Localization of Ca^{2+} + Mg^{2+}-ATPase of the Sarcoplasmic Reticulum in Adult Rat Papillary Muscle

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ABSTRACT Localization of the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum in rat papillary muscle was determined by indirect immunofluorescence and immunoferritin labeling of cryostat and ultracryotomy sections, respectively. The Ca^{2+} + Mg^{2+}-ATPase was found to be rather uniformly distributed in the free sarcoplasmic reticulum membrane but to be absent from both peripheral and interior junctional sarcoplasmic reticulum membrane, transverse tubules, sarcolemma, and mitochondria. This suggests that the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum is antigenically unrelated to the Ca^{2+} + Mg^{2+}-ATPase of the sarcolemma. These results are in agreement with the idea that the sites of interior and peripheral coupling between sarcoplasmic reticulum membrane and transverse tubules and between sarcoplasmic reticulum and sarcolemmal membranes play the same functional role in the excitation-contraction coupling in cardiac muscle.

In cardiac muscle the sarcoplasmic reticulum is the intracellular membrane system which, together with the transverse tubular system (T-system) and the sarcolemma, regulates the Ca^{2+} concentration of the myofibril and thereby the state of contraction and relaxation of the myocardium. Ultrastructural examination of cardiac muscle (9, 29, 32, 34) has shown that the sarcoplasmic reticulum forms a separate intracellular compartment in the muscle cells surrounding the myofibrils like a laced sleeve. The portion of the sarcoplasmic reticulum membrane that is located in close apposition to the T-system is called interior junctional sarcoplasmic reticulum membrane while that portion in close apposition to the sarcolemma is called peripheral junctional sarcoplasmic reticulum membrane. The remainder of the membrane system is referred to as the free or nonjunctional sarcoplasmic reticulum membrane (34).

The mechanism for myofibril contraction in the cardiac muscle is similar to that in skeletal muscle. Contraction is induced when depolarization of the sarcolemma leads to an increase in the Ca^{2+} concentration of the myofibril (36); in turn, relaxation sets in when Ca^{2+} is removed from the myofibril. It is generally agreed that in mammalian cardiac muscle (33, 36) calcium is removed from the myofibril by active transport across the sarcoplasmic reticulum membrane to the lumen of the sarcoplasmic reticulum. However, the site of storage of Ca^{++} during relaxation as well as the origin of Ca^{2+} required for the induction of contraction is a controversial issue. Cardiac muscle, in contrast to skeletal muscle, is very sensitive to changes in extracellular Ca^{2+} concentration (23). Thus, although it is believed that some of the Ca^{2+} required for contraction is released from the lumen of the sarcoplasmic reticulum (8, 10), it is almost certain that some of the Ca^{2+} is also of extracellular origin (23). Accordingly, some Ca^{2+} must be stored in the lumen of the sarcoplasmic reticulum during relaxation, while some must be transported to the extracellular space. So far it has not been possible to determine whether the sites where the sarcoplasmic reticulum membrane is in close apposition to the T-tubules and/or the sarcolemma are sites of excitation-contraction coupling and/or sites of Ca^{2+} transport in and out of the muscle cells. To understand the possible specific functions of the morphologically specialized regions of the sarcoplasmic reticulum in the transport of Ca^{2+} to and from the myofibril during the contraction-relaxation cycle, it is important to know the function of the sarcoplasmic reticulum proteins as well as the precise distribution of these proteins in the various regions of this membrane system.

The presence of a Ca^{2+} + Mg^{2+}-dependent ATPase in mammalian cardiac sarcoplasmic reticulum (Ca^{2+} + Mg^{2+}-ATPase) has been reported and it is generally agreed that this protein, as in skeletal muscle, actively transports Ca^{2+} from the myofibril to the lumen of the sarcoplasmic reticulum when relaxation sets in (36). The biochemical characteristics of the protein from cardiac muscle are similar to those of the protein of skeletal muscle (4, 15, 25, 33, 44, 45) although the rate of Ca^{2+} uptake by the cardiac protein is slower than the rate of Ca^{2+} uptake by

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the skeletal protein (36). Several investigators have reported that there is also a Ca^{2+} + Mg^{2+} ATPase in the sarcolemma of myocardial cells (3, 7, 14, 28, 35). While some of the Ca^{2+} + Mg^{2+}-ATPase activity reported to be associated with isolated sarcolemmal membrane vesicles might represent contamination with sarcoplasmic reticulum membrane vesicles, Caroni and Carafoli (2, 3) have recently isolated a Ca^{2+} + Mg^{2+}-dependent ATPase from purified sarcolemma and have shown that it is distinct from the sarcoplasmic reticulum enzyme in size and in that it is calmodulin-dependent.

To determine (*a*) whether the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum is confined to the sarcoplasmic reticulum or whether it is also present on the sarcolemmal membrane and (*b*) whether this Ca^{2+} + Mg^{2+}-ATPase is uniformly distributed in the various regions of the sarcoplasmic reticulum membrane system, we have used affinity-purified antibodies against the Ca^{2+} + Mg^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum to localize the Ca^{+2} + Mg^{+2}-ATPase in the adult rat papillary muscle by the indirect immunofluorescence and immunoferritin labeling techniques.

We have found that the Ca^{2+} + Mg^{2+}-ATPase is confined to the sarcoplasmic reticulum in adult rat papillary muscle. Furthermore we have found that the Ca^{2+} + Mg^{2+}-ATPase is rather uniformly distributed within the nonjunctional region of the sarcoplasmic reticulum membrane but is apparently absent from the region where the sarcoplasmic reticulum membrane is located in close apposition to either the transverse tubules or the sarcolemma.

MATERIALS AND METHODS

Purification of Rat Ca^{2+} + Mg^{2+}-ATPase

Rat skeletal muscle Ca^{2+} + Mg^{2+}-ATPase was prepared as previously described for the purification of rabbit ATPase (27), except that the fractionation in ammonium acetate was carried out at pH 8.35. The ATPase was separated from minor contaminating proteins by preparative SDS PAGE according to the procedure described by Laemmli (22). The Ca^{2+} + Mg^{2+}-ATPase protein band was then excised from the polyacrylamide gel and the protein eluted electrophoretically as previously described (20). Isolation of the microsomal fraction from rat ventricular muscle enriched in sarcoplasmic reticulum was carried out according to the procedure of Harigaya and Schwartz (11) as modified by Jones et al. (15).

Preparation and Characterization of Antisera to Rat Ca^{2+} + Mg^{2+}-ATPase

Antisera to the Ca^{2+} + Mg^{2+}-ATPase from rat skeletal muscle were produced in rabbits as previously described (13) except that a total of only 600 μg of antigen was used for the immunization. The specificity of the antiserum used in the present study was demonstrated by reacting the antiserum in Ouchterlony double diffusion tests against solubilized rat sarcoplasmic reticulum and purified rat Ca^{2+} + Mg^{2+}-ATPase. Only a single precipitin line was obtained in both cases. No precipitin line was observed when the preimmune rabbit serum was used. Although no precipitation line was observed when the antiserum to the Ca^{2+} + Mg^{2+}-ATPase from rat skeletal muscle was tested against a microsomal fraction isolated from rat ventricular muscle, the ability of this antiserum to cross-react specifically with the Ca^{2+} + Mg^{2+}-ATPase of sarcoplasmic reticulum from rat myocardium was demonstrated by the indirect ^{125}I–protein A immunostaining on Western blots (see below).

Immunostaining of Western Blots

The indirect ^{125}I–protein A assay on Western blots was carried out as described by Towbin et al. (42). Protein samples to be examined by this technique for the presence of the Ca^{2+} + Mg^{2+}-ATPase were first separated by SDS PAGE (22) and then transferred electrophoretically to nitrocellulose sheets. The sheets were first incubated with 3% bovine serum albumin (BSA) in buffer E (0.15 M NaCl, 10 mM Tris-HCl, pH 7.2) for 1 h at 37°C and then with affinity-purified rabbit antibodies to rat Ca^{2+} + Mg^{2+}-ATPase (1 μg/ml in buffer E containing 3% BSA). Subsequently, the sheets were washed in buffer E and then incubated with ^{125}I–protein A diluted to 10^6 cpm/ml in buffer E containing 3% BSA for 5–16 h at room temperature. Finally, the sheets were washed in buffer E, air-dried, and exposed to Kodak AR-5 x-ray film.

Dissection and Sectioning of Unfixed Papillary Muscle

Adult female Wistar rats were killed by cervical dislocation. The hearts were removed and rinsed in buffer A (0.15 M NaCl, 10 mM phosphate, pH 7.4). The papillary muscle was dissected from the left ventricle together with a 1-mm strip of the ventricular wall. The dissected tissue was frozen in liquid N_2, cooled in isopentane, and 4- to 6-μm-thick transverse and longitudinal sections were cut and postfixed in ethanol as previously described (17).

Dissection, Fixation, and Sectioning of Papillary Muscle

Adult female Wistar rats were killed by cervical dislocation. The hearts were removed and rinsed in buffer A (0.15 M NaCl, 10 mM phosphate, pH 7.4). The papillary muscles were dissected from the left ventricle and fixed for 1 h at 4°C in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in buffer B (0.1 M sodium cacodylate, pH 7.2, and 4.5 mM $CaCl_2$). Subsequently, the tissue was washed for 0.5 h in buffer B and then for 3 h in buffer C (0.08 M lysine-HCl and 0.02 M Tris-HCl, pH 7.5). The fixed tissue was stored at 4°C for up to 1 wk in buffer B. Autofluorescence of semithin sections (≤1 μm) was minimal. The ultracryotomy and immunoferritin labeling of fixed papillary muscle was performed as described by Tokuyasu and Singer (40, 41). Tissues were infused for 30–60 min with either 0.6 M sucrose in 0.1 M cacocylate buffer, pH 7.2 (for cutting semithin sections for immunofluorescence labeling), or 2.3 M sucrose in cacodylate buffer (for cutting thin sections for immunoferritin labeling). The sucrose-infused tissue was then frozen in liquid N_2, and sectioned either at −80°C (semithin sections) or at −110°C (ultrathin sections) with a Sorvall MT-2B ultramicrotome (DuPont Co., Wilmington, DE). For immunofluorescence labeling, the thickness of the sections was ~500 nm and for immunoferritin labeling ~100 nm as judged by the interference reflection colors of the sections. The frozen sections were collected on a droplet of buffer D (2.0 M sucrose and 9.75% gelatin in 0.1 M Tris-acetate, pH 7.4) and transferred to either glass slides for immunofluorescence labeling or to Formvar-carbon-coated copper grids for immunoferritin labeling.

Indirect Immunofluorescence Labeling

The immunofluorescent staining of the cryostat sections from fixed and unfixed papillary muscle was carried out as previously described (17). Cryostat sections were first incubated with affinity-purified antibodies to the Ca^{2+} + Mg^{2+}-ATPase for 30 min at room temperature (anti-ATPase, 30 μg/ml in buffer A), and then with the fluorescein-labeled γ-globulin fraction of goat anti-rabbit serum (0.1 mg/ml; Hyland Laboratories, Costa Mesa, CA) for 30 min at room temperature. Finally, the sections were washed four times in buffer A, and mounted in 50% glycerol in buffer A. The cells were examined in a Zeiss photomicroscope provided with an epi-fluorescence attachment and a phase-contrast condenser. The fluorescence and phase contrast pictures were taken on Kodak film S0115.

Adsorption

For adsorption studies, 25 μg of affinity-purified antibodies to Ca^{2+} + Mg^{2+}-ATPase in 0.3 ml of buffer A were incubated for 2 h at room temperature and then 6 h at 4°C with either 0 or 10 μg of lipid-free Ca^{2+} + Mg^{2+}-ATPase dissolved in 0.5% Triton X-100. The supernatants obtained after centrifugation were diluted threefold and used in the indirect immunofluorescence staining test.

Indirect Immunoferritin Labeling

The immunoferritin labeling technique was carried out according to procedures developed by Tokuyasu and Singer (37, 41). Ultrathin frozen sections on grids were treated with 2% gelatin in buffer F (0.1 M Tris-HCl, pH 7.4) for 2–3 min before immunoferritin labeling (41). The sections were incubated for 15 min at room temperature with affinity-purified antibodies to the Ca^{2+} + Mg^{2+}-ATPase (18) at a concentration of 20–30 μg/ml in buffer F. The samples were then rinsed four times with buffer F and incubated for 15 min at room temperature with affinity-purified goat anti-rabbit γ-globulin conjugated to horse spleen ferritin as previously described (19, 21) at an antibody concentration of 30 μg/ml in 0.1% BSA in buffer F. Samples were then washed four times in buffer F. The

immunoferritin-labeled sections were postfixed first for 10 min in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and then for 10 min in 2% uranyl acetate, neutralized to pH 7.0 with potassium oxalate, to stabilize the membranes (38). Finally, the sections were adsorption stained and postembedded in a mixture of 0.02% uranyl acetate, pH 4, 0.1% methylcellulose (1,500 cp [centipoise]) (40) and 1.9% polyethylene glycol (1,540 daltons) as described by Tokuyasu (39). After 1 min, excess staining solution was withdrawn and the sections were air-dried at room temperature. Sections were examined with a Philips 300 electron microscope.

RESULTS

Antibody Specificity

The specificity of the rabbit antiserum to the electrophoretically purified Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum from rat skeletal muscle was demonstrated by Ouchterlony's double diffusion test as previously described (13). However, the antiserum did not produce a precipitation line when tested against a microsomal fraction isolated from rat ventricular muscle. The ability of the rat skeletal Ca^{2+} + Mg^{2+}-ATPase antiserum to bind specifically to the Ca^{2+} + Mg^{2+}-ATPase from rat ventricular muscle was nevertheless demonstrated by indirect ^{125}I–protein A immunostaining on Western blots (42) as shown in Fig. 1. The antiserum or affinity-purified antibodies were bound to a single band at 100,000 daltons, corresponding to the Ca^{2+} + Mg^{2+}-ATPase from skeletal muscle when tested against either a homogenate from rat skeletal muscle or a microsomal fraction from rat ventricular muscle. These results strongly suggest that the antibodies to skeletal muscle Ca^{2+} + Mg^{2+}-ATPase also cross-react specifically with the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum in cardiac muscle.

Immunofluorescence Studies

The position of the free and junctional sarcoplasmic reticulum membrane in cardiac muscle has been studied by electron microscopy (9, 29, 34). Although no quantitative analysis has been carried out on the relative amount of sarcoplasmic reticulum membrane surface present in the A- and I-bands, qualitative observations suggest that the distribution of the sarcoplasmic reticulum membrane in the A-band region is quite uniform, with the exception of the H-band region, which appears to have more sarcoplasmic reticulum than the rest of the A-band region (43). In the I-band region there appears to be more sarcoplasmic reticulum membrane close to the Z-line. It has been reported that the sarcoplasmic reticulum membrane closely apposed to the sarcolemma (peripheral junctional sarcoplasmic reticulum membrane) is frequently observed in cardiac muscle from many species (1, 9), including the rat (31).

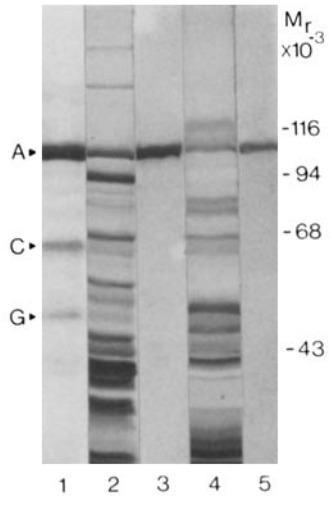


FIGURE 1 Detection of the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum in a microsomal fraction from rat ventricular muscle. Lanes *1*, *2*, and *4*, Coomassie Blue staining pattern of proteins separated on a 7.5% polyacrylamide gel (23). Lanes *3* and *5*, autoradiogram of Western blot of muscle proteins labeled with affinity-purified antibodies to the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum from skeletal muscle by the indirect ^{125}I-protein A immunoassay. Lane *1*, 20 μg of sarcoplasmic reticulum protein purified from rat skeletal muscle. Lanes *2* and *3*, 100 μg of postmitochondrial supernatant from rat skeletal muscle. Lanes *4* and *5*, 100 μg of the microsomal fraction isolated from rat ventricular muscle as described in Material and Methods. (*A*) Ca^{2+} + Mg^{2+}-ATPase; (*C*) calsequestrin, and (*G*) 53,000-dalton glycoprotein.

These observations make it possible to examine, at the light-microscope level, whether the Ca^{2+} + Mg^{2+}-ATPase is uniformly distributed within the sarcoplasmic reticulum membrane or whether it is confined to certain regions of this membrane. This was done by comparing the fluorescent staining pattern with the position of the A- and I-bands in the same field as determined by phase-contrast microscopy in longitudinal 4- to 6-μm cryostat sections of a cardiac myofiber after labeling with antibodies to the Ca^{2+} + Mg^{2+}-ATPase. In longitudinal cryostat sections of unfixed adult rat papillary muscle ranging in sarcomere length from 1.9 to 2.6 μm, an intense, regular, fluorescent staining was present throughout the I-band region (Fig. 2*A* and *C–E*). A regular staining pattern, limited to the center of the A-band region, could frequently be seen with this antibody (Fig. 2*A* and *D*). Faintly stained strands running parallel to the longitudinal axis of the myofiber could occasionally be observed in the A-band region (Fig. 2*C* and *D*). It should be noted that the intercalated disk remained unlabeled (Fig. 2*A* and *D*).

The resolution of the staining pattern after anti-ATPase labeling of longitudinal semithin sections of fixed papillary muscle (Fig. 3) was greatly improved compared to that obtained from the 4- to 6-μm cryostat sections of unfixed tissue (Fig. 2). Intense fluorescent staining was still present in the I-band region (Fig. 3*A*) but the continuous fluorescent strand observed throughout the I-band in the 4- to 6-μm cryostat sections (Fig. 2) was, in the semithin sections, frequently observed to be composed of a transversely oriented row of short fluorescent rods perpendicular to the longitudinal axis of the cell (Fig. 3*A*), because the presence of the mitochondria in the interfibriller spaces (Fig. 3*B*) interrupted the continuity of the staining pattern in the I-band region. Intense fluorescent labelings located between myofibrils was frequently observed (Fig. 3*A*). Comparison between the phase-contrast micrograph(s) and the immunofluorescent micrograph(s) of the same field in the semithin sections (Fig. 3*A* and *B*) clearly indicated that the staining pattern observed after labeling with anti-ATPase was confined to the interfibrillar spaces while intercalated disks, mitochondria, nuclei, and blood vessels (endothelial cells, erythrocytes) were free of fluorescent staining (Figs. 3*A* and *B*).

In transverse, semithin sections of fixed papillary muscle treated with antibodies to rat skeletal muscle Ca^{2+} + Mg^{2+}-ATPase, a polygonal staining pattern was observed within each myofiber (Fig. 3*C*). The number of sides as well as the length of the sides varied from polygon to polygon. The fluorescently stained sides of the polygons were frequently observed to be composed of a row of fluorescent spots. In addition, a discontinuous labeling pattern was observed in the periphery of each myofiber.

To demonstrate the specificity of the staining patterns observed in rat papillary muscle with the antibodies to the Ca^{2+} + Mg^{2+}-ATPase of sarcoplasmic reticulum from rat skeletal muscle, the supernatant from Ca^{2+} + Mg^{2+}-ATPase-adsorbed antibodies to skeletal muscle Ca^{2+} + Mg^{2+}-ATPase were used in the indirect immunofluorescent staining procedure. As

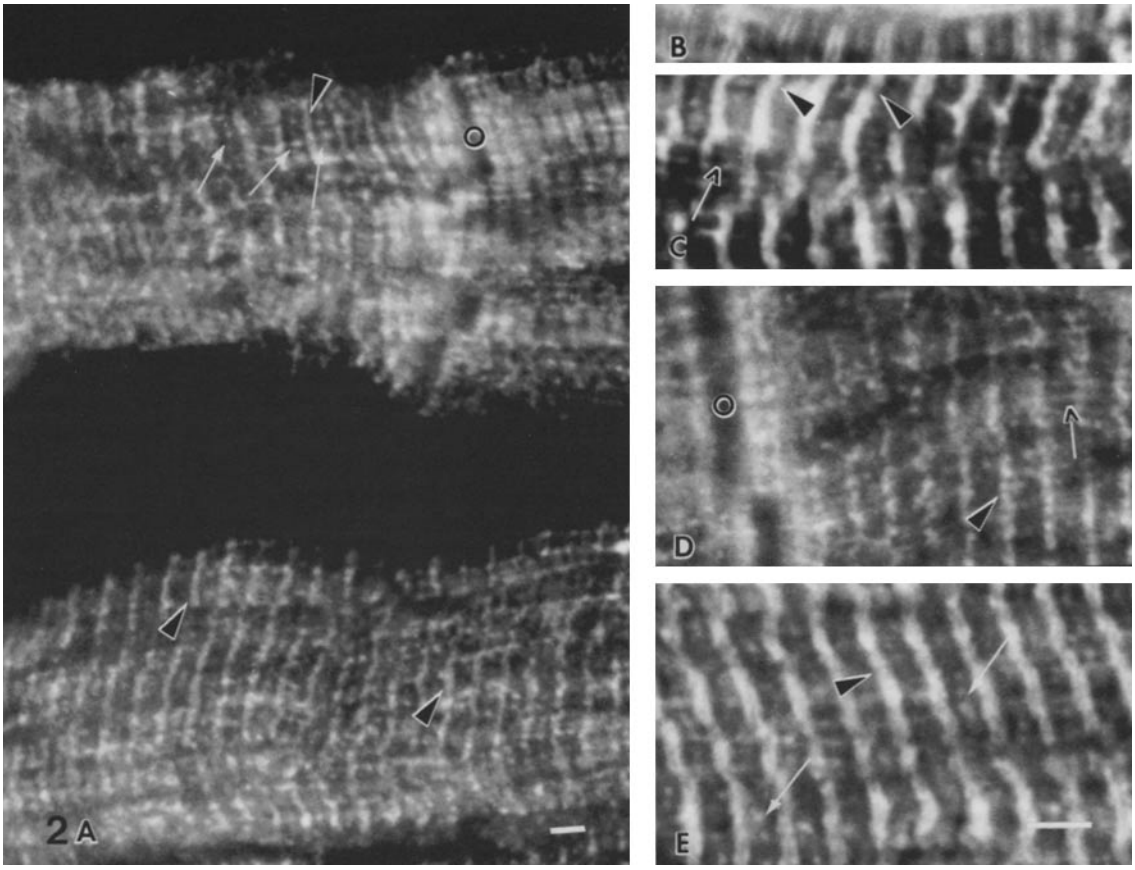


FIGURE 2 Light micrograph of longitudinal cryostat sections (4–6 μm) of unfixed rat papillary myofibers, labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies (*A* and *C–E*). The fluorescent staining pattern in *C* was compared to the position of the A and I bands in the same field as seen by phase-contrast microscopy in *B*. The width of the fluorescent staining present in the I-band regions (black arrowhead) of *A* and *C–E* equalled the width of the corresponding I-band (only shown for *C*). Staining of the center of the A-band region (white arrow) was apparent in *A* and *E*. Faintly fluorescent strands running parallel to the longitudinal axis of the myofiber could be seen in *C* and *D* (black arrows). It should be noted that the intercalated discs (black circles) remained unlabeled. Bar, 2 μm.

shown in Fig. 3 *D* the staining pattern described above could not be detected. It should be noted that a faint nonspecific fluorescent labeling was observed on the red cells and the myofibrils after adsorption of the specific labeling of Ca^{2+} + Mg^{2+}-ATPase. Similarly, no fluorescent staining was observed when the affinity-purified rabbit antibodies to the Ca^{2+} + Mg^{2+}-ATPase were substituted with preimmune rabbit γ-globulin in the immunofluorescence tests (not shown).

Immunoferritin Labeling

The distribution of the Ca^{2+} + Mg^{2+}-ATPase, as observed by the indirect immunofluorescence staining technique, corresponded in general to the density of the sarcoplasmic reticulum membrane surface area in the various band regions. However, in order to determine the localization and distribution of the Ca^{2+} + Mg^{2+}-ATPase more precisely, these studies were extended to the ultrastructural level.

Longitudinal ultrathin frozen sections of fixed adult rat papillary muscle, were first labeled with antibodies to the Ca^{2+} + Mg^{2+}-ATPase by the indirect immunoferritin-electron microscopic labeling technique and subsequently adsorption stained with 0.02% uranylacetate in a mixture of 1.9% polyethylene glycol (1,540 daltons) and 0.1% methylcellulose (1,500 cp). This positive staining procedure resulted in a clear definition of cellular membranes in cardiac muscle, including those of mitochondria (*M*; Figs. 4 and 5 *b*), transverse tubules (*T*; Figs. 4 and 5), the sarcolemma (*SL*; Figs. 4 and 6), subsarcolemmal vesicles (Figs. 4 and 6), longitudinal sarcoplasmic reticulum (SR; Fig. 5 *a*), and interior junctional sarcoplasmic reticulum (*i-J-SR*; Fig. 5 *b*).

To date, adsorption staining of ultrathin frozen sections has not visualized the "feet" which in Epon sections have been shown to bridge interior and peripheral junctional sarcoplasmic reticulum membrane with transverse tubular membrane and sarcolemma, respectively. Although interior junctional sarcoplasmic reticulum membrane could quite easily be identified due to its close apposition to the transverse tubules (Fig. 5 *b*), it was not possible, with certainty, to determine which of the subsarcolemmal vesicles were caveolae, free sarcoplasmic reticulum membrane, or junctional sarcoplasmic reticulum membrane.

Assuming that the center of the ferritin core labeling an antigen marker on a membrane by the indirect immunoferritin labeling technique is <30 nm away from the membrane (6) we can conclude that the sarcoplasmic reticulum membrane was densely labeled with ferritin particles while the mitochondria (*M*; Figs. 4 and 5 *b*) and the sarcolemma (*SL*; Figs. 4 and 6), including the caveolae (*C*; Fig. 6) were labeled only at background levels (Fig. 7). Although it appears that the number of

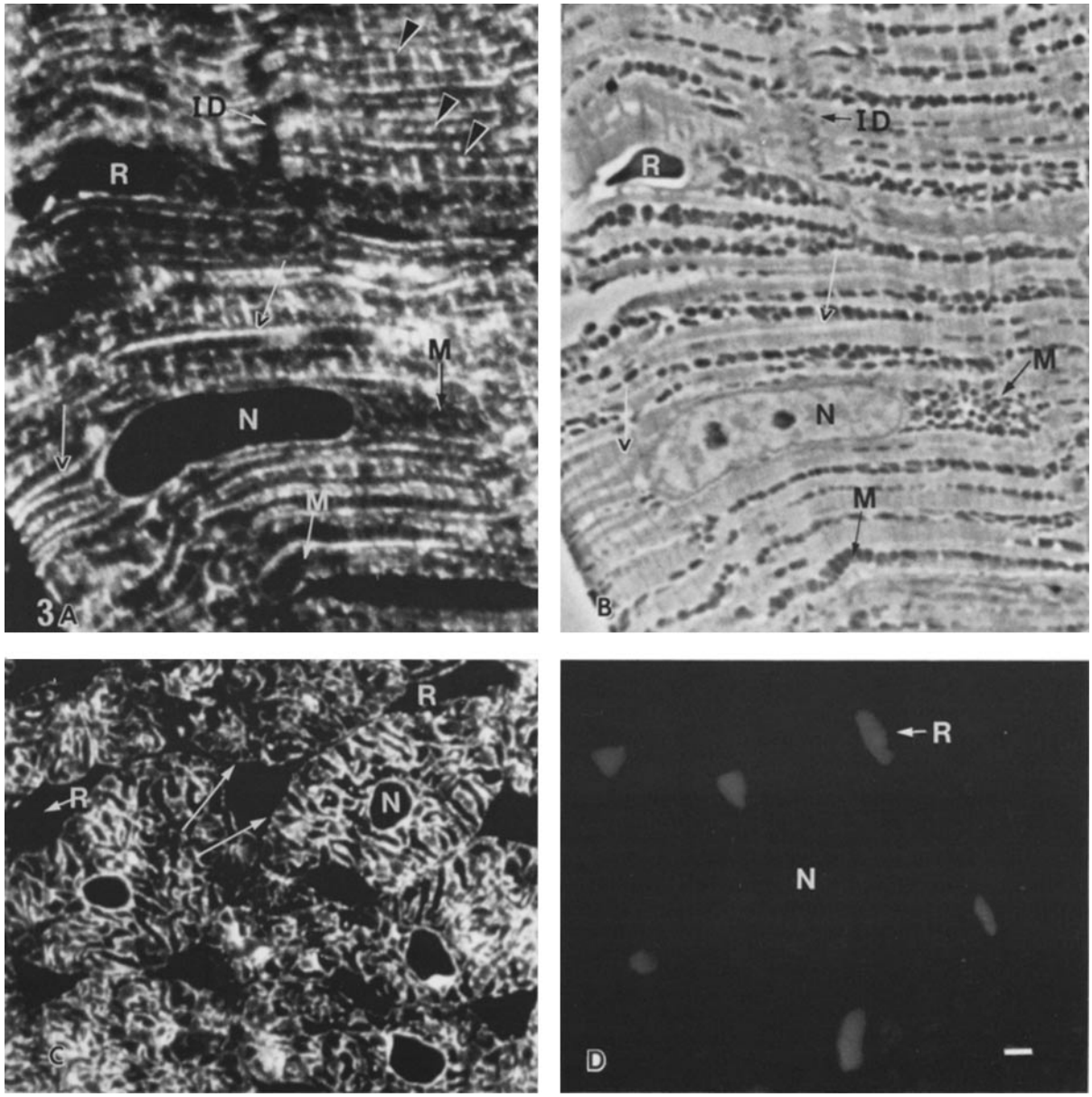


FIGURE 3 Light micrographs of longitudinal (*A* and *B*) and transverse (*C* and *D*) semithin sections (0.5–1.0 μm) of fixed rat papillary muscle. (*A*) A section labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies by the indirect immunofluorescent staining technique is compared with *B*, a phase-contrast micrograph of the same field. The fluorescent staining pattern observed after labeling with Ca^{2+} + Mg^{2+}-ATPase antibodies was confined to the I-band region (black arrowheads) and interfibrillar spaces (black arrows), whereas intercalated disks (*ID*), mitochondria (*M*), nuclei (*N*), and blood vessels (*R*) were only labeled at background level. (*C*) A section labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies is compared with (*D*) a section labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies adsorbed with Ca^{2+} + Mg^{2+}-ATPase from rat skeletal muscle. The polygonal staining pattern observed in each myofiber in *C* was very similar, but the number and length of the sides of the polygons was varied. Notice that the fluorescent labeling in the cell peripheries was discontinuous. After adsorption (*D*) the specific staining pattern observed in *C* is absent, however a low level of nonspecific fluorescent staining was present on the erythrocytes (*R*) and myofibrils. To demonstrate this nonspecific fluorescent staining, the field shown in *D* was exposed twice as long as that shown in *C*. Bar, 2 μm.

ferritin particles on the mitochondrion at the lower left of Fig. 4 as well as in certain regions of the myofibril is somewhat higher than could be accounted for by nonspecific background staining, it is suggested that, as previously discussed (19), the excess ferritin particles in these regions most probably are examples of labeling of the Ca^{2+} + Mg^{2+}-ATPase present in obliquely and tangentially cut membranes which are difficult to visualize.

Simultaneous visualization of the sarcoplasmic reticulum membranes and of ferritin particles showed that the density of ferritin particles throughout the free sarcoplasmic reticulum membrane was quite uniform (Figs. 4–6), whereas the interior junctional sarcoplasmic reticulum membrane was only labeled at background level (Fig. 5 *a* and *b*). These results are comparable to our earlier results on the localization of the Ca^{2+} + Mg^{2+}-ATPase in skeletal muscle (19) in which we observed that the density of ferritin particles on the free sarcoplasmic reticulum membrane was much higher than on the interior junctional sarcoplasmic reticulum membrane.

In the subsarcolemmal region of the myofiber three kinds of

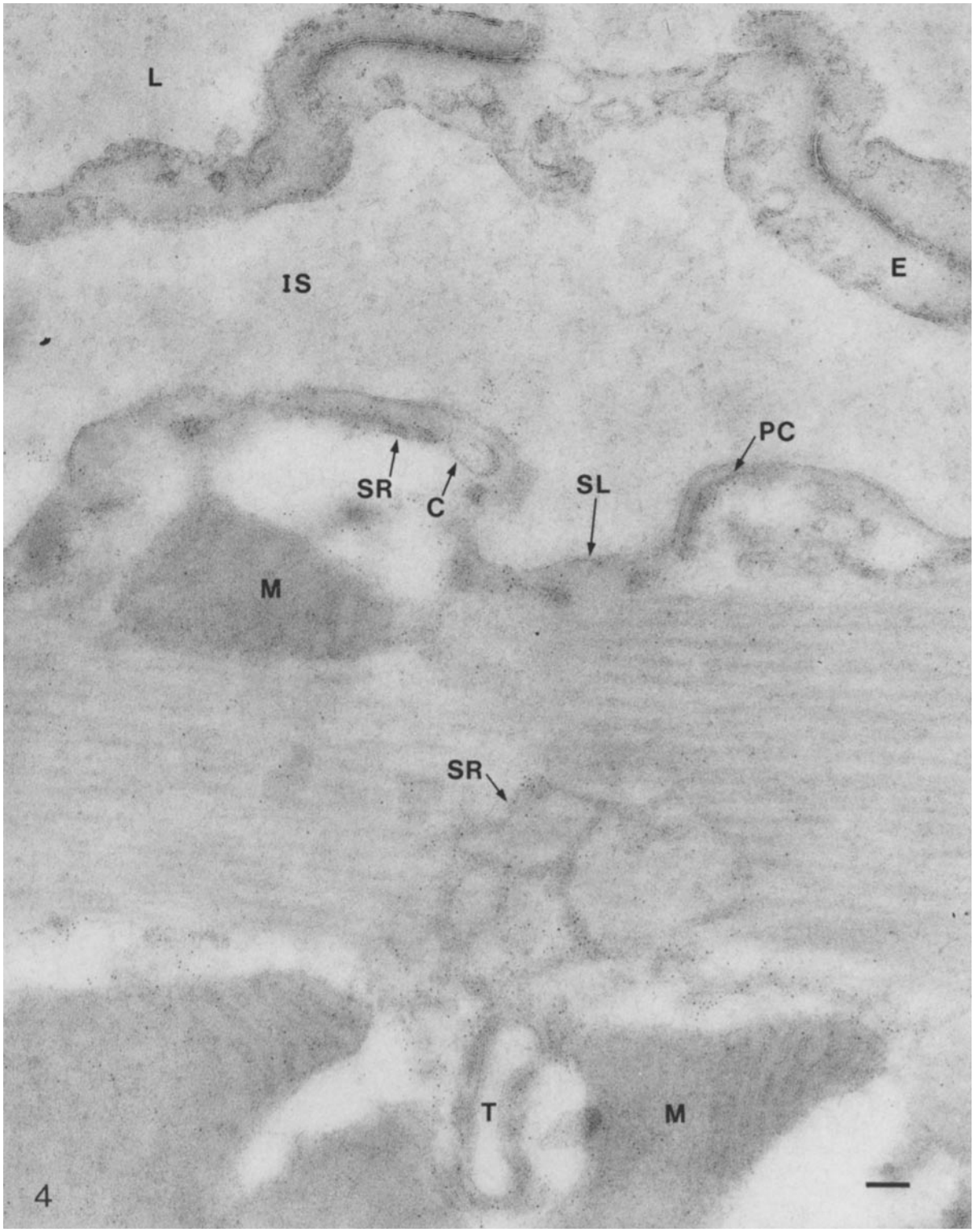
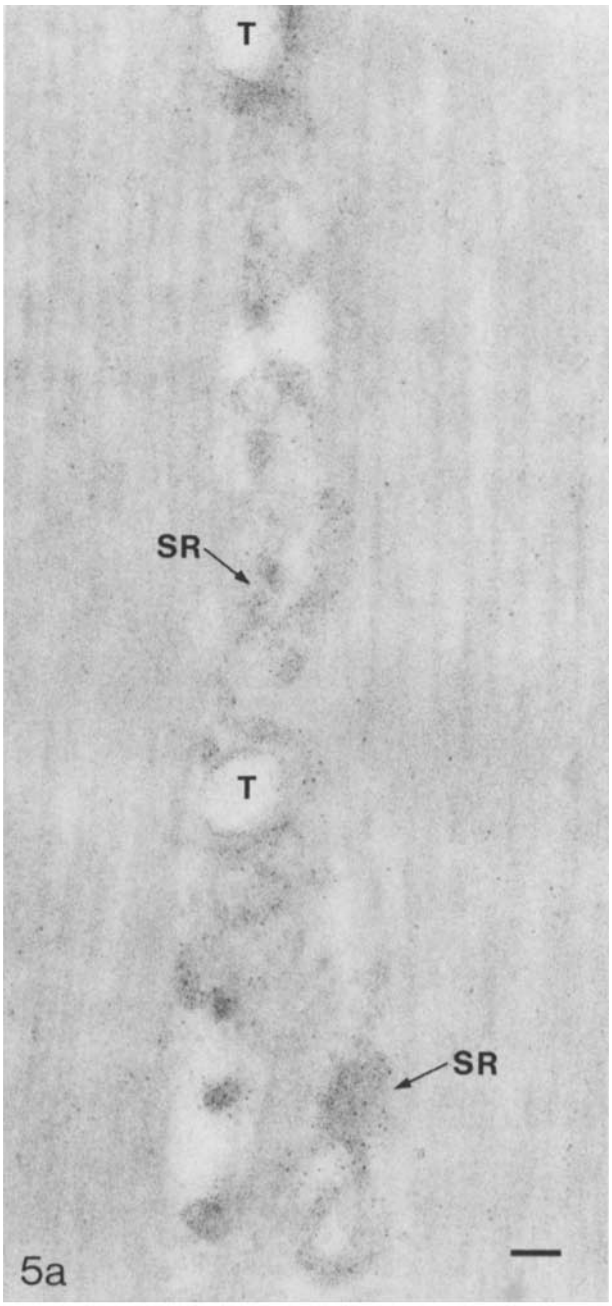
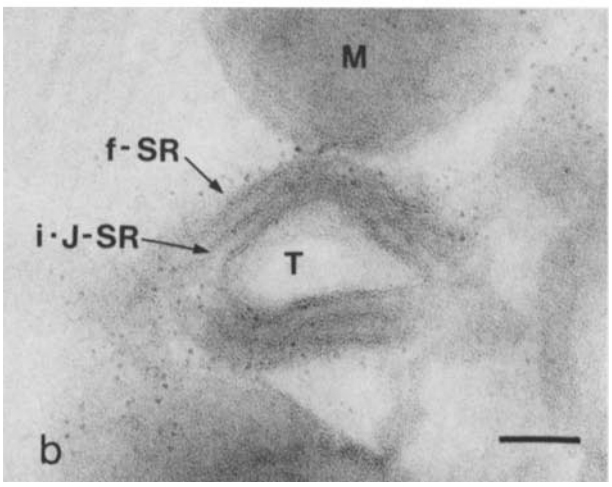


FIGURE 4 Electron micrograph of a longitudinal section of fixed rat papillary muscle labeled with antibodies to the Ca^{2+} + Mg^{2+}-ATPase by the indirect immunoferritin staining technique and then adsorption stained to visualize membranes. Many ferritin particles were present on sarcoplasmic reticulum (*SR*), the myofibrillar side of peripheral junctional complex (*PC*) and some (for example *SR* and *PC*) but not all (*C*) subsarcolemmal vesicles, while few were observed over the transverse tubules (*T*), the sarcolemmal side of peripheral junctional complex (*PC*), the mitochondria (*M*), the intercellular space (*IS*), the endothelial cell (*E*), the lumen of the capillary (*L*) and the sarcolemma (*SL*). Bar, 0.1 μm.

membrane-bound vesicles were distinguished by their content and distribution of ferritin particles. One type of vesicle was uniformly labeled with ferritin particles on both the sarcolemmal and the myofibrillar side of the vesicle (*SR*; Fig. 6). Another was observed to be more densely labeled on the myofibrillar side than on the sarcolemmal side (*PC*; Fig. 6),





while the third type was only labeled at background level (*C*; Fig. 6).

DISCUSSION

The presence of the Ca^{2+} + Mg^{2+}-ATPase in cardiac sarcoplasmic reticulum membranes is well-documented and it is generally believed that this protein, as in skeletal muscle, actively transports Ca^{2+} from the myofibril to the lumen of the sarcoplasmic reticulum when relaxation sets in. The Ca^{2+} + Mg^{2+}-ATPase from cardiac muscle is similar biochemically to that of the skeletal muscle, although several recent reports support the idea that the regulation of the Ca^{2+} + Mg^{2+}-ATPase in cardiac and skeletal muscle differs (24, 26, 36).

The Ca^{2+} + Mg^{2+}-ATPases from cardiac skeletal muscle are related antigenically as demonstrated by the ability of the affinity-purified antibodies to the Ca^{2+} + Mg^{2+}-ATPase from rat skeletal muscle to cross-react specifically with a component of the microsomal fraction from rat ventricular muscle that coelectrophoreses with the Ca^{2+} + Mg^{2+}-ATPase from rat skeletal muscle. This finding strongly indicates that the affinity-purified antibody to skeletal muscle Ca^{2+} + Mg^{2+}-ATPase is binding to the Ca^{2+} + Mg^{2+}-ATPase of the cardiac sarcoplasmic reticulum. This result is in agreement with previous studies using Ouchterlony's double diffusion test (12) and complement fixation (5), which reported that the Ca^{2+} + Mg^{2+}-ATPase from cardiac and skeletal muscle are antigenically related.

The immunofluorescent studies of 4- to 6-μm sections suggested that the Ca^{2+} + Mg^{2+}-ATPase is confined to the sarcoplasmic reticulum. In agreement with this interpretation, fluorescent labeling was absent from the intercalated disks, which are specialized regions of sarcolemma (9). Labeling of semithin sections confirmed these findings and, in addition, demonstrated that the mitochondria were also free of fluorescent labeling. Furthermore, the fluorescent labeling observed in the periphery of the cell in transverse sections was discontinuous and this suggested that the Ca^{2+} + Mg^{2+}-ATPase was absent from the sarcolemma but present in subsarcolemmal vesicles.

The immunoferritin labeling of ultrathin frozen sections provided far more detailed information regarding the precise localization of the Ca^{2+} + Mg^{2+}-ATPase in rat papillary muscle. As suggested by the immunofluorescence studies, the immunoferritin labeling of the Ca^{2+} + Mg^{2+}-ATPase was almost exclusively confined to the sarcoplasmic reticulum membranes and was absent from the mitochondrial, transverse tubular, and sarcolemmal membranes. This result is in agreement with some recent, elegant biochemical studies (2, 3) that have demonstrated that the Ca^{2+} + Mg^{2+}-ATPase enzyme in cardiac sarcolemmal vesicles is distinct from the Ca^{2+} + Mg^{2+}-ATPase in the cardiac sarcoplasmic reticulum (16, 24, 36, 46). Although

FIGURE 5 Electron micrographs of longitudinal sections of rat papillary muscle labeled with antibodies to the Ca^{2+} + Mg^{2+}-ATPase and adsorption stained. (*a*) Many ferritin particles were present along the entire longitudinal sarcoplasmic reticulum membrane (*SR*) while the transverse tubular membrane (*T*) was only labeled at background level. (*b*) Most ferritin particles in the dyad region were found over the free sarcoplasmic reticulum (*f-SR*) while very few were present over the interior junctional sarcoplasmic reticulum (*i-J-SR*) and the transverse tubule membrane. Note that the mitochondria (*M*) were only labeled at background level. Bar, 0.1 μm.

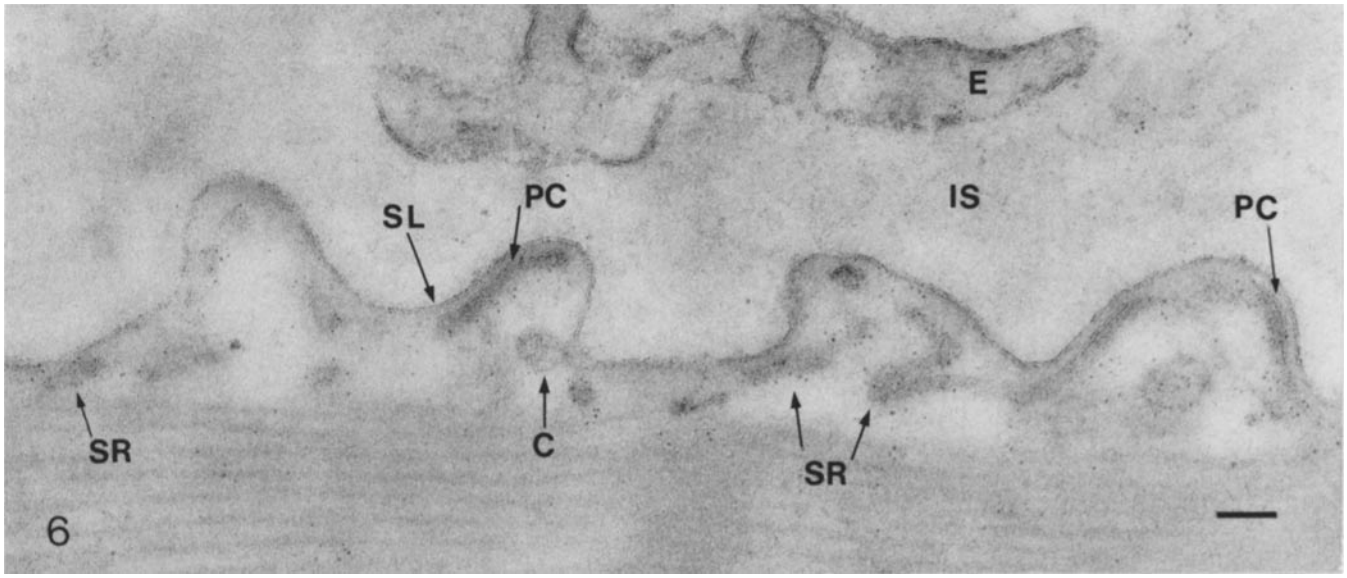


FIGURE 6 Electron micrograph of a longitudinal section of rat papillary muscle labeled with antibodies to the Ca^{2+} + Mg^{2+}-ATPase and adsorption stained. In the subsarcolemmal region most ferritin particles were only found over the subsarcolemmal membrane vesicles (*PC* and *SR*), whereas the sarcolemma (*SL*), the intercellular space (*IS*), and the endothelial cell (*E*) were only labeled at background level. In this region of the myofiber three types of membrane-bound vesicles were observed as characterized by the distribution of the ferritin particles on the vesicles. One type of membrane-bound vesicle (*SR*) was uniformly labeled with ferritin particles on both the sarcolemmal and the myofibrillar side of the vesicle and was tentatively identified as free sarcoplasmic reticulum. Type two (*PC*) was observed to be more densely labeled on the myofibrillar side than on the sarcolemmal side of the vesicles and was tentatively identified as peripheral junctional complex between sarcoplasmic reticulum and the sarcolemma while the third type of vesicle (*C*) was only labeled at background level and was tentatively identified as caveolae. Bar, 0.1 μm.

several investigators have previously reported on the presence of a Ca^{2+} + Mg^{2+}-ATPase in the cardiac sarcolemma (7, 14, 28, 35), it is probable that some of these findings were due to contamination of the sarcolemmal membrane preparation with sarcoplasmic reticulum, as suggested by other investigators (8, 15).

With regard to the localization of the Ca^{2+} + Mg^{2+}-ATPase within the various regions of the sarcoplasmic reticulum membrane, our results have shown that the Ca^{2+} + Mg^{2+}-ATPase was fairly uniformly distributed throughout the sarcoplasmic reticulum membrane, with the exception of the region of the interior and peripheral junctional sarcoplasmic reticulum membrane where less-dense labeling was observed.

The interior junctional sarcoplasmic reticulum membrane is easily identified in the positively stained, ultrathin frozen sections because of its close apposition to T-tubules. However, since the "feet" were not visualized by the positive staining technique, used in these studies, it was more difficult to determine with certainty which of the subsarcolemmal membrane vesicles were indeed peripheral junctional sarcoplasmic reticulum membranes. The distribution of ferritin particles on the subsarcolemmal vesicles suggested that there were three kinds of vesicles. Some vesicles were uniformly labeled, others were preferentially labeled on the myofibrillar side of the vesicle, while the remaining vesicles were only labeled at background level. We suggest that the uniformly labeled membrane vesicles correspond to the free sarcoplasmic reticulum membrane present in this region of the cell (9, 34), the vesicles preferentially labeled on the myofibrillar side correspond to peripheral junctional sarcoplasmic reticulum, and the unlabeled vesicles correspond to caveolae, which have been demonstrated to be of sarcolemmal origin. This suggestion is supported by the observations that most of the uniformly labeled vesicles were not in close apposition to the sarcolemmal membrane, that membrane vesicles preferentially labeled on the myofibrillar side were closely apposed to the sarcolemmal membranes and that most of the unlabeled vesicles were spherical as are caveolae membranes.

This interpretation implies that the Ca^{2+} + Mg^{2+}-ATPase of the sarcoplasmic reticulum is absent from both the internal and peripheral junctional sarcoplasmic reticulum membrane but that it is quite uniformly distributed in the free sarcoplasmic reticulum membrane. Furthermore, until differences in composition between internal and peripheral junctional sarcoplasmic reticulum membrane can be demonstrated, one might conclude that both types of junctions have the same function. The lumen of both peripheral and interior junctional sarcoplasmic reticulum has been reported to contain electron-dense material (9, 34). In skeletal muscle this electron-dense material is believed to correspond to calsequestrin (30).

In a recent study, Wendt-Gallitelli et al. (47) reported that the Ca^{2+} concentration in the lumen of the interior junctional sarcoplasmic reticulum in papillary muscle is high during relaxation and below the level of detection during contraction. This finding suggests that Ca^{2+} is stored in this region of the cardiac sarcoplasmic reticulum during relaxation as it is in skeletal muscle. However, the Ca^{2+} concentration of the lumen of the sarcoplasmic reticulum in the peripheral junction was not reported in this study.

In a recent study we have demonstrated the presence of a cardiac form of calsequestrin in dog ventricular muscle.[1] Several biochemical characteristics of cardiac calsequestrin were

[1] Campbell, K. P., D. H. MacLennan, and A. O. Jorgensen. Identification of calsequestrin and the intrinsic glycoprotein in canine cardiac sarcoplasmic reticulum. Submitted for publication.

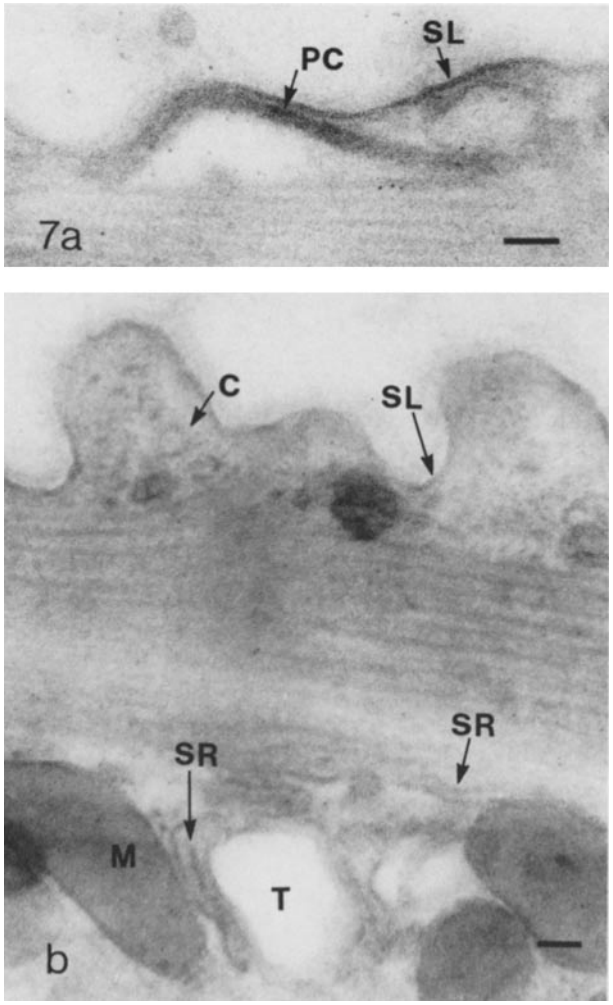


FIGURE 7 Electron micrographs of longitudinal sections of fixed rat papillary muscle labeled with normal rabbit γ-globulin by the indirect immunoferritin staining technique and then adsorption stained to visualize membranes (*a* and *b*). Note that very few ferritin particles are observed on the membranes present on the ultrathin frozen sections including the sarcoplasmic reticulum (*SR*) and the peripheral complexes (*PC*). For interpretation of the letters on the electron micrographs see the legend to Fig. 4.

found to be similar to those of skeletal calsequestrin. In addition cardiac calsequestrin appeared to be related antigenically to skeletal calsequestrin as judged by the ability of dog ventricular calsequestrin to bind rabbit antisera to rat calsequestrin in the indirect ^{125}I–protein A immunoassay on Western blots. An attempt is presently being made to use affinity-purified antibodies to calsequestrin from rat skeletal muscle to determine the localization of calsequestrin in rat cardiac muscle by immunohistochemical labeling techniques.

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