Ultrastructural Localization of the Ca^{2+} + Mg^{2+}-dependent ATPase of Sarcoplasmic Reticulum in Rat Skeletal Muscle by Immunoferritin Labeling of Ultrathin Frozen Sections

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ABSTRACT   The ultrastructural localization of the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum in rat gracilis muscle was determined by indirect immunoferritin labeling of ultrathin frozen sections. Simultaneous visualization of ferritin particles and of adsorption-stained cellular membranes showed that the Ca^{2+} + Mg^{2+}-ATPase was concentrated in the longitudinal sarcoplasmic reticulum and in the nonjunctional regions of the terminal cisternae membrane but was virtually absent from mitochondria, plasma membranes, transverse tubules, and junctional sarcoplasmic reticulum. Ferritin particles were found preponderantly on the cytoplasmic surface of the membrane, in agreement with published data showing an asymmetry of the Ca^{2+} + Mg^{2+}-ATPase within the sarcoplasmic reticulum membrane. Comparison of the density of ferritin particles in fast and slow myofibers suggested that the density of the Ca^{2+} + Mg^{2+}-ATPase in the sarcoplasmic reticulum membrane in a fast myofiber is approximately two times higher than in a slow myofiber.

The sarcoplasmic reticulum is the intracellular membrane system of skeletal muscle that controls sarcoplasmic Ca^{2+} concentrations, thereby regulating the contraction-relaxation cycle (4, 11, 28). It creates a separate membranous compartment in muscle cells that surrounds each myofibril like a fenestrated sleeve (8, 10, 21). The membrane system *in situ* is composed of voluminous, matrix-filled terminal cisternae abutting each tranverse tubule, and essentially empty, longitudinal elements. Fragmented sarcoplasmic reticulum has been separated by density gradient centrifugation into heavy and light fractions (3, 19). Both heavy and light fractions contained the Ca^{2+} + Mg^{2+}-dependent ATPase (Ca^{2+} + Mg^{2+}-ATPase), the protein concerned with Ca^{2+} uptake (16, 17), but only the heavy fraction contained calsequestrin, the protein that probably functions as a luminally located, calcium-sequestering agent (17, 18). Meissner (19) noted a correlation between the presence of matrix material in the heavy fraction and the presence of calsequestrin and proposed that the matrix was composed of calsequestrin. Moreover, since matrix material is limited to the terminal cisternae *in situ*, Meissner (19) proposed that the heavy

vesicles originated in the terminal cisternae and that calsequestrin was localized in the terminal cisternal region of the sarcoplasmic reticulum.

These findings strongly support the idea that certain functions of the sarcoplasmic reticulum are restricted to specific regions of this membrane system. To determine whether the heterogeneity of sarcoplasmic reticulum vesicles is also reflected in a nonuniform distribution of sarcoplasmic reticulum proteins *in situ*, we previously localized the two major protein components of this membrane system, the Ca^{2+} + Mg^{2+}-ATPase and calsequestrin, on cryostat sections from adult rat skeletal muscle by the indirect immunofluorescence technique (13). In agreement with the biochemical findings, we found that the Ca^{2+} + Mg^{2+}-ATPase was rather uniformly distributed throughout the sarcoplasmic reticulum whereas calsequestrin was located mainly in the regions that corresponded to the terminal cisternae of the membrane system (18). To determine the distribution of calsequestrin and the Ca^{2+} + Mg^{2+}-ATPase more precisely we have extended our studies to the ultrastructural level in rat skeletal muscle. In this paper, we report on

the ultrastructural localization of the $Ca^{2+} + Mg^{2+}$-ATPase by indirect, immunoferritin surface labeling of ultrathin, frozen sections (23-27).

MATERIALS AND METHODS

Dissection and Fixation of Myofibers

Bundles of myofibers (1-2 mm diameter), dissected from the gracilis muscle of adult female rats, were tied to applicator sticks and fixed for 1 h in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in buffer A (0.1 M sodium cacodylate buffer, pH 7.2, and 4.5 mM $CaCl_2$) and then washed as previously described (13). Fixed muscle tissue, stored at 4°C in buffer A for up to 4 d, was used for ultracryotomy.

Ultracryotomy

Procedures were carried out as described by Tokuyasu and Singer (23, 27) except that the tissue was infused for 20-40 min with buffer B (0.6 M sucrose in 0.1 M sodium cacodylate, pH 7.4). The temperature for sectioning was −80°C.

Purification of Rat ATPase

Rat $Ca^{2+} + Mg^{2+}$-ATPase was prepared by procedures similar to those used for the purification of rabbit ATPase (16), except that the fractionation in ammonium acetate was carried out at pH 8.35.

Preparation of Antibodies

The rabbit anti-rat ATPase serum previously characterized (12) was used to localize the ATPase. The specificity of this antiserum for the ATPase was demonstrated by reacting the antiserum in Ouchterlony double-diffusion tests against solubilized rat sarcoplasmic reticulum or purified rat ATPase. Only a single precipitin line was obtained in both cases. No precipitin line was observed when normal sera were used. The antiserum did not cross-react with purified rat calsequestrin or with the high affinity Ca^{2+}-binding protein (16). Specific antibodies to the ATPase were isolated from the antiserum, using insoluble, purified, rat ATPase as previously described (14).

Ferritin-conjugated goat anti-rabbit antibodies were prepared by conjugating horse spleen ferritin (a gift from Dr. S. J. Singer, Department of Biology, University of California at San Diego, Calif.) to affinity-purified, goat anti-rabbit antibodies (15). Goat anti-rabbit antibodies were purified by affinity chromatography of goat anti-rabbit serum on a rabbit globulin affinity column (1). The goat anti-rabbit serum was a gift from Dr. J. F. Ash (Department of Anatomy, College of Medicine, University of Utah, Salt Lake City, Utah).

Indirect Immunoferritin Labeling of the $Ca^{2+} + Mg^{2+}$-ATPase

In general, the immunoferritin labeling technique was carried out according to procedures developed by Tokuyasu (23-27). Ultrathin frozen sections on grids were treated with 2% gelatin in 0.1 M Tris-HCl, pH 7.4, for 2-3 min before immunoferritin labeling (27). The sections were incubated with affinity-purified antibodies to the $Ca^{2+} + Mg^{2+}$-ATPase at a concentration of 20-30 μg/ml in buffer C (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.2) for 15 min at room temperature.
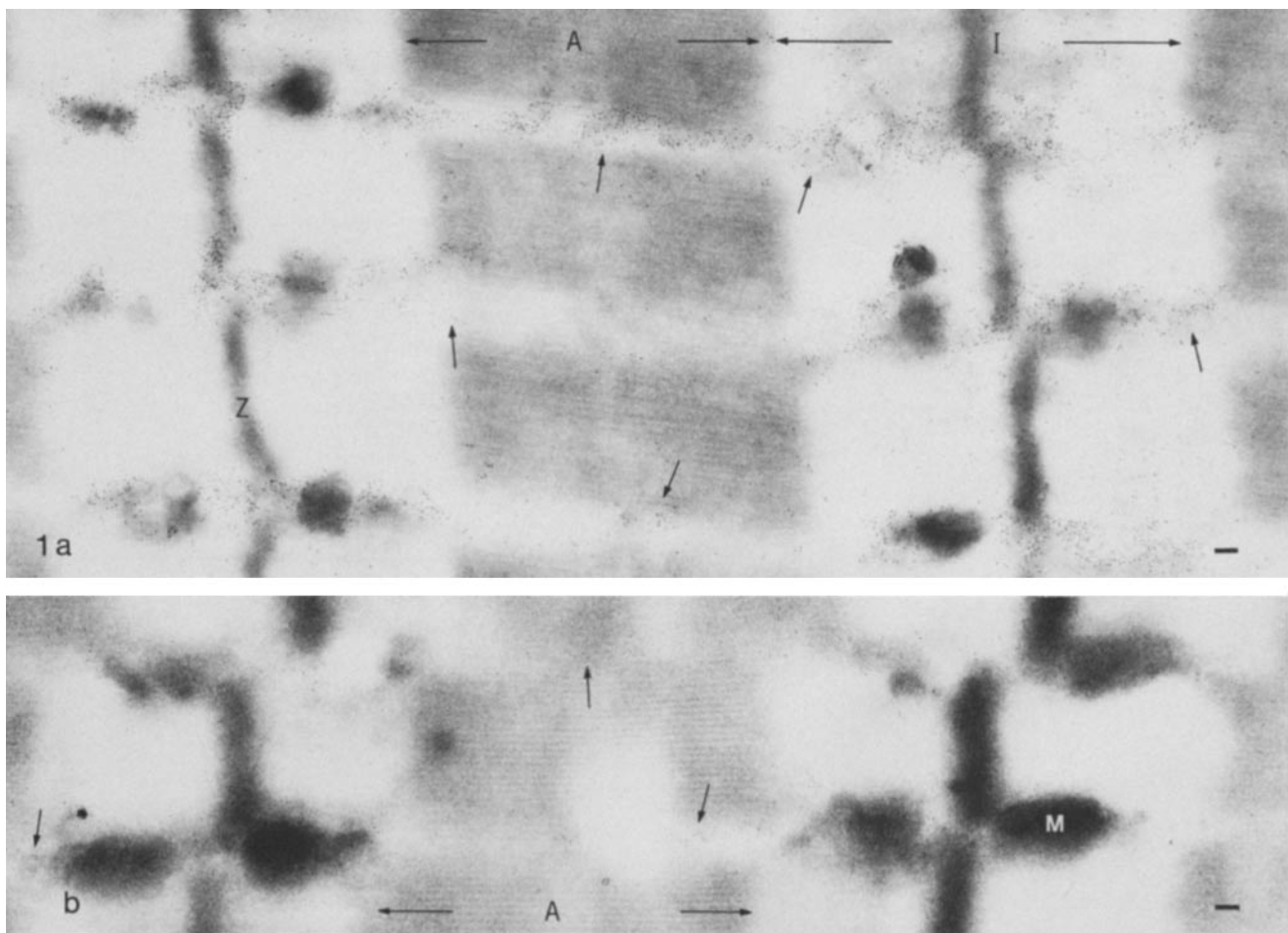

FIGURE 1 Electron micrographs of longitudinal sections of fast (a) and slow (b) rat skeletal muscle fibers labeled with antibodies to the $Ca^{2+} + Mg^{2+}$-ATPase. Membranes were not visualized in these sections because the adsorption staining was done in the absence of polyethylene glycol. In general, dense ferritin labeling was present in the interfibrillar spaces (arrows) and more ferritin particles were seen in the I band region (I) than in the A band region (A). Note that the density of ferritin particles in the fast fiber (a) was ~3.5-fold higher than in the slow fiber (b). M, mitochondrion; Z, Z line. Bars, 0.1 μm.

The samples were then rinsed four times with buffer C and incubated for 15 min at room temperature with affinity-purified goat anti-rabbit globulin conjugated to horse spleen ferritin at an antibody concentration of 30 μg/ml in 0.1% bovine serum albumin in buffer C. Samples were then washed four times in buffer C. 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 (24). Finally, the sections were adsorption stained and postembedded in a mixture of either (a) 0.02% uranyl acetate, pH 4, and 1.0% methylcellulose (15 cp [centipoises]) (24), or (b) 0.02% uranyl acetate, pH 4, 0.8% polyethylene glycol (1,540 daltons) and 0.2% methylcellulose (400 cp) as described by Tokuyasu (25). After 1 min, excess staining solution was withdrawn and the sections were air-dried. Sections were examined with a Philips 300 electron microscope.

RESULTS

Longitudinal ultrathin frozen sections of adult rat gracilis muscle, containing both slow and fast myofibers, 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% uranyl acetate in 1% methyl cellulose. This positive staining procedure resulted in a clear definition of the Z line, the A band, the I band, and the myosin filaments located in the A band. The membranes of the sarcoplasmic reticulum and other subcellular organelles were not always clearly delineated, however. Densely stained oval structures were tentatively identified as mitochondria (Fig. 1).

The distribution of anti-Ca^{2+} + Mg^{2+}-ATPase labeling in skeletal muscle myofibers was found to be highly nonrandom. The majority of the ferritin particles were present in the interfibrillar regions of the myofibers where the sarcoplasmic reticulum membrane was located (Fig. 1 a and b). Labeling was more pronounced in the I band region than in the A band region. While ferritin particles were present throughout the width of the I band region, they were present mainly in the central region of the A band. Occasionally, ferritin particles

were found to be densely located in a relatively large area of the myofibril (Fig. 2). The faintly stained, reticular structures underlying this area of ferritin particles suggested that they represented tangential sections of the longitudinal sarcoplasmic reticulum. Very few ferritin particles were present on the sections labeled with normal rabbit γ-globulin (Fig. 3 b).

Examination of the density of specific ferritin labeling on neighboring myofibers present in the same thin section, showed that while some myofibers were strongly labeled with anti-Ca^{2+} + Mg^{2+}-ATPase (Fig. 1 a), others were weakly labeled (Fig. 1 b). The general distribution of ferritin particles in the two types of fiber was very similar, and the ferritin labeling of the weakly labeled fiber (Fig. 1 b) was well above the level of background labeling seen in controls (Fig. 3 b).

Results that we obtained previously by combining indirect immunofluorescent labeling of the Ca^{2+} + Mg^{2+}-ATPase with a histoenzymatic assay for alkaline stable myosin ATPase on adjacent sections of adult rat gracilis muscle, showed that both strongly and weakly stained myofibers were present in gracilis muscle and that all of the myofibers strongly labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies corresonded to fast myofibers, whereas myofibers weakly labeled with Ca^{2+} + Mg^{2+}-ATPase antibodies corresponded to slow myofibers. Thus we have concluded that a myofiber strongly labeled with the anti-Ca^{2+} + Mg^{2+}-ATPase by the indirect immunoferritin labeling technique corresponds to a fast myofiber, whereas a myofiber weakly labeled by this technique corresponds to a slow myofiber.

Studies of guinea pig skeletal muscles have demonstrated that the width of the Z line in a slow myofiber is approximately twice the width of the Z line in a fast myofiber (6). In the present studies we observed that the width of the Z line in strongly labeled myofibers (Fig. 1 a) is also approximately twice the width of that in weakly labeled myofibers (Fig. 1 b). This
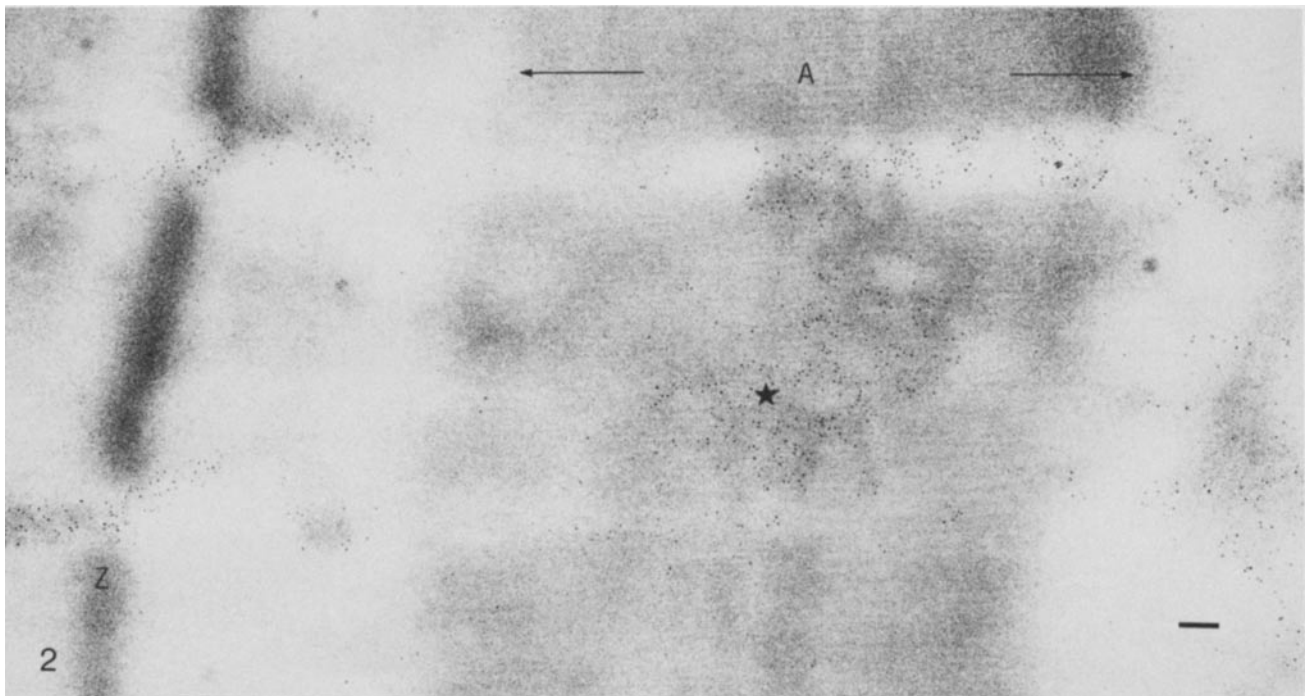

FIGURE 2   Electron micrograph of a longitudinal section of a rat skeletal muscle fiber labeled with antibodies to the Ca^{2+} + Mg^{2+}-ATPase and adsorption stained in the absence of polyethylene glycol. In general, the ferritin labeling pattern corresponds to that shown in Fig. 1. Occasionally ferritin particles were densely clustered in a relatively large area of the myofibril ($\star$) corresponding to a tangential cut of the myofibril that passed through the sarcoplasmic reticulum. Z, Z line; A, A band, Bar, 0.1 μm.

finding supports our conclusion, stated above, that a myofiber strongly labeled with the anti–Ca^{2+} + Mg^{2+}–ATPase by the indirect immunoferritin labeling technique corresponds to a fast myofiber, whereas a myofiber weakly labeled by this technique corresponds to a slow myofiber.

Because of the inefficiency of observing a reasonable number of both strongly and weakly labeled myofibers on the same thin section by the indirect immunoferritin labeling of ultrathin frozen sections, we did not attempt a rigorous quantitation of the differences in density of specific ferritin labeling between the two types of myofibers. On the basis of a limited number of electron micrographs from such thin sections, the density of specific ferritin labeling on sections of strongly labeled myofibers was estimated to be ~3.5-fold higher than that of weakly labeled myofibers. However, this value must be corrected for the difference in densities of the sarcoplasmic reticulum in slow and fast myofibers. The density of sarcoplasmic reticulum in fast myofibers has been found to be ~1.7-fold higher than that of slow myofibers (5–7). Therefore, division of 3.5 by a factor of 1.7 suggests that the density of the Ca^{2+} + Mg^{2+}–ATPase in the sarcoplasmic reticulum membrane of fast myofibers is approximately two times higher than in slow myofibers.

To determine the distribution of the Ca^{2+} + Mg^{2+}–ATPase in the sarcoplasmic reticulum of skeletal muscle *in situ* more

precisely, it is important to visualize the cellular membranes and the electron-dense ferritin antigen marker simultaneously. The procedure for positive staining of cellular membranes in ultrathin tissue sections by adsorption staining (24, 26) has recently been modified so that it is now possible to delineate cellular and intracellular membranes in skeletal muscle (25). Figs. 3–6 demonstrate that adsorption staining clearly delineated the terminal cisternae (*T-SR* and *J-SR*, Figs. 3, 5, and 6), the longitudinal reticulum (*L-SR*, Figs. 3, 5, and 6), the transverse tubules (*T*, Figs. 3–6), the mitochondria (*M*, Fig. 4), and the sarcolemma (*P*, Fig. 4 a) in ultrathin frozen sections. Under conditions that provide optimal membrane staining, the procedure produced a grainy appearance in the sectioned material, which decreased the contrast between the ferritin particles and the stained section, making it more difficult to visualize ferritin (compare the contrast of ferritin particles in Fig. 1 with those in Figs. 5 and 6). Thus, for the simultaneous visualization of membranes and ferritin particles, a less than optimal adsorption staining for membranes was used. As a result, the longitudinal sarcotubules were visualized less frequently than the triadic structure composed of the terminal cisternae and transverse tubules (Fig. 3). In longitudinal sections of rat skeletal muscle labeled first with antibodies to the Ca^{2+} + Mg^{2+}–ATPase by the indirect immunoferritin staining technique and then ad-
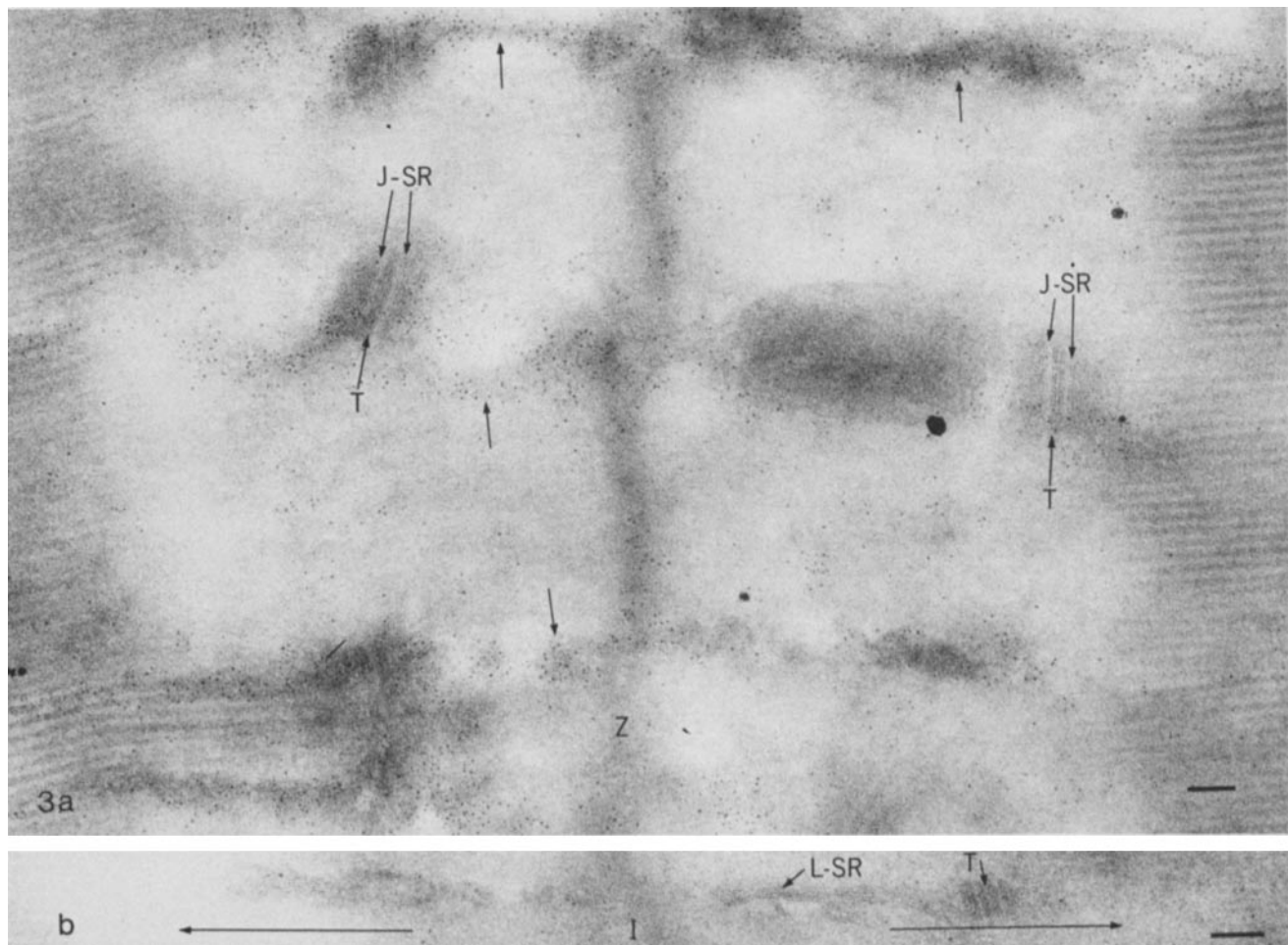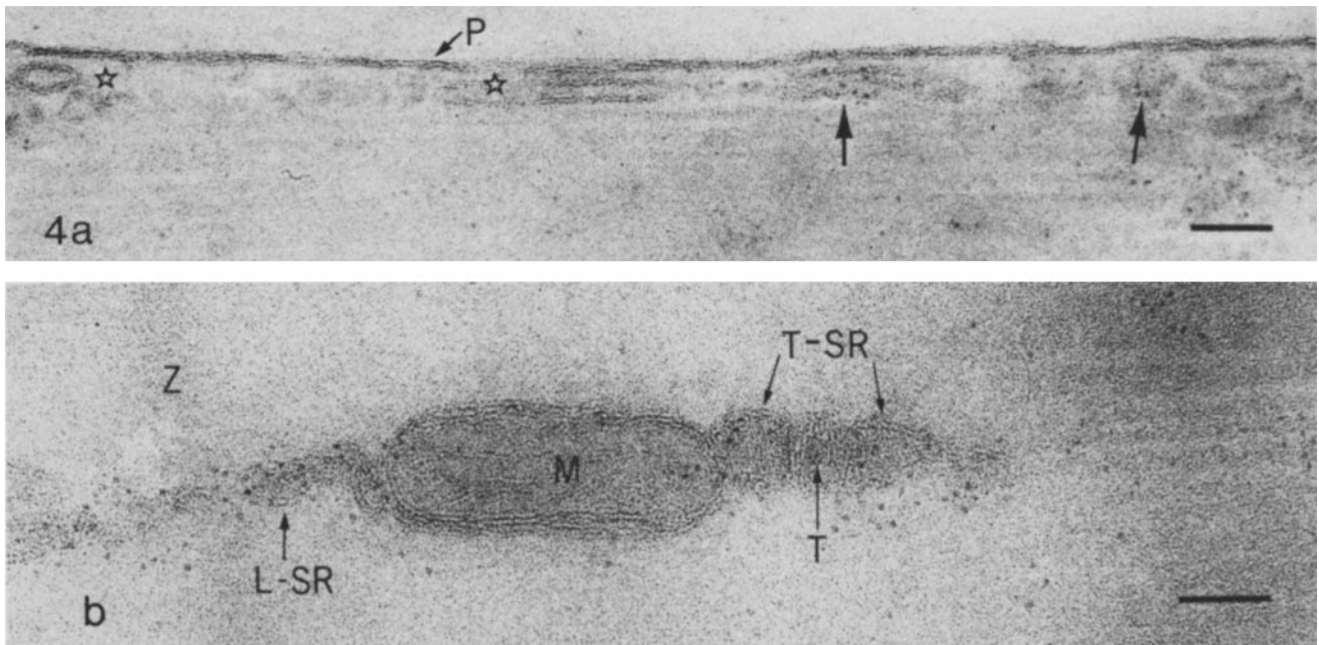

FIGURE 3   Electron micrographs of longitudinal sections of rat skeletal muscle labeled (a) with antibodies to the Ca^{2+} + Mg^{2+}–ATPase and (b) with normal rabbit γ-globulin. The sections were adsorption stained in the presence of polyethylene glycol to visualize membranes. (a) Ferritin labeling in the region of the transverse tubules (T) and the junctional sarcoplasmic reticulum membrane (J-SR) was sparse in comparison with ferritin labeling in the interfibrillar spaces occupied by longitudinal sarcotubules (arrows). (b) Note that very few ferritin particles were located on the section labeled with normal rabbit γ-globulin. Z, Z line; I, I band; L-SR, longitudinal sarcotubules. Bars, 0.1 μm.

FIGURE 4   Electron micrographs of longitudinal sections of rat skeletal muscle labeled with antibodies to Ca^{2+} + Mg^{2+}-ATPase and stained to visualize membranes. (a) In the region of the plasma membrane, some of the subsarcolemmal vesicles were strongly labeled with ferritin particles (arrows), whereas others (☆) were free of ferritin particles. Note that the plasma membrane (P) was also free of ferritin particles. (b) The free sarcoplasmic reticulum membrane (the lontigudinal region [L-SR] and the nonjunctional region of the terminal cisternae [T-SR]) was densely labeled with ferritin particles, whereas the mitochondrion (M) and the transverse tubular membranes (T) were only labeled at background levels. Z, Z line. Bars, 0.1 µm.

sorption-stained to delineate membranes, the general distribution of ferritin particles (Fig. 3) was indistinguishable from that observed without the visualization of membranes (Fig. 1 a).

If we assume 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 (20), then we can conclude that the sarcoplasmic reticulum membrane was densely labeled with ferritin particles, whereas the transverse tubular membranes (T, Figs. 3–6), the sarcolemma (P, Fig. 4a), and the mitochondria (M, Figs. 4b and 5) were labeled only at background levels (Fig. 3b). Simultaneous visualization of sarcoplasmic reticulum membranes and ferritin particles showed that the density of ferritin particles throughout the longitudinal reticulum (L-SR) and the portion of the terminal cisternae not closely apposed to the transverse tubules (T-SR) was quite uniform and much higher than that of the junctional membrane (J-SR) (that portion of the terminal cisternae closely apposed to the transverse tubule) (Figs. 3–6). Moreover, most of the ferritin particles labeling the terminal cisternae membrane in sections where the luminal space could clearly be discerned were present on the cytoplasmic side of the membrane (Figs. 5 and 6).

We observed that some of the membrane vesicles in the subsarcolemmal region were strongly labeled with ferritin particles whereas others were labeled only at background level (Fig. 4a). It is possible that the ferritin-labeled vesicles corresponded to sarcoplasmic reticulum, whereas the unlabeled membrane corresponded to invaginations of the sarcolemmal membrane.

DISCUSSION

Ultrastructural data presented in this paper show that the Ca^{2+} + Mg^{2+}-ATPase in skeletal muscle in situ is almost exclusively associated with the sarcoplasmic reticulum membrane and is

absent from the transverse tubule, the plasma membrane, and the mitochondrial membrane. If the Ca^{2+} + Mg^{2+}-ATPase is confined to the sarcoplasmic reticulum membrane, we would expect that all specific ferritin labeling of the Ca^{2+} + Mg^{2+}-ATPase should be located within 30 nm of the sarcoplasmic reticulum membrane (20). Examination of the electron micrographs of ultracryotome sections in which both ferritin particles and sarcoplasmic reticulum membranes were visualized indicated that the number of ferritin particles that were >30 nm away from the membrane was somewhat higher than could be accounted for by nonspecific background staining. This probably occurred because obliquely cut sarcoplasmic reticulum membranes present in a thin section were difficult to visualize by positive staining, whereas the Ca^{2+} + Mg^{2+}-ATPase exposed to the surface of the thin section could still be labeled by the indirect immunoferritin technique.

The results presented also showed that the Ca^{2+} + Mg^{2+}-ATPase was fairly uniformly distributed throughout the sarcoplasmic reticulum membrane, with the exception of the region of the junctional sarcoplasmic reticulum membrane, which appeared to be less densely labeled. This interpretation is in agreement with freeze-fracture studies of sarcoplasmic reticulum membranes in intact skeletal muscle (9), which showed that the 8-nm intramembranous particles, believed to corresond to the Ca^{2+} + Mg^{2+}-ATPase, were densely and uniformly distributed throughout the free membrane portion but were absent from the junctional membrane region.

Biochemical and morphological studies have established that the Ca^{2+} + Mg^{2+}-ATPase is an integral protein which is asymmetrically distributed across the sarcoplasmic reticulum membrane, with >60% of its bulk on the cytoplasmic surface but with virtually no exposure on the luminal side of the sarcoplasmic reticulum (17, 22). In agreement with these findings, we observed that most of the ferritin particles associated with the terminal cisternae of the sarcoplasmic reticulum were
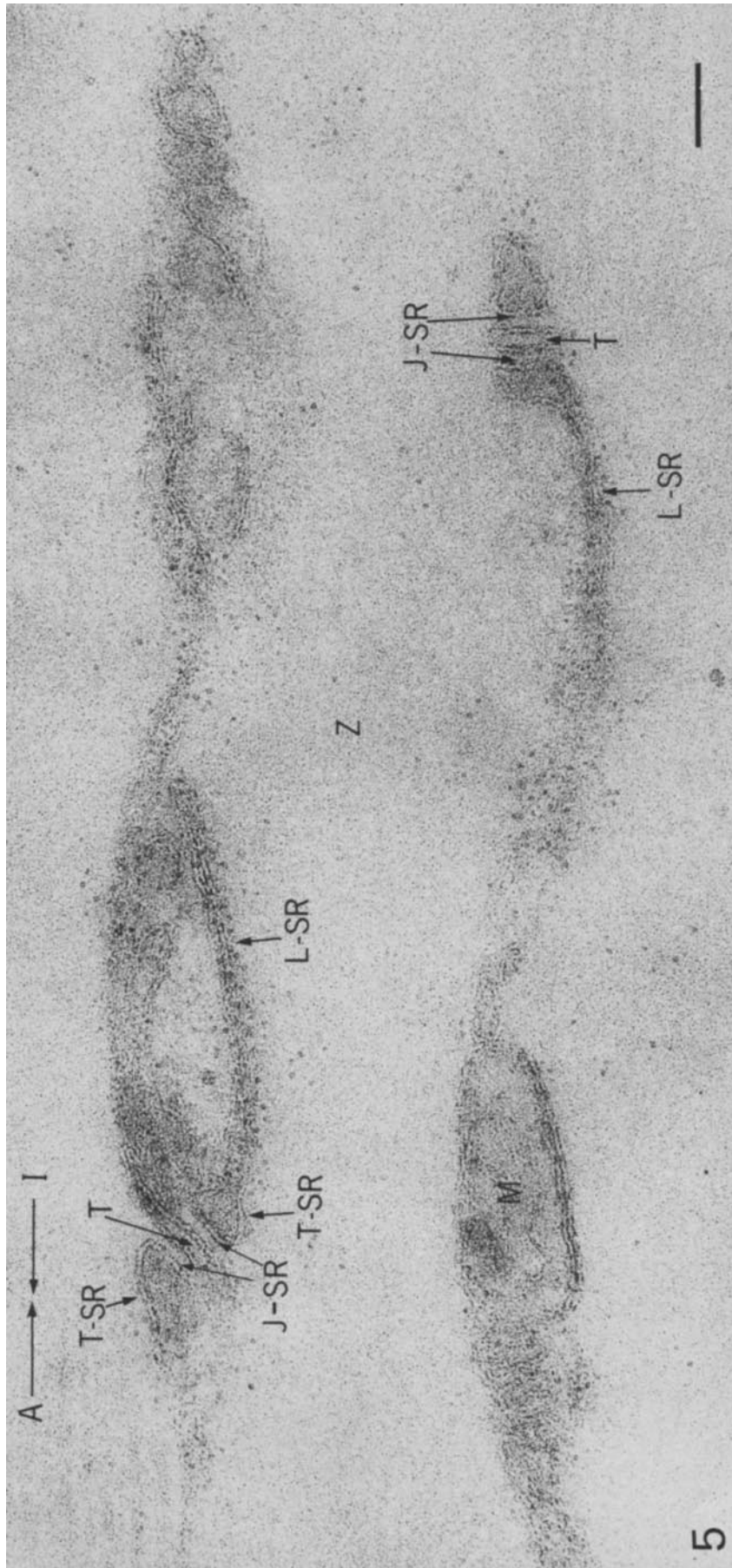
FIGURE 5 Electron micrograph of a longitudinal section of rat skeletal muscle labeled with antibodies to the $Ca^{2+} + Mg^{2+}$-ATPase and then adsorption stained to visualize membranes. Many ferritin particles were present along the entire length of the longitudinal sarcotubules (L-SR) and in the nonjunctional regions of the terminal cisternae membrane (T-SR), whereas few were found over the transverse tubules (T), in the region of the junctional membrane (J-SR), and over the lumen of the terminal cisternae (TC). Note that the mitochondrion (M) was also very sparsely labeled in comparison with the sarcoplasmic reticulum. I, I band; A, A band; Z, Z line. Bar, 0.1 μm.
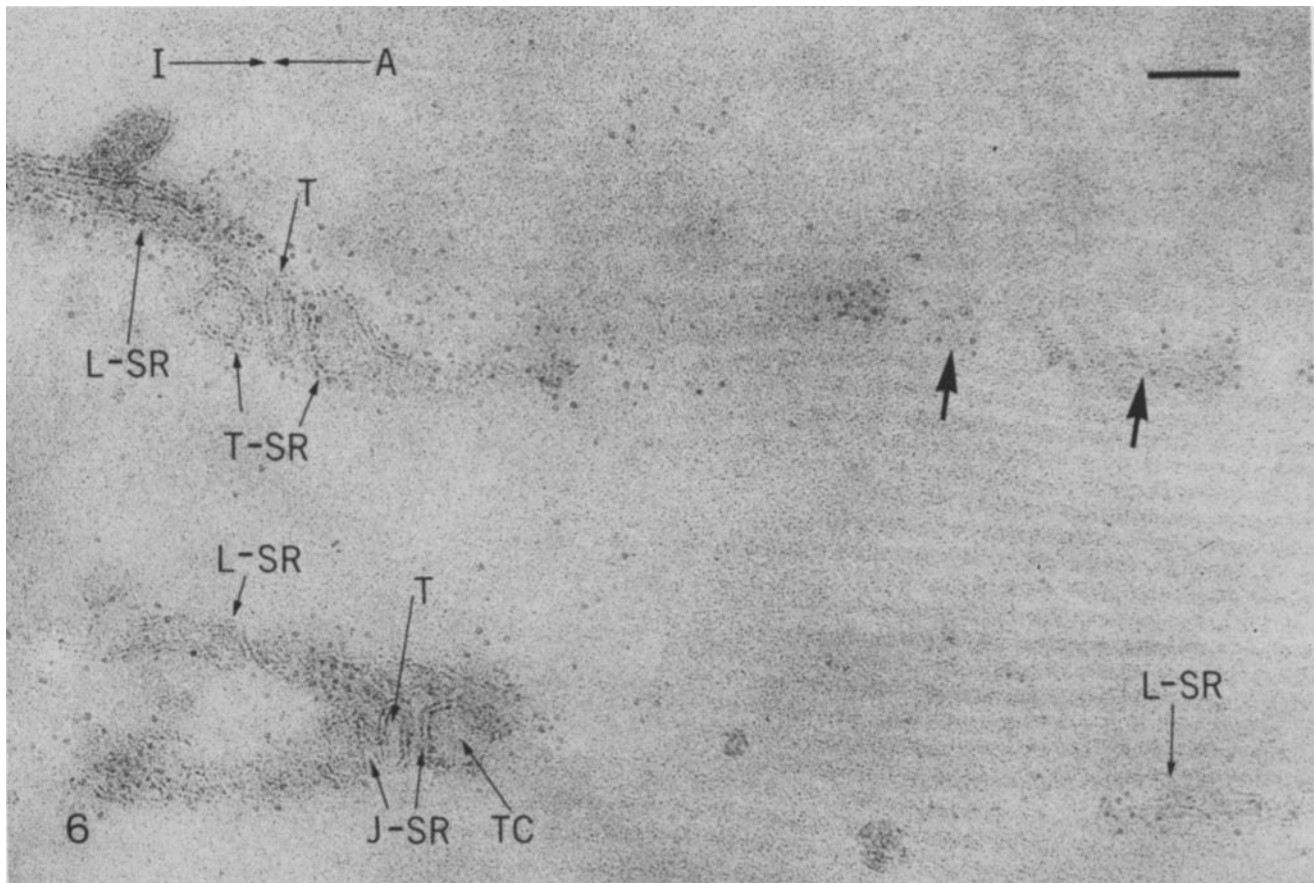
FIGURE 6 Electron micrograph of a longitudinal section of rat skeletal muscle labeled with antibodies to the $Ca^{2+} + Mg^{2+}$-ATPase and adsorption stained to visualize membranes. Many ferritin particles were present along the entire length of the longitudinal sarcotubules (large arrows and L-SR) and on the cytoplasmic side of the nonjunctional regions of the terminal cisternae membrane (T-SR), whereas only a few particles were located in the regions of the transverse tubules (T), in the regions of the junctional membrane (J-SR) and over the terminal cisternae (TC). I, I band; A, A band. Bar, 0.1 μm.

located on the cytoplasmic side of the membrane while the lumen was almost free of ferritin particles, suggesting that the $Ca^{2+} + Mg^{2+}$-ATPase or its antigenic sites were only exposed to the cytoplasmic side and not to the luminal side of the sarcoplasmic reticulum membrane. However, it cannot be excluded that an antigenic site on the $Ca^{2+} + Mg^{2+}$-ATPase is exposed to the luminal side of the sarcoplasmic reticulum and that antibodies to such a site are absent from our antibody preparation.

Our estimate of the density of ferritin particles in the slow and fast myofiber suggests that the density of the $Ca^{2+} + Mg^{2+}$-ATPase in the sarcoplasmic reticulum of fast myofibers is approximately two times higher than in slow myofibers.

Intramembranous particles believed to represent the $Ca^{2+} + Mg^{2+}$-ATPase, were recently shown (2) to be two times more concentrated in sarcoplasmic reticulum membranes from fast myofibers than from slow myofibers in adult, chicken skeletal muscle. Results obtained in a biochemical study (Zubrzycka-Gaarn, Korczak, and Osinska, manuscript in preparation) suggested that the amount of $Ca^{2+} + Mg^{2+}$-ATPase in heavy sarcoplasmic reticulum vesicles isolated from slow muscle was three to four times smaller than that isolated from fast muscle. In the same study, it was shown that the $Ca^{2+} + Mg^{2+}$-ATPases from slow and fast muscles were equally antigenic against the antibodies to the $Ca^{2+} + Mg^{2+}$-ATPase used in the present study. Thus our findings correspond favorably with other morphological and biochemical estimates of the differences in

density of the $Ca^{2+} + Mg^{2+}$-ATPase molecule in the sarcoplasmic reticulum membrane between slow and fast myofibers.

In conclusion, our evidence strongly suggests that the $Ca^{2+} + Mg^{2+}$-ATPase is localized in the sarcoplasmic reticulum membrane and is absent from the plasma membrane, the transverse tubular membrane, and mitochondrial membranes, and that it is uniformly distributed throughout the sarcoplasmic reticulum, except for the region of junction between sarcoplasmic reticulum and transverse tubules. In addition, our data tentatively suggests that the antigenic sites of the $Ca^{2+} + Mg^{2+}$-ATPase are exposed to the cytoplasmic side of the sarcoplasmic reticulum and that the density of the $Ca^{2+} + Mg^{2+}$-ATPase in the sarcoplasmic reticulum of fast myofibers is approximately two times higher than in slow myofibers.

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