Structural Evidence for Direct Interaction between the Molecular Components of the Transverse Tubule/ Sarcoplasmic Reticulum Junction in Skeletal Muscle

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Abstract. The architecture of the junctional sarcoplasmic reticulum (SR) and transverse tubule (T tubule) membranes and the morphology of the two major proteins isolated from these membranes, the ryanodine receptor (or foot protein) and the dihydropyridine receptor, have been examined in detail. Evidence for a direct interaction between the foot protein and a protein component of the junctional T tubule membrane is presented. Comparisons between freeze-fracture images of the junctional SR and rotary-shadowed images of isolated triads and of the isolated foot protein, show that the foot protein has two domains. One is the large hydrophilic foot which spans the junctional gap and is composed of four subunits. The other is a hydrophobic domain which presumably forms the SR Ca^{2+}-release channel and which also has a fourfold symmetry. Freeze-fracture images of the junctional T tubule membranes demonstrate the presence of diamond-shaped clusters of particles that correspond exactly in position to the subunits of the feet protein. These results suggest the presence of a large junctional complex spanning the two junctional membranes and intervening gap. This junctional complex is an ideal candidate for a mechanical coupling hypothesis of excitation–contraction coupling at the triadic junction.

CONTRACTION in skeletal muscle fibers is regulated by the movement of calcium ions in and out of an internal store: the sarcoplasmic reticulum (SR).[1] Release of calcium from the SR follows depolarization of the surface membrane and is a steeply graded function of the potential across the transverse tubule (T tubule) membrane (Schneider, 1981). Interaction between the two separate but closely apposed membrane systems (T tubule and SR) occurs at specific junctions where evenly disposed components with four subunits and a tetragonal structure, called feet, span the junctional gap (Franzini-Armstrong, 1970; Franzini-Armstrong and Nunzi, 1983; Ferguson et al., 1984). Feet are one of the most conserved structural features of muscle fibers occurring with the same basic disposition and spacing at the junctions between internal and surface membranes of all types of muscle fibers.

Considerable advance in the understanding of the coupling at T tubule–SR junctions has recently come from identification of the molecular components of the junction. Two proteins, which are likely to play a major role in the process of signal transduction at this junction, have been isolated from the SR and T tubule membranes, respectively. A high molecular mass protein with an intramembranous domain, was initially identified as a component of the junctional feet and localized to the T tubule–SR junction (Kawamoto et al., 1986; Zorzato et al., 1986). More recently ryanodine, a plant alkaloid which binds with high affinity to the Ca^{2+}-release channels in junctional SR (jSR) (Fleischer et al., 1985) and has a complex effect on whole muscle and the SR Ca^{2+}-release channel (Jenden and Fairhurst, 1969; Sutko et al., 1985; Meissner, 1986; Lattanzio et al., 1987; Rousseau et al., 1987), has been used to isolate a "ryanodine receptor" (Pessah et al., 1986; Inui et al., 1987*a*; Lai et al., 1987, Campbell et al., 1987; Imagawa et al., 1987). The ryanodine receptor has been identified with the junctional feet and is composed of four apparently identical subunits. Each of the subunits appears to be composed of a single 450-kD polypeptide. The ryanodine receptor/foot protein has been reconstituted into a functional calcium-release channel (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988) with pharmacological properties similar to the calcium-release channel of heavy SR (Smith et al., 1986, 1988). In the following description we will use the term foot to indicate the cytoplasmic component of the molecule, calcium channel (or intramembrane bump) for the portion spanning the SR mem-

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1. *Abbreviations used in this paper*: jSR, junctional sarcoplasmic reticulum; jT tubule, junctional transverse tubule; mAB-XA7, anti-ryanodine receptor monoclonal antibody; SR, sarcoplasmic reticulum; T tubule, transverse tubule.

brane, and foot protein (or ryanodine receptor) for the entire molecule.

On the T tubule side of the junction, receptors of the voltage-dependent Ca^{2+}-channel blocker 1,4-dihydropyridines are abundant in T tubular membranes of skeletal muscles (Fosset et al., 1983) and isolated triads (Leung et al., 1987, 1988). The high affinity dihydropyridine receptors of skeletal muscle have been purified from T tubule membranes from a number of laboratories (Curtis and Caterall, 1984; Borsotto et al., 1985; Flockerzi et al., 1986) and the exact subunit structure has been more recently identified (Leung et al., 1987, 1988). In skeletal muscles, very few of the abundant dihydropyridine receptors are active calcium channels (Schwartz et al., 1985). The receptors which are not active as calcium channels have recently been implicated in excitation–contraction coupling by the finding that dihydropyridines inhibit charge movements and Ca^{2+} release from the SR in parallel (Rios and Brum, 1987). This has led to the hypothesis that dihydropyridine receptors may be the molecules which initiate transduction of the voltage change across the T tubule membrane to Ca^{2+} release from the SR (Schneider and Chandler, 1973).

We have examined the architecture of the jSR and junctional T (jT) tubule membranes in triads of skeletal muscle, and the structure of the two protein components isolated from them: the ryanodine receptors (feet molecules) and the dihydropyridine receptor. From these results we infer a structural model of the triad in which a direct link exists between an intrinsic component of the T tubule membrane and the calcium-release channel of the jSR. The relevance of these findings to various hypotheses of excitation–contraction coupling at the triadic junction is discussed.

Materials and Methods

Preparation of Skeletal Muscle Membranes

Heavy microsomes or triads were isolated from adult rabbit skeletal muscle by a modification of the method of Mitchell et al. (1983) as described by Sharp et al. (1987) in the presence of the following protease inhibitors: aprotinin (76.8 nM), benzamidine (0.83 nM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and PMSF (0.23 mM). All membrane preparations were stored frozen at −135°C in 0.25 M sucrose, 20 mM Tris-maleate (pH 7.4), 0.83 mM benzamidine, 1 mM iodoacetamide, and 58 μM PMSF. Protein was measured using the method of Lowry et al. (1951) as modified by Peterson (1977), with BSA as a standard. [^{3}H]PN-200-110 binding and [^{3}H]ryanodine binding to isolated membranes was previously described by Leung et al. (1987) and Campbell et al. (1987).

Purification of Dihydropyridine Receptor

Dihydropyridine receptor was purified in the presence of digitonin from heavy microsomes or isolated triads using wheat germ agglutinin-Sepharose-affinity chromatography and DEAE-cellulose ion exchange chromatography as described by Leung et al. (1987). All buffers used in the preparation contained 0.5 M sucrose and the solubilization buffer contained the following protease inhibitors: pepstatin A (0.6 μg/ml), aprotinin (0.5 μg/ml), iodoacetamide (18.5 μg/ml), leupeptin (0.5 μg/ml), benzamidine (0.75 mM), and PMSF (0.1 mM). All other buffers contained 0.1 mM PMSF and 0.75 mM benzamidine. Detergent-solubilized proteins were quantitated by the method of Lowry et al. (1951) as modified by Peterson (1977) after the proteins were precipitated with 5% TCA in the presence of 0.5 mg sodium deoxycholate.

Purification of Ryanodine Receptor

Ryanodine receptor was purified from heavy microsomes or isolated triads by immunoaffinity column chromatography (Imagawa et al., 1987) in the presence of Chaps (Inui et al., 1987*a*; Smith et al., 1988). Anti-ryanodine receptor monoclonal antibody (mAb-XA7) was purified and the immunoaffinity adsorbent (mAb-XA7-Sepharose) was prepared as described previously by Imagawa et al. (1987). Isolated triads were solubilized with 1% (16 mM) Chaps, 0.5% asolecithin (L-phosphatidylcholine from soybean) (Type II-S; Sigma Chemical Co., St. Louis, MO) in the presence of 0.5 M NaCl in buffer A (0.5 M sucrose, 0.75 mM benzamidine, 0.1 mM PMSF, and 50 mM Tris-HCL (pH 7.4), at the protein concentration of 1 mg/ml in the presence of the following protease inhibitors: aprotinin (3.8 μg/ml), antipain (7.2 μg/ml), chymostatin (7.2 μg/ml), and pepstatin A (0.6 μg/ml). Solubilized triads were applied to mAb-XA7-Sepharose column (20 ml), and recycled overnight. The column was washed with 20 ml of buffer A containing 1% Chaps, 0.5% asolecithin, and 0.5 M KCl, followed by 40 ml of 0.3% (5.3 mM) Chaps, 0.15% asolecithin, and 0.5 M KCl in buffer A. Ryanodine receptor was eluted from the mAb-XA7-Sepharose column with 0.3% Chaps, 0.15% asolecithin, and 0.5 M KSCN in buffer A. Immediately after the elution, DTT was added to the eluted ryanodine receptor fraction to a final concentration of 5 mM. The purified ryanodine receptor was applied to a Pharmacia Fine Chemicals (Piscataway, NJ) PD-10 column preequilibrated with 0.3% Chaps, 0.15% asolecithin, 5 mM DTT, and 0.5 M KCl in buffer A to remove KSCN. Protein was quantitated as described above for the dihydropyridine receptor. Ryanodine receptor was also purified from digitonin-solubilized membranes as previously described by Imagawa et al. (1987).

Electron Microscopy

Isolated triads were deposited on a freshly split mica surface and either sequentially fixed in 0.1% glutaraldehyde in 0.1 M cacodylate buffer for 15 min and 2% uranyl acetate for 30 s, or directly fixed in uranyl acetate for 30 s. After a brief rinse in water, the liquid was withdrawn with filter paper to a very thin film and the mica was frozen in liquid nitrogen, freeze-dried, and rotary shadowed at 25°. Isolated molecules were laid on mica, washed either with 50 mM Tris or 100 mM ammonium acetate, fixed for 30 s in 1% uranyl acetate, rinsed in 3% glycerol, and freeze-dried as above. Rotary shadowing was at 15°. Protein concentrations of ~0.05 mg/ml for the dihydropyridine receptor and 0.25 mg/ml for the ryanodine receptor gave an appropriate density of molecules on the mica. Sections and replicas were examined in JEOL USA (Peabody, MA) JEM 100S and Philips Electronic Instruments, Inc. (Mahwah, NJ) model 410 electron microscopes.

Muscle fibers from the swimbladder of the toadfish (*Opsanus tau*) were fixed by perfusion through the major artery supplying this muscle with variable concentrations (0.1–6%) of glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. For freeze-fracture, bundles of fibers were infiltrated with 30% glycerol, frozen in freon, fractured, and unidirectionally shadowed at 45° in a 400D unit (Balzers, Hudson, NH). For embedding, muscles perfused in either 3 or 6% glutaraldehyde were fixed afterward for 1 h in 2% OsO_4 in cacodylate buffer, enblock stained for 1 h in saturated aqueous uranyl acetate, and embedded in Spurr. Thin sections were stained in lead and uranyl acetate solutions.

Results

In this section we will describe in order, (*a*) the structure of junctional feet as seen in thin sections; (*b*) the structure of intramembrane components of SR and T tubules as revealed by freeze-fracture; (*c*) the structure of junctional feet as seen after freeze-drying and rotary shadowing on the surface of isolated heavy SR vesicles; (*d*) the structure of isolated ryanodine receptor (foot protein) and dihydropyridine receptor; and (*e*) the correlation between the above components and their contribution to the structure of the triadic junction.

Thin Section and Freeze-fracture Images of the Triad

The fish muscles chosen for this study were examined because their triads are formed by long straight segments of T tubule and SR, running between the flat ribbon-like myofibrils. This arrangement of the membranes makes it easier to catch long regions of jSR and jT tubule membranes and the intervening junctional gap in sections. The fast contracting swimbladder muscle fibers from toadfish are useful for these

studies because membrane components are abundant and precisely oriented. Since the junctions are located in planes transverse to the fiber long axis, cross sections of the fiber provide the best view of the junctional gap and often graze the junction allowing examination of the feet in some detail (Fig. 1, *A* and *B*). The feet have a tetragonal arrangement and form double (occasionally triple) rows parallel to the long axis of the jSR and jT tubule membranes. The center-to-center distance between feet is ~30 nm along the longitudinal axis of the junctional membranes. The shape of individual feet varies between a diamond and a circle depending on the location of the section relative to the junctional gap.

Higher magnification reveals that the two axes of each diamond-shaped foot are not oriented parallel and perpendicular to the long axis of the T tubule profile, but are slightly twisted so that the corner of each foot slightly overlaps with the corner of its neighbors. Individual feet are in contact with adjacent feet along and across each row. Further details on the structure and disposition of feet are given in previous publications (Franzini-Armstrong and Nunzi, 1983; Ferguson et al., 1984).

The four leaflets derived from the fracture of the junctional membranes are the luminal and cytoplasmic leaflets of jSR (jSR_l and jSR_c), and the luminal and cyoplasmic leaflets of jT tubule (jT_l and jT_c; see Franzini-Armstrong and Nunzi, 1983; Franzini-Armstrong, 1984, for criteria on leaflet identification). The distinguishing characteristics are as follows: jSR_l has prominent bumps with a central pit; the jSR_c has numerous small particles; jT_l and jT_c have a population of tall intramembranous particles of equal size and shape, which are more frequent on the cytoplasmic than on the luminal leaflet. We describe these structures in more detail below.

The jSR_l has two rows of evenly spaced bumps at an average center-to-center distance of 28 nm (range 26–31 nm, from 182 spacings, 23 triads, 21 micrographs, and 8 freeze-fractures; Figs. 1, *C–E*, and 2). The periodicity and disposition of bumps and junctional feet are identical indicating that each foot lies on top of a bump. This spatial relationship between intramembranous protrusions in the SR and the feet was first recognized in amphibian muscles by Kelly and Kuda (1979).

Details of the substructure of the bump are visible only in replicas with very finely grained shadows (Fig. 3 *A*) and are emphasized in stereomicrographs (Fig. 4). In these, each bump in the jSR_l is composed of four rounded subunits (see circle in Fig. 3 *A*) creating a tetrameric substructure with a small, but easily visible pit in the center. In most images the outlines of just two or three of the subunits, rather than all four, are clearly visible. The central pits are always visible, even when all the subunits are barely noticeable, and can be used to measure the distance between bumps. The small tetrameric bumps measure 18–20 nm on the side. The sides of the tetramer do not parallel the long axis of the jSR membrane but are instead twisted, in a manner similar to that described above for the junctional feet.

In the jSR_c there are numerous small round particles but it is difficult to detect any regularity in their disposition (Figs. 1 *E* and 3 *B*). Occasionally small clusters of four particles measuring 16–21 nm on the side, and thus comparable in size to the bumps in the jSR_l leaflet, are visible (Fig. 3 *B*).

The tall intramembranous particles of the jT_c leaflet are clustered in orderly groups of four, marking the corners of small diamonds (Fig. 5, *A* and *B*). The diamond-shaped particle clusters (referred to below as jT tubule tetrads) are aligned in two rows parallel to the long axis of the T tubule, at an average center-to-center distance of 58 nm along the rows (range 52–64 nm, from 96 spacings, 14 micrographs, 23 triads, and 3 freeze-fractures). The disposition is alternate in the two adjacent rows, so that a tetrad in one row faces an empty space in the adjacent row (see Fig. 5 *B*). The spacing and disposition of jT tubule tetrads is such that they match the position of alternate feet and jSR bumps. The axes of the diamonds delineated by the jT tubule tetrads are twisted relative to long and short axes of the T tubules in a similar manner to the feet. Particles of adjacent groups align with each other forming lines oblique to the T tubule axis (Fig. 5 *B*).

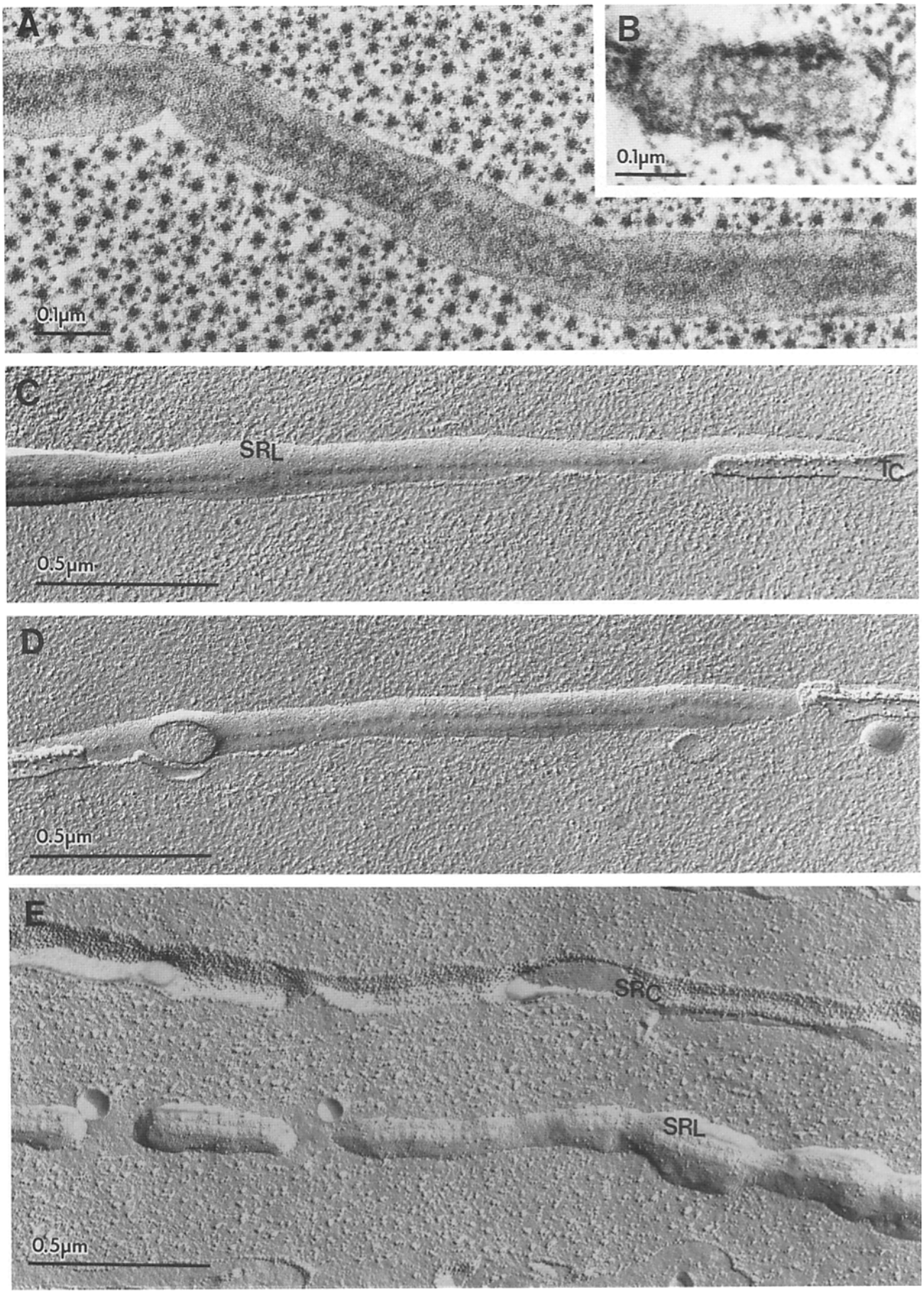
The distribution of the jT tubule particles varies systematically with the concentration of glutaraldehyde used in fixation. At very low (0.1%) glutaraldehyde concentrations most of the particles appear on the luminal leaflet, at concentrations equal to or higher than 3% all (or most) particles appear on the cytoplasmic leaflet. At glutaraldehyde concentrations between the two, the particles partition unequally between the two leaflets. The disposition of the jT tubule particles is thus easier to see at extreme concentrations, particularly at the higher end, where the shape of the tubules is better preserved. In muscles fixed at intermediate glutaraldehyde concentrations, the pattern of T tubule particles is less clear, but the oblique alignment lines described above are still detectable, indicating that the disposition is the same although the pattern is less complete obviously due to the lack of some particles. Previous descriptions of this less complete disposition were obtained in muscles fixed by immersion rather than by perfusion (Franzini-Armstrong and Nunzi, 1983).

The clustered particles described above are the only components of the jT tubule membrane. The rest of the T tubule membrane, or free T tubule membrane, has a variable content of smaller, randomly distributed particles.

Freeze-drying of Isolated Triads: the Junctional Feet

Terminal cisternae of SR isolated from a rabbit triad preparation, have a surface covered in part by the cytoplasmic "tails" of the calcium ATPase and in part by junctional feet (*arrows*, Fig. 6). T tubules (Fig. 6 *T*) have a much finer grain shadow, presumably because none or very few proteins project over its cytoplasmic surface. Although the overall shape of the organelles is distorted in such preparations, the ability to identify surface landmarks such as the pump and the feet allow easy identification of the components of the fraction.

The feet, as seen on SR vesicles, are composed of four apparently identical subunits, symmetrically disposed around a central depression. The central depression and the approximately spherical shape of the subunits are clearly visible in stereomicrographs (Fig. 7). The entire large quatrefoil structure is 26–28 nm side to side and 33–36 nm along the diagonal. Where the nearest neighbor relationship of adjacent feet is preserved, the feet abut corner to corner, but with the partial overlap of one subunit. Thus, although the feet are aligned diagonally, their center-to-center distance (~30 nm)



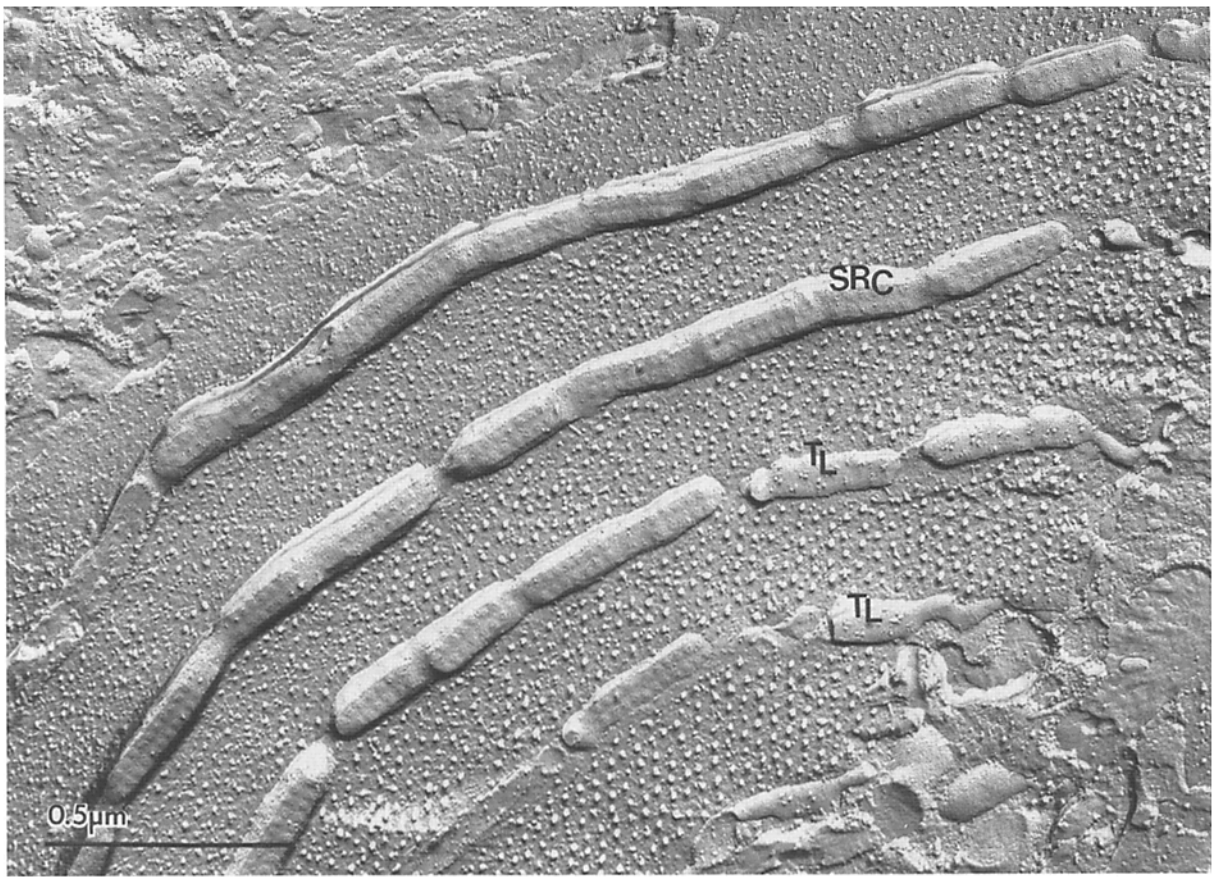


Figure 2. The periodic array of bumps on the jSR_I is particularly visible in this image from toadfish. The interruptions in the rows of bumps are regions of nonjunctional SR. T_L is the luminal leaflet of the jT tubule membrane. In this fixation, jT tubule tetrads are mostly on the luminal leaflet.

is less than the foot size along the diagonal (see Ferguson et al., 1984, for details).

Individual feet exhibiting the same size and shape as those illustrated here were seen in preliminary results with heavy SR vesicles isolated from the toadfish swimbladder muscle, using the same methodology as in rabbit. This extends the similarity of the foot protein structure from mammalian and amphibian muscles to fish muscle.

Freeze-drying of Purified Dihydropyridine and Ryanodine Receptors

The purified ryanodine receptor consists of a single high molecular mass protein with an apparent molecular mass of 450 kD (Fig. 8 *A*). In contrast, the purified dihydropyridine receptor consists of four polypeptides of 175 (α_2), 170 (α_1), 52 (β), and 32 kD (γ). When analyzed under nonreducing conditions the apparent molecular mass of the subunit shifts upon reduction from 175 to 150 kD (Fig. 8 *B*). Densitometric scanning has shown that the four polypeptide components exist in a 1:1:1:1 stoichiometric ratio (Leung et al., 1988).

Freeze-drying and rotary shadowing of the purified ryanodine receptor preparation shows a uniform population of large molecules, with a distinctive fourfold symmetry (Fig. 9 *A*). The molecules isolated after solubilization with Chaps are more intact than the digitonin-solubilized preparations, and will be described first. At higher magnification and in stereomicrographs the ryanodine receptor shows four apparently identical rounded subunits centered around a prominent central bump (Fig. 9, *A–D*, and *F*). The size and height of the entire molecule varies slightly from one preparation to the other, presumably as a result of flattening of the molecule over the mica substrate. Slightly taller molecules are 29 nm on the side, other less high molecules are up to 31 nm

Figure 1. (*A*) Thin section view grazing the junctional gap in toadfish swimbladder muscle. In this view, two rows of junctional feet run parallel to the T tubule long axis. The feet have a 30-nm center-to-center spacing. (*B*) In this image, the diamond-shaped profile of the feet is more clearly visible. Photomicrograph taken from a black mollie. (*C* and *D*) Freeze fracture of jSR membranes in toadfish muscle showing the luminal leaflet (SR_L). The distinctive feature of the jSR_I membrane is a double row of evenly spaced periodic bumps. Cytoplasmic leaflet (T_c) of the jT tubule membrane. Photomicrograph taken from a black mollie. (*E*) Cytoplasmic (SR_C) and luminal (SR_L) leaflets of the jSR from toadfish. On the cytoplasmic leaflet, small particles crowd the surface and evidence for periodicity is less clear.

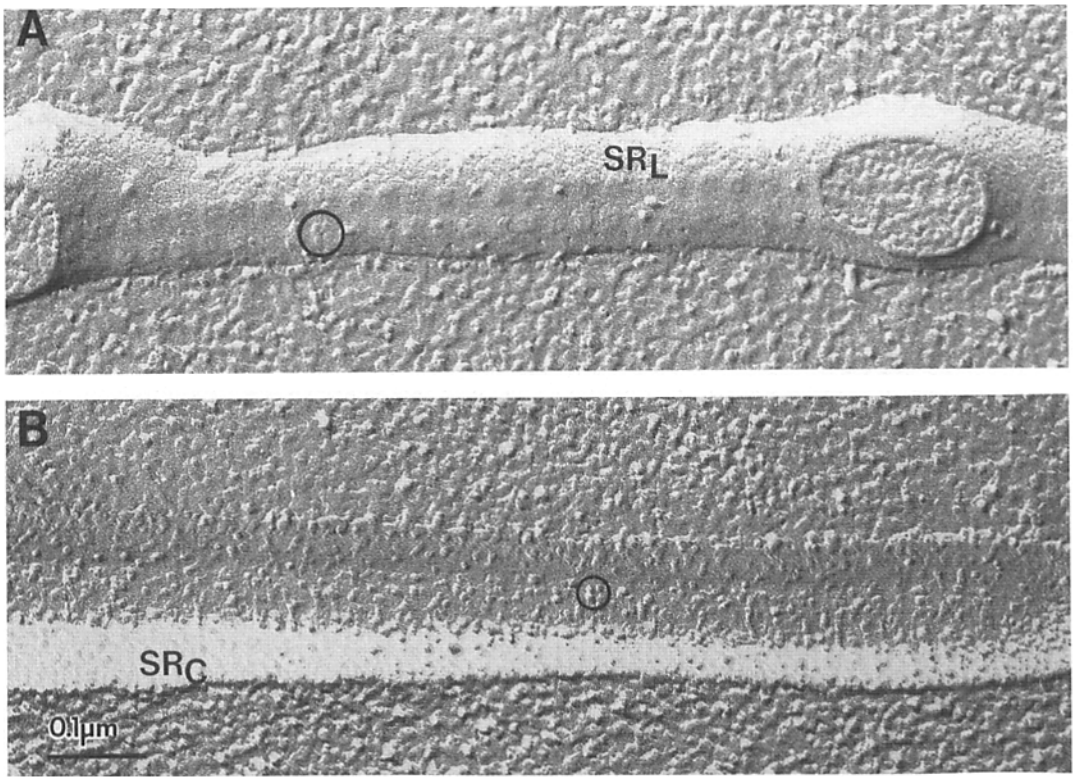


Figure 3. (*A*) Higher magnification of the luminal jSR leaflet showing a fourfold structure of the bumps (*circled area*). At the center of each bump there is a small depression or pit. The bumps are best seen by holding the micrograph at eye level and glancing along the long axis of the junction. (*B*) On the cytoplasmic leaflet (SR_C) the fourfold symmetry (*circled area*) is less frequently observed. From assorted fish.

on the side. The central bump is 18–20 nm in diameter. Some structural variation from the above description occurs and this is most likely due to the presence of dissociating or damaged molecules. One or more of the subunits may appear indented, or separated into two components, so that the edge of the molecule has a scallopped appearance (Fig. 9 *C*, *arrowheads*). In some molecules the central bump is clearly cleaved into four parts (Fig. 9 *D*).

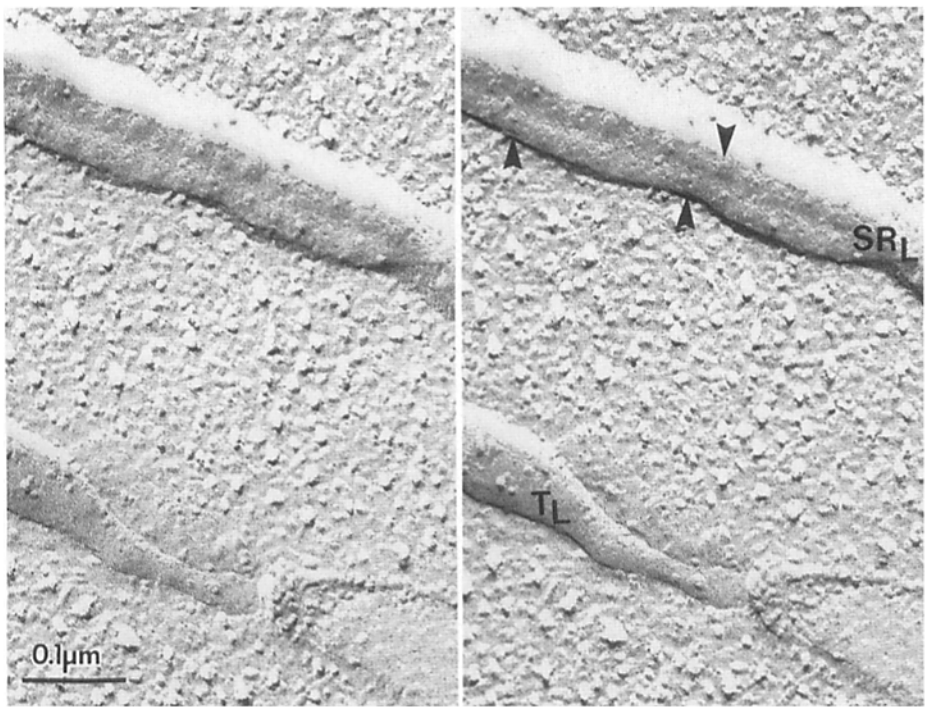


Figure 4. The periodic bumps of jSR_l and their substructure are more visible in stereomicrographs (*arrowheads*). T_L, luminal leaflet of jT tubule. Stereomicrographs taken from toadfish.

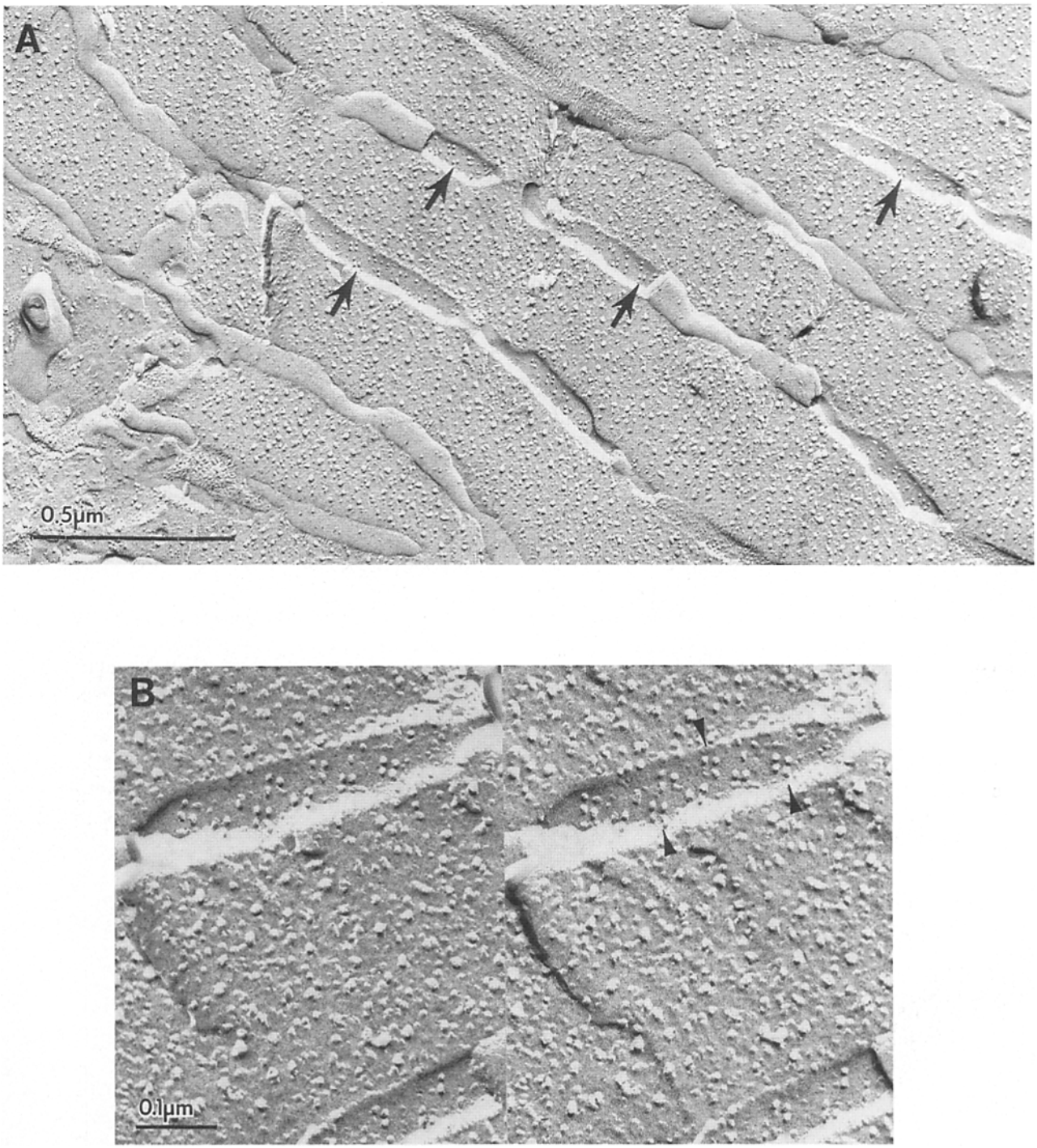


Figure 5. (*A*) Most of the profiles are from cytoplasmic and luminal leaflets of jT tubule membranes. *Arrows*, the cytoplasmic leaflet where clusters of large particles are seen. The luminal leaflet has few particles belonging to tetrads on it in this fixation. (*B*) Stereomicrographs, showing a detail of jT_c leaflet. jT tubule tetrads (*arrowheads*) are groups of four particles delineating small diamonds. The tetrads have alternate positions along two rows parallel to the T tubule long axis, so that each tetrad faces an empty space. The spacing between tetrads is exactly double that between feet and jSR bumps.

The foot protein solubilized by digitonin generally has the same structure as the Chaps-extracted protein, but it is less stable, and this allows observation of stages in the dissociation of the molecule into four subunits (Fig. 9 *E*). In the partially dissociated molecule four subunits of apparently equal size are separated by a distinct cross-shaped cleft. The central bump visible in the intact molecule is missing and we assume that it also splits equally with the four subunits. An unfolding of the four subunits to form scallopped profiles is often seen in these molecules, suggesting that this shape is taken by the molecule as it begins to dissociate.

The isolated dihydropyridine receptor (Fig. 10 *C*) is a

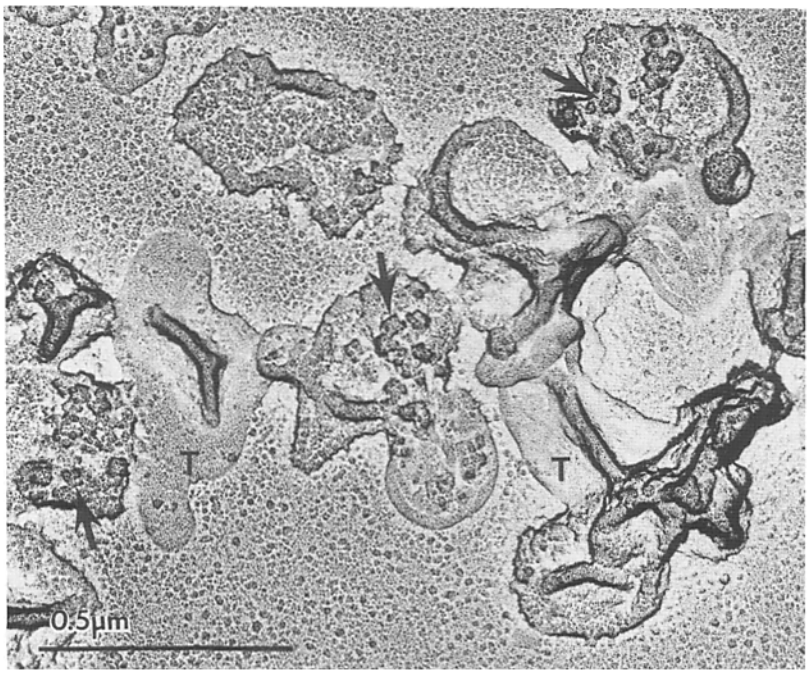


Figure 6. Freeze-dried vesicles from a triad fraction isolated from rabbit muscle. T tubules (*T*) and SR (*arrows*) are distinguishable on the basis of surface features. The jSR is decorated by feet, which have lost their precise disposition.

slightly elongated ovoid 16 × 22 nm in diameter, and often has a cleft between two portions of the molecule of similar size as described in Leung et al. (1988). There is no apparent symmetry in the overall outline of the molecule as seen in shadowed images.

Comparison between Isolated and In Situ Molecules

Fig. 10, *A–F*, shows a composite gallery of the various details described above. The images are printed at the same magnification, so that direct comparisons can be made, as follows. (*a*) The isolated and intact foot molecules have approximately the same size and four leaf clover profile, but differ in one significant detail. The intact molecule has a central depression, while the detergent-solubilized molecule has a central projection (Fig. 10, compare *A* and *B*). We assume that the central bump is the hydrophobic component of the molecule and that the isolated foot protein adheres to the mica sheet with its hydrophobic component away from the mica surface. When attached to the jSR membrane the molecule is situated such that the hydrophilic component forming the fourfold foot molecule is uppermost and visible, while the hydrophobic component is included within the membrane and not visible. (*b*) The bump seen in freeze-fracture of the jSR membrane is similar in size to the central bump of the isolated foot protein (Fig. 10, compare *B* and *D*). This is in direct confirmation of the assumption that the central portion of the molecule is intramembranous. (*c*) The jSR membrane bumps have a tetrameric substructure and an ob-

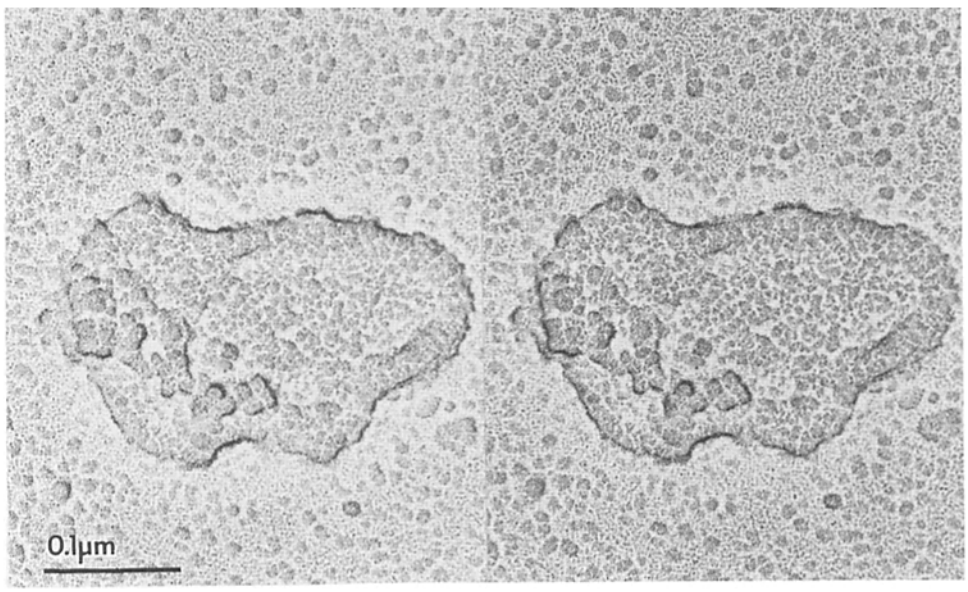


Figure 7. Stereomicrographs of a heavy SR vesicle from a triad fraction from rabbit. Feet have four symmetrical subunits, surrounding a central depression.

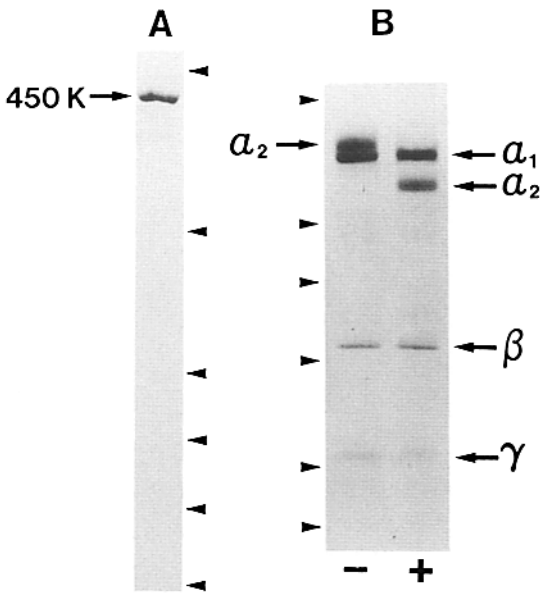


Figure 8. Purified ryanodine receptor (*A*) or purified dihydropyridine receptor (*B*) were subjected to SDS-PAGE and stained with Coomassie Blue. (*A*) 3 μg of purified ryanodine receptor separated on a 3–12% gradient polyacrylamide gel. The arrow (*450 K*) indicates the position of the 450-kD ryanodine receptor. (*B*) 15 μg of purified dihydropyridine receptor separated on a 5–16% gradient polyacrylamide gel under nonreducing (−) or reducing (+) conditions. Subunits of the dihydropyridine receptor are indicated by arrows; 170-kD subunit (α_1); 175-kD nonreduced (150-kD reduced) subunit (α_2); 52-kD subunit (β); 32-kD subunit (γ). Molecular mass standards (nebulin, 500 kD [*A* only]; myosin, 200 kD; phosphorylase b, 97 kD; BSA, 68 kD; ovalbumin, 45 kD; α-chymotrypsinogen, 25 kD; β-lactoglubulin, 18 kD [*B* only]) are indicated by arrowheads in *A* and *B*.

vious central pit (Fig. 10, *D*, *circled*). (*d*) The jT tubule has distinct clusters of four particles and the center-to-center distance between the components of the jT tubule tetrads is the same as that between the subunits of the foot (Fig. 10, *E* and *F*). However, note that one jT tubule tetrad is available only for every other foot. The skew of feet, jSR bumps, and jT tubule tetrads relative to the long axis of T tubules is similar (Fig. 10, compare *D* and *F*). (*e*) The size of the isolated dihydropyridine receptor is equal to or smaller than that of the subunits of the junctional feet, so that it is possible for four dihydropyridine receptors to be associated with each foot (Fig. 10, compare *B* and *C*). (*f*) The size of the isolated dihydropyridine receptor is larger than the size of the individual particles in the jT tubule tetrads. However, four closely apposed dihydropyridine receptor molecules would superimpose precisely over a jT tubule tetrad, indicating that the dihydropyridine receptor has the appropriate size for being a putative component of the tetrads. (*g*) In a very thin section located where the T tubule membrane and the junctional gap are enclosed within the thickness of the section, alternate feet are more dense (not shown). This may be the result of superimposition of jT tubule tetrads and feet in the thickness of the section.

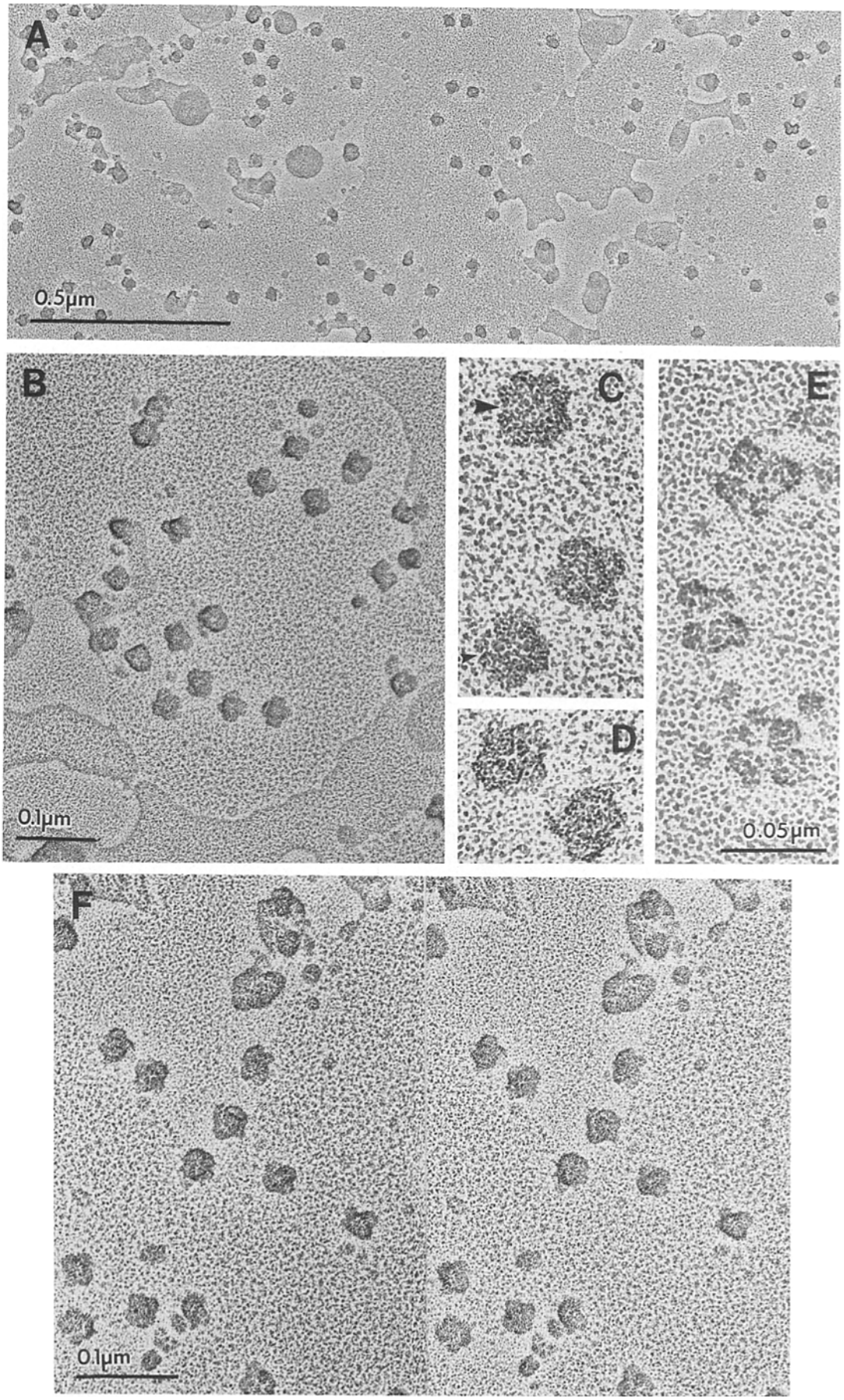
The results above are consistent with a specific relationship between jT tubule tetrads and feet subunits, illustrated in Fig. 11. Each foot is represented by four equal shaded spheres, and the relationship between neighboring feet is as modeled in Ferguson et al., 1984. A smaller tetrameric structure (Fig. 11, *white spheres*) representing the proposed intramembranous portion of the molecule is superimposed on the profile of each foot. The orientation of the four subunits of the intramembranous portion of the molecule are also skewed relative to the axis of the T tubules, in a manner consistent with that of the small projections in the jSR_1 leaflet. The similar skew of the four subunits of the intramembranous portion of the foot molecule indicates a relationship between the intramembranous and cytoplasmic component of the junctional feet. Alternate feet are covered by four black discs, one at the center of each foot subunit, representing the position of the jT tubule tetrads relative to the feet. The direct fit between the components illustrated in this diagram and the results presented above strongly corroborate the interdependence between the feet protein and jT tubule tetrads at the junction.

Discussion

From the images shown in this report, we propose a molecular model of the T tubule–SR junction with the following salient features (Fig. 12). (*a*) The foot protein (ryanodine receptor) has a large cytoplasmic domain composed of four subunits of equal size and approximately spherical shape. Comparison of negatively stained (Lai et al., 1988) and shadowed images of the isolated molecules indicates that the four subunits may actually be hollow spheres. The four cytoplasmic subunits together form the junctional foot and join jSR membrane to the cytoplasmic surface of the jT tubule membrane. Notice that, due to their spherical shape, the foot subunits contact the T tubule membrane approximately at their centers. (*b*) The hydrophobic, intramembranous domain of the foot protein, which penetrates the jSR membrane, is also divided into four subunits, which are rotated by 45° relative to the cytoplasmic domains. This information is obtained by combining the freeze-fracture images from this paper with negatively stained images by Lai et al. (1988), and with the images of feet by Ferguson et al. (1984). This representation of the structure of the foot protein is more detailed than that given in Saito et al. (1988), since it adds information on the relationship between cytoplasmic and intramembraneous domains of the molecule. (*c*) The four components of jT tubule tetrads are located directly above the center of the foot subunits and we expect that the jT tubule protein and the foot subunits are in direct contact at the jT tubule membrane interface with the cytoplasm. The disposition of the tetrads presents an apparent anomaly; they do not establish contact with alternate feet. (*d*) Components of jT tubule tetrads are quite tall, and we expect them to protrude into the lumen of the T tubules. This is not illustrated in Fig. 12.

The structural results provide strong evidence for molecular continuity across the SR–T tubule junctional membranes. The large junctional complex formed by a jT tubule tetrad and a foot protein spans the entire junction, going from lumen of the T tubules, across the T tubule membrane, the junctional gap, and the SR membrane to the lumen of the SR. The complex maintains a fourfold symmetry throughout.

The channel formed by the reconstituted foot protein has characteristics similar to the calcium-release channel of the



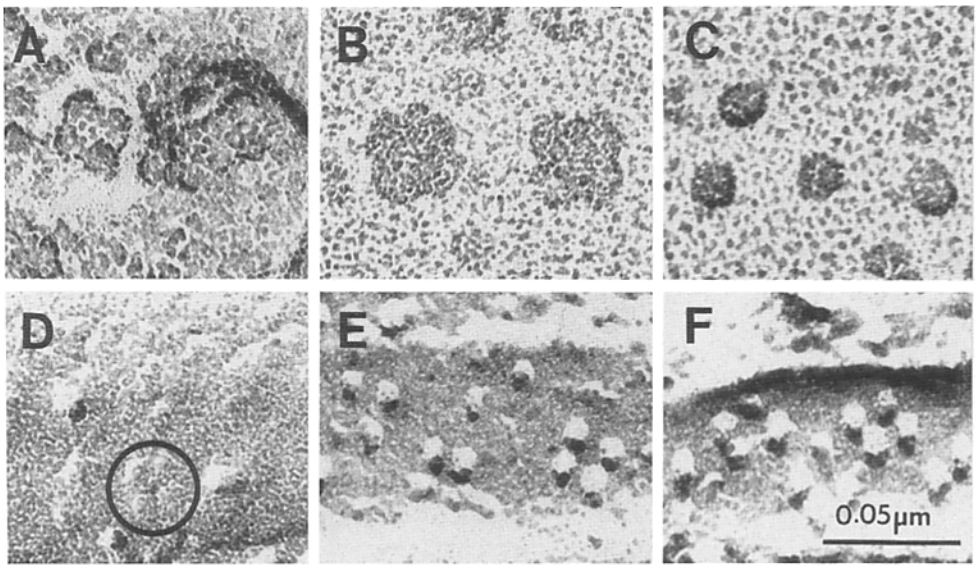


Figure 10. Comparison between a foot associated with the SR membrane (*A*), isolated foot proteins (*B*), dihydropyridine receptors (*C*), jSR_1 bumps (*D*), and jT tubule tetrads (*E* and *F*). The foot protein has the same subunits as the foot, but is slightly flattened on the mica substrate. See text for the relationship between the various components of the junction.

heavy SR (Lai et al., 1988). In particular, ATP, Ca, and Mg, and low concentrations of ryanodine and ruthenium red affect calcium release from heavy SR channels (Smith et al., 1986, 1988) and from channels produced by the reconstituted molecule in a similar manner. Calcium and magnesium competition in affecting calcium release of the isolated heavy SR and permeability of the purified molecules is consistent with that observed in skinned fibers (Endo, 1975). On the basis of these experiments it is assumed that the foot protein represents the channel through which calcium is released during excitation–contraction coupling.

The components of the jT tubule particles of the junctional complex have not been identified. The following data argue in favor of an identification of the jT tubule proteins with the dihydropyridine receptor. jT tubule membranes are the richest source of the dihydropyridine receptor (Brandt et al., 1985). The protein is a large molecular mass T tubule membrane component and it has a large glycosylated polypeptide side chain, which must protrude into the lumen of the T tubule (Leung et al., 1987, 1988). The jT tubule particles are the only intramembrane protein of the jT tubule membrane, they are tall, large, and leave a pit on the luminal leaflet. These results indicate that the jT tubule particle arises from a large protein that is a prominent component of the jT tubule membrane with the same characteristics as the dihydropyridine receptor. The jT tubule particles are located over the centers of the foot subunits, and, thus, they represent a protein which may be smaller, but must not be larger, than a foot subunit. The isolated dihydropyridine receptor has a size approximately equal to one of the foot subunits. Immunocytochemistry indicates that the dihydropyridine receptor is located in the T tubule membrane, although the resolution is not yet sufficient to assign it to the jT tubule membrane. Presumed calcium channels in postsynaptic membranes have a freeze-fracture profile very similar to that of the jT tubule particles (Heuser et al., 1979). The estimated densities of dihydropyridine receptors in frog T tubules (230/μm^2; Schwartz et al., 1985) and that of jT tubule particles (808/μm^2 calculated on the basis of four particles per tetrad, a 60-nm spacing for jT tubule tetrads, and a T tubule perimeter of 330 nm) are within an order of magnitude. Considering that the T tubule surface density reported by Mobly and Eisenberg (1975) and used in the above calculation by Schwartz et al. (1985) may include some nonjunctional tubules, the discrepancy is not large.

Whatever the exact nature of the jT tubule particles, they provide evidence for a direct molecular coupling between the T tubule membrane and the SR calcium-release channel. This molecular continuity provides the structural basis for a hypothesis of T tubule–SR coupling which is appealing in its simplicity and represents the most direct explanation for the tight and quantitative coupling between T tubule depolarization and calcium release (Schneider and Chandler, 1973). The hypothesis proposes that the initial step in excitation–contraction coupling is a voltage-dependent rearrangement of a molecule located in the T tubule membrane resulting in a detectable "charge movement." Direct interaction between the voltage sensor and a component of the SR membrane, mediated by the junctional feet, would result in opening of a calcium-release channel. We have established that the continuity between jT tubule and jSR components needed for this mechanism of long range interaction exists. In this scheme, the jT tubule tetrads are the voltage detectors and foot proteins have the dual role of receiving the signal from the jT tubule particles and of constituting the calcium-release channel. The large cytoplasmic domains of the foot protein

Figure 9. Purified foot protein from rabbit muscle, solubilized in Chaps (*A–D*, and *F*) and in digitonin (*E*). Purity of the fraction is demonstrated by homogenous presence of square-looking molecules (*A* and *B*). At higher magnification, the molecules show four rounded subunits and a central projection (*C* and *D*). The central projection is best visible in stereo (*F*). Various stages of dissociation are shown in the small clefts in the subunits (*C*, *arrowheads*), cleaving of the central projection (*D*), and the clear separation into four subunits and disappearance of the central projection (*E*). The latter is most often seen in digitonin-solubilized samples.

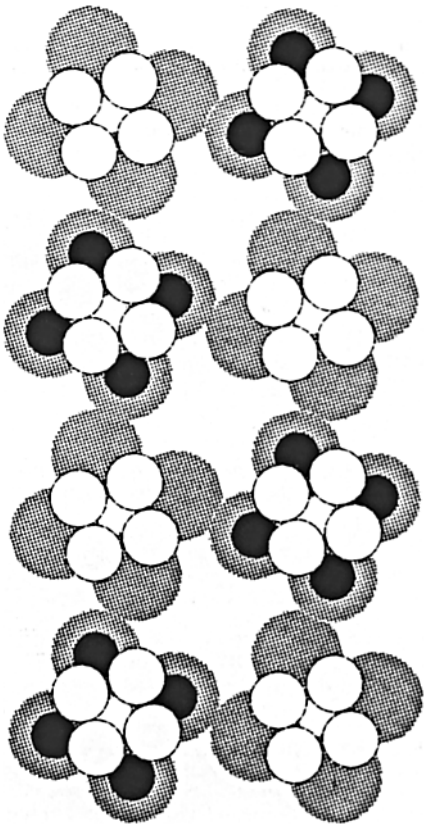


Figure 11. Diagram of the spatial relationships between the junctional complex components. Four large shaded spheres represent each foot; small white spheres are the intramembranous portion of the foot protein (jSR_1 bumps); black discs represent the position (but not necessarily the size) of the jT tubule tetrads. In this diagram tetrads interact with alternate feet. We have considered alternate interpretations of the geometry of this relationship, trying to find one in which each tetrad could interact with two adjacent feet. However, this always necessitated a distortion of either shape or position of the tetrads.

perform the first function, and the smaller hydrophobic regions the second. The unusual interaction of alternate feet with jT tubule tetrads has three possible functional implications. The first suggestion is that the foot protein/Ca^{2+} channels are activated in a cooperative fashion. Secondly, alternate feet might be affected by two different activating mechanisms; and finally it is possible that every other foot is silent.

The next step in understanding the junction is the identification of the jT tubule tetrads. It has been proposed that the dihydropyridine receptor is the T tubule voltage sensor (Rios and Brum, 1987). The best way to confirm this identification is to isolate the junctional complex intact. However, efforts at establishing whether the foot protein binds to the dihydropyridine receptor have had either negative or only partially promising results. Perhaps, the interaction is mediated by an intermediary protein.

Other mechanisms for transmission at the triad have been proposed and all need anchorage of T tubule to SR in order to maintain a narrow junctional gap, particularly those involving a chemical message (Ca^{2+}; Endo, 1975; Fabiato, 1983; or IP3; Vergara et al., 1985). However these mechanisms for transmission do not require molecular continuity across the junction. It is not clear why such an elaborate structure as the junctional complex would exist if transmission only occurred by an indirect interaction between the membranes.

Feet are common components to all muscle fibers, including cardiac and smooth muscle, and extend throughout the animal kingdom. Despite the fact that the effect of ryanodine on cardiac muscle is different from that on skeletal muscle, the isolated ryanodine receptor of cardiac muscle is structurally indistinguishable (at a limited resolution) from that of skeletal muscle (Inui et al., 1987*b*). Whether the rest of the junction is built on the same general plan in all muscle fibers remains to be demonstrated. jT tubule tetrads have been identified in freeze-fracture images of the surface membranes in frog muscle (see Figs. 16–18 in Franzini-Armstrong, 1984). The particles have a similar distribution and show the same 60-nm alternate spacing described above. So far, we have failed to find jT tubule tetrads on surface membrane of smooth and cardiac muscle, or in some muscles of invertebrates. The lack of the particles in the jT tubule membranes in smooth and cardiac muscle is consistent with the hypothesis that the particles represent the dihydropyridine receptors

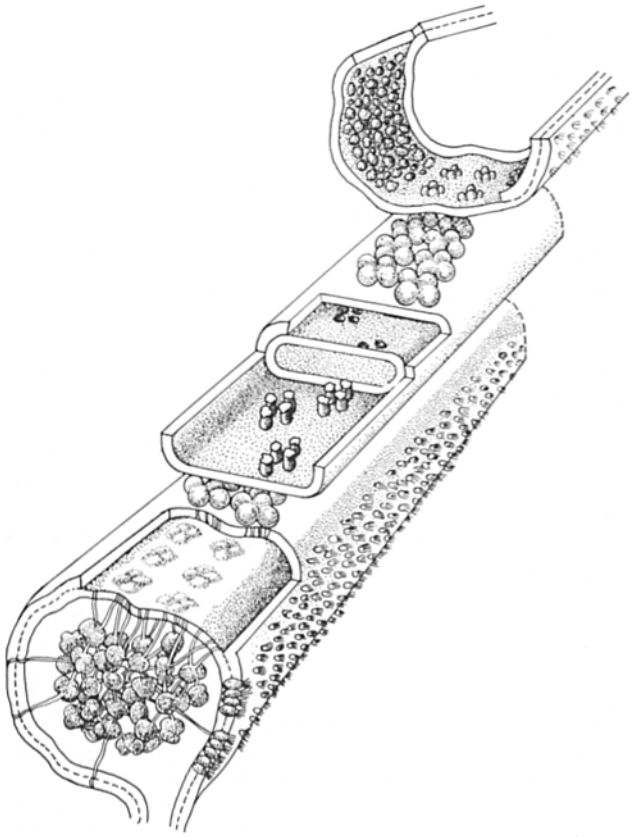


Figure 12. Three-dimensional reconstruction of a triad, showing relative positions of foot proteins, jT tubule tetrads, calsequestrin, and calcium ATPase. Feet (four balls with a central depression) occupy the T tubule–SR junctional gap, and the intramembranous portion of the foot protein penetrates into the jSR membrane, forming bumps with a fourfold structure. The tall particles of the jT tubule tetrads are located in the jT tubule membrane, opposite alternate feet.

which are present in a much lower number in these muscles. However, in view of the effect of fixation protocols on these components, we cannot yet be sure that these negative results must be taken seriously.

The purified foot proteins vary somewhat in their pharmacological properties. About 50% of the molecules solubilized by the use of Chaps (Lai et al., 1988), and all the molecules solubilized by digitonin (Imagawa et al., 1987) have lost sensitivity to magnesium and in the latter case also are not blocked by ruthenium red. This may be, in part, due to the fact that the isolated tetrameric complex is quite unstable. In particular, the complex isolated using digitonin rather than Chaps easily dissociates into four subunits. Permeability and gating properties of the tightly aggregated foot tetramer thus may be different from those of the loosely associated tetramer.

The two macromolecules discussed in this report both behave as calcium channels, and both have design characteristics typical of channels, although this is more directly obvious in one case (SR Ca^{2+} channel) than the other. Most channels have been designed with a symmetric disposition of components around a center (Unwin, 1986). The foot protein fits this pattern, particularly in view of the fact that the stoichiometry of ryanodine binding would indicate one molecule per four chains (Lai et al., 1988), indicating that an entire foot protein may be needed to form a functional channel. If this is the case, the channel would have an unusually large molecular mass (~1,800 kD) partly due to an unusually large cytoplasmic domain. In the dihydropyridine receptor we detect two subunits, presumably representing the two large subunits, but no outward symmetry. The underlying symmetry however, has been demonstrated in the cloning of the dihydropyridine-binding subunit, which has enough homology to the sodium channel to allow prediction of a fourfold symmetry around a center produced by four identical or very similar domains (Tanabe et al., 1987).

The association demonstrated here between a component of the T tubule membrane, and the junctional feet would provide the physical basis for a direct long range molecular interaction between the two membranes, of the type initially formulated by Schneider and Chandler (1973). This unique molecular arrangement, allowing direct coupling between two sets of membranes belonging to the same cell has evolved in skeletal muscle fibers for the rapid and precise control of intracellular release of large quantities of calcium under the influence of changes in the surface membrane potential.

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