

# Ultrastructural Localization of Calsequestrin in Rat Skeletal Muscle by Immunoferritin Labeling of Ultrathin Frozen Sections

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**ABSTRACT** The ultrastructural localization of calsequestrin in rat skeletal muscle (gracilis) was determined by indirect immunoferritin labeling of ultrathin frozen sections. Calsequestrin was found in the lumen of the transversely and longitudinally oriented terminal cisternae but was absent from most of the longitudinal sarcotubules and the fenestrated sarcoplasmic reticulum. Calsequestrin was occasionally observed in vesicular structures found in the central region of the I band. Since calsequestrin is believed to provide the major site of  $\text{Ca}^{2+}$  sequestration in the sarcoplasmic reticulum, the present results support the view that  $\text{Ca}^{2+}$ , transported to the lumen of the sarcoplasmic reticulum, is preferentially sequestered in the terminal cisternae, but they also suggest that additional  $\text{Ca}^{2+}$  sequestration may occur near the center of the I band.

The sarcoplasmic reticulum is the intracellular membrane system that, together with the transverse tubular system, regulates the  $\text{Ca}^{2+}$  concentration of the myofibril and, thereby, the state of contraction and relaxation of skeletal muscle fibers (5, 10, 34). Ultrastructural studies of skeletal muscle (7, 23) have shown that the sarcoplasmic reticulum forms a separate, membranous compartment in muscle cells, surrounding each myofibril like a fenestrated sleeve. This membrane is composed of longitudinal sarcotubules which, at regular intervals corresponding to the A-I junction in mammals, anastomose to form transversely oriented terminal cisternae. The lumen of the longitudinal sarcotubules is electron lucent while the lumen of the terminal cisternae contains an electron-dense matrix. The transverse tubular system consists of tubular invaginations of the sarcolemma which extend from the surface to the center of the myofiber. These tubules are, for the most part, oriented perpendicular to the longitudinal axis of the myofiber. The transverse tubules intercept, and are closely apposed to, the two terminal cisternae at the A-I interface in mammals, thus forming a triad. Regularly spaced densities (feet) cross the 10-nm space between the two membrane systems. Although depolarization of the sarco-

lemma and the transverse tubular system causes the sarcoplasmic reticulum to release  $\text{Ca}^{2+}$  from the terminal cisternae to the cytoplasm (11), the signal that is transmitted from the transverse tubular system to the junctional sarcoplasmic reticulum is unknown.

The major intrinsic membrane protein of highly purified sarcoplasmic reticulum vesicles is the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase, which is responsible for the active transport of  $\text{Ca}^{2+}$  from the cytoplasm to the lumen of the sarcoplasmic reticulum (19, 27). The major extrinsic protein, calsequestrin, is located in the lumen of this membrane system where it probably functions as a  $\text{Ca}^{2+}$ -sequestering agent (20).

Purified sarcoplasmic reticulum vesicles have been separated into light and heavy membrane fractions by sucrose density centrifugation (2, 21). Both of these fractions were found to contain the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase, but only the heavy fraction contained calsequestrin. Since the heavy fraction vesicles, like the terminal cisternae, contained an electron-dense matrix, it was suggested that the heavy membrane fraction originated from the terminal cisternae and that calsequestrin was confined to that region. On the basis of these results it was proposed that the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase is

uniformly distributed in the sarcoplasmic reticulum membrane while calsequestrin is confined to the lumen of the terminal cisternae (21).

In previous studies we have localized the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase and calsequestrin in adult rat skeletal muscle by immunocytochemical staining techniques (13, 16). Our immunofluorescence (13) and immunoferritin (16) labeling studies showed that the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase is confined to the sarcoplasmic reticulum membrane, that it is uniformly distributed throughout the free sarcoplasmic reticulum, and that it is absent from, or very sparse in, the junctional sarcoplasmic reticulum. These findings are in agreement with freeze-fracture studies that have shown that 90-Å intramembrane particles, believed to correspond to the ATPase, are absent from the junctional sarcoplasmic reticulum (8). By contrast, immunofluorescence labeling has suggested that calsequestrin is largely confined to the A-I junction of the myofiber, the region where the terminal cisternae are localized in mammalian skeletal muscle (13).

In this paper, we report on the ultrastructural localization of calsequestrin in adult rat skeletal muscle by indirect immunoferritin labeling of ultrathin frozen sections.

## MATERIALS AND METHODS

### Preparation of Antibodies to Rat Skeletal Calsequestrin:

Antiserum to rat skeletal muscle calsequestrin was produced in rabbit by injecting glutaraldehyde-fixed calsequestrin as previously described (3). Affinity-purified antibodies to calsequestrin were prepared from this antiserum using an insoluble calsequestrin albumin complex as described previously (14). The specificity of the antiserum and of affinity-purified antibodies toward calsequestrin was demonstrated by the indirect  $^{125}\text{I}$ -Protein A immunostaining of Western blots (33) of rat skeletal muscle sarcoplasmic reticulum proteins separated by two-dimensional SDS PAGE (22).

### Dissection, Fixation, and Ultracryotomy of Myofibers:

Dissection and fixation of bundles of myofibers from the gracilis muscle of adult female Wistar rats were carried out as previously described (13, 16). The fixed myofibers were infused for 30–60 min with 1 M sucrose in 0.1 M sodium cacodylate buffer, pH 7.2. The sucrose-infused tissue was then frozen in liquid  $\text{N}_2$  and ultrathin frozen sections were cut at  $-90^\circ\text{C}$  according to the procedure of Tokuyasu (28, 31) and Tokuyasu and Singer (32).

**Immunoferritin Labeling:** The immunoferritin labeling of ultracryotomy sections of skeletal myofibers with affinity-purified antibodies to calsequestrin (15  $\mu\text{g}/\text{ml}$ ) as primary antibodies was carried out according to procedures developed by Tokuyasu (28, 31) and Tokuyasu and Singer (32). Specific details of the various steps in this procedure have already been reported (16). Immunoferritin-labeled ultrathin frozen sections were adsorption stained and postembedded as previously described (15, 16) following the technique developed by Tokuyasu (29, 30). Sections were examined with a Philips 300 electron microscope.

## RESULTS

### Antibody Specificity

The specificity of the rabbit antiserum and affinity-purified antibodies to rat skeletal calsequestrin was demonstrated by indirect immunostaining. Sarcoplasmic reticulum proteins were separated by two-dimensional SDS PAGE prior to blot-

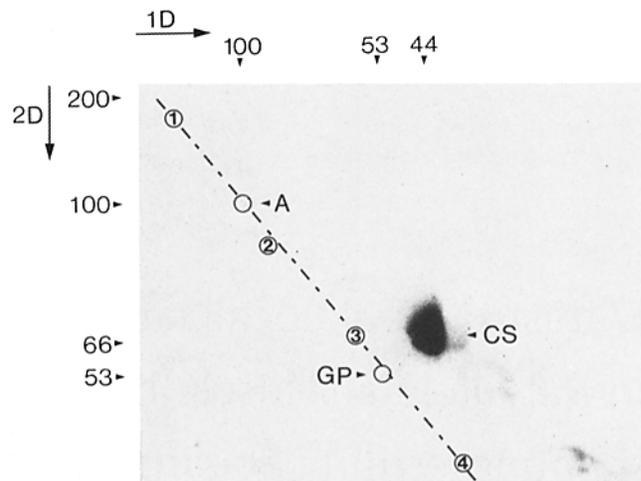


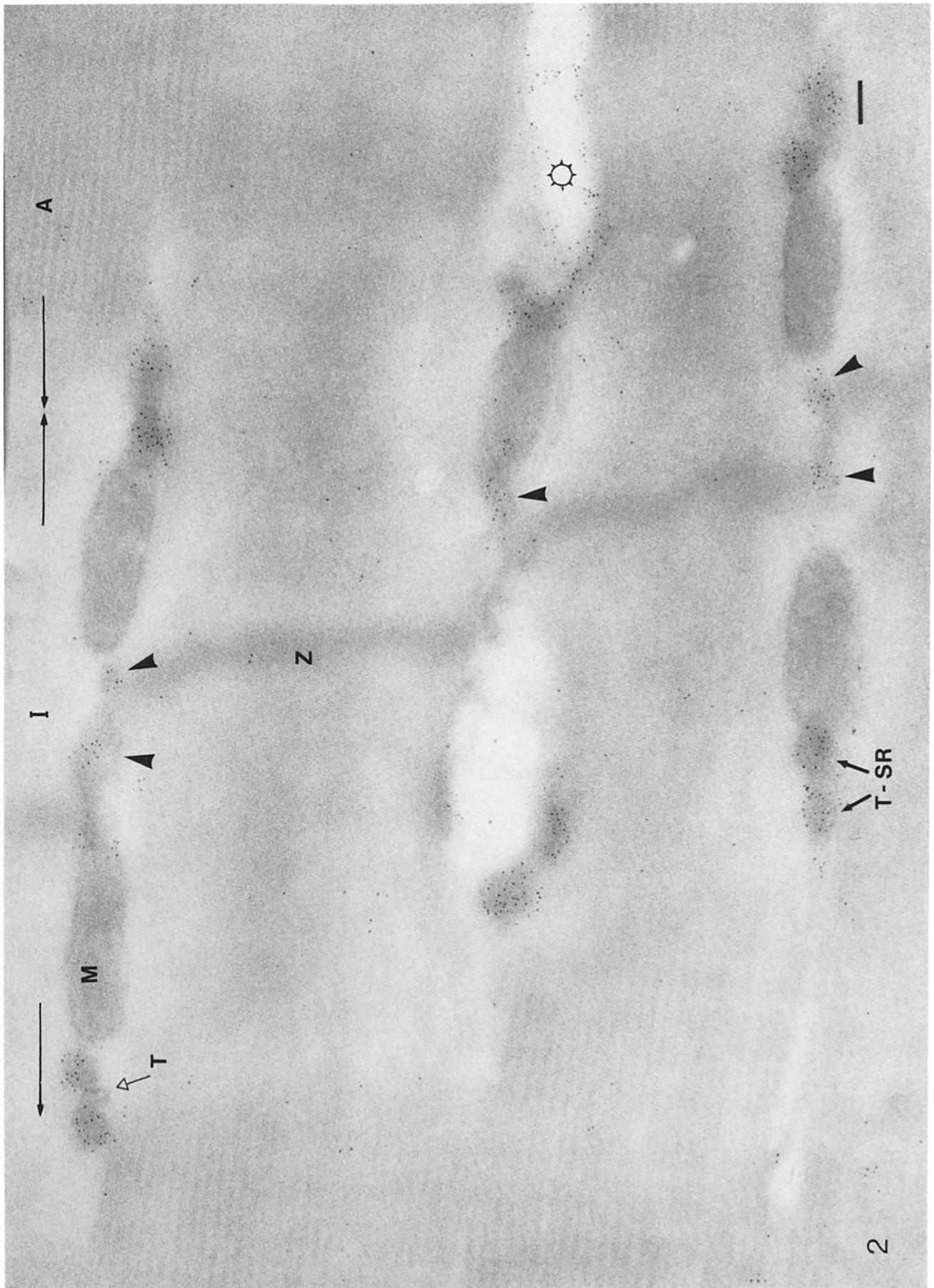
FIGURE 1 The specificity of affinity-purified antibodies to rat skeletal sarcoplasmic reticulum. Rat skeletal muscle sarcoplasmic reticulum proteins were separated by two-dimensional (2D) gel electrophoresis as previously described (17) and electrophoresed onto nitrocellulose (Western blot). The Western blot was first incubated with affinity-purified antibodies to rat skeletal calsequestrin and then with  $^{125}\text{I}$ -Protein A. The autoradiograph shows that the affinity-purified antibodies to rat calsequestrin specifically bound to rat calsequestrin. The Coomassie Blue-stained protein standards are: (1) heavy chain of myosin ( $M_r$  210,000), (2) phosphorylase ( $M_r$  94,000), (3) BSA ( $M_r$  68,000), and (4) ovalbumin ( $M_r$  44,000). A,  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase; CS, calsequestrin; GP, 53,000 mol-wt glycoprotein.

ting (22), transferred to nitrocellulose, and reacted sequentially with antibodies and with  $^{125}\text{I}$ -protein A (33). As shown in Fig. 1, the antiserum or affinity-purified antibodies bound to the spot identified as skeletal muscle calsequestrin. Small amounts of antibodies also bound to a few spots having a lower molecular weight than calsequestrin. Since these spots, like calsequestrin, were located to the right of the diagonal on the two-dimensional acrylamide gel, we believe that they corresponded to degradation products of calsequestrin, which have been shown to run to the right of the diagonal on two-dimensional SDS polyacrylamide gels (Campbell, K. P., unpublished results). We have recently shown that these affinity-purified antibodies to calsequestrin cross-react specifically with calsequestrin in cardiac muscle (3).

### Immunoferritin Labeling

Longitudinal ultrathin frozen sections of adult rat gracilis muscle were labeled with antibodies to calsequestrin by the indirect immunoferritin labeling technique and subsequently adsorption stained to delineate cell membranes. To obtain simultaneous visualization of ferritin particles and cellular membranes, it was necessary to use an adsorption staining procedure that resulted in a less than optimal visualization of the sarcoplasmic reticulum (16). Consequently, the longitu-

FIGURE 2 Electron micrograph of a longitudinal section of fixed rat gracilis muscle first labeled with antibodies to calsequestrin by the indirect immunoferritin staining technique and subsequently adsorption stained. Most of the ferritin particles were present on the terminal cisternae (T-SR) of the sarcoplasmic reticulum. Ferritin particles were occasionally present on the interfibrillar spaces in the central region of the I band where they appeared to be confined to two foci (arrowhead) equidistant from the Z line. The ferritin particles in the white space (spiked circle) in the right side of the picture represents nonspecific ferritin labeling of the edges of a small hole in the section. I, I band, A, A band, T, transverse tubule, Z, Z line, M, mitochondrion. Bar, 0.1  $\mu\text{m}$ .  $\times 80,000$ .



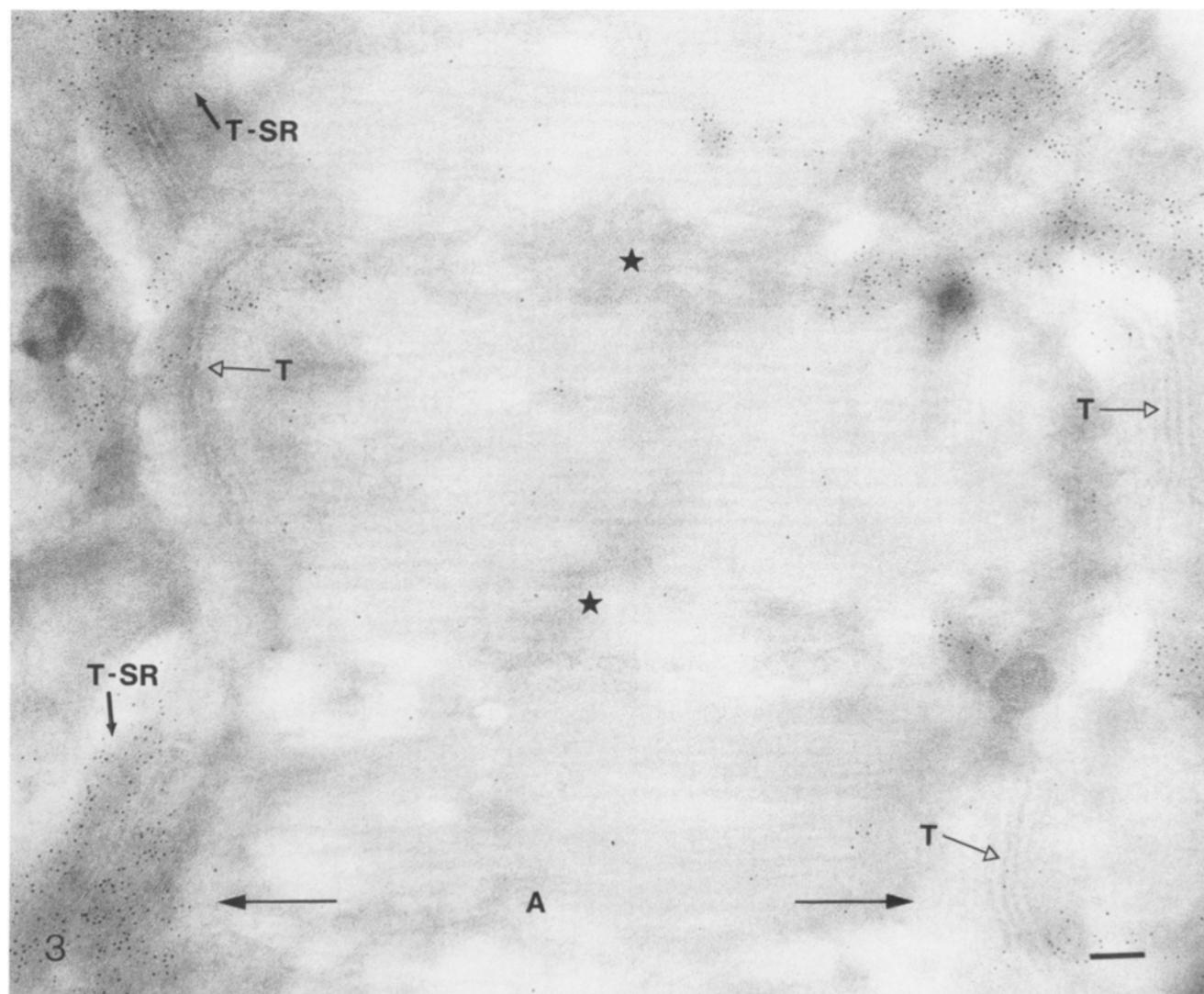


FIGURE 3 Electron micrograph of a longitudinal section of rat skeletal muscle labeled with antibodies to calsequestrin. Most of the ferritin particles were confined to the lumen of the terminal cisternae (T-SR) which is frequently seen in longitudinal section. Note that the fenestrae of the sarcoplasmic reticulum in the A band (A) region (star) was only labeled at the background level. T, transverse tubule. Bar, 0.1  $\mu$ m.  $\times$  81,000.

dinal sarcotubules were visualized less frequently than the triadic structures which were composed of the terminal cisternae and transverse tubules (7).

Examination of longitudinal sections of rat skeletal muscle showed that most of the ferritin particles were localized over the sarcoplasmic reticulum (Figs. 2–6), while the mitochondria (Figs. 2 and 5), the myofibrils (Figs. 2–6), and the transverse tubules (Figs. 2, 3, 4*b*, 5, and 6) were labeled only at background levels (Fig. 7). Within the region of the sarcoplasmic reticulum, ferritin particles were densely distributed on all transversely cut terminal cisternae (Figs. 2, 4, and 6*b*), and on approximately half of the longitudinally cut terminal cisternae (Fig. 3). A likely explanation for the absence of calsequestrin labeling in some parts of the longitudinally cut terminal cisternae is that the cytoplasmic side and not the luminal side of the terminal cisternae was exposed to the surface of the section. Simultaneous visualization of sarcoplasmic reticulum membranes and of ferritin particles in sections, including transversely cut triadic structures, showed that ferritin particles were rather uniformly distributed over

the luminal side but absent from the cytoplasmic side of the terminal cisternae (Fig. 4*a*). By contrast, the longitudinal sarcotubules in the I-band region (Fig. 4*a*) and the A-band region (Fig. 4*b*) and the fenestrated sarcoplasmic reticulum in the H zone of the A-band region (Figs. 3 and 5) were, in general, labeled only at background level.

Elongated patches of densely distributed ferritin particles were occasionally observed over the interfibrillar spaces in the I-band region and less frequently over the A-band region (Fig. 5). Since longitudinally oriented transverse tubules were sometimes (Fig. 6*a*), but not always (Fig. 6*b*), observed to be closely apposed to the membranous structures (terminal cisternae) that were densely labeled with ferritin particles, it is possible that some, if not all, of the patches of ferritin particles were localized in obliquely and longitudinally oriented terminal cisternae.<sup>1</sup>

<sup>1</sup>The terms “longitudinally” and “transversely oriented” terminal cisternae are used to describe terminal cisternae that are components of triads oriented longitudinally and transversely, respectively, with regard to the long axis of the muscle fiber.

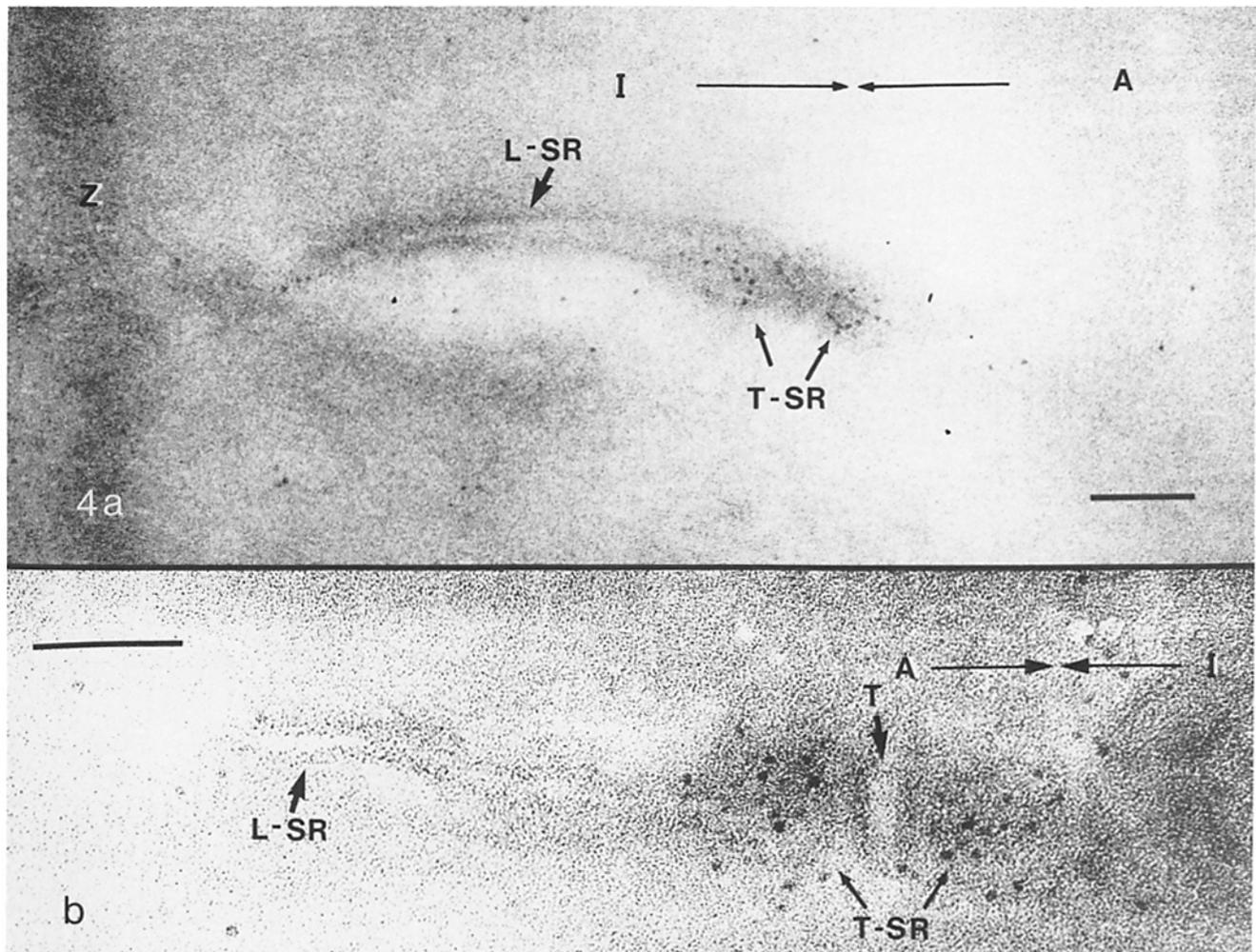


FIGURE 4 Longitudinal sections of rat skeletal muscle labeled with antibodies to calsequestrin. The ferritin particles were almost exclusively confined to the lumen of the terminal cisternae (T-SR) while the lumens of the longitudinal sarcotubules (L-SR) in the I band (I) region of a and the A-band (A) region of b as well as the transverse tubules (T) were only labeled at background level. Z, Z line. Bar, 0.1  $\mu$ m. (a)  $\times$  144,000; (b)  $\times$  210,000.

Ferritin particles were also observed occasionally in interfibrillar spaces in the central region of the I band where they appeared to be confined to two foci equidistant from the Z line (Fig. 2). Transverse tubules were never visualized in close apposition to these ferritin-labeled foci. In our previous study of the localization of calsequestrin in rat skeletal muscle (13), we observed fluorescent foci at the border between the A and I bands which occasionally appeared to be dumbbell shaped. It is possible that one end of these dumbbells might have corresponded to the terminal cisternae of the triad and that the other end might have corresponded to one of the ferritin-labeled foci in the central region of the I band.

#### DISCUSSION

We used indirect immunoferritin labeling of ultrathin frozen sections of rat skeletal muscle to localize calsequestrin *in situ*. This protein, like the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase (16), is almost exclusively associated with the sarcoplasmic reticulum membrane and is absent from the transverse tubules and the mitochondrial membrane. However, the distribution of calsequestrin within the sarcoplasmic reticulum is very different from that of the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase. We have previously shown, by immunoferritin labeling, that the  $\text{Ca}^{2+} + \text{Mg}^{2+}$ -

ATPase is rather uniformly distributed throughout the free sarcoplasmic reticulum but is apparently absent from the junctional sarcoplasmic reticulum (16). The present study showed that most of the calsequestrin was confined to the lumen of the terminal cisternae of the sarcoplasmic reticulum and was absent from the longitudinal sarcotubules and the fenestrated sarcoplasmic reticulum. These conclusions are in agreement with previous deductions from biochemical and morphological studies by Meissner (21) and Campbell et al. (2) and from our previous immunofluorescence studies of the location of calsequestrin in skeletal muscle (13).

Although calsequestrin was observed occasionally in the interfibrillar spaces of the I-band region and less frequently in the A-band region, the incidence of calsequestrin labeling in these regions was too small to contribute to a regular staining pattern at the light microscopic level of resolution. By contrast Junker and Sommer reported (17) that calsequestrin is frequently present in the I-band region and in the center of the A-band region (the M line) as well as in the terminal cisternae. It has been reported that longitudinally oriented transverse tubules in mammalian skeletal muscle traverse the I band and the A band, connecting the transverse tubular system in neighboring A-I junctions (1, 9, 12). In the present

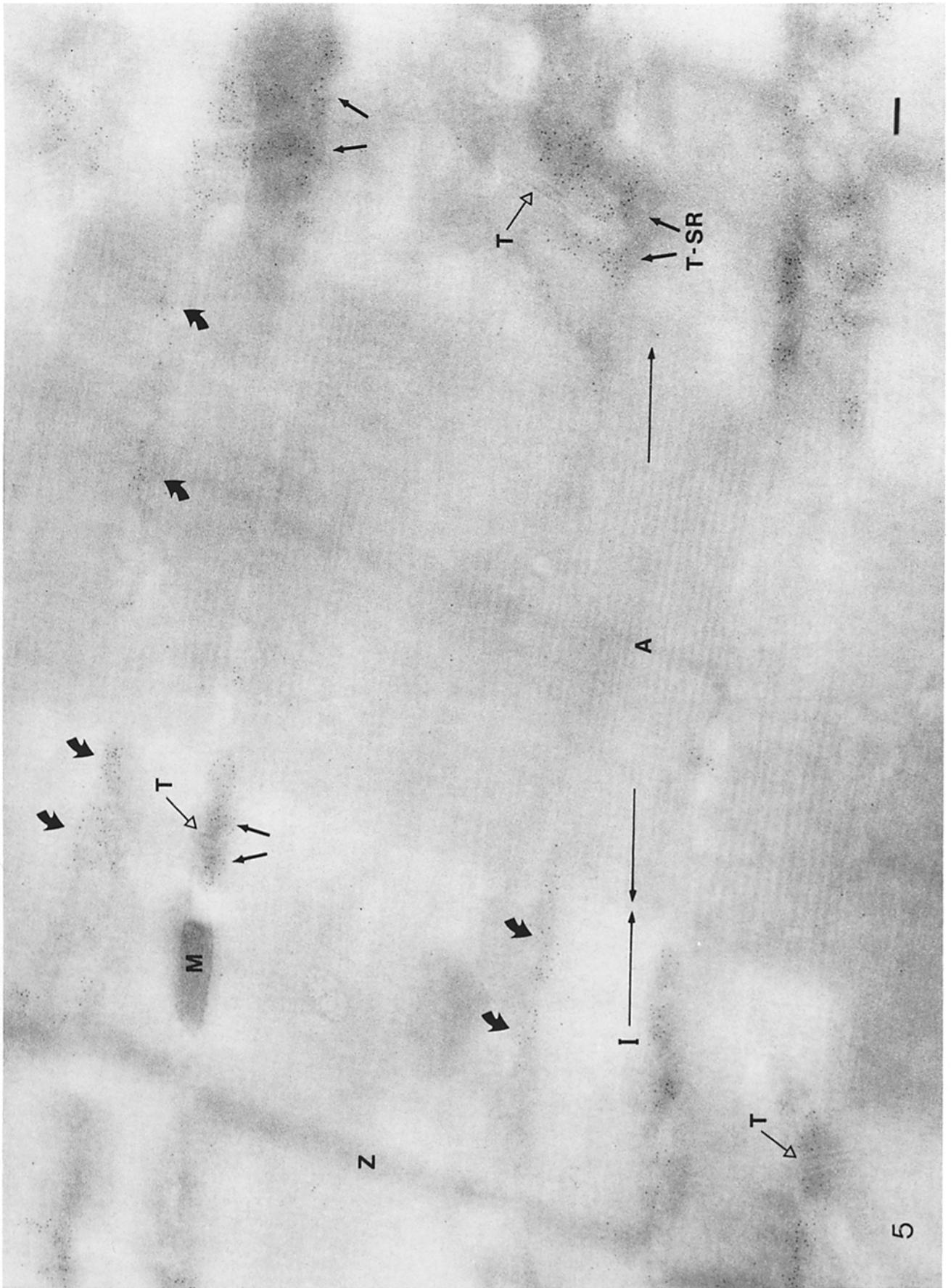
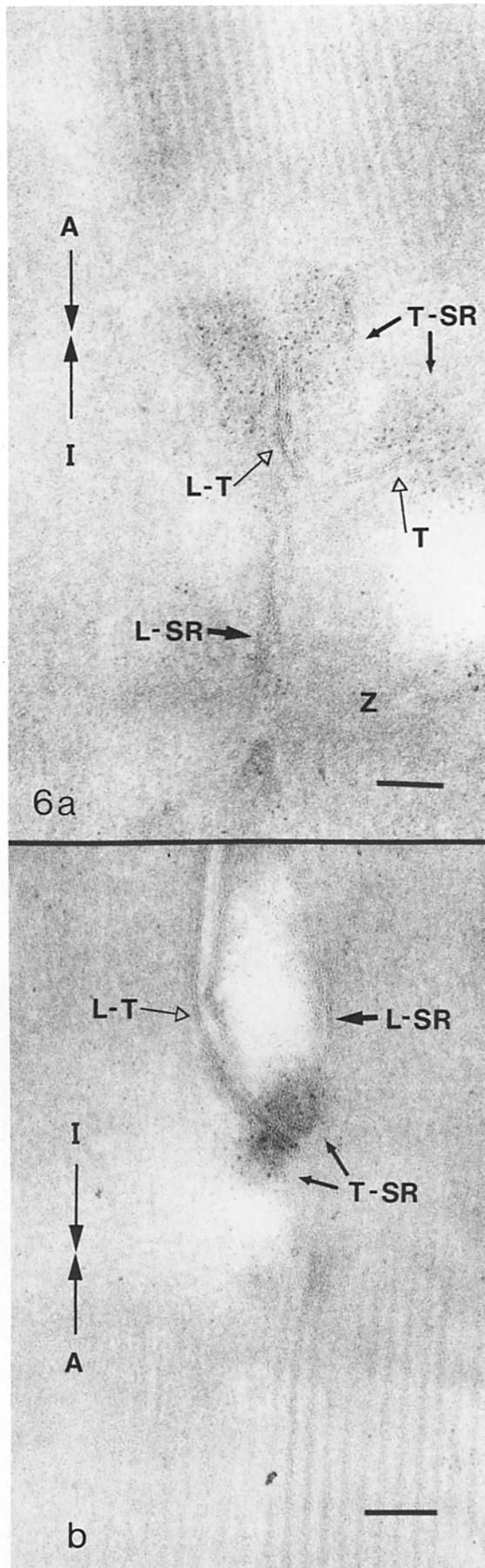


FIGURE 5 Longitudinal section of rat skeletal muscle labeled with calsequestrin antibodies. As shown in Figs. 2 and 3, most of the ferritin particles were confined to the lumen of the terminal cisternae (T-SR, arrows). Occasional ferritin particles were also present in the intermyofibrillar spaces of the A bands and I bands (curved arrows). Z, Z line; M, mitochondrion; T, transverse tubule. Bar, 0.1  $\mu\text{m}$ .  $\times 67,000$ .



study we occasionally observed calsequestrin in the lumen of membrane-bounded structures in close apposition to longitudinally oriented tubules. Therefore, it is possible that at least some of the calsequestrin present in the interfibrillar spaces in the I- and A-band regions was, indeed, localized in longitudinally oriented terminal cisternae. It is also possible that two kinds of longitudinal sarcotubules exist and that one contains calsequestrin in its lumen, while the other does not. This interpretation is supported by ultrastructural studies of frog skeletal muscle which suggest that junctional granules, similar to those present in the lumen of the terminal cisternae of the sarcoplasmic reticulum, are also present in some, but not all, of the longitudinal sarcotubules in frog skeletal muscle (25).

Occasionally we observed calsequestrin staining in what appeared to be the luminal space of vesicular structures present in the central region of the I band. In no case were transverse tubules identified in close apposition to these calsequestrin-containing structures. However, since the membrane visualization in our studies was less than optimal we cannot exclude the possibility that transverse tubules were present, but not visualized.

At present we have no knowledge of the structural relationship between these calsequestrin-containing structures in the central region of the I band and the remaining free and junctional sarcoplasmic reticulum. It is possible that they are similar to the "coated vesicles" previously observed in the Z-line region of mammalian cardiac muscle cells (6, 18, 26). High voltage electron microscopic studies (26), as well as conventional electron microscopic studies of osmium-ferrocyanide-stained specimens of cardiac muscle (6), have clearly demonstrated that these coated vesicles are fused with, and form blind ends on, the sarcoplasmic reticulum. Furthermore the lumens of these structures, like the lumen of the terminal cisternae, have been shown to contain electron-dense granules (26). However, similar structures have not so far been observed in the central region of the I band in mammalian skeletal muscle.

Determination of the subcellular distribution of  $\text{Ca}^{2+}$  (4, 24) has clearly shown that  $\text{Ca}^{2+}$  is stored in and released from the terminal cisternae in frog skeletal muscle cells, but these studies have not provided evidence for any other  $\text{Ca}^{2+}$  storage sites. Since our present studies show that most of the calsequestrin is confined to the lumen of the terminal cisternae in situ, it is probable that  $\text{Ca}^{2+}$  transported to the lumen of the sarcoplasmic reticulum in rat skeletal muscle is also preferentially sequestered in the lumen of the terminal cisternae.

The possibility that the calsequestrin-containing vesicular structures in the central region of the I band represent additional  $\text{Ca}^{2+}$  storage sites might be determined by studying their capacity for  $\text{Ca}^{2+}$  storage and release during the various

FIGURE 6 Longitudinal sections of rat skeletal muscle labeled with antibodies to calsequestrin. a shows an apparent branch point of a transverse tubule (T) in which one branch proceeds longitudinally (L-T) while the other proceeds transversely (T). Both branches of the transverse tubule are closely apposed to ferritin-labeled terminal cisternae (T-SR). b also shows a longitudinally oriented transverse tubule without closely apposed terminal cisternae visible in the section. L-SR, longitudinal sarcotubules; Z, Z line. Bar, 0.1  $\mu\text{m}$ . (a)  $\times 94,000$ ; (b)  $\times 109,000$ .

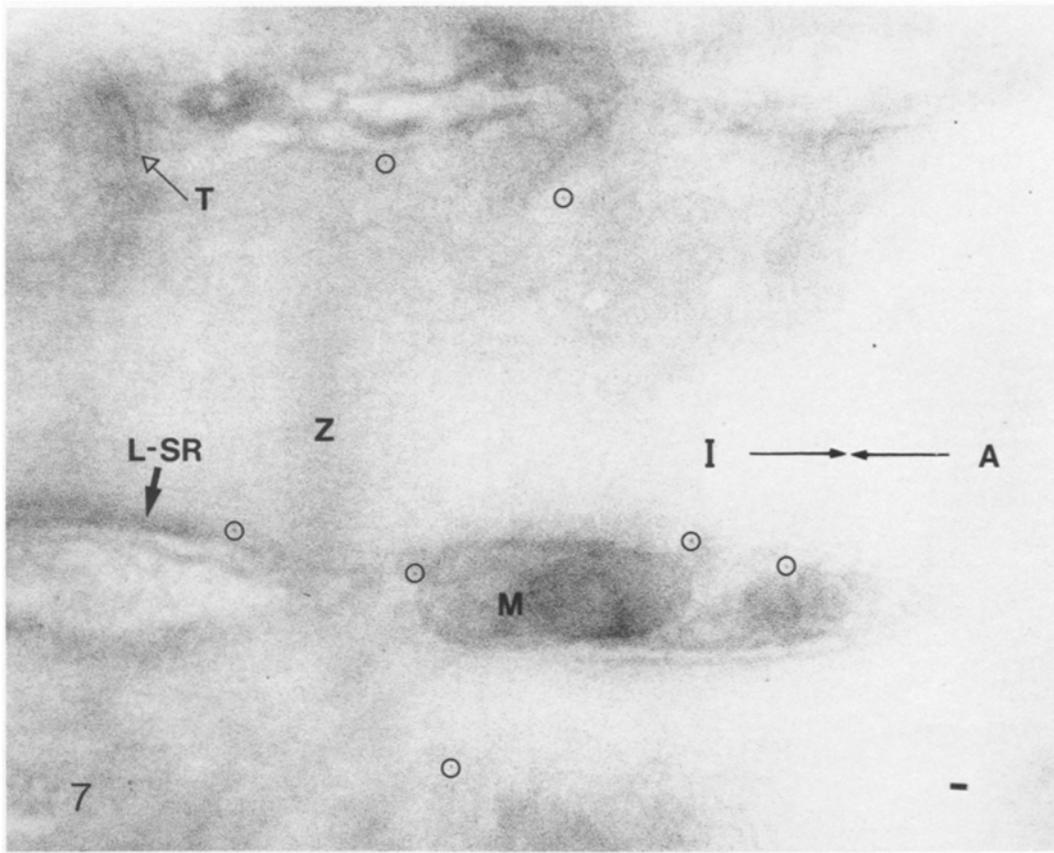


FIGURE 7 Longitudinal section of skeletal muscle labeled with normal rabbit  $\gamma$ -globulin by the indirect immunoferritin labeling technique and then adsorption stained to visualize membranes. Note that very few ferritin particles (circles) were observed on the membranes. L-SR, longitudinal sarcotubules; M, mitochondrion; T, transverse tubules; Z, Z line. Bar, 0.1  $\mu$ m.  $\times$  24,000.

stages of the excitation-contraction-relaxation cycle. Alternatively, these calsequestrin-containing vesicles might represent calsequestrin storage sites. To determine whether the calsequestrin-containing vesicular structures also contain  $\text{Ca}^{2+}$  +  $\text{Mg}^{2+}$ -ATPase, characteristic of the free sarcoplasmic reticulum, it will be necessary to carry out double labeling with antibodies to the  $\text{Ca}^{2+}$  +  $\text{Mg}^{2+}$ -ATPase and to calsequestrin.

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