

FREEZE FRACTURE OF SKELETAL MUSCLE FROM THE TARANTULA SPIDER

Structural Differentiations of Sarcoplasmic Reticulum and Transverse Tubular System Membranes

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ABSTRACT

The structure of the membranes of sarcoplasmic reticulum (SR), tubular (T) system, and sarcolemma has been studied by freeze fracture in leg muscles of the Tarantula spider. Two regions of the sarcoplasmic reticulum can be differentiated by the distribution of particles on the fracture faces: a junctional SR, at the dyads, and a longitudinal SR, elsewhere. The dyads are asymmetric junctions, the disposition of particles in the apposed membranes of SR and T tubules being different from one another and from the regular arrangement of feet in the junctional gap. It is concluded that no channels can be visualized to directly connect SR- and T-system lumina.

INTRODUCTION

The membranes of the sarcoplasmic reticulum (SR) and transverse tubular (T) system of striated muscle fibers perform a number of well-characterized functions in the processes of excitation-contraction coupling and relaxation (see Sandow, 1965; Weber, 1966, for reviews). The aim of this study is to explore, by freeze fracture, the structure of surface and internal membranes of a striated muscle and to identify in them areas which are dedicated to specific steps in the fiber's activity cycle. A spider muscle was selected for this investigation mostly because it has large, flattened areas of junction between the SR and T system in longitudinally oriented dyads (Sherman and Luff, 1971).

Freeze-fracture studies of a variety of membranes have shown that a correlation exists between the number and distribution of particles on the exposed fracture faces and the activity of the membrane

(Branton, 1966, 1971). It is increasingly evident that the particles that one sees are spatially related to proteins occupying the interior of the membrane and that they may represent lipoprotein complexes (see Marchesi et al., 1972). The demonstration of different and sometimes highly organized arrays of particles in the fracture faces of functionally specialized membranes leads to the hope that one can eventually assign a role to each family of particles. Striated muscle is particularly suited for this type of investigation, because there is good evidence that some of the properties of the membranes are located in specific areas. It is hoped that freeze fracture will allow identification of these areas and further understanding of their function. A useful comparison can be made between the structure of the dyadic junction and that of areas of mechanical, electrical, and metabolic junctions between cells (e.g., see Stachelin et al., 1969;

Friend and Gilula, 1972; Gilula et al., 1972; Peracchia, 1973; Claude and Goodenough, 1973).

Although this study falls very short of its ultimate goals, it reveals structural details which bring insight into the functional specializations of the SR and T-tubule membranes and of their junction. Differences between plasma membrane and T tubules may be interpreted as showing less involvement of the latter in general metabolic functions. The SR membrane is composed of two distinct portions: one along the longitudinal tubules (longitudinal SR) and the other at the triads and dyad (junctional SR). Two major functions of the reticulum, calcium accumulation and the initiation of calcium release, are probably performed by the two portions of the membrane. A comparison of junctional SR and junctional T tubule, where they face each other, reveals that the dyads are junctions with structural asymmetry of the apposed membranes and that there is no apparent pathway joining the lumina of the SR and T system.

MATERIALS AND METHODS

Tarantula bird spiders (*Eurypelma californicum*) were obtained from Carolina Biological Supply, Burlington, N. C. Levator pretarsi and depressor pretarsi muscles were fixed by injecting 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2–7.4, containing hydrogen peroxide (Peracchia and Mittler, 1972), into the excised leg.¹ For freeze fracture the muscles were dissected out after 20–40 min fixation and gradually infiltrated in glycerol in water, up to a concentration of 30% (vol/vol). Freezing was carried out in Freon 22, at a temperature slightly above that of liquid nitrogen and fracture, shadowing and replication were performed in a Denton DFE-3 freeze-etch unit (Denton Vacuum Inc., Cherry Hill, N. J.) fitted over a Kinney (KSE-2A-M) evaporator, Kinney Vacuum Co., Boston, Mass. (see Moor, 1971 for details of the technique). The replicas were handled as described by Peracchia (1973). For thin sectioning, muscles were fixed for about 2 h, rinsed in buffer, postfixed in 2% osmium tetroxide in the same buffer, dehydrated in ethyl alcohol and propylene oxide, and embedded in Epon. Sections were cut on a Cambridge microtome, Cambridge Scientific Instr. Ltd., Cambridge, England. (of A. F. Huxley pattern), stained with uranyl acetate and lead salts (Sato, 1968), and examined in an AEI 801 microscope.

¹ In my experience with a number of muscle fibers I found that, at the level of resolution achieved here, fixation with or without hydrogen peroxide produces similar results.

Estimates of surface areas of membranes were obtained by tracing the areas on tracing paper of uniform weight and then weighing the cut shapes. Curvature affects only the peripheral rim of each junctional area, no more than 17% of the total, and it was disregarded.

RESULTS

Foreword

The disposition of membranes in the leg muscles of the tarantula spider, which has been elegantly described by Sherman and Luff (1971), makes them admirably suited for a freeze-fracture study of the structure of SR- and T-system membranes, particularly at the dyadic junctions. The most relevant features of these muscles are: (a) the longitudinal SR is abundant, (b) the transverse tubular system has an unusual profile, in which flat junctional cisternae alternate with smaller tubules, (c) the invaginations of the plasma membrane, which continue into the T-system tubules, are relatively large and frequent, (d) the dyads are oriented longitudinally. Thus identification of SR- and T-system membranes is immediate in freeze-fracture replicas, and comparison of the junctional and nonjunctional portions of the membranes (see below) can be made on the same replica, a considerable advantage when fine differences want to be emphasized. Some muscles from insects (Pasquali-Ronchetti, 1969; Smith and Aldrich, 1971) as well as from scorpions (Gilai and Parnas, 1972) have a similar disposition of internal membranes.

A brief description of some of the structural features of the spider's muscle fibers, as observed in thin sections, is given here only where necessary for the understanding of the freeze-fracture images. The reader is referred to the original description (Sherman and Luff, 1971) for other details.

In interpreting the micrographs it is assumed that the fracture plane always follows the interior of the membranes, thereby dividing them into two leaflets: a cytoplasmic leaflet (whose exposed face is termed face A, McNutt and Weinstein, 1970) and an exterior (or, in the case of internal membranes, luminal) leaflet (whose exposed surface is face B). The assumption is based on the well-known demonstrations by Pinto da Silva and Branton (1970) and by Tillack and Marchesi (1970) that in red blood cells the true cell surfaces are never exposed by the fracture.

The Plasma Membrane and T-Tubule Invaginations

The surface of the fiber is sulcated by frequent, narrow, longitudinally oriented invaginations (Fig. 1). These small clefts usually penetrate for a short distance into the fiber, and then give rise to the T system (see Sherman and Luff, 1971, Fig. 8). The length of the clefts is variable. The shortest clefts (800–3,000 Å along the long axis of the fiber) give rise to single T tubules, and they are aligned in transverse bands which repeat longitudinally at a distance of approximately 1–1.2 μm (Fig. 1, arrows).

The longer clefts represent the site of concomitant invagination of two to three tubules. Some of these arise at the appropriate level, i.e., in register with the single openings. Other tubules originate in a position at random, relative to the bands of the sarcomere, and then course diagonally for some distance before becoming transversely oriented (Fig. 2). By this means a single tubule may form

dyadic contacts with the SR wrapping two adjacent sarcomeres. Many of these obliquely running T tubules have been noticed both in freeze-fractured and in sectioned specimens.

The cytoplasmic and outer leaflets of the plasma membrane (Figs. 3 and 4) differ in the number of particles, in a manner similar to that of most biologically active plasma membranes, i.e., face A has more particles than face B (Branton, 1969; Weinstein, 1969). One major difference between muscle plasmalemma and the membrane limiting other cell types (i.e., the frequently described red blood cells) is that face A has a heterogeneous population of particles. Both leaflets have a population of particles which are not only large in diameter (80–100 Å), but also project farther out of the fracture plane, as indicated by the elongated shadow. The number of large (80–100 Å) particles on the two fracture faces of the plasmalemma was estimated by counts on photographs at a final magnification of 48,000. The major source of error in a counting of this type is the often encountered

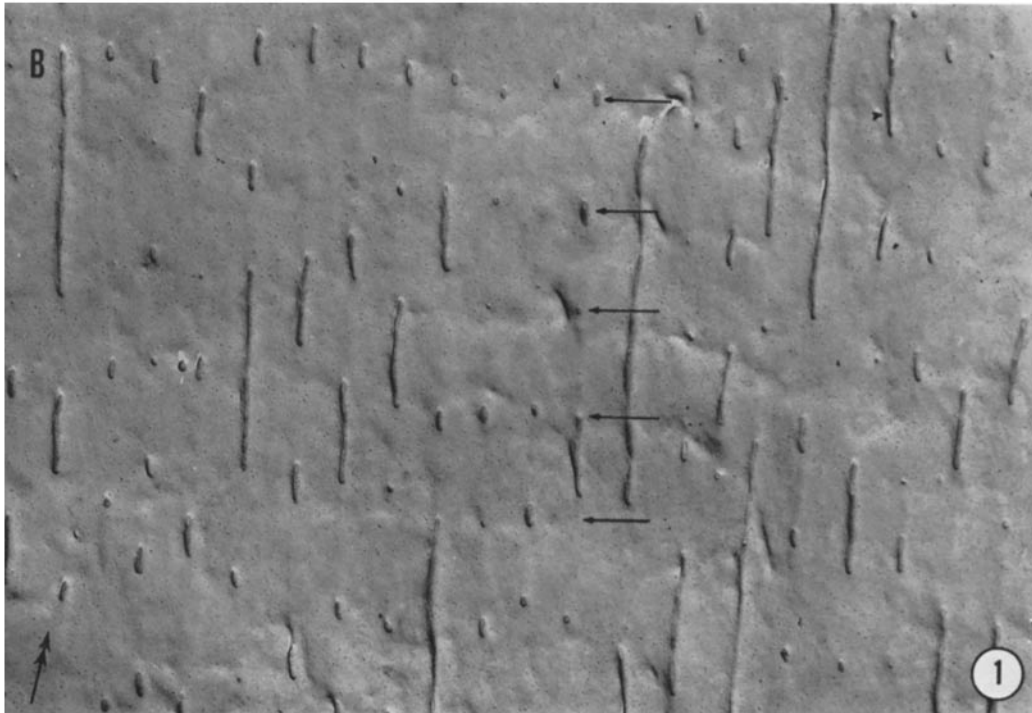


FIGURE 1 Exterior leaflet (face B) of the plasma membrane. The long axis of the fiber is vertical. Numerous narrow clefts invaginate into the fiber. The T system arises from these. Arrows at right give approximate position or rows of T-system openings. In this as in following figures, double arrows indicate direction of shadowing. $\times 12,000$.

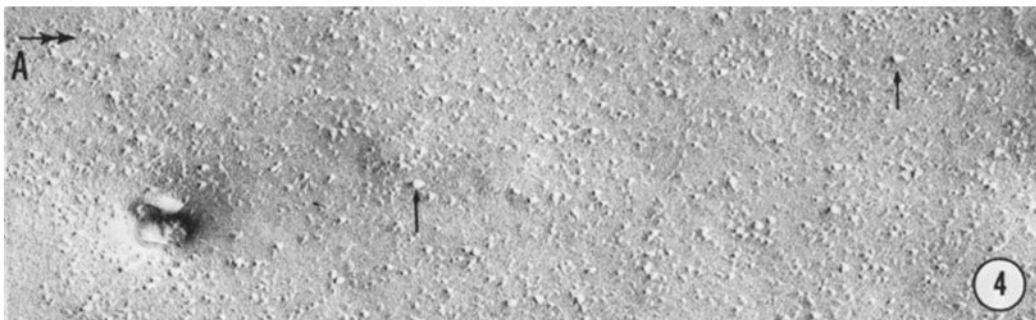


FIGURE 2 At left is a portion of A face (*A*) of the plasma membrane. Couples of small arrows mark the level at which T tubules arise from a small cleft and the A face changes structure (see text). T tubule in the middle is initially oblique to the long axis of the fibril (large arrow). $\times 56,000$.

FIGURES 3 and 4 B and A faces of the plasma membrane. The latter contains a larger number of small and intermediate size particles. Both faces have large particles, some of them marked by arrows. $\times 90,000$.

uncertainty in placing individual particles in the appropriate category, and the numbers obtained are only approximately correct (see Table I).

The sites of origin of the T tubules from the small clefts are visible in freeze fracture because at that point the clefts become narrower and a subtle change in the structure of the membrane occurs (arrows, Fig. 2): the cytoplasmic leaflet has fewer small and intermediate size particles than the corresponding leaflet of the plasma membrane, but it has about the same density of large particles. The luminal leaflet of the T tubule is almost identical to the cytoplasmic leaflet.

In spider muscle fibers, as is often the case in arthropods, some dense material is attached to the sarcolemma at the level of the Z line (forming a so called hemi-desmosome), and by that means an apparent continuity, with probable mechanical function, is provided between the fibrils and the plasma membrane. Interestingly, this dense material is not anchored to the interior of the membrane, since the fracture faces of the plasma membrane do not display any specific structure at the Z-line level. This is in contrast to epithelial desmosomes in which the membrane is differentiated (McNutt and Weinstein, 1973).

Structure of Membranes in the Interior of the Fiber

Figs. 5 and 6 illustrate the general geometry of SR and T system in the interior of the fiber as seen in sectioned material. The longitudinal SR is in the form of round tubules, mostly oriented longitudinally, forming a complex labyrinthine network with interruptions at the Z line. Dyads occur in rows along the T-system path (Fig. 5). They are formed by the apposition of flattened cisternae of the SR (junctional SR) and correspondingly widened portions of the T tubules (Fig. 6). "Feet" cover the junctional SR surface (short lines, Fig. 6). Notice that the T system is smaller than the apposed SR and that the latter is slightly concave. Both details are useful for the identification of the membranes in freeze fracture. The dyads have an unusual feature: the flattened junctional SR is covered by feet, even where no T-system membrane is immediately adjacent to it. Apart from this detail, the structure and disposition of the junctional feet are indistinguishable from that described in the triads and dyads of slow and twitch fibers from vertebrates (Franzini-Armstrong, 1970, 1972, 1973). Thus, the feet form a square array,

with a center-to-center distance of approximately 300 Å. This square array is faintly visible in Fig. 5 and is more clearly seen in Figs. 11 and 13 of Sherman and Luff (1971).

Freeze-fracture images presented here focus on four areas of the internal membranes: (*a* and *b*) the longitudinal and the junctional SR, which are continuous with one another, (*c*) the junctional T-tubule surfaces facing the SR at the dyads, and (*d*) the nonjunctional T tubule, at the same level, but facing in opposite direction. Each membrane is split into two leaflets by the fracture, thereby exposing A and B faces. The alternate tubular and flattened portions of the T tubule (T) are clearly identifiable in fracture replicas (Fig. 7). The longitudinal SR tubules are concave when the A face is exposed and convex when the B face is exposed. Several tubules join the flat junctional cisternae (junctional SR) which are seen around the T tubules.

Fig. 8 schematically illustrates the splitting of membranes at the level of a dyad which is oriented as in Fig. 6. Two fracture possibilities are illustrated and both encountered in the replicas. In one (dashed line) the fracture follows the junctional SR and then the junctional T system membrane. In dyads thus fractured, either the A face of junctional SR and B face of junctional T tubule (Fig. 9, left), or B face of SR and A face of T tubule (Fig. 9, right) are alternatively exposed. In other cases, the fracture jumps from the junctional SR to the nonjunctional surface of the T system (dotted line, see also Fig. 11). Notice that the fracture does not follow the junctional SR throughout the dyad, even though the SR does not curve abruptly. The possible reason for this unusual fracture pattern will be dealt with in the discussion.

At the level of resolution obtained here, the B face of longitudinal SR elements is smooth except for a few particles (Fig. 9). The B face of the junctional SR, on the other hand, is occupied by numerous particles and shallow pits (arrows) (Figs. 9-11). Particles and pits are variable in number per unit area, and they are randomly disposed. The patches of junctional SR membrane are roughly circular or oval in shape, and a comparison between Figs. 5 and 7 readily demonstrates that this area coincides with that covered by the junctional feet.

In the A face of the longitudinal SR, numerous, tightly packed particles occupy the surface.

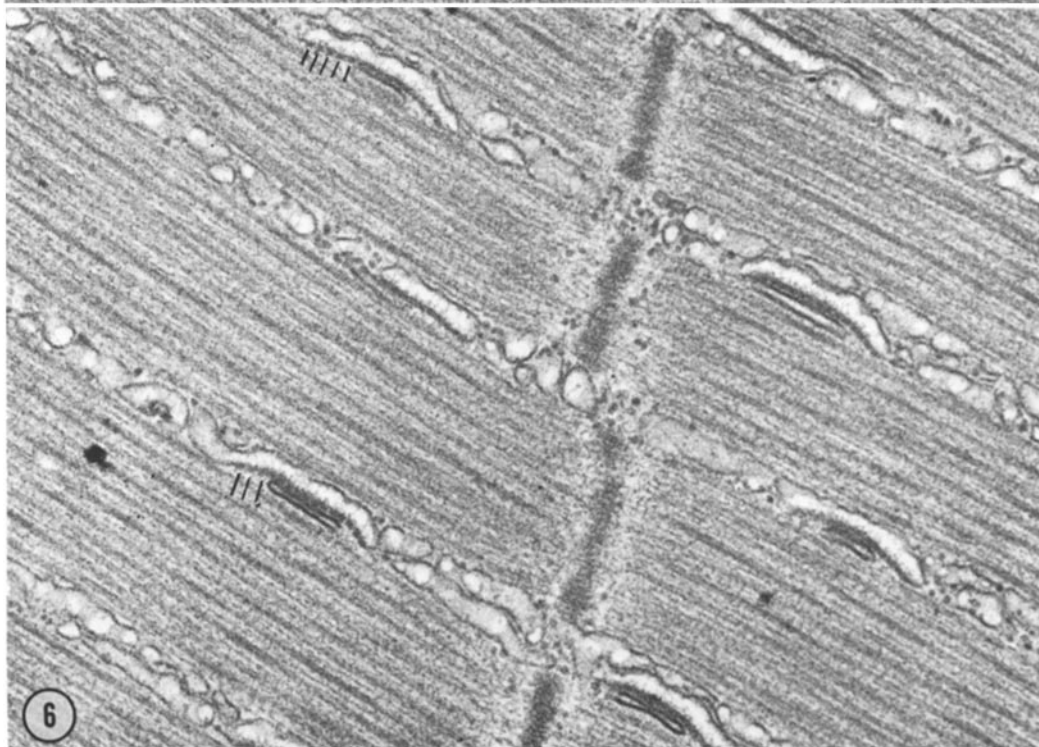
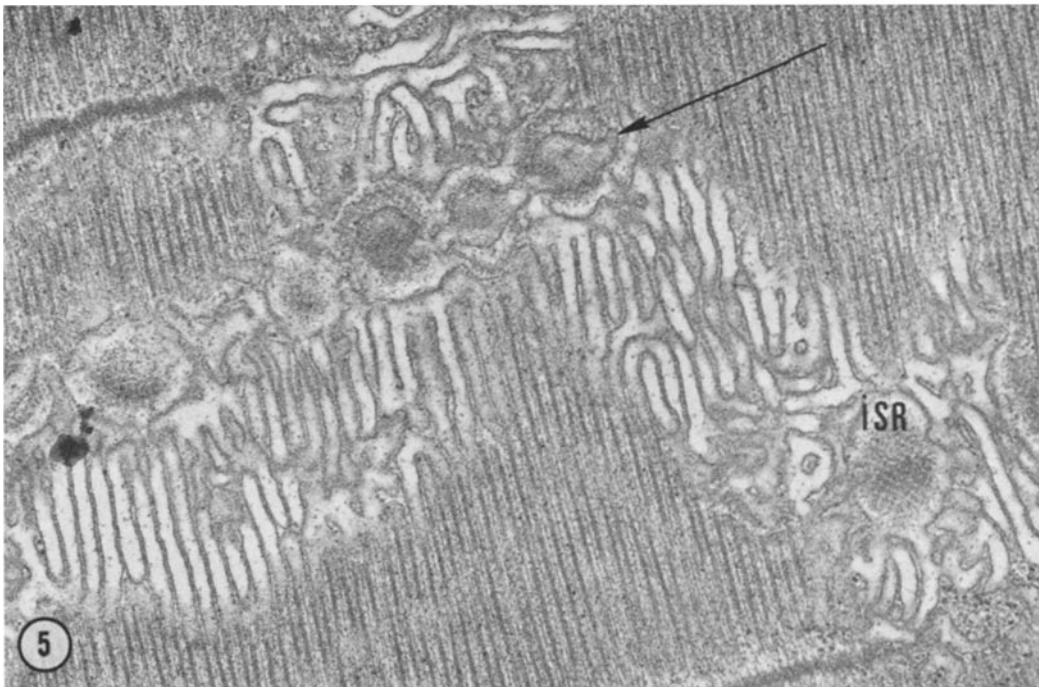


FIGURE 5 Thin longitudinal section, tangent to a fibril. The T system (arrow) has a characteristic shape; the SR is abundant. Notice square array of feet covering the junctional SR (*jSR*). $\times 30,000$.

FIGURE 6 Several dyads cut in cross section. The junctional SR, covered by feet (small parallel lines), extends farther than the T system. $\times 47,000$.

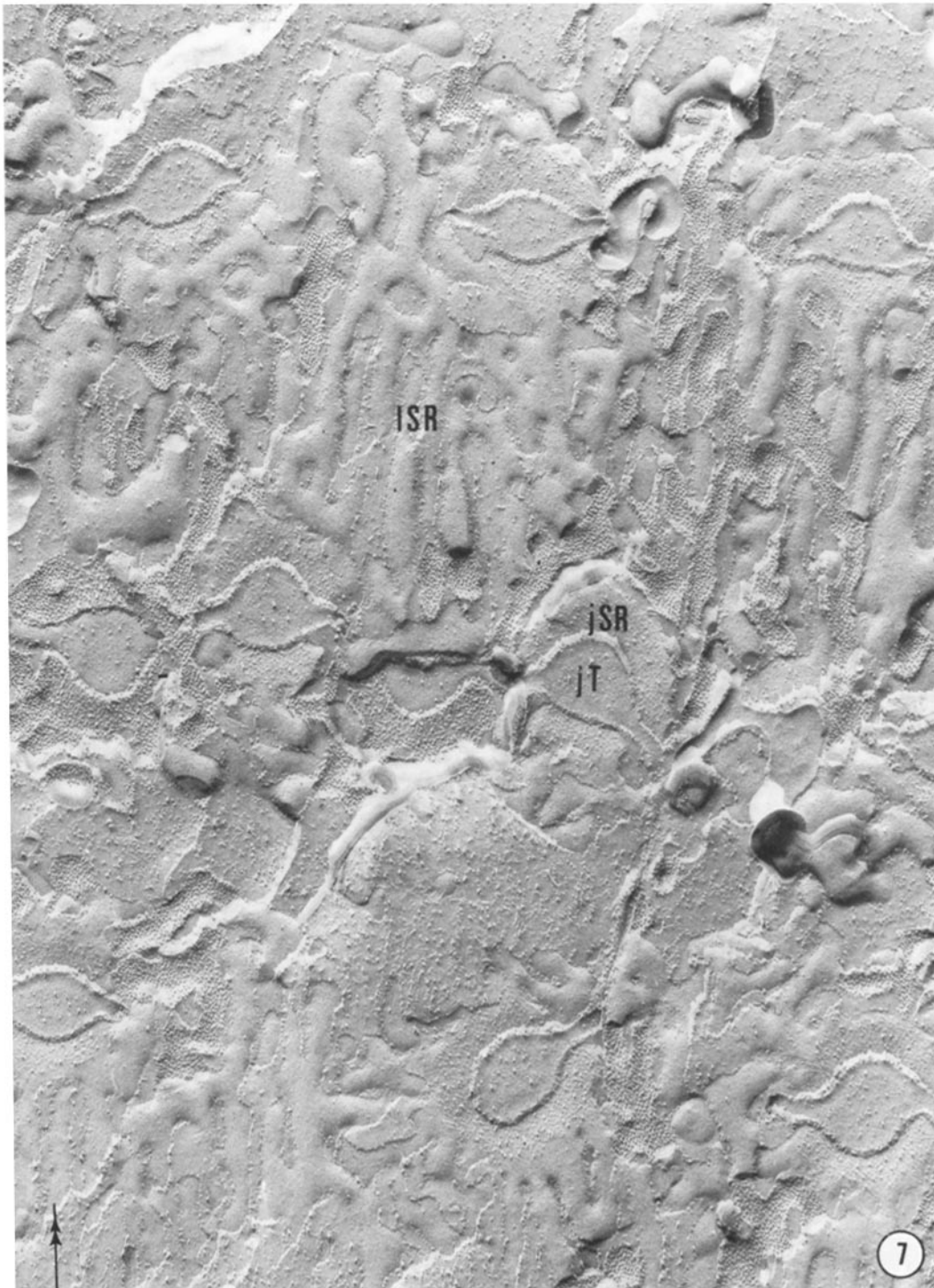


FIGURE 7 Overall view of a fracture area of SR and T system. The junctional T system (*jT*) is recognizable by its shape. A round patch of junctional SR membrane (*jSR*) surrounds it. Tubules of the longitudinal SR (*lSR*) occupy most of the picture. $\times 48,000$.

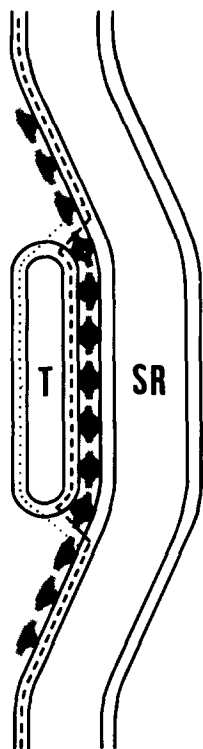


FIGURE 8 Diagram of a dyad in a view similar to that obtained in Fig. 6. Lumina of SR and T tubules are marked. The shaded structures in the junctional gap are the feet. The dashed and dotted lines follow the two commonly found fracture planes (see text).

Judging from the irregularity of the packing, the size of the particles is probably variable. The mean center-to-center separation is about 80 Å. The exact location of the transition between longitudinal and junctional SR is not clearly marked in the A face, but it is clear that the junctional areas are covered by less numerous and larger particles than the immediately adjacent longitudinal SR (Fig. 9). These particles are probably responsible for the pits which appear in the complementary B face. The spaces between A face particles may be occupied before fracture by particles from B face. Since the disposition of the particles and the spaces between them are quite variable in different dyads, it is likely that the particles can fracture on either leaflet at random.

As mentioned earlier, one feature of the T-tubule membrane is that both faces are similar (Fig. 9): both have a few small and medium size particles and some large particles. The nonjunctional T system is much the same (compare Figs. 10 and

11). The number of large particles (80–100 Å) in junctional and nonjunctional surfaces of the T system is given in Table I. Although the particles seem to be more numerous in the junctional region, counting is difficult as mentioned in the section on the sarcolemma, and the difference in Table I is of doubtful significance. The large particles are found in slightly higher numbers in the B face of both T system and sarcolemma.

The large particles of the T system do not leave a visible pit on the opposite fracture face, in contrast to the similar looking particles of the junctional SR which leave very evident pits (Fig. 9). Also, the particles on the junctional T system are less numerous than those on the apposed SR (see Discussion).

DISCUSSION

The major conclusion of this study is that there are structural differences between the membranes of the junctional and longitudinal portions of the SR and between the membranes of the T tubules and the sarcolemma. It is tempting to relate these morphological differences to the functions attributed to these portions of membrane.

In the longitudinal SR the vast majority of the particles are on the cytoplasmic leaflet, and no pits are present on the luminal leaflet (Bertaud, et al. 1970; Rayns, 1971). The luminal leaflet, in fact, closely resembles an uninterrupted lamellar lipid fracture face (Deamer et al., 1970). The numerous particles on the SR cytoplasmic leaflet have been said to represent the calcium activated ATPase (Deamer and Baskin, 1969; Sommer and Steere, 1970; Baskin, 1971, 1973), a proposal which is consistent with the facts that: (a) the ATPase is a major component of the SR membrane (Martonosi, 1969; MacLennan, 1970) and, more significantly, purified ATPase from SR can form membranes showing globular subunits in freeze fracture (MacLennan et al., 1971); (b) the major part of the pump is thought to be on the cytoplasmic surface of the SR (Hasselbach and Elfvin-Lars, 1967); and (c) X-ray diffraction shows the SR as having a markedly asymmetric density profile (Liu and Worthington, 1973). The SR membrane has ATPases other than the calcium pump, and it may also contain other enzymes participating in the general metabolism of the fiber (see Martonosi, 1972, for a review). The observed variability in size of the particles in the longitudinal SR may be an indication of their

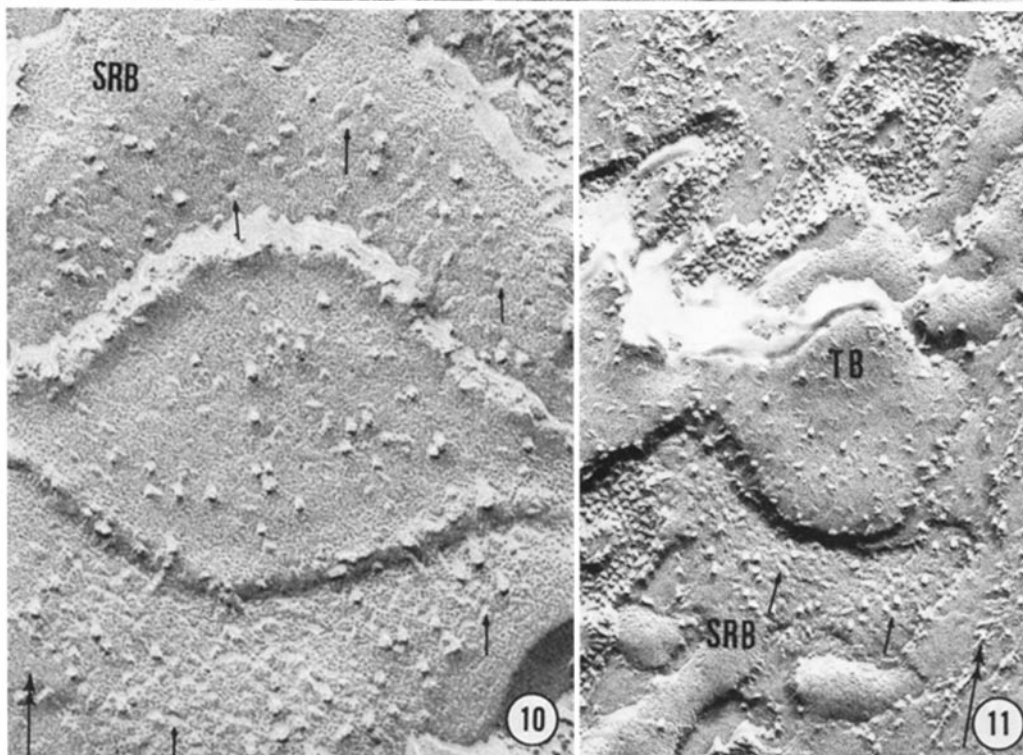
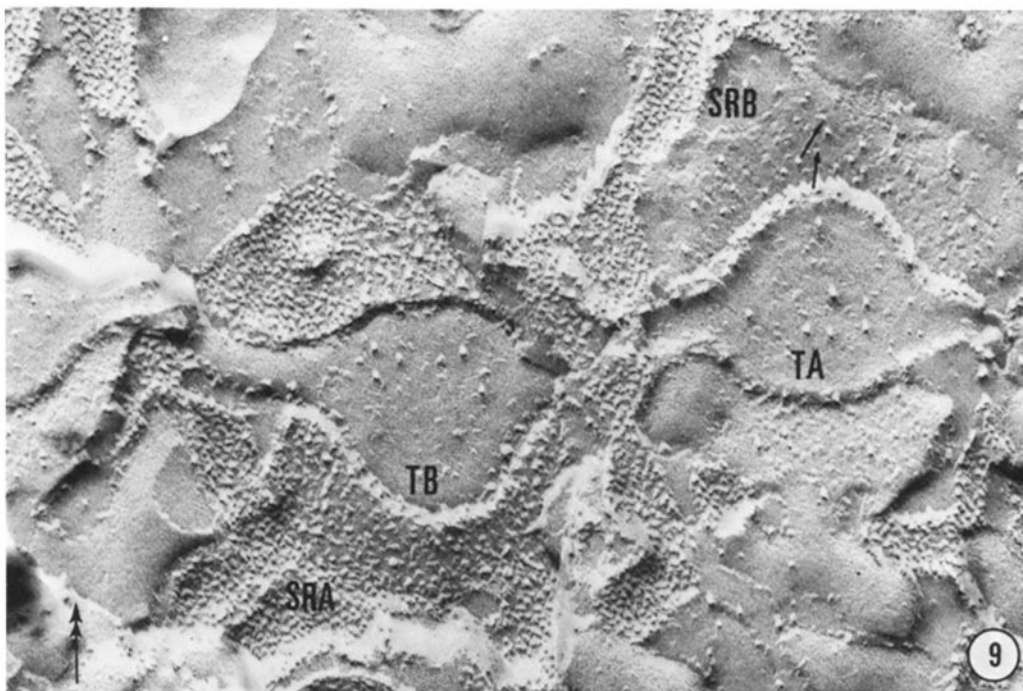


FIGURE 9 In the dyad at left, the B face of the junctional T system (*TB*) and the A face of the junctional SR (*SRA*) are shown. In the dyad at right, the complementary leaflets are visible (*TA* and *SRB*). Notice how the B face of the SR changes in structure in the junctional region. In this and following images, small arrows point to pits. Luminal and cytoplasmic leaflets of the T system are apparently identical. $\times 90,000$.

FIGURE 10 Details of the B face of the junctional SR (*SRB*). Shallow pits produce the rough appearance of the membrane in the lower portion of the *jSR*. $\times 153,000$.

FIGURE 11 The fracture plane here follows the dotted line (Fig. 8), thus exposing the nonjunctional region of the T system. Both SR and T tubule show a B face (*SRB* and *TB*). Notice similarity with B face of junctional T tubule (*TB*, Fig. 10). The junctional SR has evident pits (arrows). $\times 90,000$.

TABLE I
*A Comparison of the Distribution of Large Size
 Particles on Sarcolemma and T-System
 Fracture Faces*

Membrane	Number of particles on outer (or luminal) leaflet	Number of particle on inner (or cytoplasmic) leaflet
Plasma mem- brane	76	64
Junctional T sys- tem	108	97
Nonjunctional T system	106	64

varied roles. It is interesting that the SR is noticeably more asymmetric in the distribution of particles between the two fracture faces than the endoplasmic reticulum (Bullivant, 1969; Orci et al., 1971).

The particles of the junctional SR are found on both A and B faces and there is some indication that they can fracture at random on either one. This may mean that the particles are located symmetrically, relative to the two leaflets. Also, unlike those in the longitudinal SR, they leave pits on the complementary face. The pits are not simply due to the size of the particles, since large particles on sarcolemma and T system do not, in these preparations, produce an obvious pit in the complementary leaflet. Also, as smooth and pitted areas of the SR are seen side by side in the same replica, the presence or absence of pits cannot be attributed to the technique, but it must depend on the composition of the particles and/or their interaction with the surrounding lipids (cf. Flower, 1973; Peracchia, 1973). The unusual fracture properties of the dyads can also be interpreted in terms of the structure of the junctional SR membrane. The fracture plane preferentially jumps across a 120-Å thick layer of cytoplasm, rather than following the gently curving SR, even when the muscle is fractured obliquely to the fiber axis. It must be concluded that the SR membrane is much more resistant to fracture than the T-system membrane, and this is consistent with the idea that large protein complexes cross the center of the bilayer and are anchored on both leaflets of the membrane. This morphological evidence for the molecular architecture of the junctional SR differing from that of the longitudinal SR is consonant with the finding that a low density subfraction of the SR, containing the triads, has a

different composition in protein and phospholipids from a high density subfraction, identifiable with the longitudinal SR (Heuson-Stiennon et al., 1972).

Release of calcium from the SR is governed by the membrane potential of the T tubules (Huxley and Taylor, 1958; Hodgkin and Horowitz, 1960), and transmission of information between the two membrane systems is likely to occur at the triad

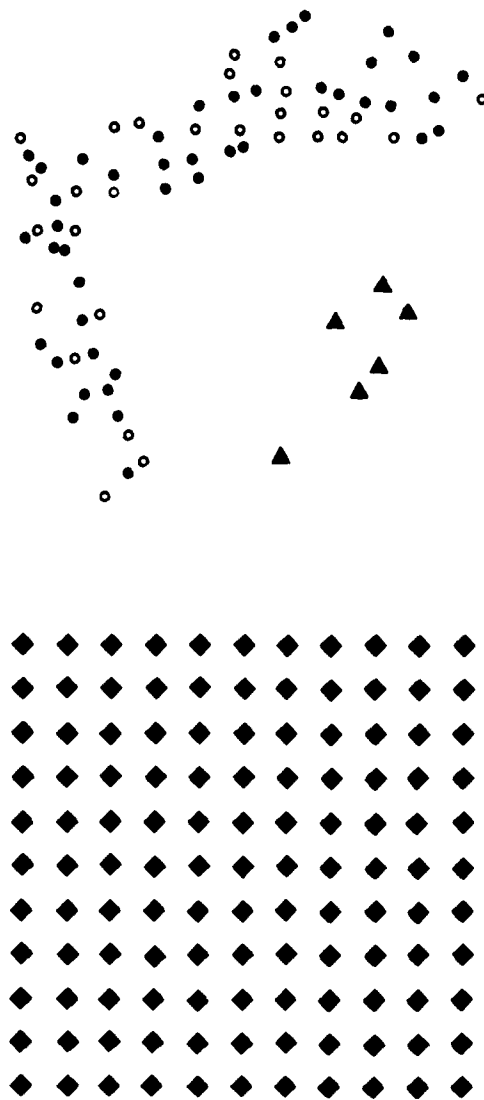


FIGURE 12 The disposition of particles and pits in the junctional SR (filled and empty circles) and of particles in the T-system membranes (triangles) are drawn from a photograph. Disposition of the feet (bottom) is drawn to scale. See text for details.

(or, in spider muscle fibers, at the dyad). The large molecular complexes occupying the junctional SR membrane are possibly involved in the transmission of information and in the initiation of calcium release from the SR. It is frequently postulated that the signal for calcium release may be a flow of ionic current between the lumina of SR and T tubules, a mechanism which would require the existence of preformed channels similar to those existing between the cytoplasm of electrotonically coupled cells (see Bennett, 1973, for a review). The following comparison between the architecture of the dyadic junction and that of low resistance junctions between cells indicates that at the dyads it is not possible to formulate the existence of preformed channels and confirms previous conclusions that an alternate mode of transmission is most likely to exist (cf. Franzini-Armstrong, 1970, 1971).

In the top half of Fig. 12, the disposition of particles (filled circles) and pits (open circles) on the B face of the junctional SR and of large particles (triangles) on the junctional T system is traced from a micrograph. Only the most evident pits are marked. In the bottom half of the figure, the square array of feet is drawn to scale. The T system's particles are very few ($205/\mu\text{m}^2$, see Table I), far less than the number of feet covering the same surface $1,089/\mu\text{m}^2$. The SR's particles and pits, on the other hand, are more numerous than the feet and less regularly disposed. Thus it is not possible to match disposition of feet and particles to one another,² in strong contrast to the situation occurring in low resistance junctions. In the latter, the regular hexagonal array of pits and particles on the exposed fracture faces of the junctional membranes matches the disposition of hexagonally arranged intercellular structures in the junctional gap, so that continuity between particles and such structures may exist, and thus the existence of continuous hydrophilic channels can be postulated (McNutt and Weinstein, 1970; Steere and Sommer, 1972; Peracchia, 1973).

Being structurally asymmetric, as described above, the dyadic junction is different from most types of junctions between cells, where the two membranes participating in the junction have the

² The ordered arrangement of feet is probably not disturbed by infiltration with glycerol and freezing, since regularly spaced structures, presumably the feet, can be occasionally visualized in replicas of freeze-fractured specimens.

same distribution of particles and the junction is symmetric (see McNutt and Weinstein, 1972 and Staehelin, 1974, for reviews). A general feature of intercellular junctions is that particles within the membranes seem to be located in correspondence of either extra- or intracellular material, with which they are probably in continuity. At the dyads, it is possible that the feet are anchored into the thickness of the membrane of the junctional SR, but they certainly do not penetrate into the T tubules' membrane, since the particles there are very few.

The A face of the T tubules has noticeably fewer small and intermediate type particles than the A face of the plasma membrane. This may indicate less involvement of the T tubules in the general metabolic functions of the surface membrane. The large particles, on the other hand, are slightly more numerous in both A and B faces of T tubules than they are in the SR and the possibility exists that at least some of these particles are involved in the excitation-contraction coupling process. A comparative study of T tubules in a variety of muscles, now in progress, is aimed at exploring the possibility that the specific role of some of these particles can be identified on the basis of their number and disposition.

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