The Structure of Calsequestrin in Triads of Vertebrate Skeletal Muscle: A Deep-etch Study

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Abstract. We have examined the structure of calsequestrin in three-dimensional images from deep-etched rotary-replicated freeze fractures of skeletal muscle fibers. We selected a fast-acting muscle because the sarcoplasmic reticulum has an orderly disposition and is rich in internal membranes. Calsequestrin forms a network in the center of the terminal cisternae and is anchored to the sarcoplasmic reticulum membrane, with preference for the junctional portion. The anchorage is responsible for maintaining calsequestrin in the region of the sarcoplasmic reticulum close to the calcium-release channels, and it corroborates the finding that calsequestrin and the spanning protein of the junctional feet may interact with each other in the junctional membrane. Anchoring filaments may be composed of a protein other than calsequestrin.

CALSEQUESTRIN (or calcium-binding protein) is an acidic protein which is contained within the lumen of the sarcoplasmic reticulum (SR)[1] of skeletal, cardiac, and smooth muscle (see MacLennan et al., 1983, for a review). Estimates of its molecular weight have ranged between 44 and 65 kD (Meissner et al., 1973; Campbell et al., 1980; Campbell and MacLennan, 1981; Campbell et al., 1983b). A more precise determination by gel filtration in the presence of guanidine hydrochloride gives a value of 42 kD (Cozens and Reithmeier, 1984). Calsequestrin has a low affinity ($\sim$1 mM^{-1}) but high capacity ($\sim$50 calcium ions per molecule) for calcium and it is considered to be responsible for the ability of the SR to maintain a high calcium content in vivo (Endo, 1977; Somlyo et al., 1977).

It is widely accepted that calsequestrin constitutes most if not all the content of the terminal cisternae of the SR seen in electron micrographs of thin-sectioned or negatively stained triads (Meissner, 1975; Lau et al., 1977; Campbell et al., 1980; Jorgensen et al., 1979, 1983). In isolated heavy SR fractions there is a tendency for the calsquestrin to aggregate on one side of the vesicles, usually in association with the junctional portion of the membrane, where the feet are located. This has led to the hypothesis that some relationship exists between calsequestrin and junctional feet (Campbell et al., 1980; Brunschwig et al., 1982). The hypothesis has received considerable support from the demonstration that under the appropriate conditions calsequestrin and feet and other minor components of the junctional membrane remain associated with each other when heavy SR is extracted with

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1. *Abbreviations used in this paper*: jSR, junctional sarcoplasmic reticulum; SR, sarcoplasmic reticulum.

Triton X-100 (Caswell and Brunschwig 1984; Costello et al., 1986). These results would suggest some direct attachment of calsequestrin to the junctional membrane. Thin section electron microscopy has provided images suggestive of such a connection, by showing that the internal proteins form a dense band, or plate, parallel to the junctional SR (jSR) membrane and joined to it across an intervening space by small particles or granules (Johnson and Sommer, 1967; Sommer and Johnson, 1979; Walker et al., 1971). This so-called coextensive density is particularly visible in cardiac muscle (Johnson and Sommer, 1967; Waugh and Sommer, 1974), and in rapid-frozen skeletal muscle (Somlyo, 1979; Nassar et al., 1986) and has been described in skeletal muscle fixed during K contractures (Eisenberg and Gilai, 1979) and in isolated SR fractions (Saito et al., 1984).

Three limitations of images from thin sections restrict the amount of information they can give on the structure of calsequestrin and/or other proteins forming the SR content. (*a*) The appearance is quite variable. The protein can be either fairly uniformly distributed in the available volume, or collected more or less in the center of the cisternae, leaving an empty peripheral rim. This variation indicates some lability of the structure either as a result of changes in calcium concentration during primary and secondary fixations, or due to direct damaging effects of osmium and dehydration. (*b*) The smaller components of the structure are not visible due to contrast limitations. (*c*) The images are two-dimensional. The technique of deep etching affords a three-dimensional view of structures which have not been exposed to osmium and dehydration. We have examined the structure of calsequestrin by this approach and report on the presence of specific connections between calsequestrin and SR membrane, which are predominantly found in the junctional region of the SR.
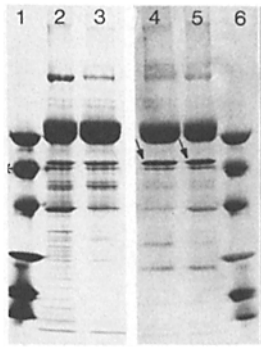
Figure 1. Coomassie Blue–stained (lanes *2* and *3*) and "stains all"–stained (lanes *4* and *5*) Laemmli gels of a crude SR fraction from toadfish swimbladder muscle. Arrows point to the only band that had a blue color in the "stains all" gels, identifying it as a calcium-binding protein, most likely calsequestrin. The large band above it is Ca ATPase. The standards (lanes *1* and *6*) and their molecular masses are as follows: lysozime (14.4 kD); soybean trypsin inhibitor (21.5 kD); carbonic anhydrase (31 kD); ovalbumin (45 kD); BSA (66.2 kD), asterisk; phosphorylase B (92.5 kD).

Materials and Methods

Muscles from the swimbladder of toadfish (*Opsanus tau*), the body of the glass fish (*Chanda randa*), and the sartorius muscle from the frog (*Rana pipiens*) were used. Muscles were fixed in 3% glutaraldehyde either in phosphate or cacodylate buffer for a minimum of 2 h, but more often for several days. Initial fixation was at room temperature, storage at 4°C. For thin sections muscles were either (*a*) postfixed in OsO₄, en bloc stained with uranyl acetate, dehydrated, and embedded in Spurr or Epon; or (*b*) cryoprotected in 30% glycerol, frozen in freon 12 near melting point, freeze-substituted in acetone-osmium (Van Harreveld et al., 1965), allowing the acetone to warm to room temperature in a period of 3–4 h. Once at room temperature, the specimens were rinsed in absolute MeOH to remove the remaining glycerol, en bloc stained in 2% uranyl acetate in EtOH, and embedded in Epon. Thin sections were stained in uranyl acetate and lead. For freeze fracture, small bundles of fibers were mounted on flat surfaces of small copper specimen holders to which a piece of filter paper was stuck by gelatin, immersed in 30% MeOH for 5–10 min, and frozen in freon 22. Slightly larger specimens were mounted directly in troughs excavated in the holders. After fracture the specimens were etched for 30 h at −110°C. The specimens were rotary shadowed with platinum at 45° and replicated with carbon. Images were taken in JEOL 100B and Philips 410 and 400 microscopes.

Crude SR fractions were isolated from toadfish swimbladder muscle, essentially as described by Herbette et al. (1977), but using a very fine initial mincing and gentle homogenization. In one purification procedure, the solutions contained 1 mM phenylmethylsulfonyl fluoride to prevent proteolysis, and gels were run from this fraction. SDS–polyacrylamide continuous gradient gels were run using the Laemmli (1970) buffer system (acrylamide 8–20%; 0.75-mm gels; 50–100 μg protein). Gels were stained either with Coomassie Blue (0.25%, for 1 h), or the cationic carbocyanine dye "stains all," as in Campbell et al. (1983a). Molecular weights of the proteins were determined against low molecular weight standards from Bio-Rad Laboratories, Richmond, CA in the Coomassie Blue gels (see Fig. 1).

Results

Identification of Calsequestrin in Toadfish Swimbladder Muscle

Since most of the description applies to muscle fibers from the toadfish swimbladder, we ascertained that a molecule similar to rabbit skeletal muscle calsequestrin exists in the fish muscle. Identification was based on two criteria: molecular weight and the specific blue stain of calsequestrin and other calcium-binding proteins in "stains all" (Cala and Jones, 1983; Campbell et al., 1983a). Fig. 1 shows Coomassie blue and "stains all" stained gels from crude SR fractions isolated from toadfish swimbladder muscle. A protein running at a molecular weight of 66–72 kD forms a blue band in the "stains all" gel (arrow), while the other proteins have

a red color. Although the protein has a molecular weight slightly higher than calsequestrin from skeletal muscle, its staining properties and abundance in the SR fraction identify it as the calcium-binding protein.

Thin Sections

Muscle fibers used in this study have triadic junctions between SR and T tubules; i.e., each T tubule is flanked by two terminal cisternae of the SR. The remaining SR is often referred to as longitudinal or nonjunctional SR. Junctional SR membrane (jSR) indicates the portion of membrane in a terminal cisterna, which faces directly across a T tubule. The rest of the SR membrane in the terminal cisternae, as well as in the longitudinal SR, is called free SR.

In thin sections most of the space of the terminal cisternae is filled by a visible content, consisting of calsequestrin and possibly other proteins (Figs. 2–5). The longitudinal SR is apparently empty (Figs. 2 and 3), except in areas where a terminal cisterna extends longitudinally (see asterisk in Fig. 4). The very fast acting fibers of the toadfish swimbladder have an SR with a higher protein content than other twitch fibers of more moderate speed, such as those from the body muscles in most fishes (see below) and from the frog.

The appearance of the SR content varies considerably depending on the method of preservation used after the initial glutaraldehyde fixation. In material conventionally postfixed in osmium, the protein(s) loosely fill the available space, leaving an ill-defined clear area between it and the jSR membrane (Figs. 2 and 4). In freeze-substituted material (Figs. 3 and 5) the SR content is denser, is more compact, and has a coarse, granular structure. Fairly large grains are arranged in rows parallel to the jSR membrane and the space between the proteins, and the membrane is a well-defined translucent strip. Fine, barely visible lines join the rows of grains and the jSR membrane, facing the T tubules (T). Preservation obtained with freeze-substitution after an initial fixation in glutaraldehyde is better than with standard osmium postfixation (Figs. 2 and 3), and is comparable to that shown in rapid-frozen tissue (Somlyo, 1979; Nassar et al., 1986). This is confirmed by the close similarity of the freeze-substituted and deep-etched structures (see below).
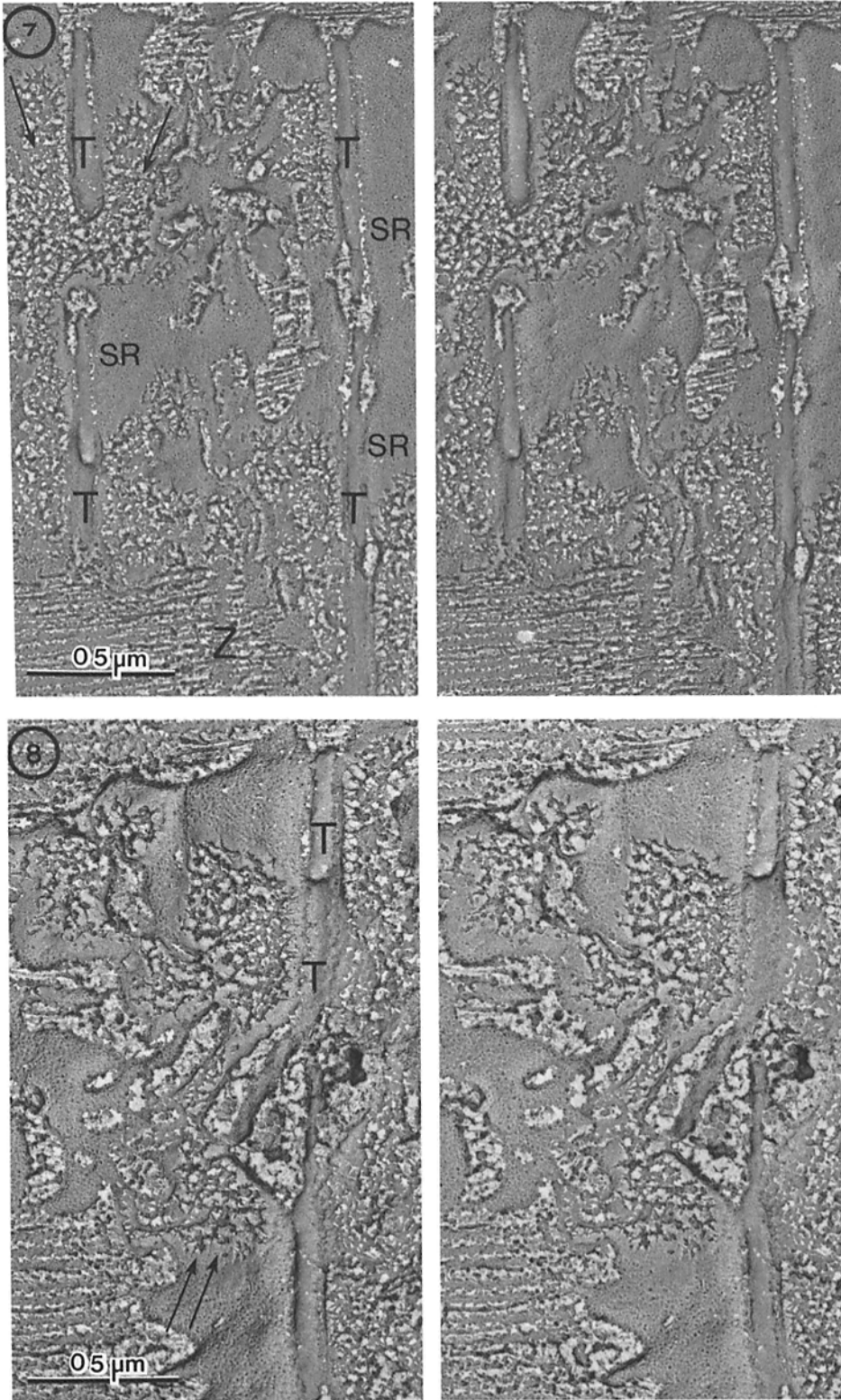
Deep Etching

The cytoplasmic leaflet of the split SR membranes is not affected by deep etching, and it shows the usual tight arrangement of particles (Fig. 6). Visibility of particles is limited due to the high angle of the rotary shadow. The content of the SR lumen is revealed wherever the fracture cuts across the interior of the vesicles and it has the same granular appearance as in the freeze-substituted images (compare Figs. 5 and 6). Notice that the granular content is not limited to the terminal cisternae, but is also found in some longitudinal extensions (see asterisk in Fig. 6).

The structure of the SR content is best observed in fractures which cut along the long axis of the T tubules and in stereo images (Figs. 7 and 8). The central region of the terminal cisternae is occupied by a three-dimensional net formed by nodal points and connecting thin strands. The nodal points of the net are granules of variable shape and size (∼16–33 nm in diameter) and they correspond to the large grains seen in thin sections of freeze-substituted material
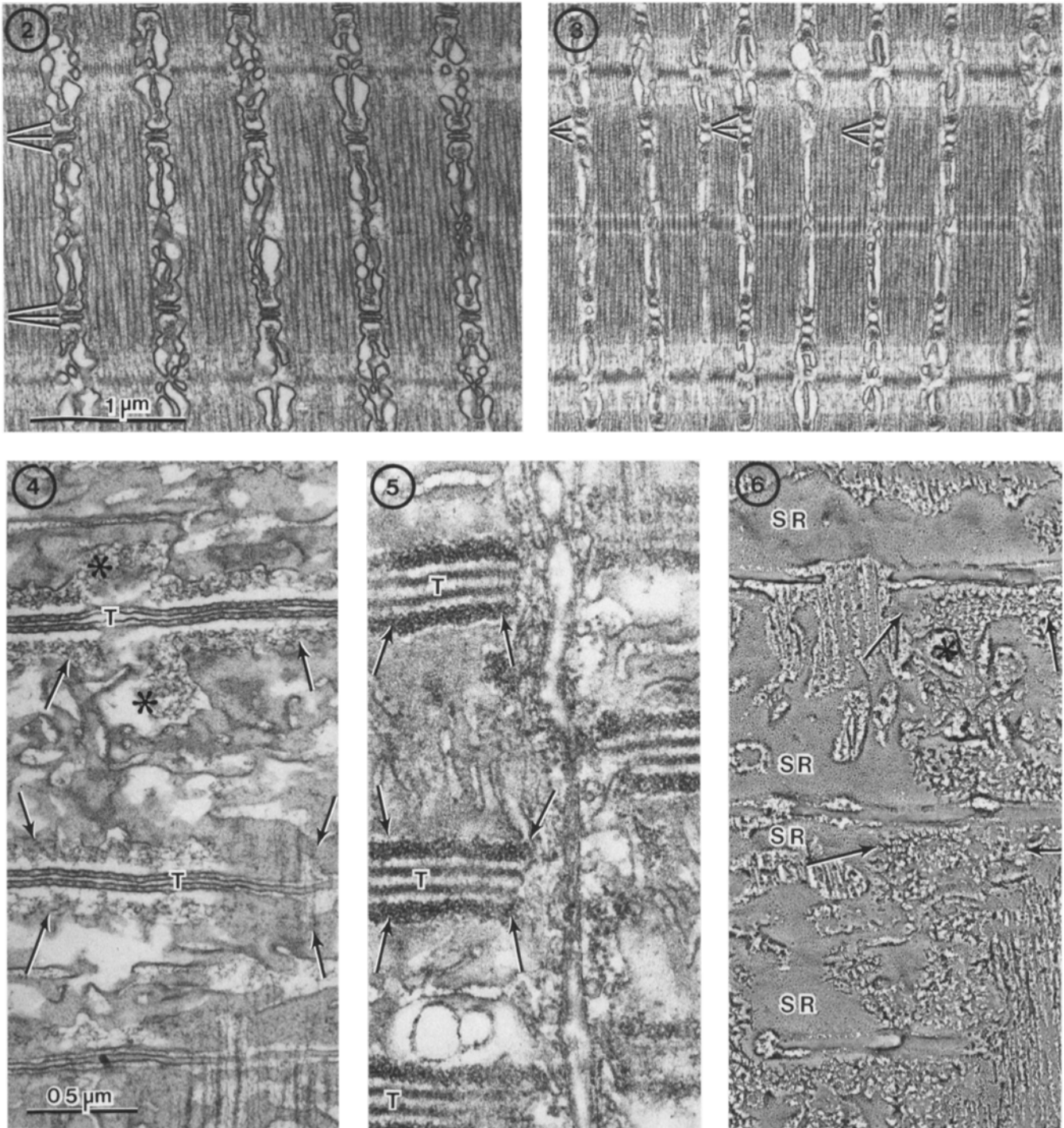
Figures 2–6. Longitudinal sections of glutaraldehyde-fixed, osmium-postfixed (Fig. 2), and glutaraldehyde-fixed, freeze-substituted (Fig. 3) muscle fibers. Both are from the toadfish swimbladder muscle. Triads are indicated by three lines. Notice that the content of the terminal cisternae is more compact and the myofilaments are better preserved in the freeze-substituted material. (*Figs. 4 and 5*) Grazing views of SR and T tubules (*T*) in longitudinal sections from the toadfish swimbladder, treated as in Figs. 2 and 3. T tubules and the accompanying terminal cisternae of the SR run from right to left in the images. In conventionally treated material (Fig. 4) the content of the lateral sacs of the triad (between arrows) is more diffuse than in freeze-substituted material (Fig. 5). The aggregates in Fig. 5 are formed by tightly arranged coarse granules located in the center of the SR cisternae, at some distance from the junctional SR membrane. Thin strands connecting these aggregates to the jSR membrane (facing T tubules) are barely visible. Notice extensions of the content away from the triad (*asterisk* in Fig. 4). (*Fig. 6*) View of SR and T tubules from a deep-etched sample. Same muscle and orientation as Figs. 4 and 5. The SR content appears as a network (between arrows). Notice extensions of the network away from the triad, similar to those in Fig. 4 (*asterisk*). *SR* indicates the areas where the SR membrane has been fractured and the ATPase particles decorating the cytoplasmic leaflet are visible.

Figures 7 and 8. Stereomicrographs of a deep-etched fiber from the toadfish swimbladder. T tubules (*T*) run vertically. The network nature of the SR filling is particularly evident between arrows, where an SR cisterna continues from one side to the other of the T network. Some of the content extends to the region of SR over the Z line (*Z*). (*Fig. 8*) View similar to Fig. 7, but at higher magnification. The internal network is excluded from the region immediately adjacent to the jSR membrane (next to T tubules, *T*). This area of the SR is occupied by thin links which connect the central net (presumably calsequestrin) to the jSR membrane. Similar, but less numerous strands connect the central network to the free SR membrane (*small arrows*). The central network is formed by nodal points of irregular size joined by short connecting strands.

(compare with Fig. 5, between arrows). The thin joining strands are not seen in thin sections, due to their small diameter. They connect each granule to several of its neighbors. Other thin strands similar in diameter to those forming the network, but of longer length, connect the periphery of the three-dimensional internal network to the surrounding SR membrane. These peripheral connections are more numerous opposite the jSR membrane than along the free SR (compare Figs. 9 and 10).

Since the cytoplasmic side of the jSR is covered by junctional feet, which join it to the T tubules, we checked whether a relationship exists between the positions of inter-
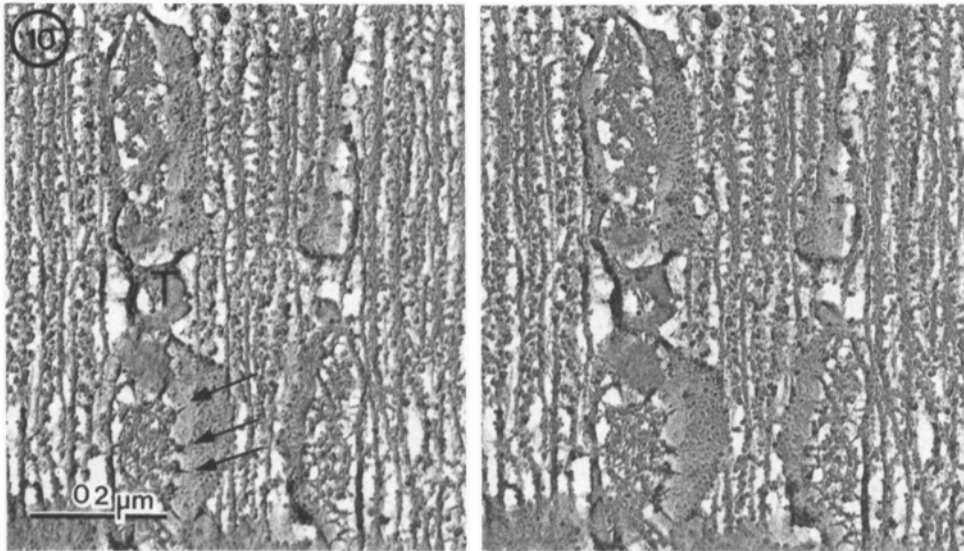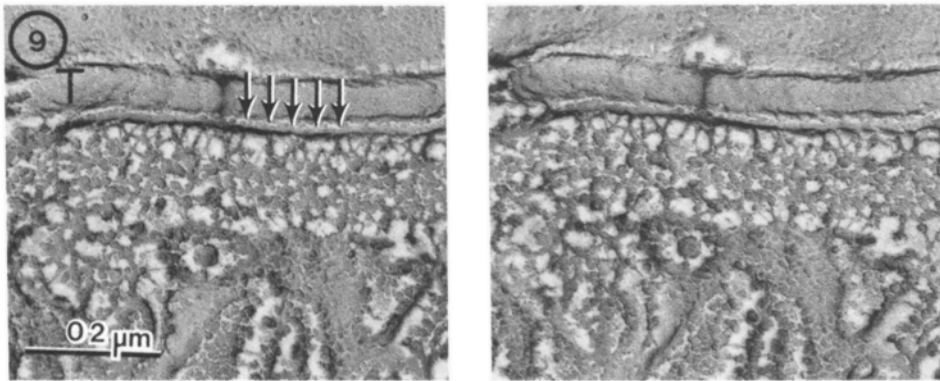
Figures 9 and 10. (Fig. 9) Detail of the joining strands at the level of the jSR. Small arrows mark the periodic distance at which junctional feet are expected to be placed over the jSR membrane. Position of joining strands does not necessarily coincide with that of feet. *(Fig. 10)* Deep-etch image of a triad fractured at right angle to T tubule *(T)* long axis, to show existence of connecting strands joining content of terminal cisternae laterally to free SR membrane *(small arrows)*.

nal joining strands and of the junctional feet. Arrows in Fig. 9 were placed at the known periodic distance between feet. It is clear that the spacing of the joining strands does not match that of the feet. Notice also that spacing of feet is constant in all muscle fibers, while frequency and spacing of junctional strands may be quite variable (compare Figs. 8 and 9 with 11 and 12).

Along the free SR, less frequent peripheral strands maintain the internal content in the center of the available space (Figs. 7 and 10). These strands initiate at a nodal point in the network and terminate at the interior surface of the SR membrane, apparently joining to it.

### Structure of Junctional SR in Other Muscle Fibers

White fibers from glass fish myotomes, which do not contract as rapidly as those from toadfish swimbladder, have considerably less content in the terminal cisternae (Figs. 11 and 12), as judged from the amount of visible structure located in the center of the cisternae. In parallel with the less extensive network, the peripheral joining strands are also less frequent, both at the jSR and at the free SR membrane. Fibers in the frog sartorius also have a less dense internal network and fewer links between it and the jSR membrane (not shown).

Does the Longitudinal SR Have a Content?

In the traditional view of longitudinal SR provided by thin sections, this portion of the SR is empty, except in areas where there is a longitudinal extension of one of the terminal cisternae (see Figs. 4 and 6). Such extensions are quite frequent in the toadfish swimbladder muscle, but rare in other fibers. In deep-etched images on the other hand, the longitudinal SR may contain small nodules closely apposed to the membrane. In Figs. 11 and 12 some of these nodules are located at some distance from the triad (arrows). Except for these small structures, the lumen of the longitudinal SR is empty.

Discussion

The major protein component of terminal cisternae is calsequestrin and thus it is likely that most of the structural components that we observe in the terminal cisternae are composed of this protein. We suggest that calsequestrin forms the nodal points in the SR content and a second protein constitutes the elongated elements which binds the components of the network to each other and to the SR membrane. The nodal points are larger than the 11–15-nm spacings in crystalline calsequestrin (Saito et al., 1984; Maurer et al., 1985), which are likely to represent molecular dimensions. Our im-
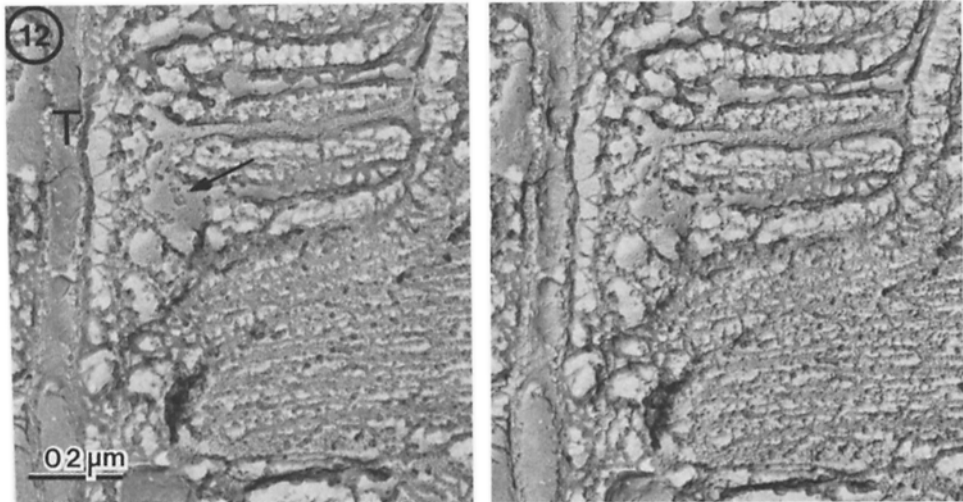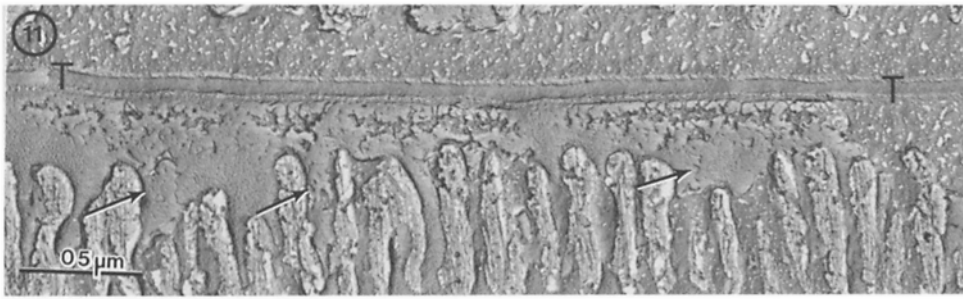
Figures 11 and 12. Deep-etch replicas from the body muscle of a small fish. These fibers are not as fast as those illustrated in the previous images, and the content of the terminal cisternae is considerably reduced. Note also less frequent connection to the jSR membrane. Arrows point to small aggregates within the SR lumen but not in the terminal cisternae. T tubules (*T*) run horizontally in Fig. 11 and vertically in Fig. 12.

ages agree quite well with those derived from rapid-frozen muscles, not exposed to chemical fixation (Somlyo, 1979; Nassar et al., 1986), except that in the latter the translucent strip may be located more centrally in the terminal cisternae.

Fig. 13 summarizes current understanding of triadic junction, combining structural and biochemical identification of junctional components from enriched fractions (Meissner, 1975; Lau et al., 1977; Campbell et al., 1980; Mitchell et al., 1983; Caswell and Brunschwig, 1984; Saito et al., 1984; Costello et al., 1986). On the cytoplasmic side of the jSR are the junctional feet (Franzini-Armstrong, 1970), large structures composed of four subunits (Ferguson et al., 1984), which are disposed in a periodic arrangement over the jSR membrane and are responsible for the constant size of the T-jSR gap. The large spanning proteins associated with the jSR membrane are the most likely candidates for feet compo-

Figure 13. Three-dimensional reconstruction of a triad. A T tubule comes towards the viewer in the center of the image and is accompanied by two terminal cisternae of the SR. The lower cisterna is occupied by a net of calsequestrin, connected to the jSR and less frequently to the free SR by small strands. The strands penetrate into the jSR membrane. The surface of the free SR is occupied by projections of the Ca ATPase, forming small aggregates. Where the membrane is fractured the Ca pump forms a tight array of intramembranous particles. Transition between free and junctional SR is marked (in vertebrates) by a double row of pits and by the absence of CaATPase in the jSR. Junctional feet are shown to have four subunits and to be either connected to or continuous with some component of the jSR membrane which is closely associated with thin strands connecting to the calsequestrin network. Modified from Franzini-Armstrong and Nunzi, 1983.
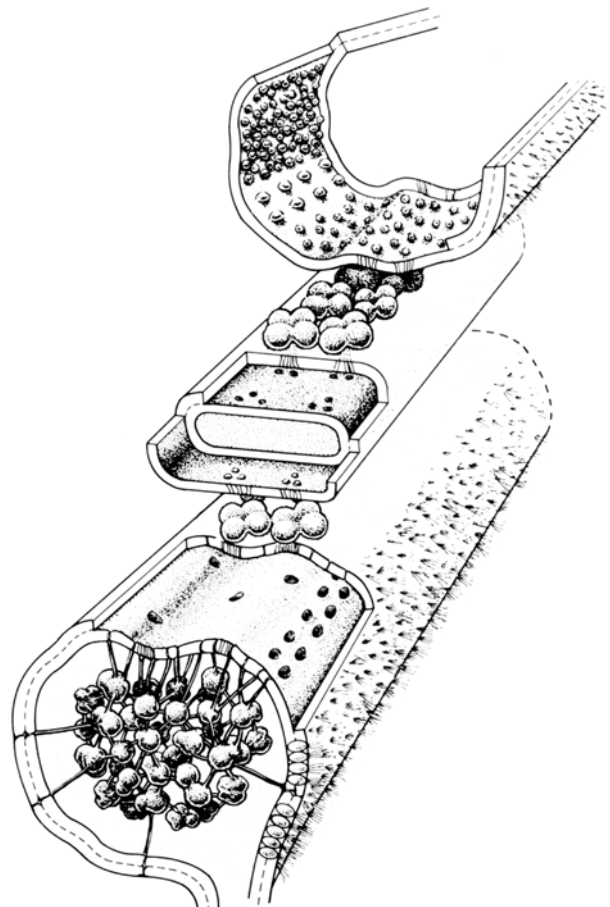
nents (Cadwell and Caswell, 1982; Seiler et al., 1984). An 80-kD intrinsic protein is thought to anchor feet to either T or jSR membranes (Caswell and Brunschwig, 1984). Labeling of the spanning proteins by photoactivateable phospholipid analogues (Volpe et al., 1987) would indicate some penetration into the membrane, as illustrated in Fig. 13. This is consistent with recent evidence suggesting a close association of the feet proteins with a ryanodine receptor (Lai et al., 1987a, b; Imagawa et al., 1987; Inui et al., 1987) or Ca receptor (Zorzato and Volpe, 1987), probably identifiable as the SR Ca-release channel. Some of the numerous intramembranous particles in jSR membrane (Kelly and Kuda, 1979; Franzini-Armstrong and Nunzi, 1983) represent feet-associated proteins (Campbell et al., 1980; Campbell et al., 1983b; Corbett et al., 1985; Kawamoto et al., 1986; Costello et al., 1986; Zorzato et al., 1986), including the highly permeable calcium channel (Smith et al., 1985, 1986; Meissner, 1986).

Calsequestrin, the major protein on the luminal side of the membrane (MacLennan and Wong, 1971), has some hydrophobic amino acids (MacLennan et al., 1983), but since it is not photolabeled its association with the membrane is peripheral (Volpe et al., 1987). We propose that association of calsequestrin with the SR membrane, and particularly with jSR, is indirect and due to the joining strands visible in the deep-etched images. In Fig. 13 the strands are shown to penetrate within the SR membrane and in the jSR region they coexist with proteins associated with the junctional feet. Here the close association between feet and SR internal proteins, which is responsible for the joint resistance of these components to Triton X-100 extraction, is probably established (see introduction).

Ca ATPase is abundant in the free SR, but excluded from the jSR (Franzini-Armstrong, 1975; Jorgensen et al., 1982; Costello et al., 1984). At the boundary between the two is a regular row of pits in the luminal leaflet.

Two structural details of triads are not included in Fig. 13. These are the dimples (Sommer and Johnson, 1979), or indentations (Dulhunty and Valois, 1983) of the terminal cisternae, and the rods (Rayns et al., 1968; Dulhunty, 1987) of the junctional membrane.

Anchoring of calsequestrin to the SR is responsible for maintaining most of the protein within the terminal cisternae. The functional significance of this location is in the fact that release of calcium during muscle activation may occur within this portion of the SR. A minor amount of calsequestrin is located in the longitudinal SR (Junker and Sommer, 1979; 1980).

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Franzini-Armstrong et al. Calsequestrin in Skeletal Muscle

55

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