LOCALIZATION OF SARCOPLASMIC RETICULUM PROTEINS IN RAT SKELETAL MUSCLE BY IMMUNOFLUORESCENCE

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ABSTRACT

Ca^{++}-Mg^{++}-dependent ATPase and calsequestrin, the major intrinsic and extrinsic proteins, respectively, of the sarcoplasmic reticulum, were localized in cryostat sections of adult rat skeletal muscle by immunofluorescent staining and phase-contrast microscopy. Relatively high concentrations of both the ATPase and calsequestrin were found in fast-twitch myofibers while a very low concentration of the ATPase and a moderate concentration of calsequestrin were found in slow-twitch myofibers. These findings are consistent with previous biochemical studies of the isolated sarcoplasmic reticulum of slow-twitch and fast-twitch mammalian muscles.

The distribution of the ATPase in muscle fibers is distinctly different from that of calsequestrin. While calsequestrin is present only near the interface between the I- and A-band regions of the sarcomere, the ATPase is found throughout the I-band region as well as in the center of the A-band region. In comparing these results with *in situ* ultrastructural studies of the distribution of sarcoplasmic reticulum in fast-twitch muscle, it appears that the ATPase is rather uniformly distributed throughout the sarcoplasmic reticulum while calsequestrin is almost exclusively confined to those regions of the membrane system which correspond to terminal cisternae.

Fluorescent staining with these antisera was not observed in vascular smooth muscle cells present in the cryostat sections of the mammalian skeletal muscle used in this study.

The sarcoplasmic reticulum is the intracellular membrane system in muscle which controls the state of contraction of the myofiber by regulating the Ca^{++} concentration in the sarcoplasm (2, 8, 29). Ultrastructural examination of the sarco-plasmic reticulum in skeletal muscle (21) shows that it forms a separate membrane compartment in muscle cells, surrounding each myofibril in a manner often likened to a laced sleeve. The protein composition of highly purified sarcoplasmic reticulum membrane vesicles, active in Ca^{++} transport, has been determined, and the function of each of these proteins has been studied extensively (17, 18, 27). The Ca^{++}, Mg^{++}-de-

pendent ATPase is the major intrinsic protein of this membrane system, and it is responsible for the active transport of Ca^{++} ions from the sarcoplasm into the membrane lumen (17, 27). Calsequestrin is the major extrinsic protein of the membrane (16). It is located in the lumen (10, 16, 20, 24, 25, 31), where it probably acts as a calcium-sequestering agent.

To understand how the various proteins interact in regulating Ca^{++} concentration in the myofibril, thereby regulating muscle contraction and relaxation, it is important to know whether they are uniformly distributed throughout this membrane system or whether some, if not all, of these proteins are confined to specific regions along the sarcomere, such as the longitudinal tubules or the terminal cisternae. In a combined biochemical and ultrastructural study, Meissner (20) showed that the sarcoplasmic reticulum could be fractionated into two populations, one containing the ATPase and calsequestrin, and the other containing mainly the ATPase. The calsequestrin-rich fraction of vesicles contained a fibrous matrix material which was believed to be calsequestrin. A similar matrix was found in vivo only in terminal cisternae. Therefore, Meissner (20) suggested that calsequestrin was located in terminal cisternae and was absent from longitudinal tubules.

In this report the in situ distribution of the two major membrane proteins, the $Ca^{++} + Mg^{++}$-ATPase and calsequestrin in adult rat skeletal muscle fast-twitch fibers was determined by immunofluorescence, and the positions of the stained regions were compared with the positions of the A and I bands. The results obtained indicate that the ATPase is rather uniformly distributed throughout the sarcoplasmic reticulum while calsequestrin is located mainly in the regions which correspond to the terminal cisternae of this membrane system.

In addition, we have shown that fast-twitch myofibers have a high concentration of both the ATPase and calsequestrin while slow-twitch myofibers have a relatively low concentration of the ATPase and a moderate concentration of calsequestrin. These findings are consistent with biochemical studies of this membrane system reported previously (references 9, 19, 22, 23, and footnote 1).

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1 Zubrzycka, E., B. Korczak, and M. G. Sarzala. 1978. The composition of sarcoplasmic reticulum membranes from slow twitch muscle. Eur. J. Biochem. Manuscript submitted for publication.

MATERIALS AND METHODS

Dissection and Fixation of Myofibers

Bundles of myofibers (1–2 mm diameter) were dissected from the gracilis muscle of adult female rats and immediately tied to applicator sticks at 80–160% of their rest length. The myofibers were then immersed in a modified Krebs-Henseleit buffer (145 mM NaCl, 2.6 mM KCl, 5.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM glucose-saturated with a mixture of 95% O_2 and 5% CO_2) and permitted to recover for 30–45 min at room temperature. They were then fixed at room temperature in 2% paraformaldehyde in buffer A (0.1 M sodium cacodylate buffer, pH 7.2, and 4.5 mM $CaCl_2$) as described by Tokuyasu and Singer (28). After 2 h, the fixative was changed to one containing 1% paraformaldehyde and 0.25% glutaraldehyde in buffer A, and the fixation was continued for an additional 3 h. Subsequently, the tissue was washed for $^1/_2$ h in buffer A and then for 12–16 h in buffer B (0.08 M lysine-HCl and 0.02 M Tris-HCl, pH 7.5) to block unreacted aldehyde groups in glutaraldehyde-fixed tissue. Autofluorescence of the sections after this type of fixation was minimal. Before freezing, the tissue was infused for 30–60 min with buffer C (0.6 M sucrose in 0.1 M cacodylate buffer, pH 7.2) to minimize the damage from freezing.

Cryostat Sectioning

Fixed or unfixed bundles of myofibers were frozen by immersion for 30 s in isopentane cooled to −150°C with liquid nitrogen. The frozen tissue was then mounted on brass pegs with Tissue-Tek II O.C.T. compound (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) and kept at −20°C.

Transverse and longitudinal sections, 7–10 μm thick, were cut at −16° to −18°C on a Tissue-Tek Microtome-Cryostat (Ames Company, Div. of Miles Lab, Inc., Elkhart, Ind.), within the following 6 h, and collected on glass slides previously covered with a film of 10% ovalbumin. The frozen sections of fixed and unfixed myofibers, cut for indirect immunofluorescent staining, were postfixed for 5 min in 70% ethanol at 0°C, air dried for 1 h, and stored dessicated at −20°C for up to 5 d. The sections of unfixed myofibers cut for histochemical staining were air dried for 1 h and stained within the following 4 h.

Purification of Rat ATPase and Rabbit Calsequestrin

Rat ATPase was prepared by procedures similar to those used for the purification of rabbit ATPase (15), except that the fractionation in ammonium acetate was carried out at pH 8.35. Rabbit calsequestrin was purified as previously described (16).

Preparation of Antibodies

The rabbit anti-rat ATPase serum previously characterized (11) 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. This antiserum did not cross-react with purified rat calsequestrin or with high-affinity Ca^{++}-binding protein (11). Specific antibodies to ATPase were isolated from this antiserum, using insoluble purified rat ATPase as previously described (13).

The sheep anti-rabbit calsequestrin serum previously characterized (32) was used to localize calsequestrin. The specificity of this antiserum for calsequestrin was demonstrated by reacting the antiserum in Ouchterlony double-diffusion tests against solubilized rat sarcoplasmic reticulum or purified rat calsequestrin. Only a single precipitin line was obtained in both cases. No precipitation line was obtained when normal sera were used. This antiserum did not cross-react with the purified rat ATPase or with the high affinity Ca^{++}-binding protein (32). Specific antibodies to calsequestrin were prepared from this antiserum by using an insoluble calsequestrin-albumin complex as described previously (13).

Fluorescein Isothiocyanate (FITC)-Conjugated Antibodies

Fluorescein-labeled goat anti-rabbit immunoglobulin was obtained from Hyland (Hyland Laboratories, Costa Mesa, Calif.) (F/P [Fluorescein/protein (male:male)] 2; 13.5 mg/ml). Fluorescein-labeled rabbit anti-sheep immunoglobulin was prepared by labeling the immunoglobulin fraction of rabbit anti-sheep IgG serum (Joe De Rose Assoc., Downsview, Ontario, Canada) with FITC, as described by The and Feltkamp (26) (F/P [Fluorescein/protein (male:male)] 2.7; 10 mg/ml).

Indirect Fluorescent Antibody Labeling

The immunofluorescent staining of the cryostat sections was carried out as previously described (13). Cryostat sections were incubated with specific antibody for 30 min at room temperature (anti-ATPase, 15 μg/ml in phosphate buffered saline (PBS), pH 7.2; anticalsequestrin, 30 μg/ml in PBS, pH 7.2), and then rinsed four times with PBS, pH 7.2. Sections previously treated with specific rabbit antibodies to ATPase were incubated with the fluorescein-labeled immunoglobulin fraction of goat anti-rabbit IgG (3.5 mg/ml) for 30 min at room temperature. Sections previously treated with specific sheep antibodies to calsequestrin were incubated with the fluorescein-conjugated immunoglobulin fraction of rabbit anti-sheep IgG (0.5 mg/ml) for 30 min at room

temperature. Finally, the sections were washed four times in PBS, pH 7.2, and mounted in 50% glycerol in PBS. 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 photographed on Kodak SO115 film and the cytochemical pictures on Ilford FP4 film (Ilford Ltd., Ilford, Essex, England).

Absorption

For absorption studies, 100 μg of specific calsequestrin antibody were incubated with 45 μg of rat calsequestrin for 72 h at 4°C in 0.3 ml of PBS. Similarly, 25 μg of specific ATPase antibody were incubated for 72 h at 4°C with 5 μg of purified, lipid-free, rat ATPase dissolved in 0.5% Triton X-100. In both cases, the supernatant solution obtained after centrifugation was used in place of the specific antibody in immunofluorescence tests.

Histochemical Studies

Transverse cryostat sections of unfixed myofibers were stained for myosin ATPase after alkaline preincubation as described by Guth and Samaha (7).

Electron Microscopy

Bundles of myofibers were fixed as for immunofluorescence, embedded in Epon, thin sectioned, and stained with uranyl acetate and lead citrate according to standard procedures. A Philips 300 electron microscope was used to examine the sections.

RESULTS

Localization of Ca^{++}-Mg^{++} ATPase and Calsequestrin in Slow-Twitch and Fast-Twitch Myofibers

Longitudinal and transverse cryostat sections of adult rat gracilis muscle, containing both slow and fast myofibers, were labeled with antibodies to either ATPase or calsequestrin by the indirect immunofluorescent staining technique. Examination of these sections to determine the distribution of the ATPase and calsequestrin showed that some of the myofibers stained strongly while others stained weakly (Fig. 1). Note that the intensity of staining in the fibers weakly stained with ATPase antibodies is just slightly above that obtained with normal rabbit immunoglobulin. Comparison of the pattern of strongly and weakly stained myofibers in sequential transverse sections, one labeled with ATPase antibodies and the other with calsequestrin antibodies, showed that any myofiber stained strongly by ATPase antibod-
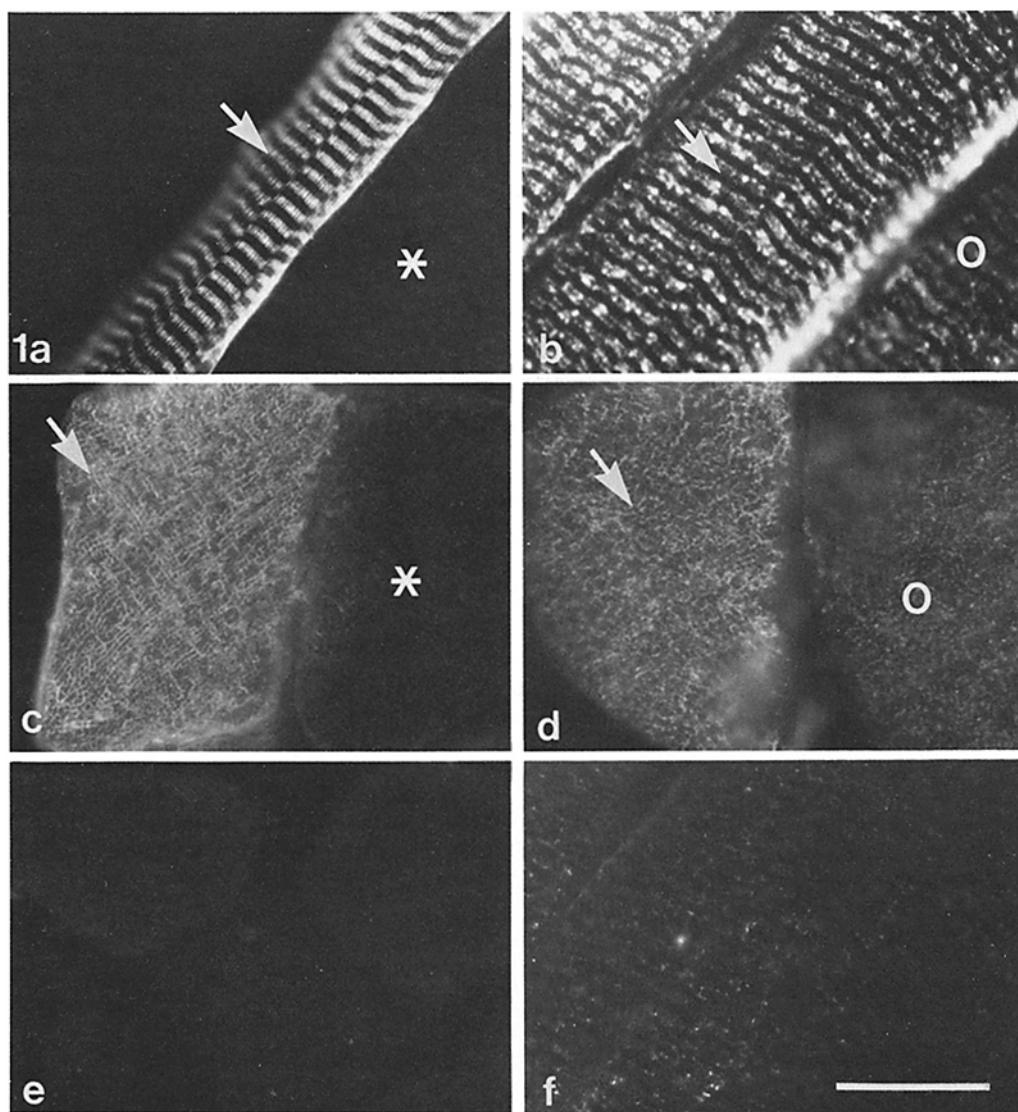
FIGURE 1 Adult rat skeletal muscle tissue (gracilis) fixed, cyrostat sectioned, and stained with antibodies to $Ca^{++} + Mg^{++}$-ATPase (a and c), antibodies to calsequestrin (b and d), antibodies to $Ca^{++} + Mg^{++}$-ATPase absorbed with $Ca^{++} + Mg^{++}$-ATPase (e), and antibodies to calsequestrin absorbed with calsequestrin (f). Longitudinal sections (a, b, and f) and transverse sections (c, d, and e) of a bundle of myofibers. Note that while one fiber is strongly stained (arrows) with antibodies to both $Ca^{++} + Mg^{++}$-ATPase (a and c) and calsequestrin (b, d), another is weakly (asterisks) (a and c) or moderately (circles) (b and d) stained with these antibodies, respectively. Bar, 25 μm. × 820.

ies was also stained strongly by calsequestrin antibodies (Fig. 2). Similarly, any myofiber stained weakly by ATPase antibodies was also stained less strongly by calsequestrin antibodies. In weakly stained fibers, the staining intensity after labeling with ATPase antibodies (Fig. 2a)

was just slightly above that observed after labeling with normal rabbit immunoglobulin (Fig. 2c). However, in the same fibers the staining intensity with calsequestrin antibodies (Fig. 2b) was well above the intensity observed after staining with normal sheep immunoglobulin (Fig. 2d).
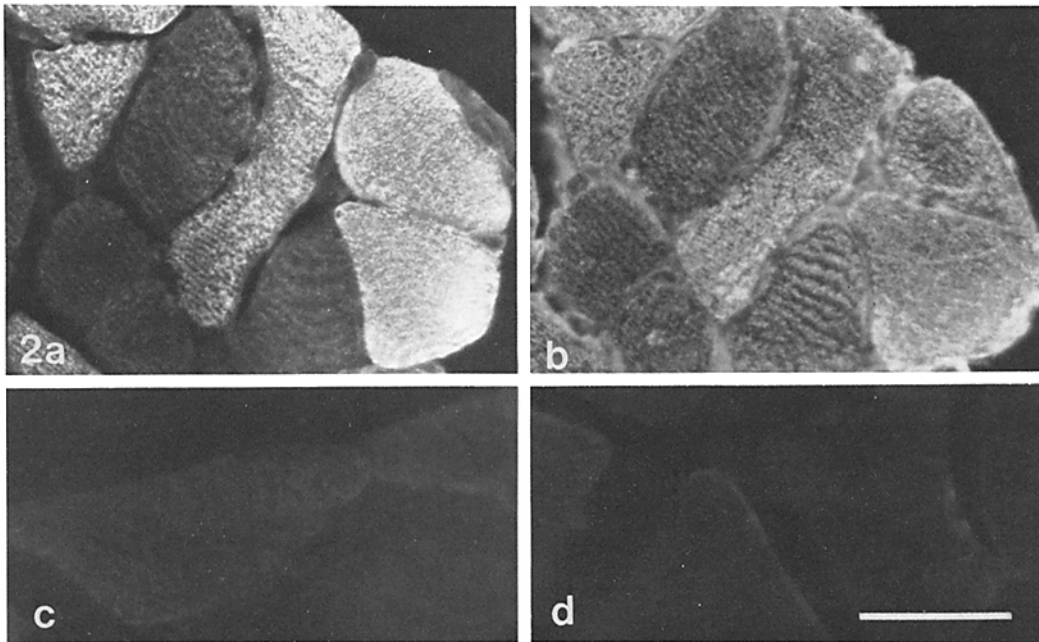
FIGURE 2 Serial transverse sections of myofibers labeled with antibodies to Ca^{++} + Mg^{++}-ATPase (a) and antibodies to calsequestrin (b). Transverse sections of myofibers labeled with normal rabbit immunoglobulin (c) and with normal sheep immunoglobulin (d). The myofibers strongly stained with the Ca^{++} + Mg^{++}-ATPase antibodies (a) are also strongly labeled with calsequestrin antibodies (b) whereas the fibers weakly stained with Ca^{++} + Mg^{++}-ATPase antibodies (a) are moderately stained with calsequestrin antibodies (b). Note that the intensity of staining in the fibers weakly stained with ATPase antibodies is just slightly above that obtained with normal rabbit immunoglobulin (c). In the same fibers, the staining intensity with calsequestrin antibodies (b) is well above that obtained with normal sheep immunoglobulin (d). Bar, 50 μm. × 400.

Myofibers can be divided into slow-twitch and fast-twitch fibers depending on whether the duration of the contraction-relaxation cycle elicited in response to an action potential is long or short (1). To determine the relationship between these two types of myofibers and the fibers showing high and low amounts of ATPase and calsequestrin, transverse cryostat sections of unfixed myofiber bundles were labeled with antibodies to ATPase and compared with adjacent serial sections stained histoenzymatically for the alkali-stable ATPase which is a specific marker for fast-twitch myofibers (7). All of the myofibers which stained strongly with Ca^{++} + Mg^{++}-ATPase antibodies (Fig. 3a) were also positively labeled for the alkali-stable myosin ATPase (Fig. 3b). It should be noted that the fast-twitch myofibers which were more strongly stained with the Ca^{++}-Mg^{++}-ATPase antibodies (Fig. 2a) were also more strongly stained with calsequestrin antibodies (Fig. 2c).

Further studies of the localization of calsequestrin and the ATPase were restricted to fast-twitch myofibers.

Localization of ATPase and Calsequestrin in Transverse Sections of Fast-Twitch Myofibers

In transverse sections of a fast-twitch myofiber from rat skeletal muscle, the ATPase staining pattern formed a continuous, polygonal network (Fig. 4a). The number of sides in each polygon varied from four to six, and the distance between the centers of neighboring polygons ranged from 1.2 to 2.0 μm. A similar, if not identical, staining pattern was observed after labeling the transverse sections with antibodies to calsequestrin (Fig. 4b). The distance between the centers of two neighboring polygons is similar to the distance between the
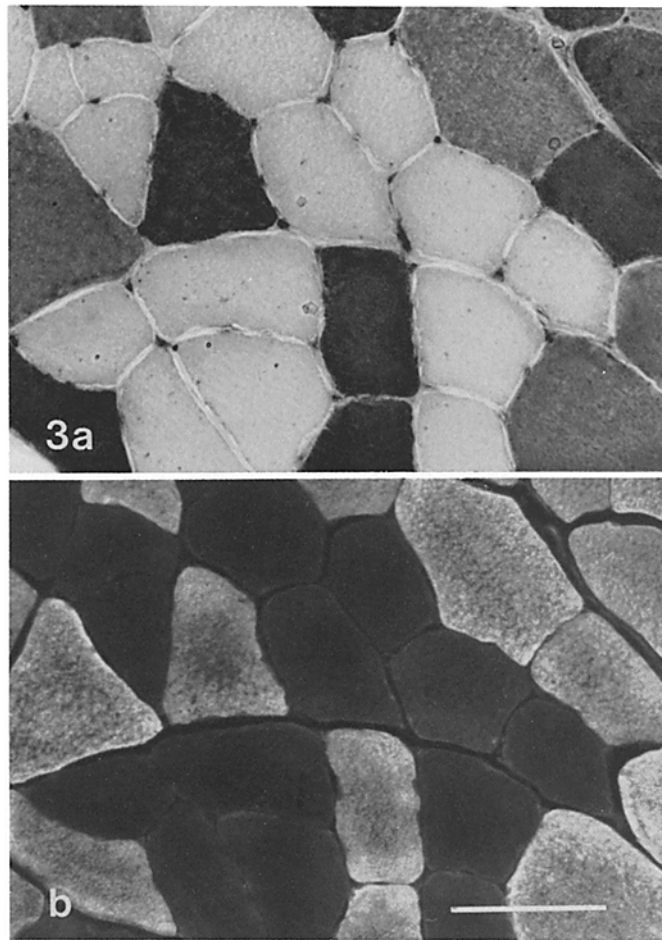
FIGURE 3 Serial cryostat sections of unfixed myofibers (a) histochemically stained for type II myosin ATPase which is a specific marker for fast-twitch myofibers, (b) stained with antibodies to Ca^{++} + Mg^{++}-ATPase by the immunofluorescent staining technique. There is a positive correlation between the fibers strongly stained with Ca^{++} + Mg^{++}-ATPase fibers (negative in contrast) and the fibers positively stained for alkali-stable myosin ATPase (positive in contrast). Bar, 50 μm. × 410.

centers of two neighboring myofibrils in a mature mammalian skeletal muscle fiber (12).

The polygonal staining pattern observed in transverse sections with ATPase antibody was obtained in fixed as well as unfixed tissue. By contrast, the polygonal staining pattern observed after labeling with calsequestrin antibodies was present only if the tissue were fixed before sectioning. This observation is consistent with the fact that the ATPase is an intrinsic component of the sarcoplasmic reticulum and is insoluble in the absence of a detergent, while calsequestrin is a water-soluble, extrinsic protein component of this membrane system (16).

Localization of ATPase and Calsequestrin in Longitudinal Sections of Fast-Twitch Myofibers

The position and the surface area of the terminal cisternae and the longitudinal tubules of the sarcoplasmic reticulum, relative to the A and I bands in mammalian skeletal muscle, have been determined from electron microscope studies (6,
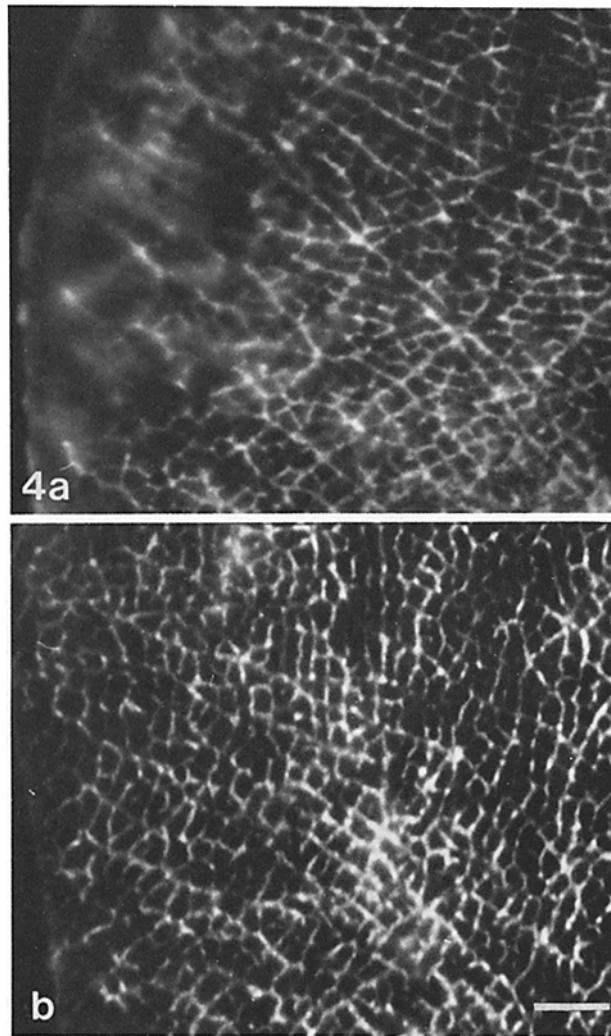
FIGURE 4 Transverse sections of fast-twitch myofibers stained with antibodies to (a) $Ca^{++} - Mg^{++}$-ATPase and (b) calsequestrin. The polygonal staining pattern observed in each of the sections is very similar. The distance between centers of neighboring polygons ranges from 1.2 to 2.00 μm. Bar, 5 μm. × 2,000.

21). Thus, it is possible to determine, at the light microscope level, whether the ATPase and calsequestrin are uniformly distributed within the sarcoplasmic reticulum or whether one or both of these proteins are confined to only certain regions of the membrane. This was done by comparing the fluorescent staining pattern, observed in longitudinal cryostat sections of a myofiber after ATPase or calsequestrin antibody labeling, with the position of the A and I bands in the same field as determined by phase-contrast microscopy.

In longitudinal sections of myofibers fixed at various sarcomere lengths ranging from 2 to 3 μm and stained with ATPase antibody, an intense, regular fluorescent staining pattern was present throughout the I-band region (Figs. 5a, 5b, and 6). In addition, a regular staining pattern limited to the center of the A band could often be seen with this antibody (Figs. 5a, 5b, and 6). While staining in the I-band region was always clearly observable, staining in the A-band region was sometimes difficult to detect.
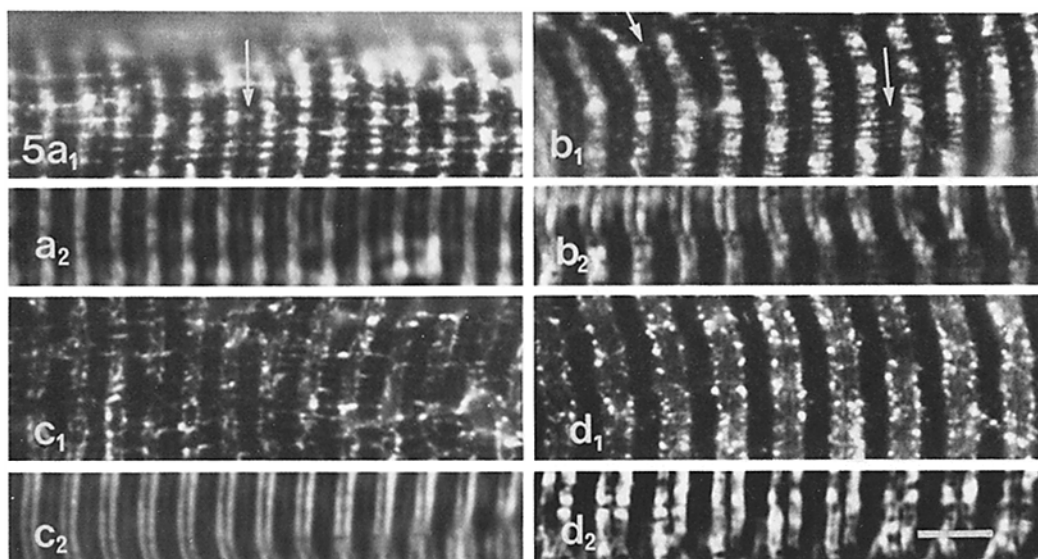
FIGURE 5 The fluorescent staining patterns observed in longitudinal cryostat sections of myofibers fixed at various sarcomere lengths and labeled with antibodies to the ATPase (a_1 and b_1) a; sarcomere length = 2.0 μm, b; sarcomere length = 3.0 μm) and calsequestrin c_1 and d_1 (c; sarcomere length = 2.5 μm and d; sarcomere length = 3.5 μm) are compared to the position of the A and I bands in the same field as seen by phase-contrast microscopy (a_2, b_2, c_2, and d_2).

After labeling with ATPase antibodies (a_1, b_1), the fluorescent staining present in the I band region consists of short strands running parallel to the long axis of the myofiber. The length of these strands equals the width of the I band irrespective of the length of the sarcomere. The staining in the A-band region appears as a single row of small spots running perpendicular to the long axis of the myofiber (arrows). After labeling with calsequestrin antibodies, the staining is confined to the I-band region and is located in parallel rows of small spots. Each row runs perpendicular to the long axis of the myofiber and is positioned at the edges of the I-band region equidistant from the Z-line. Bar, 5 μm. × 2,000.

The fluorescent staining in the I-band region was present in strands which extended throughout the length of the I band and ran parallel to the long axis of the myofiber ~0.5–1.5 μm apart (Figs. 5a, 5b, and 6). The length of these strands was equal to the length of the I band irrespective of whether the myofiber was contracted, at rest length, or stretched during fixation (Figs. 5a, 5b, and 6). The staining pattern present in the center of the A-band region consisted of a single row of small fluorescent spots, ~0.4 μm in diameter, which ran perpendicular to the long axis of the myofiber in the region of the H zone. The distance between adjacent spots in the A-band region was similar to the distance between the parallel fluorescent strands observed in the I-band regions on both sides of the A band. This range of distances between the adjacent strands and spots corresponds well with the distances one would expect to observe between the centers of neighboring

myofibrils cut at random in longitudinal sections through the myofiber.

While fluorescent staining with ATPase antibodies was observed both in the I- and the A-band regions of the myofiber, fluorescent staining with calsequestrin antibodies was confined to the I-band region of the myofiber (Figs. 5c, 5d, and 7). Moreover, even in the I-band region the staining pattern of the two proteins was different. Although some staining was observed throughout the I-band region,; most of it was concentrated in rows of brightly staining spots running perpendicular to the long axis of the myofiber at the edge of each I band, equidistant from the Z-line. The distance between the two neighboring fluorescent spots in a row varied from 0.5 to 1.5 μm. This staining pattern was observed in sarcomeres varying in length from 2.5 to 3.5 μm (Figs. 5c, 5d, and 7).
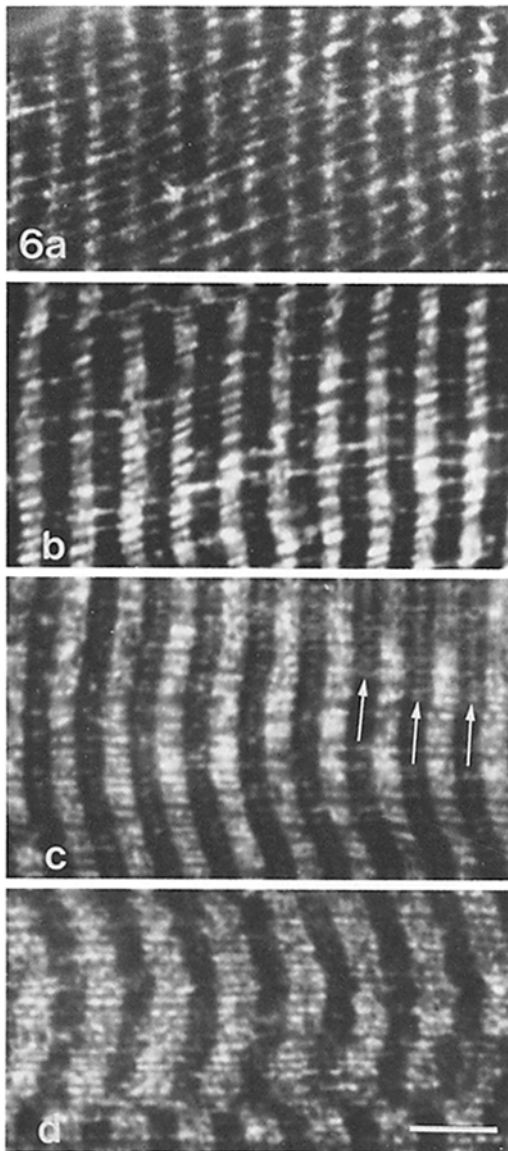
In a stretched myofiber, the majority of the

FIGURE 6 Longitudinal cryostat sections of myofibers fixed at various sarcomere lengths (a) 2.0, (b) 2.5, (c) 2.8, and (d) 3.5 μm and labeled with $Ca^{++} + Mg^{++}$ ATPase antibodies. It should be noted that the general staining pattern remains the same irrespective of the sarcomere length. The length of the positively stained strands in the I-band region equals the width of the I band at each of the different sarcomere lengths. Staining in the center of the A-band region (arrows) is also apparent in some of the sections (c). Bar, 5 μm. × 2,300.

small fluorescent spots located at each edge of the I band appeared round. Occasionally, however, some of the fluorescent spots appeared to be

dumbbell shaped, with the long axis of the dumbbell oriented parallel to the long axis of the myofiber (Fig. 7b).

Control Studies

To test the specificity of the staining patterns obtained with the two antibodies, the supernate from the ATPase antibody absorbed with ATPase and the supernate from the calsequestrin antibody absorbed with calsequestrin were used in immunofluorescent staining tests. In both cases, reduced or no staining was observed and the patterns of staining described above could not be detected (Fig. 1e and f). Similarly, no staining was observed when the monospecific rabbit antibodies to ATPase or sheep antibodies to calsequestrin was substituted with normal rabbit γ-globulin or normal sheep γ-globulin, respectively, in the immunofluorescence tests (Fig. 2c and d).

Other Cell Types

No staining was observed either in the endothelial cells or the smooth muscle cells of blood vessels present in the same sections after labeling with either ATPase or calsequestrin antibodies (results not shown).

Electron Microscopy

Our interpretation of the distribution of the ATPase and calsequestrin within the muscle cell depends on how reliably the fixation of the tissue used for immunofluorescence studies maintained these proteins at the location they occupied in the living cell. To determine the extent of preservation of normal morphology of this membrane system by our fixation technique, we have examined the ultrastructure of the myofibers fixed in the same way as those used for the immunofluorescence staining. The results obtained are shown in Fig. 8. The contractile apparatus and the sarcoplasmic reticulum were, indeed, well preserved, and longitudinal tubules, terminal cisternae, and triads could be easily distinguished. These results indicate that the fixation procedures used before the antibody labeling preserved the ultrastructure of the sarcoplasmic reticulum very well and that the distribution of ATPase and calsequestrin observed by immunofluorescence should correspond closely to their distribution in living myofibers.

DISCUSSION

The results obtained from these studies show, at the cellular level, that the $Ca^{++} + Mg^{++}$-ATPase,

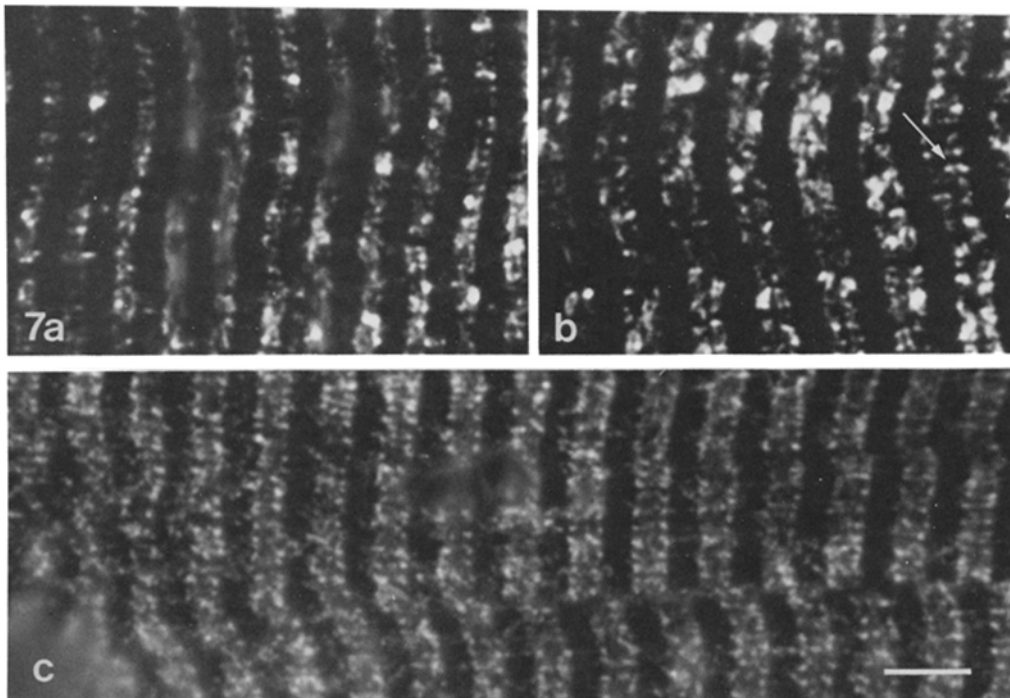FIGURE 7 Longitudinal cryostat sections of myofibers fixed at various sarcomere lengths (a, sarcomere length = 2.5 μm, b and c, sarcomere length = 3.5 μm) and stained with antibodies to calsequestrin. The staining pattern in the I-band region appears as rows of small fluorescent spots which run perpendicular to the long axis of the myofiber and are positioned at each edge of the I-band region (a,b,c). Occasionally, the fluorescent spots appear to be dumbbell shaped (b, arrow). At short sarcomere lengths (a) the staining is still confined to the I-band region of the sarcomere, but the distance between the two adjacent rows of fluorescent spots has now become shorter. A narrow dark line is noticeable at a position corresponding to the Z-line region of the sarcomere. Bar, 5 μm. × 2,300.

the major intrinsic protein, and calsequestrin, the major extrinsic protein, are differently distributed in the sarcoplasmic reticulum of mammalian skeletal muscle. In addition, they show that fast-twitch myofibers have a relatively high amount of both the ATPase and calsequestrin while slow-twitch myofibers have a very low amount of the ATPase and a moderate amount of calsequestrin.

Stereological analyses of the sarcoplasmic reticulum at the ultrastructural level have established that the density of this membrane in mammalian fast-twitch myofibers is approximately twice that in slow-twitch myofibers (3, 4, 5, 14). If the protein composition of the membrane and the antigenicity of the two proteins were the same in the two types of myofibers, one would expect approximately one-half of the staining intensity in the slow-twitch as compared with the fast-twitch myofibers after labeling with either the ATPase or calsequestrin antibodies. This may be true for the staining intensity observed after labeling with cal-

sequestrin antibodies. However, it is not true for the ATPase where the staining intensity is reduced severalfold in slow-twitch fibers. These results, therefore, suggest that the stoichiometric relationship between the ATPase and calsequestrin is different in slow and fast-twitch myofibers. This interpretation, obtained as a result of immunofluorescence studies at the cellular level, is supported by several biochemical studies which have demonstrated that the Ca^{++}-Mg^{++}-ATPase activity (references 9, 19, 22, 23, and footnote 1) in sarcoplasmic reticulum vesicles purified from slow-twitch muscle is absent or at least ten times lower than in the sarcoplasmic reticulum vesicles isolated from fast-twitch muscle. Zubrzycka et al.[1] have carried out disc gel electrophoretic analysis to determine the protein composition of the heavy fraction of sarcoplasmic reticulum vesicles purified from fast-twitch and slow-twitch muscle tissue. The ATPase was found to constitute 10–20% and 70%, respectively, of the total membrane protein
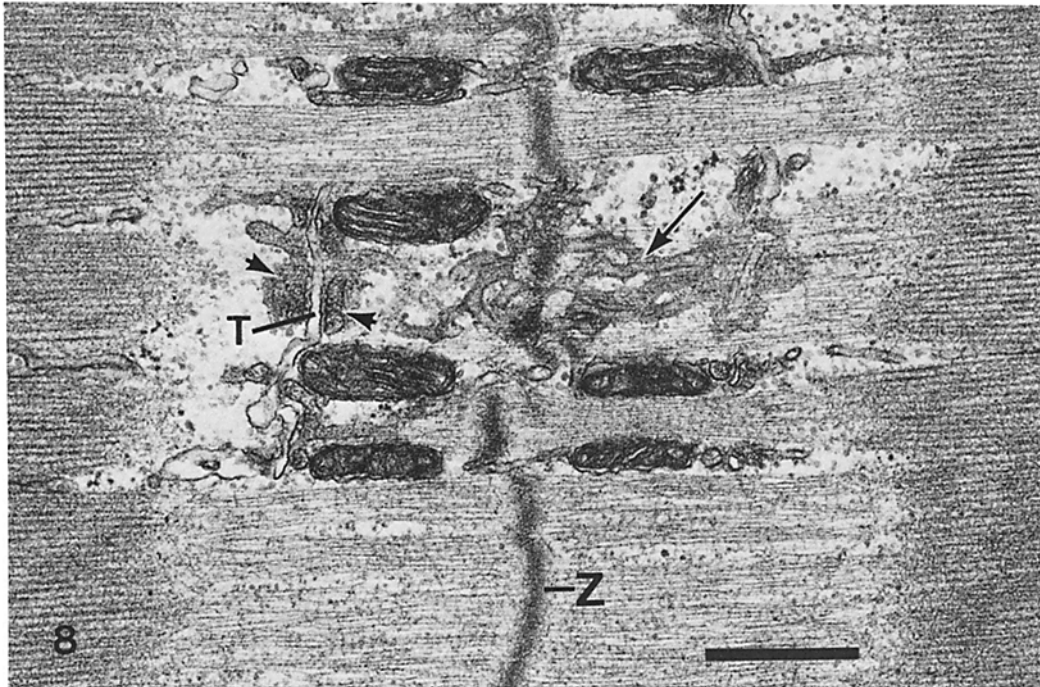
FIGURE 8 Electron micrograph of a longitudinal section of a myofiber from rat skeletal muscle fixed in the same way as the sections used for immunofluorescence. Both the longitudinal tubules (arrow) and the terminal cisternae (arrowheads) of the sarcoplasmic reticulum as well as the T-tubules (*T*) can be easily distinguished. In addition, it should be noted that much more electron-opaque material is present in the terminal cisternae than in the longitudinal tubules. The T-tubules (*T*) in particular appear to be free of electron-opaque material. Z-line (*Z*); Bar, 0.5 μm. $\times$ 40,000.

from the slow-twitch and the fast-twitch muscles, while calsequestrin constituted 9% and 15%, respectively. The ATPase and calsequestrin from both sources of muscle were shown to be equally antigenic against the very antibodies which were used in the present study.[1]

From these reports and our own data, we conclude that the content of calsequestrin per unit surface area of sarcoplasmic reticulum in fast-twitch and slow-twitch myofibers is comparable, while the content of the ATPase per unit surface area of sarcoplasmic reticulum in fast-twitch myofibers is much higher than in slow-twitch myofibers. The degree of interaction between the Ca^{2+} transport function of the ATPase and the Ca^{2+}-binding function of calsequestrin is not clear but an interaction between these functions could well affect rates of Ca^{2+} uptake and release. The finding that different stoichiometries exist between these proteins in slow and fast-twitch muscle may provide a clue about the process regula-

ting the speed of response of these two muscle types to electrical stimulation.

Previously, we described the time of appearance and distribution of the ATPase and calsequestrin in differentiating rat skeletal muscle cells from primary cultures (13). We observed that all multinucleated myotubes in cultures as old as 6 d were brightly stained with antibodies to the ATPase and calsequestrin. Therefore, it is unlikely that the cellular processes responsible for the differences in the ATPase and calsequestrin concentrations between slow-twitch and fast-twitch myofibers, seen in the adult skeletal muscle, were operating at these stages of myotube maturation. It is possible that these differences appear only after neural innervation of myotubes as suggested by Margreth et al. (19). Neural control might continuously repress the cellular functions involved in the production of the Ca^{++}-Mg^{++}-ATPase in slow-twitch myofibers.

Although both the ATPase and the calseques-

trin are present in relatively high concentration in the fast-twitch myofibers, the distribution of these two proteins, as judged from the staining patterns observed after labeling with the two antibodies, is distinctly different in these myofibers. Calsequestrin was localized in the I-band region and was concentrated at the interface between the I- and A-band regions of the sarcomere. The ATPase was found throughout the I-band region as well as in the center of the A-band region. Stereological analysis at the ultrastructural level has established that the density of the sarcoplasmic reticulum in fast myofibers varies in different regions along the sarcomere. The density of membranes in the I-band region is approximately twofold higher than in the H-zone region (3, 14, 21), while in that part of the A band where thick and thin filaments overlap the sarcoplasmic reticulum is very sparse (14). In mammalian skeletal muscle fibers, such as those examined, the triads composed of the two terminal cisternae of sarcoplasmic reticulum and the transverse tubule are always positioned close to the junction between the A and I band, and there are two of them per sarcomere (3, 14, 21).

Immunofluorescent staining patterns for the ATPase indicate that the protein is concentrated throughout the I-band region and is present also in a narrow region of the A band corresponding to the H zone. Very little if any staining was observed in the region of the A band where the actin and myosin filaments overlap. This is the region, however, where the membrane density may be so low as to preclude detection by immunofluorescence staining. Thus, the distribution of immunofluorescent staining is comparable to that of the overall distribution of the sarcoplasmic reticulum. This suggests that the ATPase is rather uniformly distributed throughout all regions of the sarcoplasmic reticulum.

By contrast, immunofluorescent staining patterns for calsequestrin indicate that the protein is located almost exclusively in the area corresponding to the junction between the A and I bands. This region is the area where the terminal cisternae of the sarcoplasmic reticulum are located. These observations indicate that calsequestrin is located mostly in the terminal cisternae of the sarcoplasmic reticulum and is not uniformly distributed throughout the membrane system.

This interpretation is in agreement with that of Meissner (20) who studied the distribution of ATPase and calsequestrin in the various parts of the sarcoplasmic reticulum by a combination of biochemical and ultrastructural approaches. Meissner separated highly purified sarcoplasmic reticulum vesicles isolated from rabbit white (fast-twitch) skeletal muscle into light and heavy fractions. Both fractions had Ca^{++} + Mg^{++}-ATPase activities and capacities to accumulate Ca^{++}. However, only the heavy fraction contained calsequestrin and the high-affinity binding protein. In addition, electron microscope analysis of the fractions showed that dense material similar to that observed in vivo in the terminal cisternae of the sarcoplasmic reticulum was present inside the heavy vesicles. On the basis of these results, it was suggested that the ATPase is uniformly distributed in the sarcoplasmic reticulum while calsequestrin and the high-affinity binding protein are confined to the terminal cisternae.

Since calsequestrin is an extrinsic protein component and the ATPase an intrinsic component of the sarcoplasmic reticulum, one might suggest that the staining intensity of calsequestrin in the various regions of the sarcomere could be proportional to the volume of the sarcoplasmic reticulum lumen, while the concentration of the ATPase could be proportional to the surface area of the sarcoplasmic reticulum. If this were true, the volume to surface ratio of the terminal cisternae in mammalian muscle would be only 30% higher than that of longitudinal tubules (3). This small difference in the volume to surface ratio in these two segments of the membrane would not account for the bright staining with anticalsequestrin in the region believed to be occupied by the terminal cisternae and the total absence of staining in the A band of the sarcomere. On the contrary, the fact that different portions of the sarcoplasmic reticulum in a fast myofiber have a different protein composition indicates that these regions are indeed specialized to perform different functions.

The possibility that the different parts of the sarcoplasmic reticulum system perform different functions was demonstrated by Winegrad (30) in elegant physiological studies on fast frog skeletal muscle. These experiments indicated that Ca^{++} was stored in, and released from, the terminal cisternae but was taken up by the whole surface of the sarcoplasmic reticulum. Our finding that calsequestrin appears to be confined to the region of terminal cisternae in situ suggests that Ca^{++} may also be preferentially stored in the terminal cisternae in mammalian fast-twitch muscle. These findings also support the view that Ca^{++} is taken up

by the Ca^{++}-Mg^{++}-ATPase in all parts of the sarcomere and may subsequently diffuse into the terminal cisternae to be bound by calsequestrin.

To determine more precisely where the ATPase and calsequestrin are located in various parts of the sarcoplasmic reticulum of the fast-twitch myofiber, we are beginning studies using antibody labeling techniques designed for the higher level of resolution obtainable by electron microscopy. This approach should allow us to make more detailed observations on the localization of these two proteins in the sarcoplasmic reticulum and to verify both the localization of calsequestrin in the terminal cisternae and the uniform distribution of the ATPase throughout the membrane. Such studies should also permit us more detailed examination of the assembly of these two proteins into a membrane during differentiation of muscle cells in culture.

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