BRIDGING STRUCTURES SPANNING THE JUNCTIONAL GAP AT THE TRIAD OF SKELETAL MUSCLE

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ABSTRACT

The membrane systems of skeletal muscle were examined after tannic acid fixation. A new structure consisting of bridges spanning the junctional gap is described, and a model is proposed in which the cytoplasmic but not the luminal membrane leaflets of the transverse tubule and of the junctional sarcoplasmic reticulum (SR) are continuous.

The globular particles (presumably the Ca-binding proteins) within the terminal cisternae were arranged in longitudinal rows and appeared adherent to the junctional membrane.

The junctional gap was present in negatively stained, frozen thin sections of fixed muscles. Negatively staining material occurred within the junctional gap.

The cytoplasmic leaflets of the longitudinal, intermediate, and terminal cisterna regions of the SR exhibited a thick coat of densely staining material compatible with the presence of the Ca-ATPase.

Similar bridges were also observed at the surface membrane-SR close coupling sites of vascular smooth muscle.

KEY WORDS striated muscle · excitation-contraction coupling · membranes · transverse tubules · sarcoplasmic reticulum

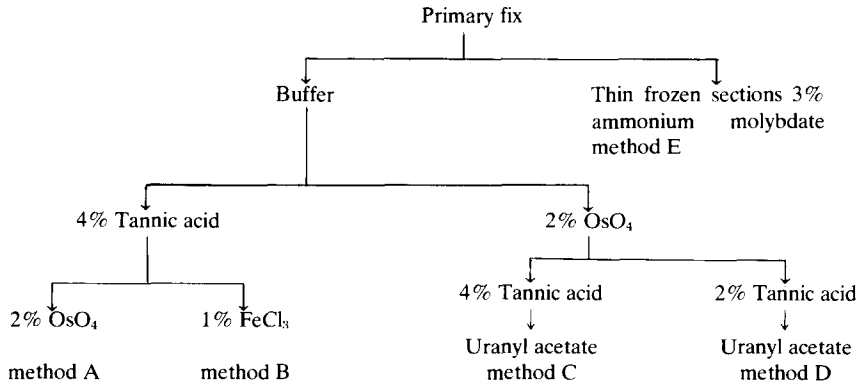
The invasion of the action potential from the surface to the center of skeletal muscle fibers via the transverse tubules (TT) (12), is followed by Ca release from the sarcoplasmic reticulum (SR), resulting in tension development. Measurements of membrane capacitance (for review see reference 3) as well as recent analysis of the elemental composition of the SR (36, 37) indicate that the lumens of the TT and SR are not in direct electrical (ionic) communication and are, therefore, separate compartments. Morphological studies also show that the signal for excitation-contraction coupling in skeletal muscle must traverse a junctional gap of ~130 Å between the electrically conducting transverse tubules and the calcium-storing terminal cisternae (TC) of the SR (9, 27). Foot processes, dimples of the TC membrane, dense bridges, and intramembrane particle sizes and distribution in these two opposing membrane systems have been described (8, 17, 19a, 29, 39a), but these features do not reveal a specific structure that could directly convey the TT signal to the SR.

In the present study a previously undescribed structure consisting of bridges which span the junctional gap is reported after the use of tannic acid during fixation procedures, and a new structural model of electromechanical coupling is proposed. A preliminary report was presented to the Biophysical Society, March 1978.

MATERIALS AND METHODS

The various methods of tissue preparation, designated A through E, are summarized in the flow diagram in Scheme I and presented in more detail below. Three to eight muscles were processed by each method, and three to four blocks from each muscle were sectioned and examined.

Fiber bundles from sartorius or semitendinosus muscles from *Rana pipiens* or from the swimbladder of the



SCHEME I

toadfish *Opsanus tau* were fixed in 2% glutaraldehyde in 0.1 M Na cacodylate buffer with 4.5% sucrose and 1.2 mM $CaCl_2$ at pH 7.2 for 2 h. The glutaraldehyde-fixed muscles were then processed in one of the following ways: (*a*) stored and refrigerated in cacodylate buffer, 6% sucrose, and 1.2 mM Ca for 3–4 d, cut into small pieces ~2 × 1 mm, postfixed with 4% tannic acid (Baker 4-0377, lot 43554, or code No. 1764, Mallinckrodt Inc., St. Louis, Mo.) in cacodylate buffer, pH 7.2, for 4 h, washed in buffer, and reacted with 2% osmium tetroxide for 90 min (method A) or 1% $FeCl_3$ (25) (method B), dehydrated, and embedded in Spurrs' resin; (*b*) stored in buffer as in (*a*) followed by 2% osmium tetroxide for 90 min, rinsed in cacodylate buffer, and subsequently reacted with 4% tannic acid, pH 7.2, for 4 h followed by 90 min of en bloc staining with saturated aqueous uranyl acetate, then dehydrated and embedded (method C); and (*c*) washed in cacodylate buffer with 6% sucrose and 1.2 mM $CaCl_2$, postfixed in 2% osmium tetroxide in cacodylate buffer, rinsed in buffer, followed by 2% tannic acid, pH 7.2, for 30 min, and subsequently en bloc stained with saturated aqueous uranyl acetate, dehydrated, and embedded (method D). Smooth muscle from the rabbit portal anterior mesenteric vein was prepared by method D. Sections were stained with lead citrate.

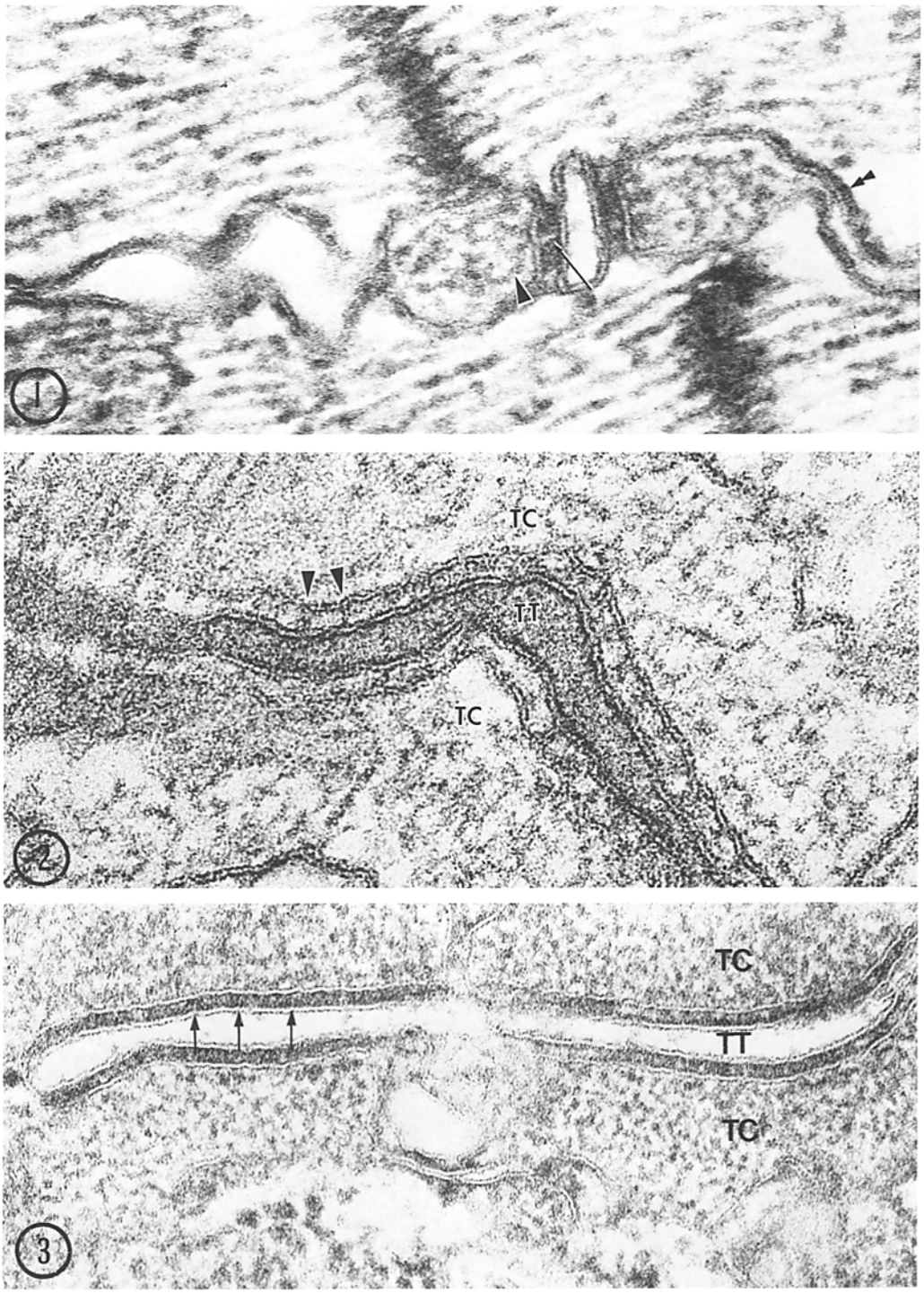
Glutaraldehyde-fixed muscles were also frozen in supercooled Freon 22 and sectioned at −130°C. (36). The frozen sections were placed on a drop of frozen saturated sucrose (38) and subsequently melted in a dry atmosphere to prevent water condensation on the surface. The sections on the sucrose drop were transferred to a glow discharged carbon foil on a copper grid by touching the grid to the surface of the drop. Sections were rinsed in water and negatively stained with 3% ammonium molybdate.

RESULTS

The intensity of the staining was highly variable especially in the preparations exposed to tannic acid before osmium. In these samples, only one or two sarcomeres at the cut ends of the fibers were adequately stained, indicating insufficient penetration of tannic acid across the sarcolemma in the remainder of the fiber. Exposure to tannic acid after osmication resulted in staining of the entire fiber, presumably due to increased permeability of the osmicated membranes (39). The density of the staining was variable.

Bridges spanning the junctional gap and joining the junctional sarcoplasmic reticulum (JSR) and the transverse tubules were observed in muscles exposed to tannic acid either pre- or postosmium as well as pre-$FeCl_3$ (Figs. 1–8). The staining of the junctional gap was variable and frequently very dense when tannic acid preceded the iron or osmium (Figs. 3, 5, 11, and 12). Excessive staining obscured the gap structures and, in general, very thin sections (50–60 nm) were required to image the bridges. The bridges were electron-lucent with stain on either side (Figs. 1–7), similar to the staining and appearance of the junctional membranes. The clear region of the bridges was continuous with the clear central lamina of the JSR (Figs. 1, 2, 4, and 7) and the TT (Figs. 3, 6, and 7). In these regions, only the outer cytoplasmic leaflets of the TT and JSR membranes appeared to make sharp right-angled turns (e.g., Fig. 4) and cross the gap. An interpretation of the images based on the similar staining properties of the bridges and the junctional membranes is shown in Fig. 9. Note the continuity of the outer leaflet of the TT membrane with the outer leaflet of the JSR membrane. The inner leaflets are not continuous and therefore the lumens of the TT and JSR are not continuous.

The bridges occurred periodically with center-to-center spacings of 29 nm ± 1.5 SEM (12 prints). This periodicity is similar to the periodicity of the foot processes (8). In some views, the bridges appeared to run within the amorphous

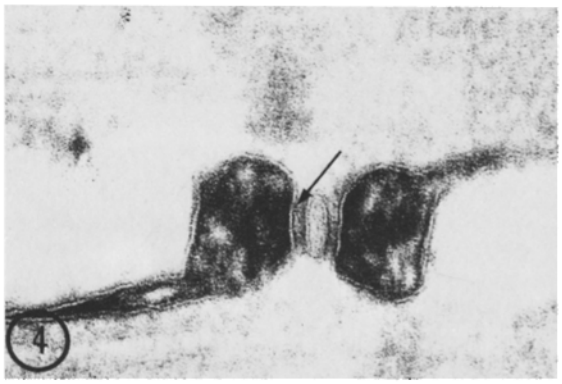


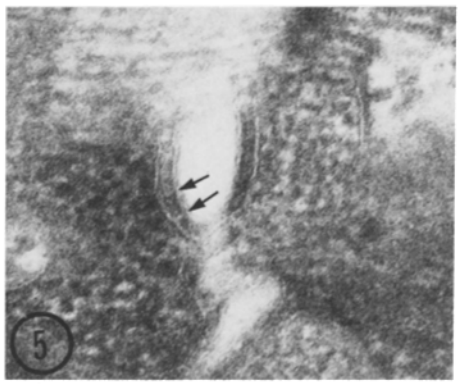
All figures except Figs. 6 and 10 show images of the triads of frog semitendinosus or sartorius muscles. The method of tissue preparation as outlined in Scheme I is indicated in each legend. *SR*, sarcoplasmic reticulum; *TT*, transverse tubule; *TC*, terminal cisterna.

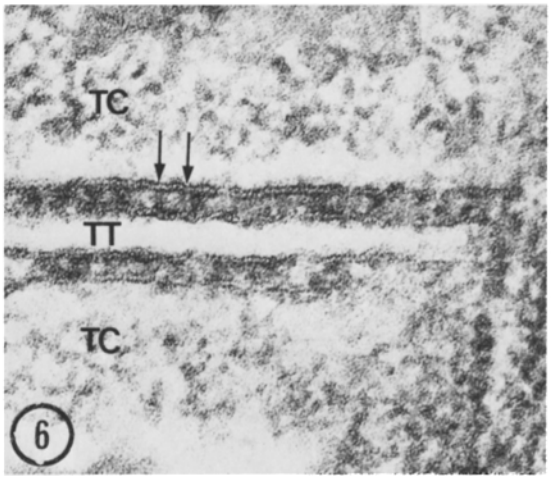
FIGURE 1 Triad illustrating a bridge (small arrow) across the junctional gap, linearly aligned particles (large arrowhead) within the TC, and densely staining material on the outer leaflet of the SR (double arrowhead) but not the TT. Method C. × 174,000.

FIGURE 2 Arrowheads indicate membrane bridges with electron-lucent central regions and stain on either side. Method D. × 152,000.

FIGURE 3 Arrows indicate periodic membrane bridges. Method B. × 124,000.



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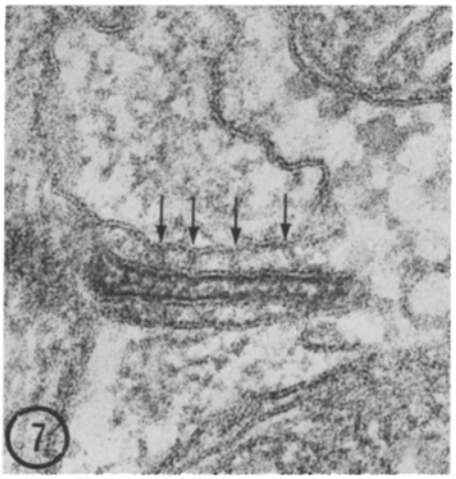

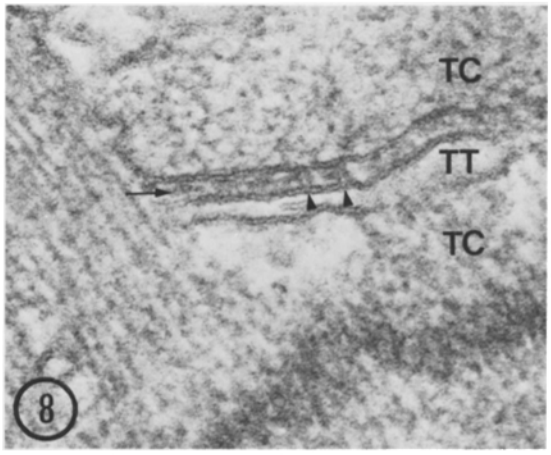
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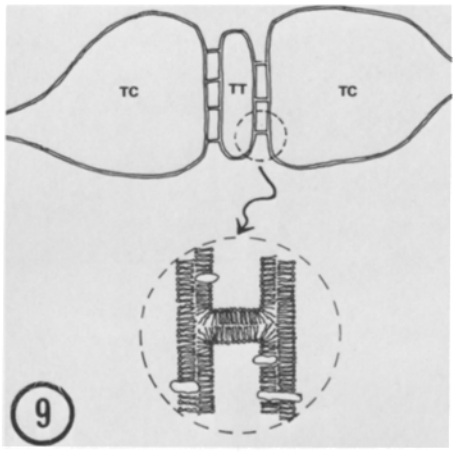
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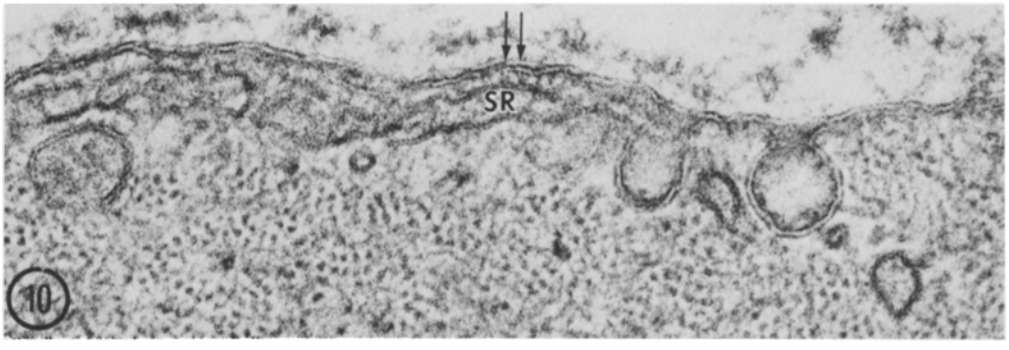

SR

foot processes (Fig. 8). The junctional gap was 18 nm ± 0.9 SEM. The bridges and junctional gap were present at various sarcomere lengths, including lengths at which there was no overlap between the thick and thin filaments. In these nonoverlapped fibers, the triads were frequently distorted and positioned in the I-band region away from their normal place at the Z line, as well as reoriented to the longitudinal axis of the fiber.

Similar bridges were observed at the close couplings of the SR with the surface membranes of smooth muscle (Fig. 10). The amorphous material of the foot processes was, in general, less obvious in smooth than in striated muscle either in the presence or in the absence of tannic acid.

The cytoplasmic leaflets of the longitudinal, intermediate, and terminal cisternal portions of the SR, but not of the transverse tubules, appeared thickened by intensely staining material (Figs. 1 and 13). Conversely, the luminal leaflets of the SR membranes did not appear thickened even when osmium, which makes the membranes leaky to tannic acid (39), preceded exposure to tannic acid. Therefore, the absence of this material on the luminal leaflet was not due to exclusion of tannic acid from the lumen.

The proteinaceous contents of the terminal cisternae appeared ordered more frequently in the preparations which were exposed to tannic acid before osmium or $FeCl_3$. The globular particles were arranged in rows and oriented longitudinally (Figs. 1, 11, 12, and 13). The average spacing of the rows was 20 nm ± 1.4 SEM. at the junctional region of the terminal cisterna. The particles appeared to adhere to the luminal membrane of the JSR (Figs. 1, 11, 12, and 13). The contents of the TC of muscles exposed to tannic acid after osmium were generally not so well organized, (exceptions are Figs. 1 and 13) and were similar in appearance to that seen in material fixed conventionally without tannic acid.

In negatively stained (glutaraldehyde-fixed) frozen sections, the junctional gap was well preserved and its diameter was consistent along its length and from one region to another (Figs. 14–16). The gap was frequently bisected by a line of negatively staining material that appeared to correspond to the (positively stained) junctional line observed in conventionally fixed frog skeletal muscle (10) (also see Fig. 8). Some negatively stained material appeared across the gap but its structure was not well resolved, possibly due to the difficulty in cutting frozen sections thinner than 100 nm at −130°C. Negatively staining material occurred within the terminal cisternae (Figs. 14–16).

DISCUSSION

Tannic acid, first used as a biological fixative by Mizuhira and Futaesaku (24), is derived from the nutgall, and consists primarily of glucosides with several orthophenol radicals that are negatively charged at neutral pH. The Simionescus (34, 35) have shown that the gallic acid moiety of the galloglucose molecule, tannic acid, is the mordanting agent between the tissue components and osmium and heavy metals such as lead. Tannic acid is also reported to stabilize structures (18, 35), preventing their extraction during dehydration (18). Kalina and Pease (18, 19) found that tannic acid formed complexes with choline bases of lecithins that were subsequently stabilized with osmium. Osmium without tannic acid is incapable of interacting directly with phosphatidylcholine,

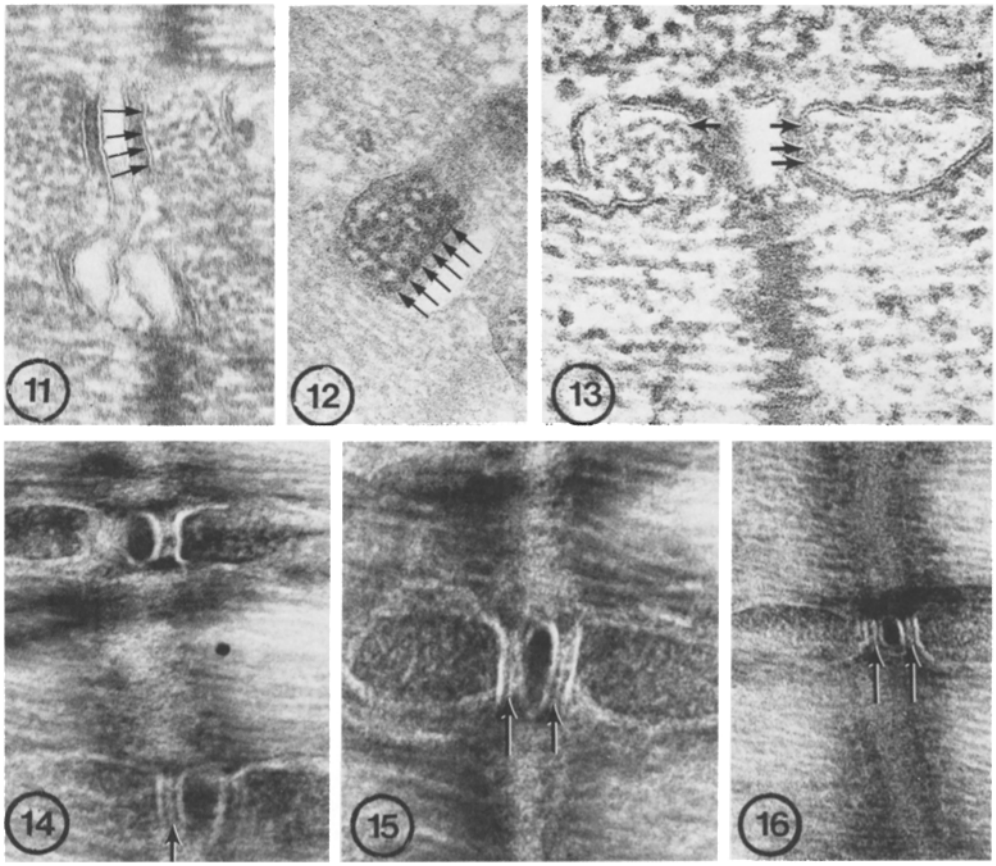
FIGURES 4–6 Arrows indicate membrane bridges. Note the diverging membrane leaflet in Fig. 4. Toadfish swimbladder muscle is shown in Fig. 6. Methods B, B, and C, respectively. Fig. 4, × 112,000; Fig. 5, × 112,000; Fig. 6, × 135,000.

FIGURE 7 Arrows indicate junctional bridges which are similar in diameter and staining properties to the TT and TC membranes. Method D. × 152,000.

FIGURE 8 Arrowheads indicate two electron-lucent bridges surrounded by amorphous foot processes. The arrow indicates dense material bisecting the junctional gap. Method D. × 153,000.

FIGURE 9 Model of the membranes at the triad. Note the continuity of the protoplasmic leaflets of the TC and TT membranes. The luminal membrane leaflets of the two structures are not continuous. The proteins depicted in one leaflet or spanning the membrane are not necessarily drawn to scale, and their distribution is not known. The amorphous foot processes surrounding the bridging structures (Fig. 8) may help to stabilize the bridge.

FIGURE 10 Transverse section from the main pulmonary artery of the rabbit showing membrane bridge (arrows) crossing the junctional gap at an SR-surface membrane coupling. Method D. × 124,000.



FIGURES 11–13 Arrows indicate the periodic longitudinally aligned particles within the TC. Note the scalloping of the junctional SR membrane in Fig. 11 and the adherence of the particles to the membrane. Methods B, B, and C, respectively. Fig. 11, × 100,000; Fig. 12, × 84,000; Fig. 13, × 135,000.

FIGURES 14–16 Glutaraldehyde-fixed, frozen sections, negatively stained (method E). A line of material occurs in all the junctional gaps (arrows). Negatively staining material bridges the gap in the upper triad of Fig. 14. Fig. 14, × 124,000; Fig. 15, × 114,000; Fig. 16, × 100,000.

and the latter does not survive subsequent dehydration for embedding. This may be the reason for the bridges not being preserved in the absence of tannic acid. These authors also reported that the tannic-acid-osmium-lead deposition occurred in the polar head groups of saturated phospholipid preparations, resulting in alternating electron-lucent and -dense lamellae. Therefore, the similar staining resulting in a trilaminar appearance of the triad membranes and the bridges (see Figs. 2, 3, and 7) suggests that the bridges may also contain phospholipids. On the other hand, the detailed chemistry of the staining reaction is only partially understood, and the continuity of the electron-lucent cores of the bridges, the SR and TT, could represent continuous hydrophobic regions of differing composition.

The similar spacing of the bridges (30 nm) and of the foot processes (30 nm; 8, 29), and the occasional images showing the bridges surrounded by amorphous osmiophilic material, suggest that the bridges run in the core of the foot processes. The bridges, however, do traverse the entire junctional gap and contact the tranverse tubule membrane.

In freeze-fracture studies, Franzini-Armstrong (8) did not find evidence of continuous structures across the junctional gap, as the number of feet was significantly greater than the number of particles per square micrometer of junctional membrane. However, Rayns et al. (29) reported "bridging particles" linking the two membrane systems. If the divergent membrane model as shown in Fig. 9 is correct, it is difficult to predict

whether the bridges would be discernible on the protoplasmic membrane fracture faces at the point where the membrane leaflets diverge and cross the junctional gap. By inspection of the model, it is apparent that the luminal leaflets require no specialized structures. As fracture faces follow the interior of membranes, a view along the center of the junctional gap has not been reported.

The existence of the junctional gap separating the transverse tubular system from the Ca-containing SR first described in osmium-fixed, plastic-embedded material (28) and later in glutaraldehyde-fixed muscle (9, 27) has been confirmed in unfixed, frozen-dried sections (36) and in the present study using glutaraldehyde-fixed, frozen and negatively stained sections. Tissues prepared by cryoultramicrotomy have been exposed only to aqueous solutions, and as a result lipids and hydrophobic substances are not extracted from the specimen by organic solvents. The presence and consistent diameter of 13–18 nm of the junctional gap found with the use of this variety of techniques substantiate its existence.

The appearance of a granular osmiophilic material on the cytoplasmic surface of the SR is similar to the negatively staining material present on the outer membrane of unfixed SR vesicles and considered to be the transport Ca-ATPase (15, 16, 21). X-ray diffraction studies of lamellae of stacked SR membranes suggest that the major portion of the Ca-ATPase activity resides on the exterior of the vesicle as well as extending into the core of the membrane (14).

The densely staining particulate material localized in the terminal cisternae is considered to be Ca-binding proteins (22; for review, see reference 20). The function of the organization of these particles into longitudinal rows closely applied to the inner membrane of the junctional SR is not understood. MacLennan and Holland (20) have suggested that the extrinsic Ca-binding proteins are bound to the membrane phospholipids. Densities closely applied to the junctional SR membrane of cardiac and skeletal muscle as well as coextensive densities parallel to the junctional SR membrane have been reported (40).

In two earlier studies (2, 17) the periodic densities across the junctional gap were interpreted as microtubules or hollow septa. Electron probe analysis of the elemental composition of the terminal cisternae has shown that the SR is not an extracellular compartment and rules out the suggestion that the lumens of the terminal cisternae and transverse tubule are continuous.

Intracellular bridges have been described, without the use of tannic acid, between parallel membrane faces separated from 7 to 35 nm in other systems, such as stacks of Golgi or ER cisternae, subsurface cisternae and plasma membrane, between the alveolar sacs of the pellicle and plasma membrane of many ciliates (see Figs. 1–16 in reference 7), as well as in actinopods (5). The tannic acid-stained junctional bridges also resemble some views of the extracellular "membrane linkers" of the septate junction (see Fig. 5 in reference 30) although the two periodicities differ (15 vs. 30 nm). Gilula et al. (11) proposed that the septa are products of the junctional membranes rather than differentiated intercellular material. A similar model as proposed here for the triad may be applicable to these inter- and intracellular bridges.

Divergence of membrane leaflets is thought to occur transiently during various forms of cell membrane fusions such as exocytosis (26) (or induced fusion of erythrocyte ghosts [4]). Similar sharp turns of divergent membrane leaflets have been observed by electron microscopy, utilizing tannic acid, in tubular myelin from lamellar bodies in the lung (32).

Whether the bridging structures are tubular or septate was not discernible in thin transverse sections and, therefore, a three-dimensional model of the structure is not proposed. Although single chain amphiphiles can form long cylindrical micelles, the predominant phospholipids of biological membranes have two hydrocarbon chains and usually form bilayers or liposomes. Low-angle x-ray diffraction studies have revealed forms other than the bimolecular leaflet, such as the hexagonal I form which consists of tubular structures with the fatty acid chains located in the interior (for review see reference 31). The manner in which the lipid molecules arrange themselves *in situ* seems to depend very much on the environment, e.g., the fractional concentration of lipid (13), the presence of cations (4), and of other molecules such as proteins (31). The amorphous foot processes and other staining material in the junctional gap may influence the arrangement and stability of the bridging structures. It should be emphasized that the suggestion that the bridges are composed of phospholipids is based solely on their staining properties. Therefore, until their composition and that of their environment is known, a definitive model of a thermodynamically stable structure cannot be proposed.

The proposed model is a specific, ultrastructural

extension of models of excitation-contraction coupling based on the voltage-sensitive charge movements of Schneider and Chandler (33) and of Adrian and Almers (1), which occur after the action potential and before tension development. It is also compatible with the membrane capacitance measurements in muscle. Chandler's "long molecules" could be replaced by a protein or ionophore moving from the T-tubule membrane to the SR membrane via the continuous membrane leaflet. Considering a diffusion rate of $\sim 10^{-8}$ cm^2/s for lipid-probe molecules, and 10^{-10} cm^2/s for proteins in membranes (6, 41) and a 15-nm junctional gap, the time for a molecule to cross the gap via the bridge is ~0.04–3.7 ms, ($\tau = \omega^2/4D$[6, 41]) which is comparable to the 1.8 ± 0.5 ms delay measured between the rise in the membrane potential and the onset of increased free cytoplasmic Ca as indicated by Arsenazo III (23).

This work was supported by grant HL15835 to the Pennsylvania Muscle Institute.

Received for publication 10 October 1978, and in revised form 11 December 1978.

Note added in proof: After the acceptance of this manuscript, Saito et al. have reported similar asymmetry of the SR membrane using tannic acid *in situ*. (*J. Cell Biol.* 1978. **79:**601–616.)

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