Subunit Structure of Junctional Feet in Triads of Skeletal Muscle: A Freeze-drying, Rotary-shadowing Study

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ABSTRACT Isolated heavy sarcoplasmic reticulum vesicles retain junctional specializations (feet) on their outer surface. We have obtained en face three-dimensional views of the feet by shadowing and replicating the surfaces of freeze-dried isolated vesicles. Feet are clearly visible as large structures located on raised platforms. New details of foot structure include a four subunit structure and the fact that adjacent feet do not abut directly corner to corner but are offset by half a subunit. Feet aligned within rows were observed to be rotated at a slight angle off the long axis of the row creating a center-to-center spacing (32.5 nm) slightly less than the average diagonal of the feet (35.3 nm). Comparison with previous information from thin sections and freeze-fracture showed that this approach to the study of membranes faithfully preserves structure and allows better visualization of surface details than either thin-sectioning or negative-staining.

The triads of vertebrate skeletal muscles are specialized junctions formed by the interposition of a T tubule between two terminal cisternae of the sarcoplasmic reticulum (SR).[1] In thin sections the junctional specializations (feet) which span the gap between the apposed membranes appear as tetragonally disposed densities, having a periodicity of ~30 nm. In twitch fibers of higher vertebrates, the feet form two- to three-long rows, parallel to the long axis of the T tubules (1).

Junctional feet are likely candidates for a primary role in the communication between surface and internal membranes which is known to occur at triads, and their structure has been extensively studied in the hope of providing a clue to the mechanism of such transmission. Thin-sectioning studies have established various appearances of the junctional components, such as those of bridging structures (2) and pillars (3) in sections across the junctional gap, and ring-like (4, 5) or diamond-like (6) shapes in tangential views. Ring and pillar shapes indicate the presence of a less-dense central core (7). Negative staining of isolated triads has clearly shown that the architecture of the junctional SR membrane reflects the sites of attachment of the feet and has confirmed the presence of feet, seen as evenly spaced, stain-excluding structures in the junctional gap (8). A relationship between feet and components of the two junctional membranes has been established in freeze-fracture replicas, where groups of particles in the junctional T tubule membrane (7) and less well defined structures in the junctional SR (8, 9) are disposed with the same spacing as the junctional feet.

Further progress has been hampered by the narrowness of the junctional gap, which makes the feet inaccessible to the recently developed deep etching techniques (10, 11). An alternative approach is to use isolated SR vesicles. The "heavy" SR fraction contains vesicles whose origin from the lateral sacs of the triad (terminal cisternae) is deduced on the basis of electron-dense contents with Ca^{2+}-binding properties (12). Under the appropriate isolation conditions, heavy SR vesicles retain structures identifiable as feet on the portion of their surface (junctional SR) which was originally participating in triadic junctions (8, 13, 14). We have developed a procedure for exposing surface details in isolated membrane vesicles that faithfully preserves three-dimensional relationships. The technique is a combination of (*a*) the classic adsorption to mica (15) which has been successfully used in various forms for the study of macromolecules (16–18); (*b*) freezing of thin water layers (19); and (*c*) "fixation" in uranyl acetate (20), which has been shown to stabilize protein structure. A very similar approach was used to study large supramolecular complexes (21).

Rotary-shadowed replicas of vesicles prepared in this manner afford the unique opportunity to visualize the feet "en face" and in three dimensions. In this paper we describe their subunit structure and their relationship to each other. These

[1] *Abbreviations used in this paper:* SR, sarcoplasmic reticulum.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 NOVEMBER 1984 1735–1742

images, used in conjunction with those seen in thin sections and in freeze-fractured specimens, allow further reconstruction of junctional detail.

MATERIALS AND METHODS

Isolation Procedures

RAT: A crude SR fraction was isolated following a procedure modified slightly from Herbette et al. (22). White muscles from hind legs of pentobarbital-anesthetized, exsanguinated rats were minced in ice-cold 10 mM D, L-histidine in 0.25 M sucrose (~5 ml of solution/g of muscle). The tissue was homogenized twelve times for 15 s at intervals of 5 min in a Sorvall omni-mixer (E. I. DuPont de Nemour & Co., Newton, CT); pH was maintained at 6.8–7.0 with 1 N NaOH. Cellular debris were pelleted at 11,250 rpm (15,200 *g*) for 20 min using a Sorvall SS34 rotor. The supernatant was strained through six layers of cheesecloth and SR membranes were pelleted using an SS34 rotor at 18,000 rpm (39,100 *g*) for 90 min. The pellet was resuspended in 0.6 M KCl and incubated for 10 min, and the centrifugation procedures were repeated. The final pellet of crude SR was resuspended in 10 mM histidine containing 0.25 M sucrose. The suspension was frozen in liquid nitrogen and stored at −70°C.

GUINEA PIG: A crude SR fraction was isolated as above except that the 0.6 M KCl wash was eliminated.

FROG: A crude SR fraction was isolated from frog skeletal muscles using a procedure modified from Castellani and Hardwicke (23). Semitendinosus, sartorius, and iliofibularus muscles were dissected from several frogs and bathed in relaxing solution (24) for 30 min. The muscles were soaked in relaxing solution containing 50 μg/ml saponin for 3 h, washed thoroughly in relaxing solution without saponin, finely minced by hand, and homogenized gently (two times for 1 s each time). After this step the centrifugation procedure was identical to that described for the rat except that the 0.6 M KCl was avoided. The suspension proved to contain too many myofilaments to allow optimum freeze-drying so the vesicles were briefly rinsed in 0.6 M KCl, repelleted, and resuspended in relaxing solution containing 0.25 M sucrose. The resultant suspension was frozen in liquid nitrogen and stored at −70°C.

RABBIT: An enriched fraction of heavy SR was donated by Dr. D. Pierce (University of Pennsylvania). This had been isolated and purified using procedures outlined in Herbette et al. (22) and Meissner et al. (25).

Freeze-drying and Replication

Droplets of suspension were placed on sheets of freshly cleaved mica, rinsed with 2% uranyl acetate and then with distilled water. The water was dried to a very thin film using filter paper. The mica plate was quickly frozen in liquid N_2, inserted into one side of a closed, precooled Balzers double replica holder (Balzers, Keene, NH), and placed into a Balzers 400D freeze-fracture unit. The holder reduces surface contamination of the specimen while in the liquid nitrogen and during transferral to the chamber. When an adequate vacuum was re-established (10^{-6} mbar), the specimens were warmed to −100°C, the cover on the specimen holder was opened, and the samples were freeze-dried for 30 min. The specimens were cooled to −150°C and rotary-shadowed with Pt at a 25° angle and replicated with carbon. Replicas were viewed in a JEOL 100B. Stereos were obtained using a Zeiss 109.

RESULTS

General Remarks

Note that throughout the following description we use the term "heavy SR vesicle" to indicate vesicles originating from the lateral sacs of the triad, even though most of the fractions we used were crude. In most of our preparations we had to use a brief wash in 0.6 M KCl to obtain isolated heavy SR vesicles. This treatment helps to separate T tubules from SR (26), but under prolonged use it results in the disappearance of feet (13). It was shown that the degradation of bridging structures is progressive (8), and by using a brief KCl treatment we preserved feet on our heavy SR vesicles, even though some were lost. We also obtained heavy SR vesicles from guinea pig muscle without using any KCl treatment, and in this preparation the feet had the same shape as in KCl washed vesicles, but were more numerous and more frequently maintained the original alignment in long rows. We conclude that KCl treatment detaches feet from the SR in toto, but does not affect the shape of those remaining attached, at least at the level of resolution of this work. We refer to vesicles from various vertebrate species to describe the structure of individual feet, but will use principally vesicles from guinea pig muscles to comment on disposition.

General Description and Structure of Individual Feet

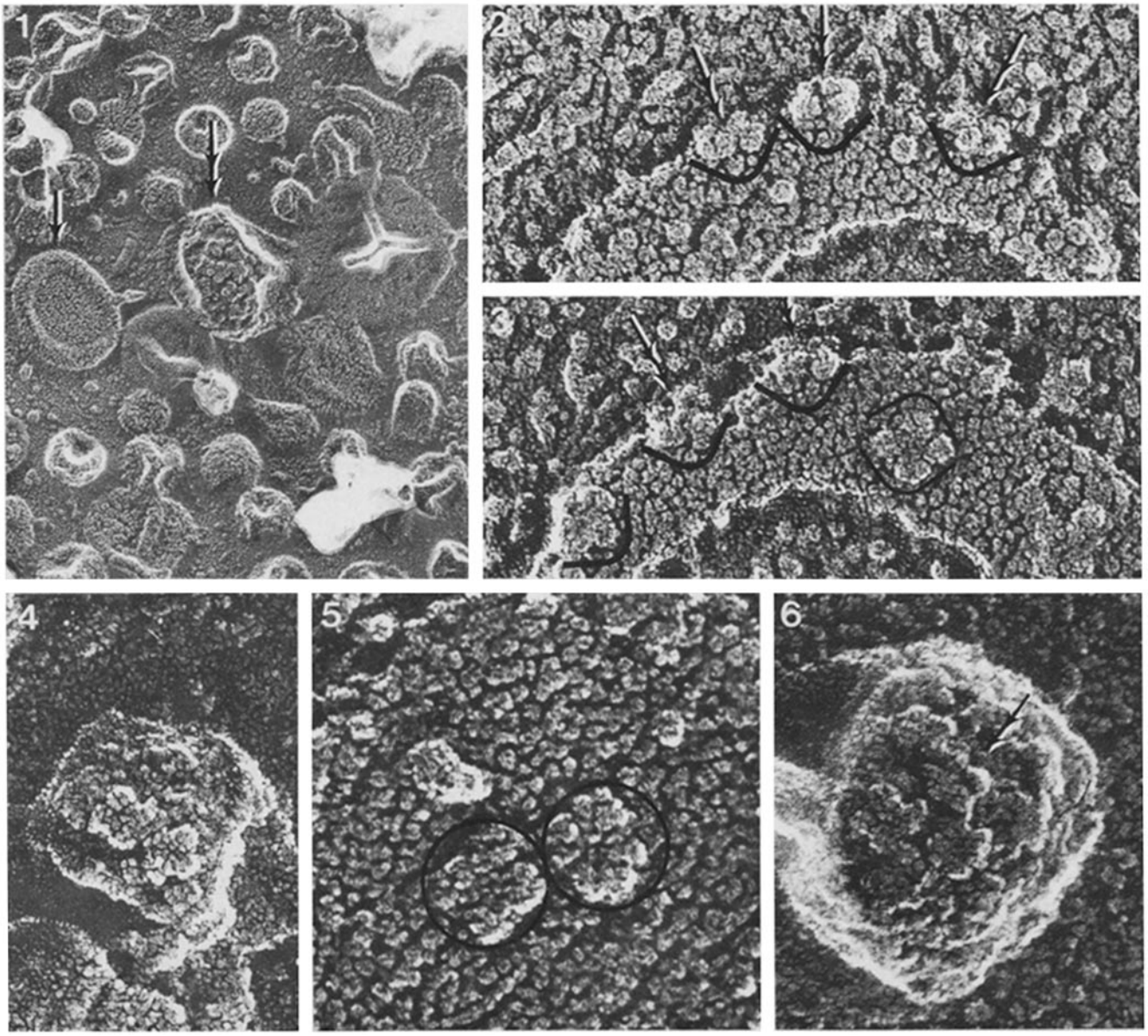
Uranyl acetate makes the vesicles somewhat rigid, so they are prominently raised above the mica surface (Fig. 1). The great majority of vesicles in the crude fraction have a surface dotted with the shadowed "tails" of the Ca-ATPase projecting into the cytoplasm (27). This fine decoration is well visible in vesicles with a flat surface (Fig. 1, arrow). A few vesicles have larger structures, located on an elevated platform (Fig. 1, double arrow). These structures are identified as feet on the basis of their shape and dimensions: namely, the quadrilateral shape and the center-to-center distance agree well with those of feet in thin sections (see below). In addition, the same structures are present in the enriched heavy SR fraction (kindly donated by Dr. D. Pierce), but not in the several enriched light SR fractions that we have examined in our studies of Ca-ATPase distribution. The elevated platform thus represents the junctional portion of the SR membrane.

Single feet or small groups of them are convenient for studying structural details. Figs. 2–6 present views of feet from frog, rat, and guinea pig, seen mostly from the top but occasionally also from the side (Figs. 2 and 3, double arows) as the membrane bends away from the observer. The feet have a tetragonal symmetry and in outline resemble four-leaf clovers (quatrefoils), being composed of four, apparently spherical subunits closely apposed to each other and surrounding a central area. Notice that the feet in Fig. 6 were not exposed to high KCl; the others were. Side views of the feet, seen at the edges of some vesicles (double arrow in Fig. 2 and 3) show that the subunits are raised ~10 nm above the surface of the membrane. In feet that are inclined slightly off the perpendicular (Fig. 6, double arrow), the four subunits are visibly rounded and a depression is visible at the center of the group.

Stereo micrographs (Figs. 7–9) confirm that the feet are elevated above the surface of the junctional SR membrane, and distinctly show the four subunits surrounding a central depression. As a first approximation, the feet may be considered to be composed of four equal, tangentially disposed, spheres. Some feet are less prominent than others (Figs. 7 and 9), even though the quatrefoil shape is still quite clear. We are not able to determine if variability in height is due to loss of some components, to collapse during drying, or to unevenness of the underlying membrane.

Disposition

Dispositions of feet most closely resembling those expected from in vivo studies were found on vesicles from the guinea pig muscle, which had not been exposed to KCl (Figs. 10–12). In these, either double (Figs. 10 and 12) or multiple rows (Fig. 11) of evenly spaced feet cover the junctional surface. To make the pattern more understandable, we have marked the position of feet centers with a dot in Fig. 12. Figs. 10 and 11 are left unmarked for comparison. The feet centers have a tetragonal disposition, slightly skewed owing to curvature of

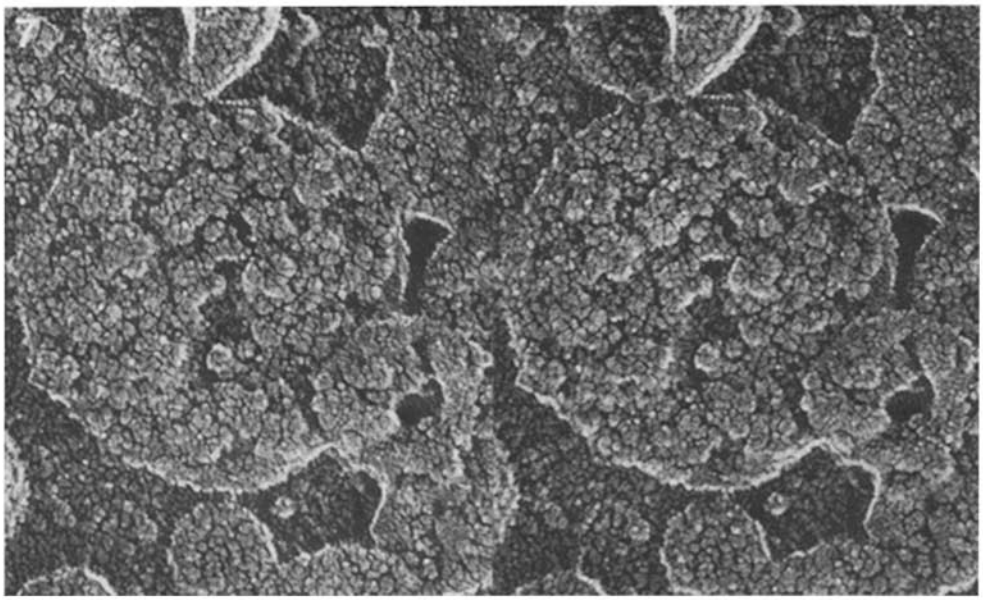


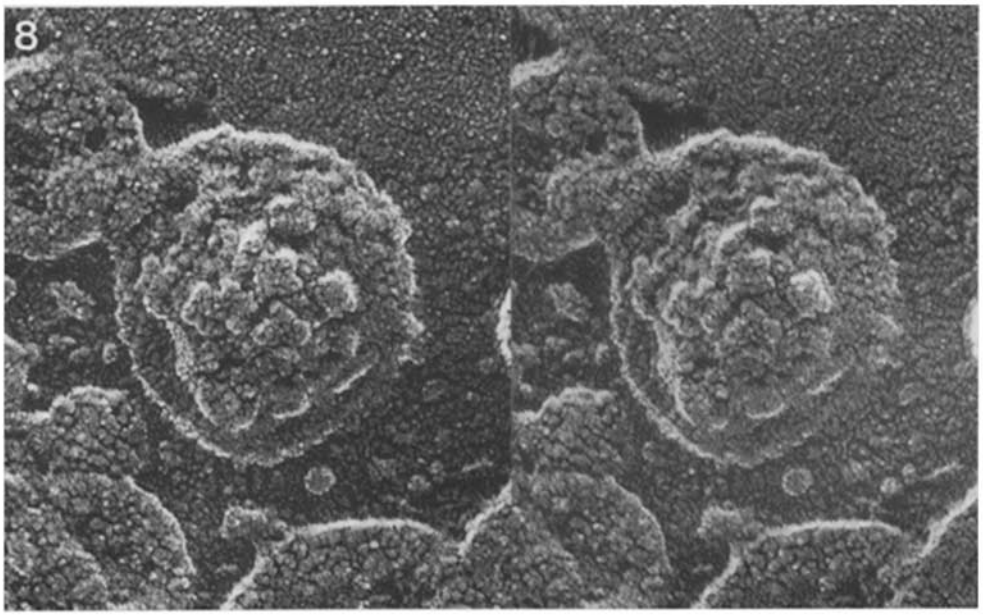
FIGURES 1–6 Fig. 1: Several vesicles from rat muscle are deposited over the mica surface, which forms the background. The vesicle indicated by an arrow is derived from the free SR and its surface is uniformly covered by very small dots, representing shadowed CaATPase "tails." The vesicle indicated by the double arrow is derived from the lateral sacs of the triad, and it is covered by several junctional feet. × 100,000. Figs. 2 and 3: Two vesicles from a frog muscle, on which only individual feet have remained. Feet seen from above have a quatrefoil configuration, with four subunits (Fig. 3, double brackets). Feet located at the edges of the vesicle are seen at varying degrees of tilt (double arrows). It is clear that the feet are raised above the vesicle surface and that subunits are approximately spherical. × 343,000. Figs. 4–6: Feet from rat, frog, and guinea pig muscles, showing particularly well-delineated shapes. In Figs. 4 and 5, two adjacent feet are so close that their subunits almost touch. The central depression is variable but is most obvious in Figs. 3 and 5, as well as in feet seen at a tilt (double arrows, Fig. 6). (Fig. 4) × 343,000; (Fig. 5) 490,000; (Fig. 6) 396,000.

the underlying membrane. Along and across the rows adjacent feet abut corner to corner, but with a slight displacement, so that the line joining centers of contiguous feet along the rows forms a small angle with the diagonals of individual feet (see lines in Fig. 12).

Arrays of feet on the surface of most vesicles have a less orderly disposition, due to several factors: (*a*) distortion of the shape of the underlying junctional SR membrane as a result of fractionation, (*b*) absent feet and/or subunits, (*c*) some shift in foot orientation, and (*d*) curvature of the membrane, which results in uneven shadowing. Absence and/or slight rotation of feet are obviously more prominent in vesicles washed with high KCl, where feet are often missing and perhaps rotated. Nonetheless, even in these vesicles, segments of the original long rows of feet can be observed. In Fig. 7, for example, two rows at right angles to each other, 4 ft each, are clearly visible along the directions indicated by arrows. During fractionation the shape of lateral sacs of the triad changes from cylindrical to spherical. We presume that where we find extensive areas covered by feet forming multiple rows, a rearrangement during isolation procedures has occurred.

Even in areas with a less regular overall arrangement of

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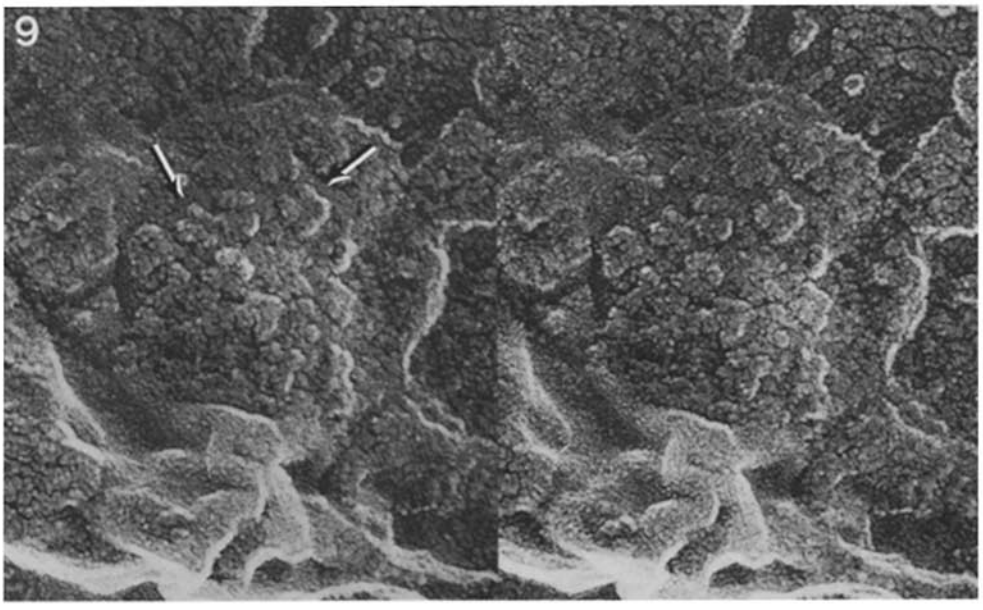
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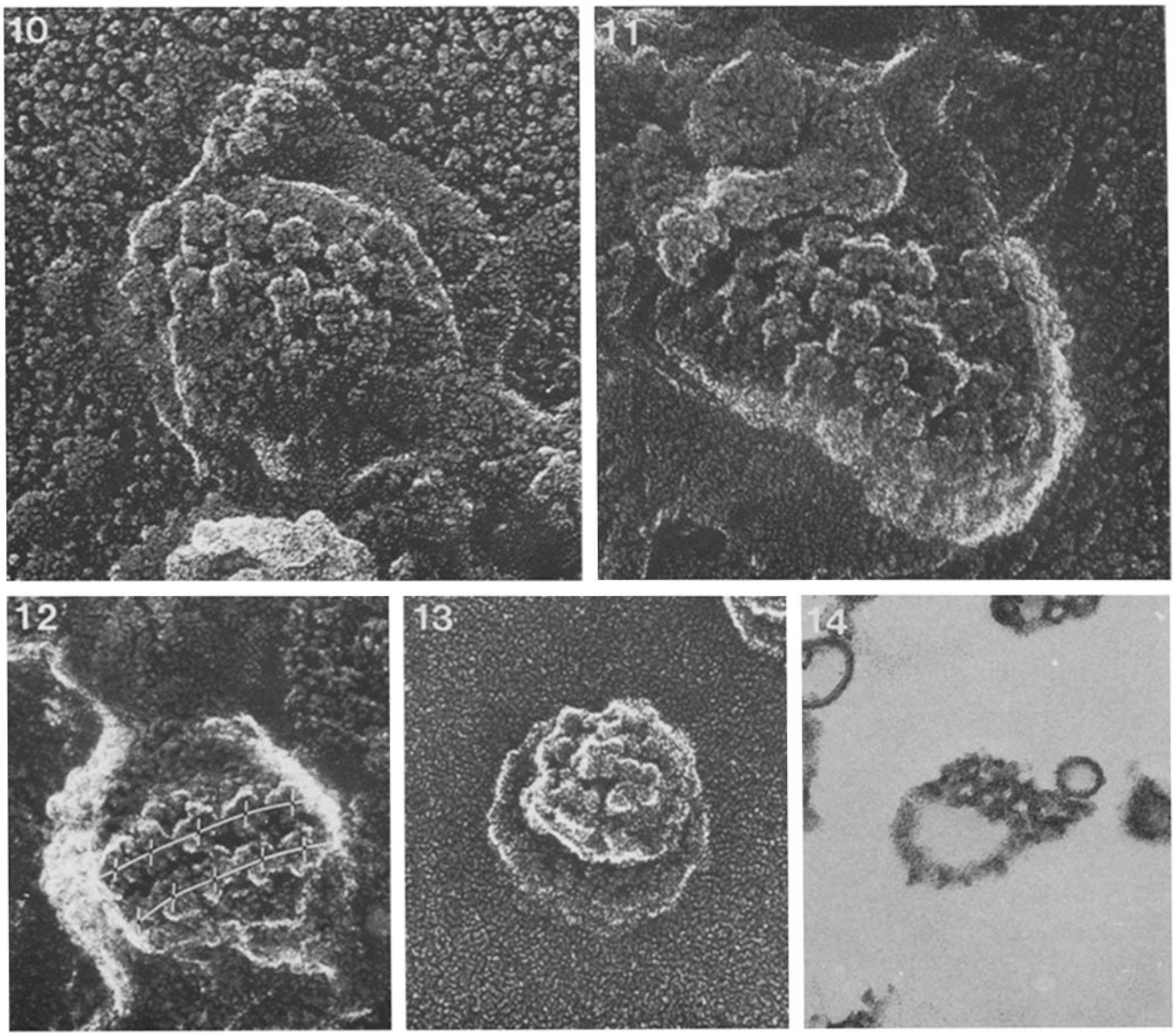


FIGURES 10–14 Figs. 10–12: Vesicles from guinea pig muscle. Well-preserved double rows of feet were relatively frequent in this preparation, which was not exposed to KCl. In Fig. 11 an incomplete third row is present. Fig. 12 is marked to show the dispositions of feet centers (dots) and of their diagonals (short lines) relative to the axes of the two rows (long lines). The predominant apposition of consecutive feet is corner to corner, but with a slight shift (cf. Fig. 19, see text). × 297,000. Figs. 13 and 14: Examples of feet in vesicles from an enriched heavy fraction, in freeze-dried and thin-sectioned view. Each diamond-like shape in thin section corresponds to a group of four subunits in shadowed images. (Fig. 13) × 264,000. (Fig. 14) × 150,000.

feet, the most frequent disposition is one in which feet that are immediately adjacent to each other are apposed diagonally, but with touching subunits offset by a half subunit (Figs. 4, 5, and 13); see also model (see Fig. 19). Two adjacent feet in Fig. 5 are outlined to emphasize this disposition.

A thin section of vesicles from one of the fractions used in this study is shown in Fig. 14. The diamond shape of the feet and their tetragonal disposition are the same as in the intact muscle (compare with Figs. 17 and 18).

Measurements

Squares circumscribed over the four subunits forming individual feet have an average width of 25.4 ± 2.1 nm (mean ± SD; $n = 34$). The average subunit diameter is half that, or 12.7 nm. Notice that the diameter of a subunit is slightly larger than its height above the SR surface, which is ~10 nm. The average center-to-center distance between feet near each other, i.e., with the subunits almost touching, is 32.5 ± 3.7

FIGURES 7–9 Stereo micrographs of vesicles from rat muscles. Fig. 8: The feet are located over a raised platform. Figs. 7 and 9: Extensive areas of membrane are covered by feet, even though the arrays are not complete. Arrows in Fig. 9 point to two rows of four feet each. × 255,000.

nm (n = 50). The diagonal of the foot, calculated from the average width, is 35.3 nm. The fact that this is slightly larger than the measured center-to-center spacing is a further confirmation that the feet do not abut exactly corner to corner, but are slightly shifted (compare with Fig. 19).

Comparisons with Thin Sections

The three images from thin sections that we use for comparison with shadowed feet are from previously published data (6, 7). See those for details. Thin sections in which the plane of section is parallel to the junctional SR and T membranes provide a view of the feet in an orientation equivalent to that seen in the replicas (cf. Figs. 10–12 and Figs. 16 and 17). Sectioned and shadowed views have the following similarities: (*a*) feet can be inscribed in a square; (*b*) the central depression between the four subunits of freeze-dried feet corresponds to the less-dense center of feet seen in very thin grazing sections (Fig. 16); (*c*) in both views feet form rows in a diagonal orientation; and (*d*) the center-to-center distance between feet in thin sections has been measured to be 28–35 nm, depending on preparative procedures (7, 8), whereas the average distance between freeze-dried feet is 32.5 nm.

Once the four subunit structure of the feet is demonstrated, some evidence for it can be found in thin sections. The circled foot in Fig. 16, for example, shows four slightly denser spots tetragonally disposed around a less-dense central region. Furthermore, two of the appearances of the feet in longitudinal sections can be directly related to the subunit structure, as indicated in the composite illustration of Fig. 15. At the right in Fig. 15 a foot has the form of a "pillar," i.e., it has a less-dense central core flanked by two dense lines (arrowheads) joining the two apposed membranes. Such an image could arise from a foot that is viewed in the direction of the two arrowheads in the inset. In this view two subunits on each side of the foot are superimposed in the line of view, forming the dense sides of the pillar, and the central core is visible. The foot at the left in Fig. 15 (three arrows) forms a bar, parallel to the SR and T tubule membrane, with a denser central region. This could arise from a foot that is viewed at right angles to one diagonal, i.e., along the direction of the three arrows in the inset. In this case two subunits would be superimposed in the center of the foot and one would be on either side. The width of the foot would correspond to its diagonal length, and the central core would not be visible.

A Model

Figs. 18 and 19 illustrate two possible dispositions of quatrefoil feet, formed by four spherical subunits and diagonally aligned to form double rows, as seen in a view equivalent to that obtained in a grazing section of the junctional gap. In one case the feet abut corner to corner (Fig. 19) in the other they are displaced one half subunit from that position (Fig. 18) mimicking the usual disposition observed in the replicas. Both arrays are tetragonal, but there are two major differences: (*a*) In Fig. 19, the center-to-center distance is the same as the diagonal length of individual feet; in Fig. 18 it is less. (*b*) Both images have spaces between the two rows of feet with a period equal that of the feet. In Fig. 19, the spaces have the same size as the feet, in Fig. 18 they are decidedly smaller. It is clear by comparison with Figs. 10–12 and 17 that of these two models, the one at left is consistent with electron micrographs, whereas the image at right is not. Thus the disposition where the feet are slightly rotated is more appropriate.

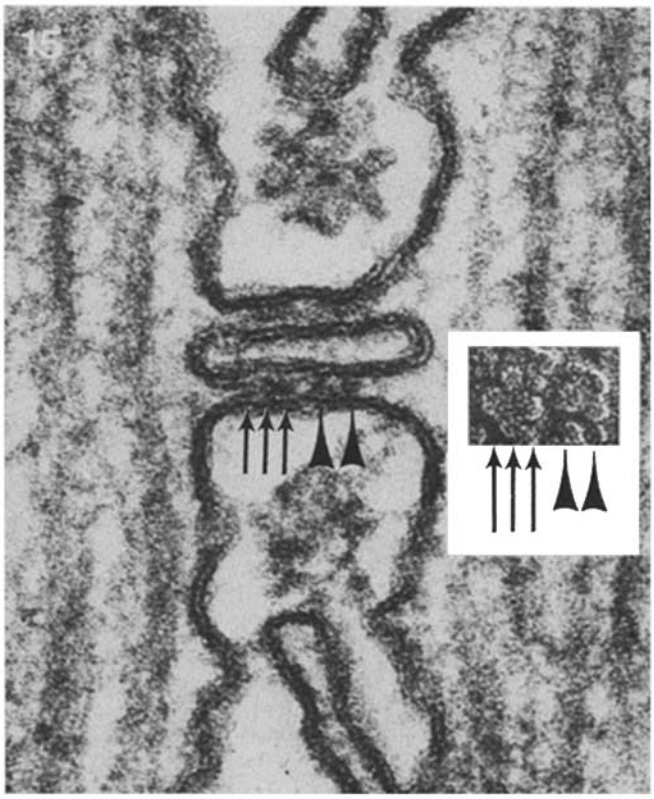
In thin sections feet often have the shape of diamonds (Fig. 17). The dotted line over some of the feet in Fig. 18 shows how this appearance may arise if some of the periphery of each foot is ignored. In thin sections, the edges of the four subunits forming the feet may not be visible, since they have much less mass than the rest of the foot. Thus the model of Fig. 18 is entirely consistent with known structure of junctional feet.

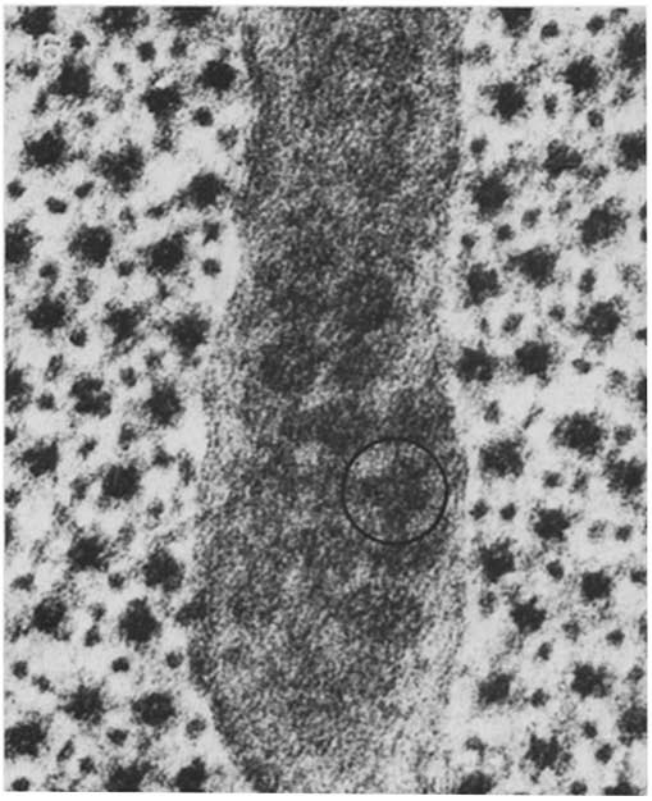
DISCUSSION

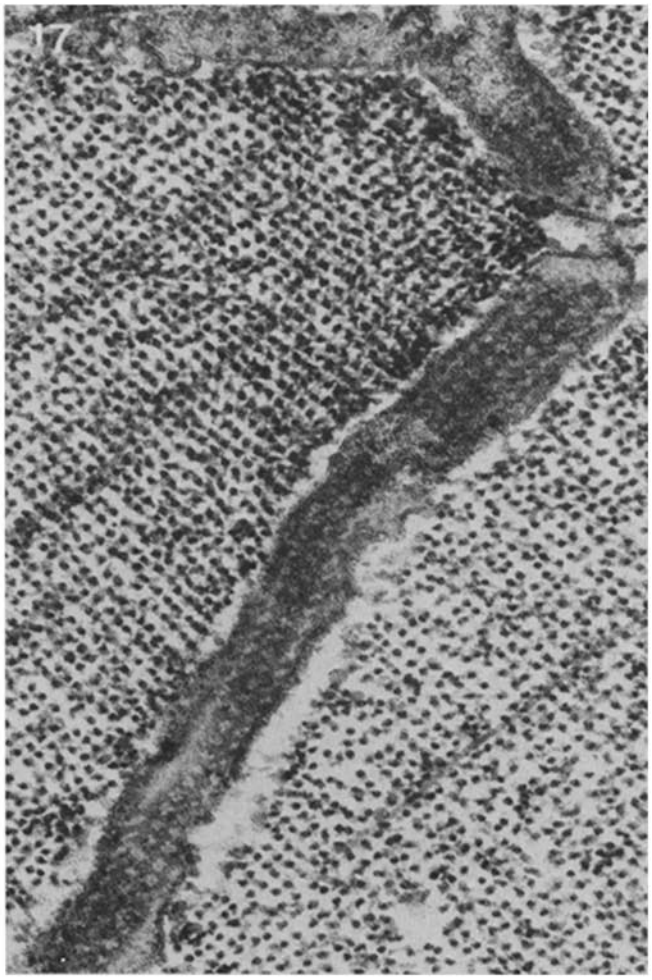
The technique used in this investigation has several advantages: (*a*) Suspended vesicles are adhered to the mica by gently depositing a droplet, rather than spraying as is done for macromolecules (16, 18). This is more appropriate for larger structures that might be sheared in spraying. (*b*) Once adhered to the mica the vesicles can be washed repeatedly with any desired solution. Thus solution changes, such as in a fixation step, can be done quickly without need for pelleting. (*c*) Biological material adhering to the replica can be cleaned after the replica is lifted from the mica surface. This step is important in the case of relatively large vesicles filled with a content, such as those used here, which would reduce image quality and contrast if they were not removed from the replica. Preservation of the vesicle shape without collapse is clearly seen in three-dimensional images, and both small surface details such as tails of the Ca-ATPase (27) and larger structures such as the feet are clearly imaged.

The disposition and four-subunit structure of feet demonstrated in this study are consistent with many previously observed details of the triadic junction. The tetragonal arrangement of feet, the disposition in rows, the center-to-center spacing, the less-dense central core, and the various appearances of individual feet in longitudinal and transverse sections

FIGURES 15–19 Fig. 15: Comparison between feet from fish muscle (7) in a thin section across the triad and in a replica from rat muscle (*inset*). Arrowheads in the inset point to a viewing direction along which the two subunits of the feet are superimposed. Feet thin-sectioned in this plane would appear as two dense lines, separated by a less dense core, or a pillar, as indicated by arrowheads in the section. Three arrows in the inset indicate a viewing direction along which two central subunits are superimposed, flanked by single subunits. This would lead to a dense bar with slightly less dense edges when seen in thin section (three arrows in Fig. 15). × 250,000. Figs. 16 and 17: Grazing thin sections of the junctional gap (fish muscles). The feet appear as long rows of diamond-shaped densities, disposed tetragonally and touching corner to corner. Subunit structure can be seen indistinctly in the circled foot in Fig. 16. From references 7 and 6, respectively. (Fig. 16) × 214,000. (Fig. 17) × 125,000. Figs. 18 and 19: Diagrams of possible dispositions of feet within rows. In Fig. 18 adjacent feet are rotated slightly and do not touch directly corner to corner. In this configuration the center-to-center separation is shorter than the diagonal of the foot, and the spaces between feet in adjacent rows are smaller than the individual feet. This is more consistent with thin-section images and shadowed images than the model in Fig. 19 where the feet touch directly corner to corner and the spaces between feet are as large as the feet themselves. Diamonds traced over feet (Fig. 18) may be compared with feet in Fig. 17 (see text for explanation).







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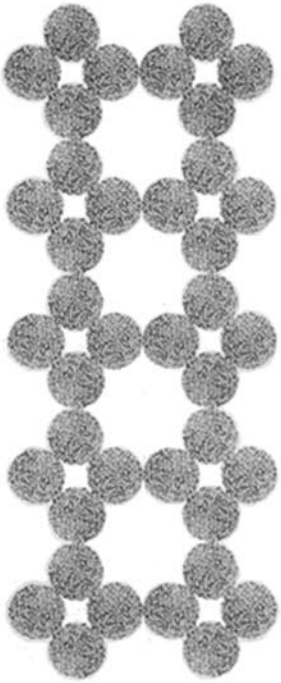

can all be accounted for if feet are composed of four equal subunits and are arrayed as diagrammed in Fig. 18. The images obtained by negative staining of isolated triads (8) can also be accounted for in terms of the shadowed structure of the feet. The good correlation of our images with the in vivo structure and with other views of isolated SR shows that during the isolation procedures we have preserved all major components of the feet and that the quatrefoil shape is a reasonable approximation to their structure. A four subunit structure had actually been predicted following the observation that groups of four intramembraneous particles mark the anchorage sites of feet into the junctional T tubule membrane (6). We also extend previous observations on the effect of high KCl (8, 13, 26) by showing that the treatment tends to extract entire feet.

There is one discrepancy between thin-section and shadowed images. When viewed in certain orientations, feet in thin sections have an apparent diameter of 15.0–18.0 nm (1). The side of the square circumscribing shadowed feet on the surface of the SR vesicles is considerably larger (~25 nm). The shadow used in these preparations was moderately heavy and was likely 1.5-nm thick. This would reduce the length of the side to ~22 nm. It is well known that, due to a relative lack of density, the edges of spherical and cylindrical structures are not seen in thin-section images. The major portion of the remaining discrepancy may well be attributable to this phenomenon. Finally, it is not unlikely that a minor flattening and spreading out of the feet occurs upon detatchment from the T tubules even though the center-to-center distances remain unchanged.

The composition of the junctional feet has not yet been determined with certainty. Likely candidates for structural proteins are the 300,000- and 325,000-dalton proteins, which mediate attachment of T tubule and SR vesicles (28, 29). Other possibilities are the 34,000- and 38,000-dalton proteins which are eluted from suspensions of heavy SR vesicles after incubation with 0.6 M KCl, concurrent with a loss of feet (13). One of the two latter proteins may be a co-factor in the attachment of the "spanning proteins" to the two membranes (30). It is interesting to compare the size of the feet subunits, as shown here, with the size of these proteins. Feet subunits are slightly less tall than wide, but as a first approximation, each subunit may be considered a sphere. Shadowed subunits have a diameter of 12.7 nm and from this we calculate a volume of 1,072.5 nm^3. Using an average specific volume for globular proteins of 0.812 cm^3/g (31), we calculate that the 300,000- and 325,000-dalton proteins in combination would occupy a total volume of 844 nm^3. The agreement between the two volumes is close, considering the numerous uncertainties; e.g., the exact shape of the subunit, thickness of the platinum shadow, and hydration of the component proteins. It can be concluded that each foot subunit may contain a doublet of the two larger proteins. The smaller proteins found in the junctional SR may either be additional components of the feet, or, as proposed (30), may mediate their attachment to the membrane.

In addition to the feet, thin bridging structures have been recently described in the junctions (2, 32). The relationship of these to feet is not yet established, even though they have a periodic arrangement (2). Bridging structures are smaller than pillars and feet. Their dimensions are such that they could be accommodated within one of the four subunits forming each foot.

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