aggregation behavior of the CC in JTC (see protocol in Materials and Methods). Triton X-100 was used to selectively extract the calcium pump membrane. When extraction was carried out after preincubation with $CaCl_2$, the CC were not extracted and remained affixed to the JFM after isolation. The complex obtained is referred to as JFM-CC. The amount of protein and phospholipid extracted as a function of Triton X-100 concentration is summarized in Fig. 2. As the detergent concentration is elevated to ~0.5%, the solubilization of protein into the supernatant increases so that approximately equal amounts of protein distribute between the supernatant and pellet. Thereafter, raising the detergent concentration to 1% does not appreciably increase the amount of protein solubilized, although it does lead to continued decrease in phospholipid content (Fig. 2). Under our solubilization conditions, 0.5% Triton X-100 corresponds to 1.5 mg detergent/mg protein or 5.3 mg detergent/mg phospholipid.

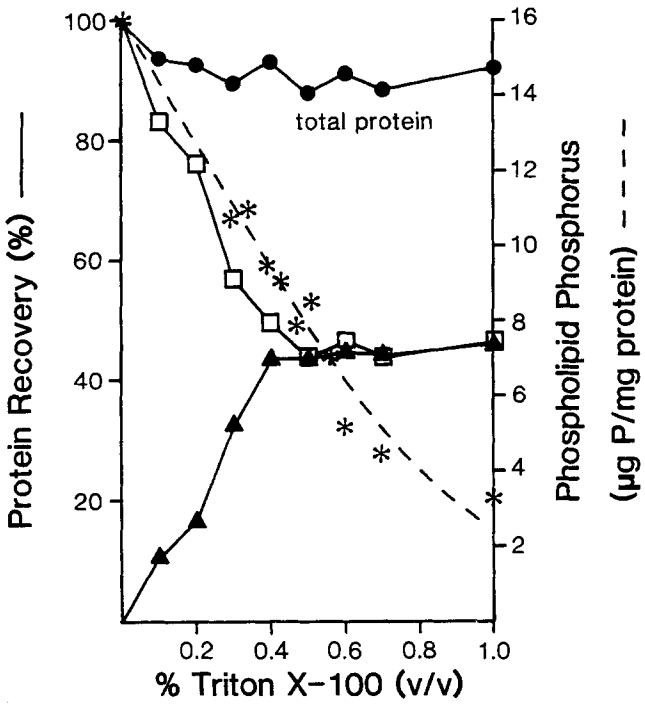
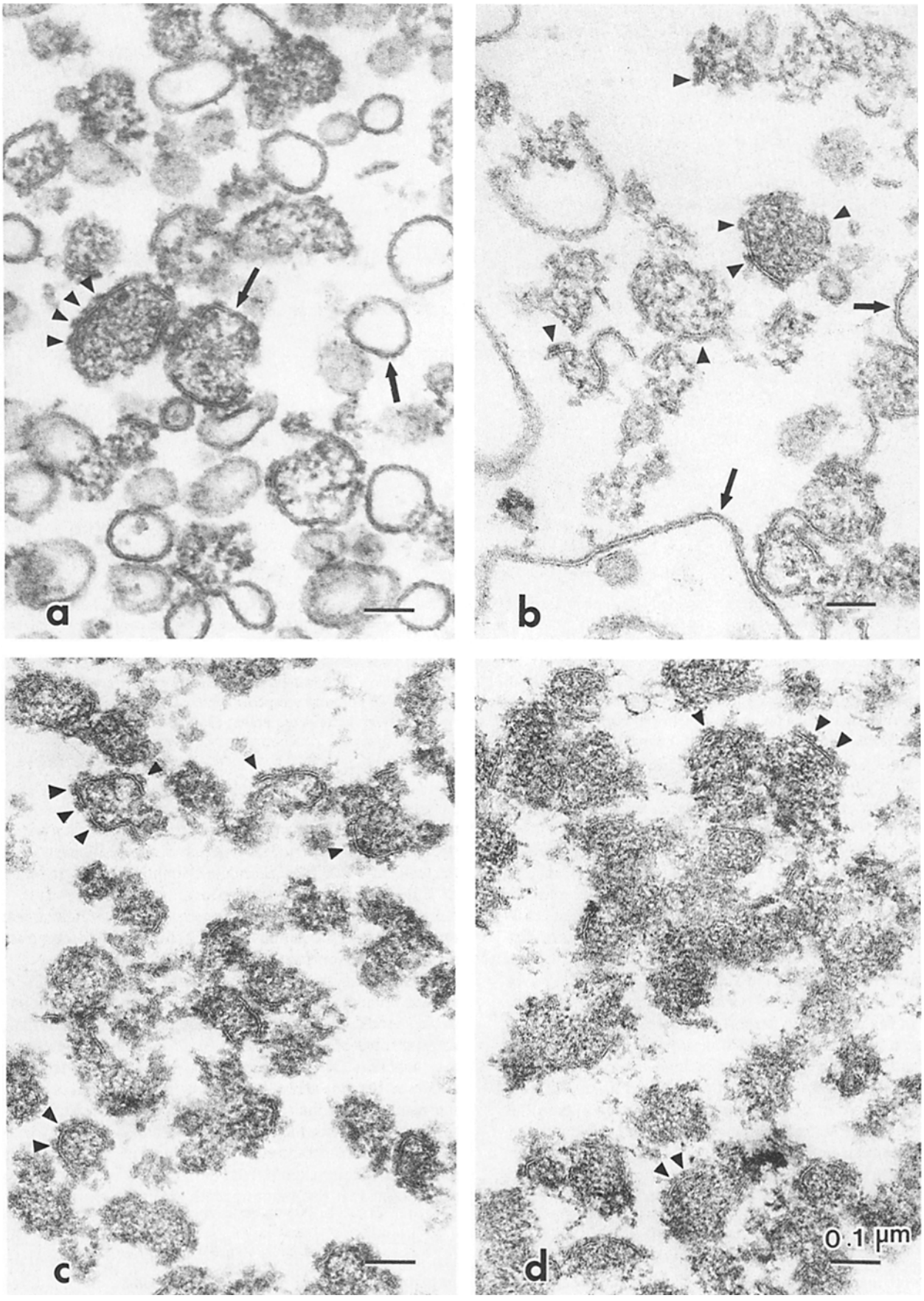


Figure 2. Extraction of JTC with Triton X-100. JTC vesicles were preincubated in buffered sucrose containing 1 mM $CaCl_2$, extracted with increasing amounts of Triton X-100, and centrifuged as described in Materials and Methods. The distribution of protein (left ordinate, solid lines) into the supernatants (▲) and pellets (□), and the phospholipid phosphorus content of the pellets (*, right ordinate, dashed line) are plotted as a function of detergent concentration. Increasing the Triton X-100 concentration to ~0.5% causes a progressive solubilization of protein into the supernatant and a corresponding decrease in the pellet. At 0.5% Triton, the amount of protein in the pellet and supernatant are about equal and further increase in detergent concentration does not result in additional solubilization of protein. There is, however, a progressive decrease in the phospholipid content of the pellets to 1.0% Triton X-100.

Electron micrographs of the pellets obtained with varying detergent concentration in the presence of 1 mM $CaCl_2$ are shown in Fig. 3. In the absence of Triton X-100, the pellet has essentially the same morphology as the Ca^{2+}-treated JTC vesicles in Fig. 1 *b*. A small amount of detergent (0.1%) significantly modifies the JTC. The JFM is observed to contain free ends, and vesicles consisting mainly of calcium pump membrane are observed which are sometimes multivesicular. That is, connections between the calcium pump membrane and the JFM become severed at low detergent concentration, and the calcium pump membrane vesiculates. This is confirmed in the later studies (see Fig. 6). At 0.3% Triton X-100, most of the calcium pump membrane has been

Figure 3. Morphology of residues obtained after extraction of JTC over a range of Triton X-100 concentrations. Pellets were obtained as described in the legend to Fig. 2 at Triton X-100 concentrations of 0.1% (*a*), 0.3% (*b*), 0.5% (*c*), and 1.0% (*d*). Junctional feet are indicated by arrowheads and calcium pump membrane by arrows. As the Triton concentration is increased, there is progressive extraction of calcium pump protein, giving rise to JFM-CC (*c* and *d*). At 0.1% Triton both JFM-CC and calcium pump membrane are recovered in the pellet separately or in the same vesicles. At 0.3% Triton X-100, the calcium pump membrane has mostly been separated from JFM-CC and sometimes takes the form of large sheets. At 0.5% and 1.0% Triton X-100, only JFM-CC remains, essentially all of the calcium pump membrane having been solubilized.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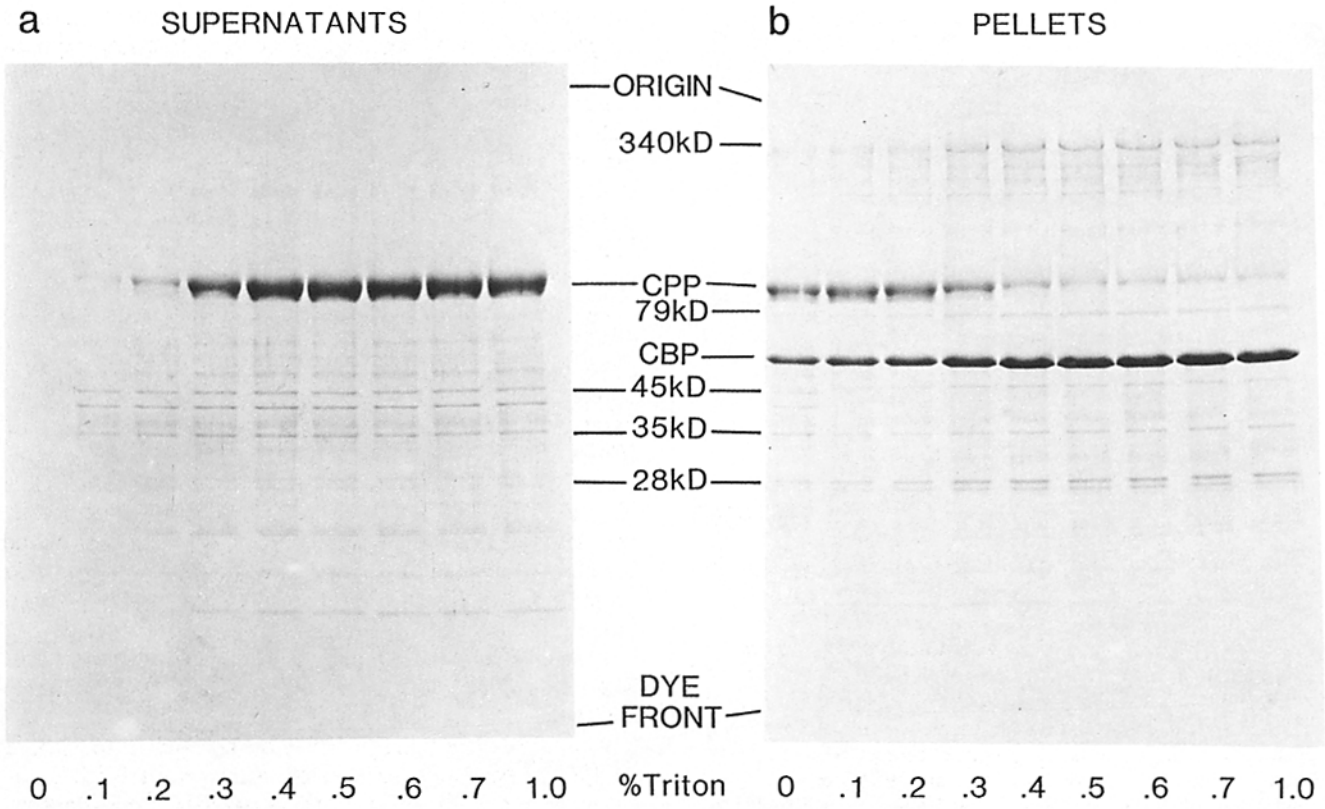


Figure 4. Protein distribution after extracting JTC over a range of Triton X-100 concentrations. Supernatants (*a*) and pellets (*b*) were prepared as outlined in the legend to Fig. 2. The calcium pump protein and CBP are the major components in the JTC; the control was pelleted in the absence of Triton X-100 (0% Triton). Other characteristic JTC proteins include the high molecular weight band at ~340 kD, the 79-kD band, four bands in the 35–45-kD range, and the 28-kD doublet. There is some variability in the number and mobility of the bands in the 35–45-kD range as well as in the high molecular weight bands (150 kD and higher). The 340-kD band sometimes appears as a doublet. Small amounts of Triton (0.1%) lead to the release of the four proteins in the 35–45-kD range. Further increase in Triton concentration leads to extraction of calcium pump protein and corresponding enrichment of other proteins in the pellet. Note that at 0.5% Triton X-100 maximal protein solubilization has been obtained.

split out or becomes solubilized. Most of the calcium pump membrane that has not been solubilized appears as large open sheets. At 0.5% detergent, only structures referable to JFM-CC are observed and the calcium pump membrane appears to have been completely solubilized. An increase in Triton X-100 to 1.0% yields JFM-CC similar to that at 0.5% except that the feet structures become somewhat less discernable.

The protein profiles of the supernatants and pellets, as a function of increasing detergent concentration, are shown in Fig. 4. In the absence of detergent there is no protein in the supernatant and the pellet contains protein bands characteristic of intact JTC. Such bands include a prominent high molecular weight component typically at ~340 kD, the calcium pump protein, a 79-kD band, CBP, four bands in the 35–45-kD range, and a doublet at 28 kD. This gel shows that Triton X-100 can be used to selectively extract the calcium pump membrane from JTC. At 0.1% Triton X-100, bands in the 35–45-kD range and a trace of calcium pump protein are extracted. As the detergent concentration is increased, calcium pump protein becomes progressively extracted. Other JTC bands remain in the pellet and become progressively enriched with increasing detergent concentration. At ~0.5% detergent, maximal solubilization has been achieved so that further increase in the detergent concentration does not change the gel patterns of the supernatant and pellet fractions. Thus, our standard JFM-CC preparation makes use of selective solubilization of the calcium pump membrane from JTC vesicles with 0.5% Triton X-100 in the presence of 1 mM $CaCl_2$ (see Materials and Methods).

In preliminary studies, we used $C_{12}E_8$ and octylglucoside as well as Triton X-100 and obtained similar selected extraction of the calcium pump membrane (not shown).

Additional insight into the disassembly of JTC with detergent was obtained by fractionating the detergent-treated mixtures on sucrose density gradients. Below 0.5% Triton X-100, two bands are obtained, one at 31%, the other at 41% sucrose, whereas Triton X-100 at 0.5% or greater yielded only the denser band at 41% sucrose. The protein profiles of the supernatants (the upper portion of the gradients where sample had been applied) and the gradient bands obtained with 0.1, 0.3, and 1.0% detergent are shown in Fig. 5. The protein profile of the supernatants from the gradient were similar to those obtained via the pelleting method (Fig. 4 *a*). In particular, it can be noted that at 0.1% Triton X-100, bands in the 35–45-kD range are enriched, and a trace of solubilized calcium pump protein can be observed. Most of the calcium pump protein appears in the 31 and 41% bands, the 31% fraction consisting predominantly of calcium pump protein in the form of calcium pump membrane vesicles (see below). With

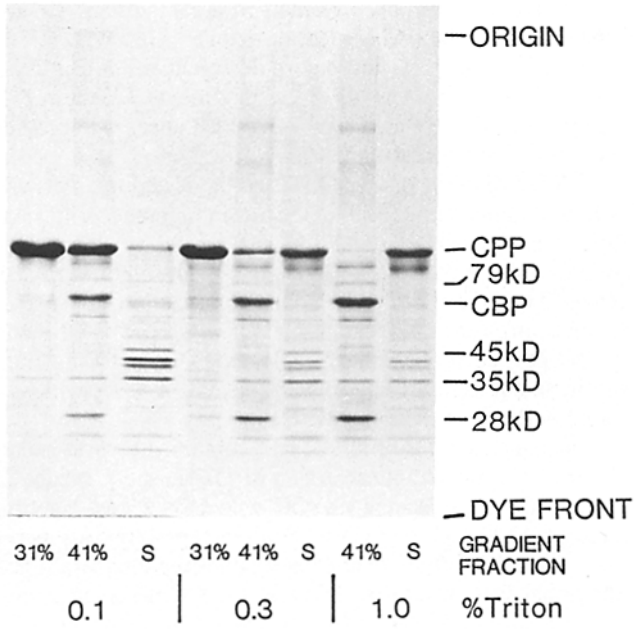


Figure 5. Protein profiles of gradient fractions after extraction of JTC with Triton X-100. JTC, treated with 0.1, 0.3, or 1.0% Triton X-100 in the presence of 1 mM $CaCl_2$, were fractionated by centrifugation on sucrose density gradients (see Materials and Methods). At the lower detergent concentrations (0.1 and 0.3%) two bands visible at 31 and 41% sucrose, and the supernatant (this is the loaded volume) were recovered. At 1% detergent, only the band at 41% sucrose and the supernatant were obtained. The bands were collected and diluted with 5 mM Hepes, pH 7.4, sedimented, and resuspended in buffered sucrose. The protein profiles obtained using an SDS PAGE, 5–17% linear gradient gel, exhibit a complex pattern that changes with detergent concentration. Solubilization of the four 35–45-kD bands occurs at low detergent concentration (0.1%). At 0.1 and 0.3% Triton, the calcium pump protein is the major component of the 31% band (see also Fig. 6). At 1.0% Triton essentially all calcium pump protein has been solubilized so that the 31% band is no longer obtained in the gradient. Notice that CBP distributes entirely with the 41% band at all detergent concentrations when 1 mM $CaCl_2$ is used. 15 μg of protein was loaded per lane.

increasing Triton X-100, the calcium pump protein becomes progressively solubilized, while the content of this component decreases in the 41% fraction. At both 0.1 and 0.3% Triton X-100, the protein content of the calcium pump protein-rich lower density fraction, is only about one-fourth that of the higher density fraction. At detergent concentrations of ≥0.5% Triton, essentially all of the calcium pump membrane has been solubilized so that no band is obtained at 31% sucrose and the band at 41% sucrose contains the proteins characteristic of JFM-CC (see Fig. 4).

Thus, the protein profiles indicate that, at lower detergent concentrations, the connection between the calcium pump membrane and the JFM becomes severed. The calcium pump membrane is recovered both as calcium pump membrane vesicles (31% band) and attached to the JFM in the JFM-CC (41% band). Electron microscopy of the two gradient fractions obtained with 0.1% Triton X-100 confirms this (Fig. 6). The 31% band consists of calcium pump membrane vesicles that have been split out but not solubilized, since the vesicles display an asymmetric membrane by tannic acid staining; the latter is characteristic of calcium pump membrane in native light SR vesicles (34).

The identification of the 31% band as calcium pump membrane is supported by measurement of Ca^{2+}-ATPase rates that are 1.9 and 5.2 μmol/mg·min (for the 0.1% Triton condition) in the absence and presence of the calcium ionophore A23187, respectively. Such values are characteristic of calcium pump membrane in purified light SR. The 2.7-fold increase in rate upon addition of ionophore indicates that these vesicles are sealed since Ca^{2+}-ATPase is coupled to Ca^{2+} transport. The 41% band has a Ca^{2+}-ATPase rate of only 2.2, which is not stimulated by ionophore, suggesting that these vesicles are leaky. This rate is only slightly lower than the ionophore-stimulated rate (2.5) obtained for native JTC vesicles (33) and reflects the lower calcium pump protein content of these membranes. The Ca^{2+}-loading activity of the 31% band is 1.45 μmol/mg·min which means that this fraction has a coupling ratio (Ca^{2+} loading rate/Ca^{2+}-ATPase rate) of 0.8. By contrast, the 41% band consists of leaky membranes since it has essentially no Ca^{2+} loading activity. The

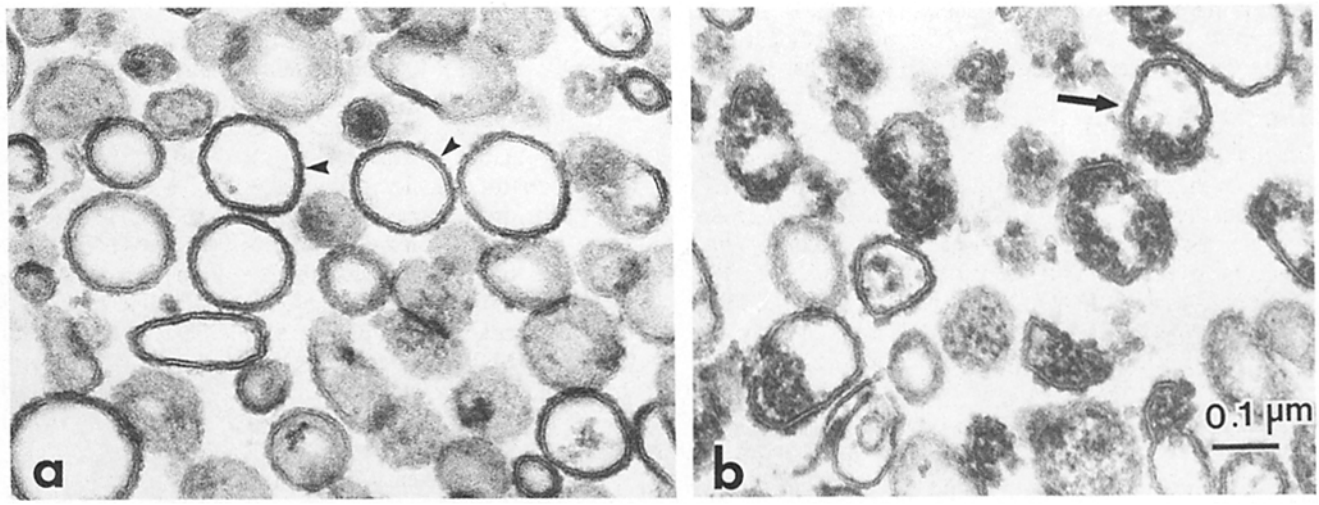


Figure 6. Morphology of the gradient fractions after extraction of JTC at low Triton X-100 concentration. JTC vesicles were extracted with 0.1% Triton in the presence of 1 mM $CaCl_2$ and fractionated by sucrose density gradient centrifugation (see Fig. 5 and Materials and Methods), yielding a supernatant and bands at (*a*) 31% and (*b*) 41% sucrose. These three fractions account for 10, 18, and 72% of the protein recovered from the gradient. The vesicles in the 31% band exhibit an asymmetric membrane (*arrowhead*) characteristic of calcium pump membrane, using tannic acid enhancement. The 41% band has a heterogeneous appearance and consists mainly of JFM-CC attached to calcium pump membrane (*small arrow*). Other vesicles consist mainly of JFM-CC.

leakiness of this fraction does not appear to be due to residual detergent since further treatment with Bio-Beads had no effect on Ca^{2+} loading. Ruthenium red at 20 μM also did not enhance Ca^{2+} loading.

Calcium preincubation before detergent extraction is important in obtaining large intact structures of JFM-CC. This can be observed in the electron micrographs in Fig. 7, *a–c*, which illustrate the effect of varying the divalent cationic composition of the solubilization mixture. JTC vesicles were incubated in either buffered sucrose (control), or in buffered sucrose supplemented with $CaCl_2$ or EDTA, before solubilization with 0.7% Triton X-100. Calcium pretreatment leads to isolation of JFM-CC which is similar in size to that observed in intact JTC vesicles (Fig. 7 *a*). They exhibit well preserved junctional feet structures and large masses of compartmental contents. By contrast, the JFM in the pellet obtained after EDTA pretreatment has been comminuted, containing only small fragments of membrane (Fig. 7 *c*). Nonetheless, feet structures are clearly discernable as squares of ~20 nm/side. The control pellet (Fig. 7 *b*) contains JFM-CC with a morphology intermediate in size between that seen with pretreatment with calcium and EDTA, containing one or several feet structures. Hence, pretreatment of JTC with calcium before detergent solubilization is essential to obtain intact JFM-CC fragments. Calcium pretreatment enables retention of CC, which appears to provide mechanical support to the JFM.

SDS PAGE of the original JTC and of the supernatants and pellets obtained under each of these three preincubation conditions are shown in Fig. 7 *d*. Differences in the protein profiles are referable mainly to changes in the amount of CBP released into the supernatant upon solubilization. Compared to the control, the EDTA supernatant is enriched in CBP and the pellet is correspondingly depleted. By contrast, calcium pretreatment yields a supernatant devoid of CBP, which is found entirely in the pellet.

Sizeable structures of JFM can be obtained by first isolating JFM-CC by Triton extraction of JTC in the presence of Ca^{2+}, and then releasing the CC with EDTA. An electron micrograph of such a JFM preparation (Fig. 8) reveals membranes of substantial size containing unidirectionally aligned junctional feet structures. Most of the CC associated with

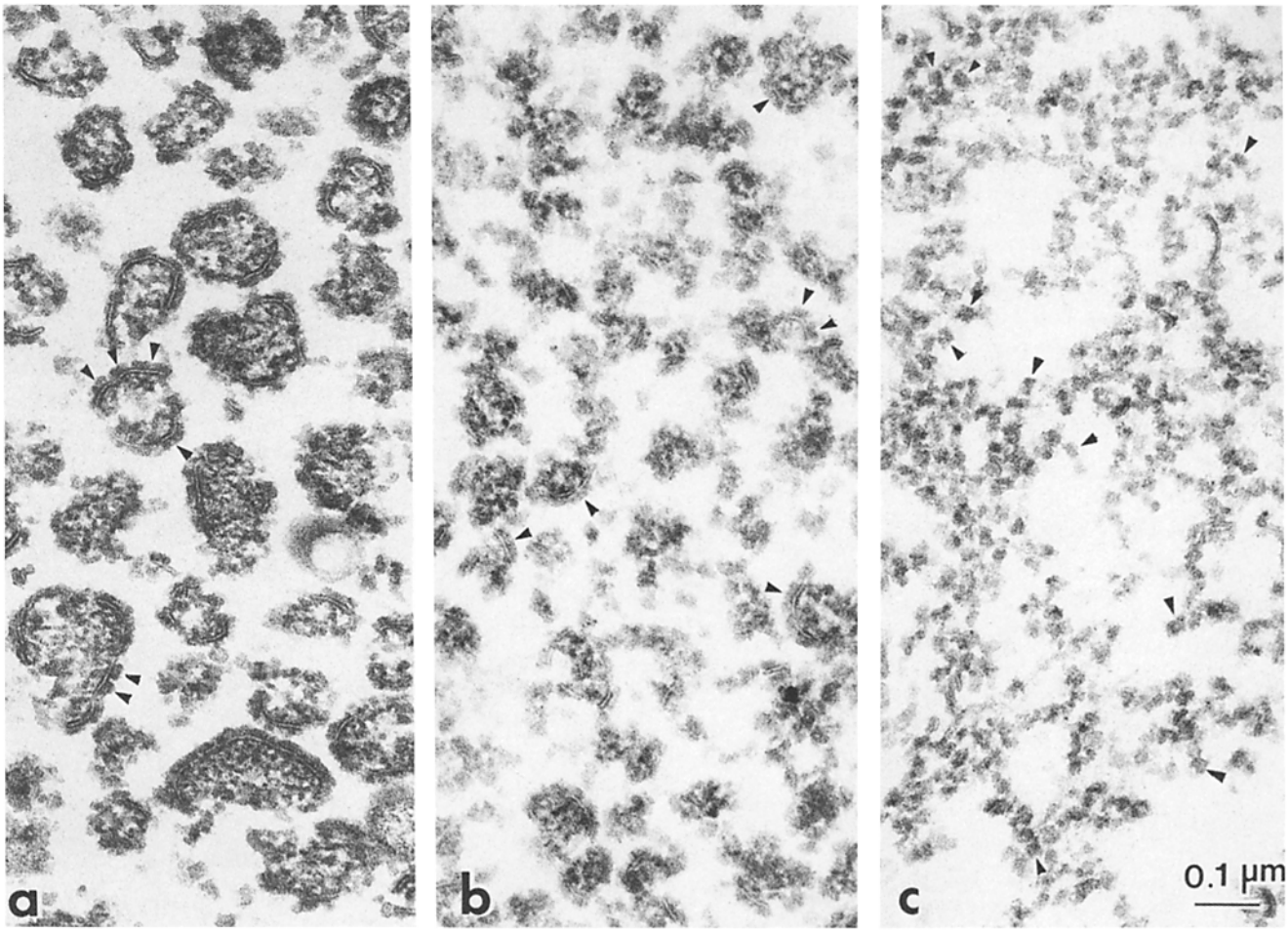


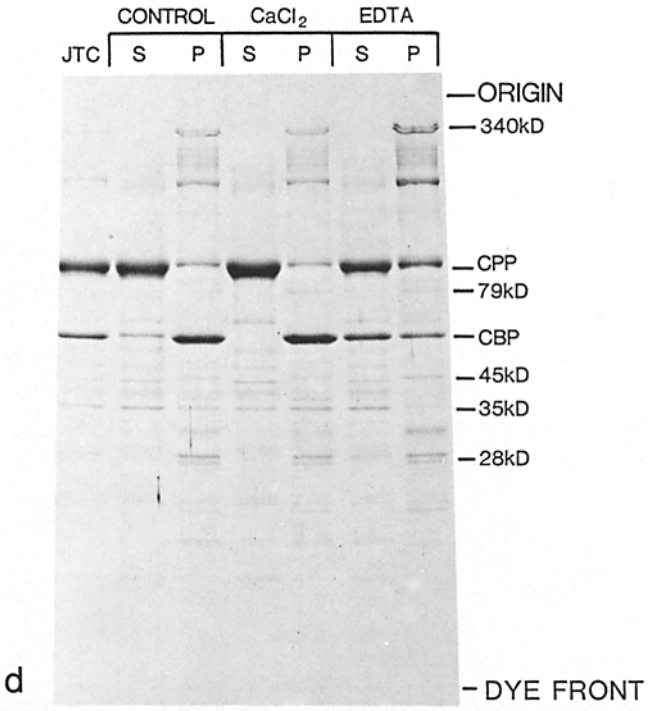
Figure 7. Morphology and protein profiles of JFM-CC prepared at different calcium concentrations. JTC were preincubated in either buffered sucrose (*b*, control) or in buffered sucrose supplemented with 1 mM $CaCl_2$ (*a*) or 2 mM EDTA (*c*), before solubilization with 0.7% Triton X-100 and sedimentation as outlined in the Materials and Methods section. JFM-CC of comparable size to that in intact JTC vesicles is obtained only when detergent extraction is carried out in the presence of added calcium. The JFM-CC fragments in the control sample (*b*) are intermediate in size between the large JFM-CC in the presence of calcium (*a*) and the small fragments in the EDTA sample (*c*). Junctional feet are readily identified in each fraction (*arrowheads*). Intact JTC along with the supernatants (*S*) and pellets (*P*) obtained

these membranes have been extracted (compare with JFM-CC, Figs. 3, *c* and *d* and 7 *a*). The JFM has the characteristic appearance of this membrane in the JTC vesicles.

Gel patterns of the JFM-CC, the EDTA extract, and the derived JFM are shown in Fig. 9. The major difference between the JFM-CC and JFM patterns is the much lower content of CBP in the JFM. In separate experiments, quantitative densitometry of gels showed that 89% ± 2% (SEM; $n = 4$) (see also Table I) of the CBP of JFM-CC has been extracted by EDTA to yield the corresponding JFM. Small amounts of the 79-kD protein and of some of the minor bands with molecular weights below CBP also are extracted with EDTA.

The portion of the CBP that remains associated with the JFM is resistant to further EDTA extraction. Varying the time of extraction of JFM-CC with EDTA from 10 min to 3 h, or the concentration of EDTA from 2 to 8 mM does not change the amount of residual CBP of the JFM. Multiple extractions of the JFM-CC with EDTA plus KCl (2 mM EDTA, 150 mM KCl, 5 mM Hepes, pH 7.4) also fails to remove the residual CBP and yields JFM with a morphology similar to that obtained after a single extraction with buffered sucrose containing 2 mM EDTA (Fig. 8).

To ascertain whether the CBP in the EDTA extract and pellet represent the same polypeptide, Cleveland protease digestion gels (6) were carried out (Fig. 10). The substrates for these gels were the CBP bands cut out from preparative gels that had been loaded with either pellet (JFM) or EDTA supernatant after extraction of JFM-CC. The peptide patterns obtained using either chymotrypsin (Fig. 10) or Staphylcoccus aureus V8 protease (not shown) are indistinguishable for CBP obtained from supernatant or pellet.



under the three conditions were analyzed by SDS PAGE (*d*). The difference in the protein patterns of the SDS PAGE for the three conditions is mainly attributable to the distribution of the CBP. The heavy band, between the 340-kD band and the calcium pump protein (*CPP*), has the mobility of myosin. It is a variable component. Hence, the EDTA extractable and membrane-bound CBP are similar, if not identical.

Our standardized procedure for preparing JFM from JTC vesicles involves an initial solubilization of calcium pump membrane with 0.5% Triton X-100 in the presence of 1 mM Ca^{2+} to obtain JFM-CC, followed by extraction of most of the CC, mainly CBP from the JFM-CC with 2 mM EDTA (the protocol is given in Materials and Methods). The JFM accounts for approximately one-fifth of the protein of JTC and as isolated has a phospholipid content of 0.16 μmol P/mg protein (Table I). The protein profile of JTC and of the four fractions obtained in the process of isolation of JFM are compared in Fig. 9. The calcium pump protein is the major protein removed by Triton extraction of the JTC, while CBP constitutes the major component extracted from the JFM-CC by EDTA. Table I presents quantitative densitometric data for the major proteins of the JTC and of the four fractions derived from it. The values represent the averages obtained from three preparations, of which the gel in Fig. 9 is typical. The 340-kD protein and the 28-kD doublet are enriched in the JFM, which also retains a small percentage of tightly bound CBP. The 340-kD band accounts for about one-fifth of the JFM proteins.

An individual JTC, a JFM-CC, and a JFM are compared at higher power in Fig. 11. The size and appearance of the JFM in each preparation is similar.

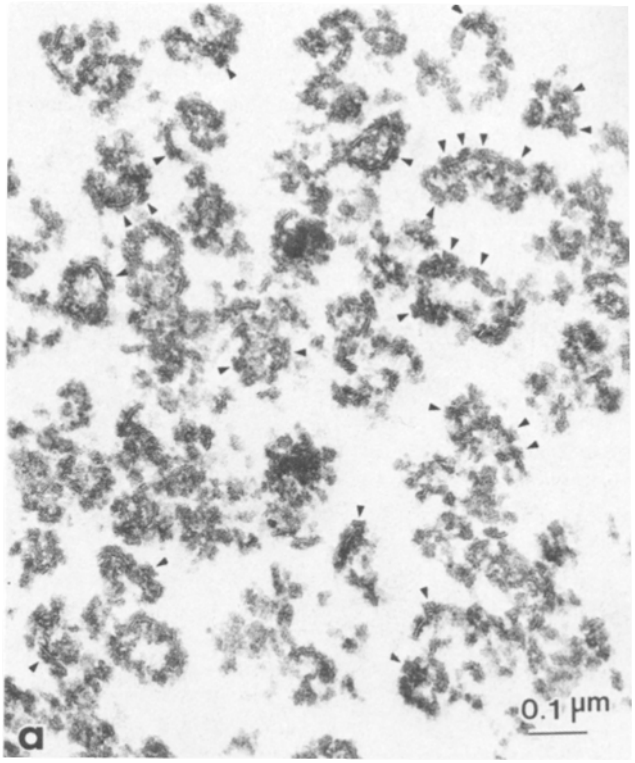


Figure 8. Morphology of the JFM. JFM-CC, obtained by solubilization of JTC with 0.5% Triton in the presence of 1 mM $CaCl_2$, was extracted for 10 min in sucrose buffer containing 2 mM EDTA. This treatment extracts most of the CBP (see Fig. 9) and the CC (see Fig. 11 also). Intact JFM containing junctional feet structures (*arrowheads*) are obtained (compare with Figs. 3, *c* and *d* and 7 *a*).

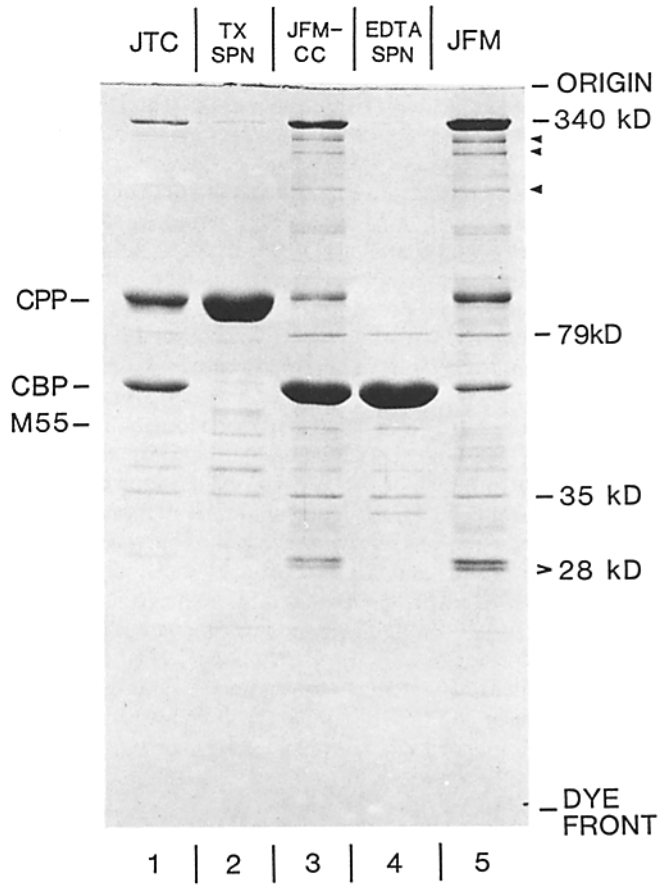


Figure 9. Protein profiles from the sequential extraction of JTC to yield JFM-CC and JFM. JFM was prepared by extracting JTC vesicles (lane *1*) with 0.5% Triton X-100 in the presence of 1 mM $CaCl_2$ to obtain a Triton supernatant (lane *2*) and pellet (lane *3*), the JFM-CC. The JFM-CC was then extracted with 2 mM EDTA for 10 min and centrifuged to obtain an EDTA supernatant (lane *4*) and pellet (lane *5*), the JFM. Protein loading was 7 μg per lane. This gel is typical for the three preparations that were used to obtain the quantitative densitometry data presented in Table I. Note that the 340-kD band and the doublet at 28 kD are highly enriched in the junctional face membrane. The molecular weights, and percentages of total protein represented by the three high molecular weight bands indicated by arrowheads are, from top to bottom: 310 kD, 6.1%; 296 kD, 2.1% and 213 kD, 1.2%, respectively.

Discussion

To study contraction and relaxation of fast skeletal muscle, our laboratory has directed its efforts toward the isolation of defined membrane fractions involved in the regulation of the intrafiber Ca^{2+} concentration. The isolation of heavy and light SR referable to terminal and longitudinal cisternae, in situ, respectively, was a first step in this direction (25). Terminal cisternae differ from longitudinal cisternae in that they contain the JFM and electron opaque contents. The heavy SR fraction contained electron opaque contents but was essentially devoid of JFM. Triads, the junctional association of terminal cisternae with transverse tubules, were then isolated (28) and made possible the study of the influence of transverse tubule on the terminal cisternae. More recently, we have succeeded in isolating a JTC fraction that consists of two membranes, the JFM (15–20% of the membrane) with morphologically well defined feet structures, and the calcium pump membrane (80–85%), as well as CC (33).

In this study, a procedure has been developed for the stepwise disassembly of the JTC. The calcium pump membrane is selectively extracted with detergent in the presence of calcium ions (1 mM) to yield JFM-CC. Sizable structures of JFM can then be prepared from JFM-CC by solubilizing the

Table I. Characterization of the JTC and Derived Fractions, Including the JFM

Fraction‡	Protein recovery§	Phospholipid‖ phosphorus		Protein bands (%)*							
		Recovery§	μg P mg prot.	340 kD	150 kD	CPP	79 kD	CBP	Proteins in the 35–45-kD range (combined)	28-kD Doublet	Others
JTC	100	100	11.4	5.4‖	1.9 ± 0.6	34.9 ± 1.4	1.4 ± 0.3	26.8 ± 5.2	9.2 ± 2.4	3.0 ± 0.5	17.4
0.5% Triton supernatant	52¶	87.8¶	19.2¶	–	–	69.2 ± 2.7	–	–	9.7 ± 1.1	–	21.1
JFM-CC (0.5% Triton pellet)	48 ± 3.9	14.1	3.3	10.2‖	–	5.2** ± 2.3	2.3 ± 0.3	47.1 ± 3.7	7.6 ± 1.1	5.3 ± 1.0	22.3
EDTA supernatant	24.8 ± 0.9	2.3	1.1	–	–	–	–	71.7 ± 3.3	8.4 ± 2.2	–	19.9
JFM	21.3 ± 1.3	9.7	5.1	22.0‖ (6.1, 2.1, 1.2)‡‡	2.4 ± 0.34	6.8‖**	2.4 ± 0.5	11.8 ± 1.1	9.7 ± 3.9	10.4 ± 2.3	25.1

* The values for each band are expressed as a percentage of the total optical density obtained by scanning Coomassie Brilliant Blue stained SDS polyacrylamide gels (an example is presented in Fig. 9). The average and standard error from three preparations are given. Dashes indicate a value too low to be measured.
‡ The JTC were extracted with 0.5% Triton in the presence of 1 mM $CaCl_2$ to yield a Triton supernatant and JFM-CC. The JFM-CC was then extracted with 2 mM EDTA to give an EDTA supernatant and a pellet, the JFM. Further details of the extraction procedures are presented in the Materials and Methods section.
§ Recoveries are expressed relative to the initial JTC, which represents 100%.
‖ Average of data from two preparations.
¶ Calculated by difference since Triton X-100 interferes with the Lowry determination of protein.
** This band appears to be distinct at least in part from the calcium pump protein (CPP).
‡‡ Values in parentheses represent the percentages of the 310-, 296-, and 213-kD bands, respectively (these bands are indicated by arrowheads in Fig. 9).

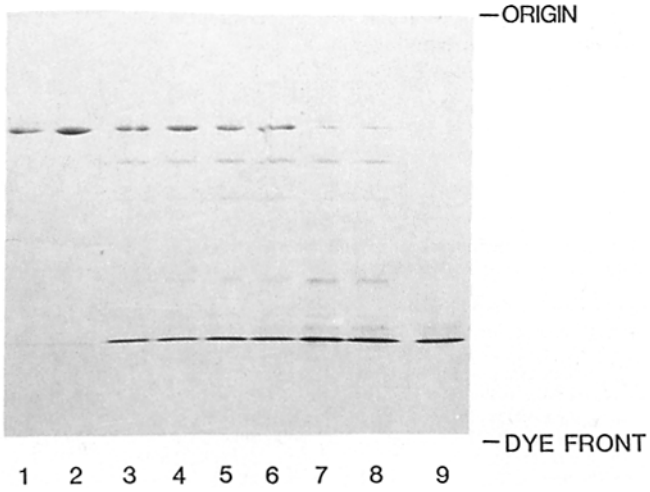


Figure 10. Comparison of CBP in the JFM vs. EDTA supernatant using peptide analysis. Gel slices containing CBP were cut from lanes of preparative SDS polyacrylamide gels that had been loaded with either the supernatant (which contains EDTA-extractable CBP) or the JFM pellet (which contains unextractable CBP), obtained by extraction of JFM-CC with 2 mM EDTA. The gel slices were loaded into the wells of SDS polyacrylamide gels along with varying amounts of α-chymotrypsin as described by Cleveland (6) and electrophoresis was initiated (see Materials and Methods). Lanes *1*, *3*, *5* and *7* contain ~5 μg of bound CBP, and lanes *2*, *4*, *6* and *8* contain ~5 μg of EDTA-extractable CBP. α-Chymotrypsin was loaded onto the gel as follows: 0.3 μg (lanes *3* and *4*); 1 μg (lanes *5* and *6*); 3 μg (lanes *7* and *8*); 4.5 μg (protease without sample, lane *9*). The two CBP samples yielded similar if not identical peptide patterns at all concentrations of protease. A similar experiment (not shown) using Staphylococcus aureus V8 protease also yielded identical peptide patterns for the two fractions of CBP.

CC with EDTA. When detergent extraction is carried out either in the absence of added $CaCl_2$ or in the presence of EDTA, the JFM is comminuted to intermediate size or small fragments, respectively.

Previous studies of Triton extraction of terminal cisternae vesicles (1) used a fraction containing intact triads. The residue retained structures seemingly referable to both the JFM and transverse tubule. In our study, isolated JTC were used and gave rise to purified JFM of approximately comparable size to that in the original vesicle. Identity of the JFM is based on morphology as the primary criterion. Junctional feet structures are more difficult to recognize when they appear singly (Fig. 7 *c*). The JFM-CC obtained by Triton extraction in the presence of millimolar divalent cations characteristically displays aligned feet and is therefore readily identified. Recognition of feet structures is also greatly enhanced by tannic acid mordanting during fixation of membranes (33). However, the exposure time and concentration of tannic acid used must be carefully controlled.

Identification of the electron dense compartmental contents of the terminal cisternae region of the SR with the CBP was made by Meissner (25) in our laboratory, who found that: (*a*) heavy SR have a high content of CBP and electron-dense compartmental contents (the latter also being a feature of the terminal cisternae, in situ); and (*b*) loss of the compartmental contents from the heavy SR could be correlated with the release of CBP. CBP can bind large amounts of Ca^{2+} (8, 18, 22, 24, 26) and thereby serves to lower the Ca^{2+} concentration within the terminal cisternae. Consistent with the localization of CBP and its high calcium-binding capacity, electron probe studies have demonstrated a much higher calcium content of the terminal cisternae compared with the longitudinal cisternae region of the SR in situ (39). This observation is consistent with the suggestion that calcium release occurs from the terminal cisternae of SR (42). However, the basis for localization of CBP to the terminal cisternae has remained unclear.

Our electron microscopic study of JTC in the presence of monovalent and divalent cations reinforces previous observations (1, 3, 28) that Ca^{2+} induces the compartmental contents to aggregate and associate with the luminal face of the JFM. The isolation of JFM-CC provides direct evidence of this association. Furthermore, this aggregation and association may have physiological relevance since it is observed in a physiological cationic mixture having concentrations of Ca^{2+}, Mg^{2+}, and K^+ mimicing those believed to exist within the lumen of the terminal cisternae at rest (9, 15, 19, 27, 41). A new finding is that ~10% of the CBP is resistant to extrac-

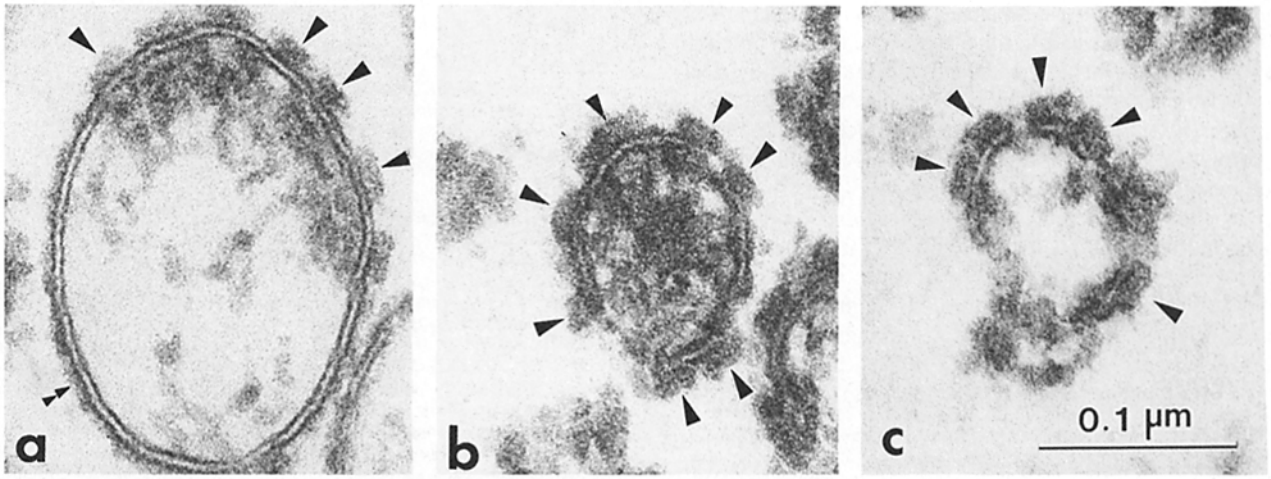


Figure 11. Electron micrographs comparing a typical vesicle of JTC (*a*), JFM-CC (*b*), and JFM (*c*). For each, the JFM is approximately of similar size and contains unidirectionally aligned feet structures. The feet structures are indicated by arrowheads and the calcium pump membrane by the double arrowheads.

tion from the membrane with EDTA. Since this small fraction appears to be identical to the extractable 90% of the CBP, the presence of a specific binding protein for CBP in the JFM is indicated. The picture that emerges is that, in the presence of Ca^{2+}, the bulk of the CBP associates with itself and to the junctional face via the bound form of the CBP. Rope-like fibers are observed by freeze-fracture electron microscopy in the hydrophobic face of the JFM which are similar in appearance and diameter to those observed within the lumen of the terminal cisternae (33). Such a connection might serve as a bridge between the feet structures and the internal compartment.

The JFM as isolated has a number of features that distinguish it from the calcium pump membrane. Morphologically, the membrane has characteristic feet structures oriented asymmetrically, and uniformly spaced, comparable to their arrangement in situ (14, 29, 33). Our standard preparation of JFM-CC (using 0.5% Triton and 1 mM $CaCl_2$) and JFM have a phospholipid content of 0.11 μmol P/mg protein and 0.16 μmol/mg protein, respectively (Table I). The JFM is insoluble under conditions that solubilize the calcium pump membrane. Characteristic proteins of the JFM include a major band at 340 kD, several (a variable number of) bands migrating between the 340-kD band and the calcium pump protein, and a doublet at 28 kD. Other bands such as the 79 and 35 kD, which are characteristic of the JFM-CC, distribute with both the JFM and CC (EDTA supernatant). JFM also contains a band that migrates at the slow edge of the calcium pump protein band. The migration of this band varies somewhat from run to run. About half the time, it migrates distinctly slower than calcium pump protein. It is either distinct from the calcium pump protein or is uniquely bound to another component in the JFM since the bulk of the calcium pump protein is readily solubilized at 0.5% Triton and this component is not released even at 1% Triton (see Fig. 4 *b*).

Both the 340-kD band and the 28-kD doublet are localized to the JFM, as indicated by its distribution during preparation from JTC, the enrichment being approximately fourfold. Since the major morphological feature of the JFM is the presence and alignment of junctional feet, both of these bands may be components of the feet structures. Our studies are consistent with the previous suggestion of Cadwell and Caswell (2) that 300- and 325-kD proteins may be components of the feet structures. It has also been suggested (4) that an 80-kD protein, present in both transverse tubules and terminal cisternae, is the anchoring protein that binds the feet proteins. Consistent with this view, we observe that the 79-kD protein distributes with the JFM-CC fraction with detergent extraction of JTC, and is only partially solubilized by subsequent EDTA extraction of the JFM-CC.

The availability of the JFM in the test tube should make possible more extensive studies with this membrane. The greater recovery of JTC by the procedure described here should facilitate studies that require substantial amounts of material, such as isolation of the components involved in junctional association and the Ca^{2+} release process, which appear to be localized in this membrane (13).

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