PARTICLE ARRAYS IN EARTHWORM POSTJUNCTIONAL MEMBRANES

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ABSTRACT

Analysis of freeze-fractured earthworm body wall muscle reveals distinctive trough-shaped concavities in the protoplasmic leaflet of the muscle cell membrane which contain diagonally oriented rows of particles sometimes in highly ordered arrays. The troughs correspond to the concave postjunctional patches of sarcolemma seen previously in thin sections of myoneural junctions identified as cholinergic, and the intramembranous particles within the troughs correspond in concentration and arrangement to granular elements present in the outer dense lamina of the postjunctional membrane which were interpreted as acetylcholine receptors. The freeze-fracture data provide a more accurate picture of the arrangement of these putative receptors within the plane of the membrane, and indicate also that they extend into the membrane at least as far as its hydrophobic layer.

KEY WORDS   myoneural junction · receptor · muscle · membrane · cholinergic

A previous study of earthworm body muscle cells has demonstrated a distinctive structural specialization of the plasma membrane at myoneural junctions consisting of concave patches in which the outermost portion of the membrane exhibits a granular substructure from which regularly spaced projections extend into the junctional cleft (16). On the basis of their location, size, and concentration, these granular elements were identified as putative transmitter receptors. This impression was supported by subsequent studies in which comparable granular elements were found in the outer dense lamina of a variety of other postjunctional membranes (17-19, 21), in particular at vertebrate skeletal myoneural junctions (19) where they occur in just the distribution of the acetylcholine receptors as determined by other methods (1, 4, 6, 8, 10).

In the specialized regions of the earthworm postjunctional membrane, the granules in the outer leaflet and the projections arising from them appear to be very regularly disposed and to exhibit a paracrystalline array in some tangential sections, but the thin sections reveal no corresponding structure either in the hydrophobic portion of the membrane or in the dense material that adheres to the cytoplasmic surface of the membrane at these specialized patches. To determine whether the substructure visible in the outer portion of the membrane is reflected deeper in the membrane, and to better visualize the arrangement of the postjunctional elements within the plane of the plasma membrane, a freeze-fracture study of earthworm muscle cells was carried out. Among the various intramembranous particles seen in the

fracture faces, one type, described in this paper, forms arrays corresponding to the postjunctional membrane specialization visible in thin sections. A preliminary report of this study has been published (20).

MATERIALS AND METHODS

Earthworms, either freshly collected or maintained in the laboratory at ~5°C, were fixed by injecting them with a mixture of 3% glutaraldehyde plus 2-3% paraformaldehyde (freshly made up) in 0.1 M phosphate buffer. Worms were then opened and eviscerated and the body wall stored in fixative. In some instances worms were cut open along the middorsal line, stretched, pinned out, and eviscerated, and the ventral nerve cord removed. They were then flooded with fixative and allowed to become stiff before being transferred to the storage vials. Specimens from either group were rinsed in Ringer's solution, trimmed into short strips, and glycerinated in stages from 5 to 30% glycerol in Ringer's solution. The specimens were then cut and mounted such that the fracture plane would pass either transversely or longitudinally across the body wall. These specimens were then frozen in Freon 22 cooled with liquid nitrogen and fractured in a Balzers freeze-fracture apparatus (Balzers AG, Balzars, Liechtenstein). Replicas were cleaned by digestion in Clorox and mounted on carbon-coated 150-mesh grids for examination. Thin sections of earthworm body wall were prepared from tissues fixed as described previously (16).

RESULTS

The longitudinal musculature of the earthworm body wall consists of pennate bundles of ribbon-shaped cells, each of which is in contact with a connective tissue septum along one edge (Fig. 1). Where the cells adjoin the connective tissue they exhibit numerous hemidesmosomes and where they are apposed to each other, typical desmosomes can be seen occasionally. At both of these sites the plasma membrane tends to be convex outwards and has conspicuous densities applied to both cytoplasmic and external surfaces. The cells also exhibit large numbers of dyads along their surface, but these, unlike the hemidesmosomes, are confined to the myofilament-containing portions of the cell and do not occur in the thin, veil-like extensions that arise from their edges. At dyads, too, the plasma membrane is convex outwards.

In addition to these surface features, the cells also form myoneural junctions with nerve bundles traveling through the connective tissue septa. Because the earthworm muscle cells are relatively short compared with vertebrate skeletal muscle fibers, and are individually innervated, junctions are readily found by random scanning. Typically, a nerve bundle in a connective tissue septum is surrounded by a number of muscle fibers in the immediate vicinity, as well as by attenuated processes from other fibers a short distance away. Thus, in one localized region, axons form multiple junctions on the same and different muscle fibers and their processes. Such junctions are of two clearly distinguishable morphological types presumed to represent cholinergic and adrenergic innervations (16). Junctions of the latter type, which occur very infrequently, are associated with virtually no morphological specialization of the sarcolemma. In contrast, the junctions identified as cholinergic, though much less common than dyads, hemidesmosomes, and desmosomes, are not difficult to find because of the very conspicuous and distinctive specialization of the postjunctional muscle cell membrane (Fig. 2). These junctions occur on both the myofilament-containing portion of the muscle cells and on their veil-like extensions. In either location they are characterized by axons containing predominantly clear vesicles and by a wide junctional gap (~900 Å). The postjunctional membrane is distinguished by dense concave patches measuring ~0.15 μm across, and containing in the outer dense lamina of the plasma membrane regularly arrayed granules which give rise to projections that extend into the junctional cleft (Fig. 2). This junctional membrane is the only specialized region of the muscle cell surface that is concave.

Freeze-Fracture Images

It is assumed that in the earthworm body wall, as elsewhere, the fracture plane tends to follow the hydrophobic layer of membranes, splitting them into inner and outer leaflets (2). In addition, the fracture plane passes frequently enough from plasma membrane through myofilament-containing portions of muscle cytoplasm (Fig. 3) to permit muscle cell membrane fracture faces to be identified positively. Presumably, as a result of etching, the myofilaments protrude above the level of the background cytoplasm, and in some instances, the band pattern characteristic of obliquely striated muscle stands out clearly.

The protoplasmic leaflet of the plasma membrane undulates considerably and exhibits many convexities whose size and frequency suggest that
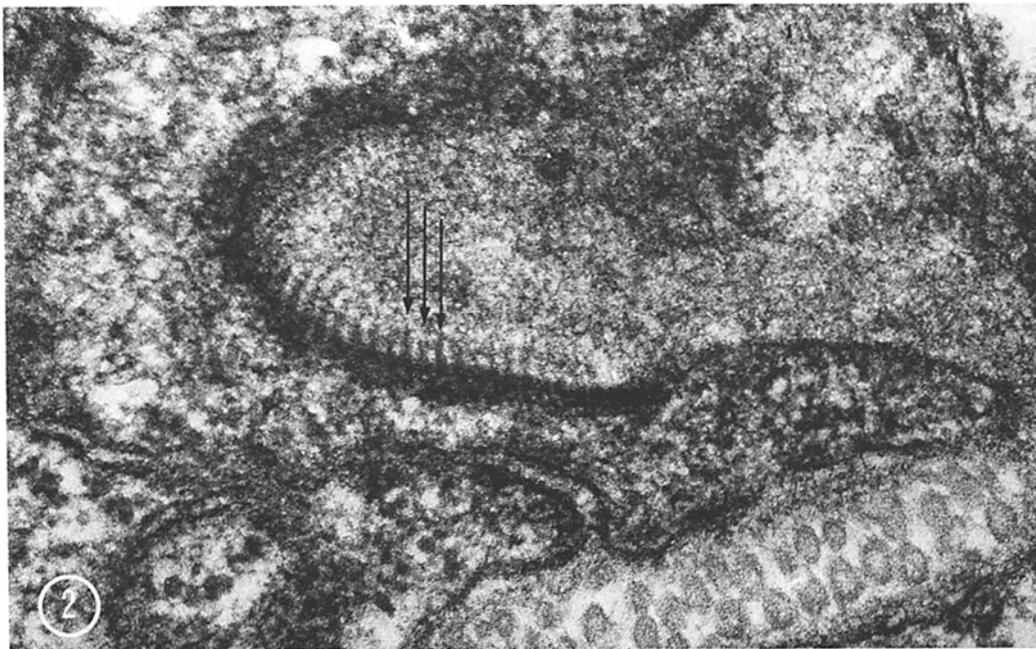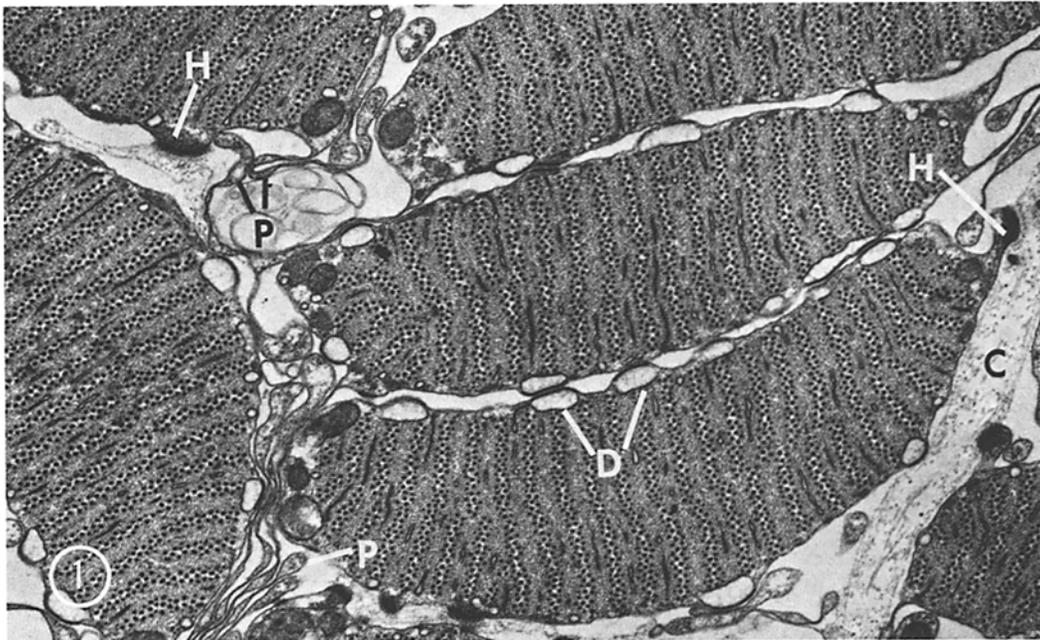
FIGURE 1 Transverse section through earthworm body musculature. Cells contain thin and thick myofilaments in the typical repeating pattern. At the edges of the cells numerous dyads (D) form convexities in the sarcolemma. Hemidesmosomes (H) are also convex outwards. The cells give rise to thin overlapping veil-like processes (P). Myoneural junctions tend to occur where the muscle cells adjoin connective tissue (C). × 20,000.

FIGURE 2 Detail of thin-sectioned muscle showing postjunctional membrane. The sarcolemma is concave and gives rise to conspicuous projections (arrows) from its outer surface. A dense material is applied to its cytoplasmic surface. × 135,000.
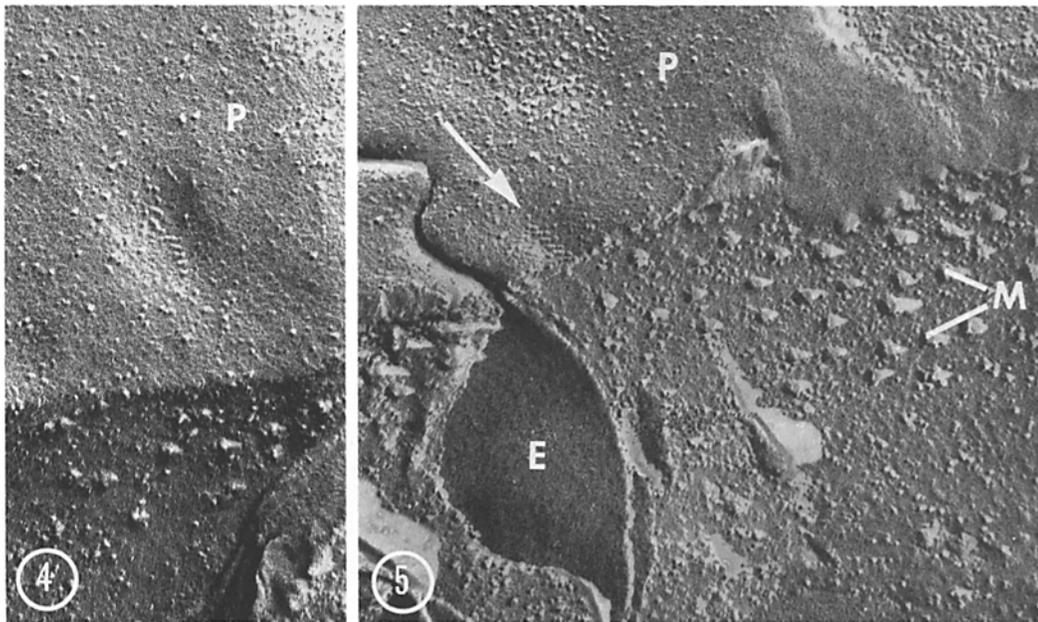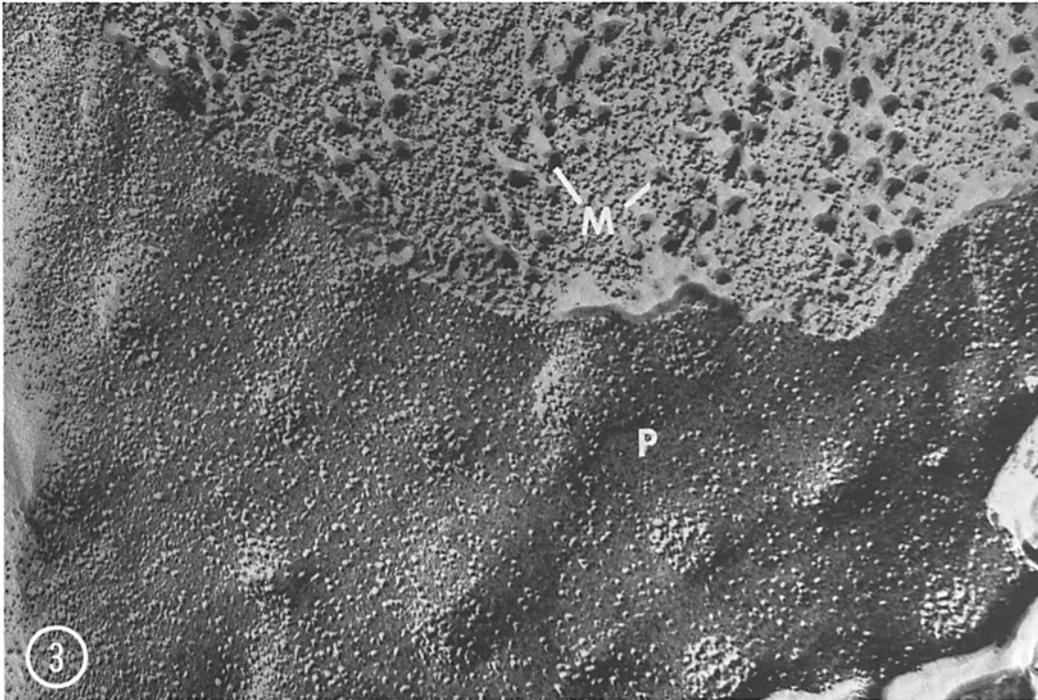
FIGURE 3   Freeze-fracture replica of earthworm muscle cell. At the top the fracture plane passes through sarcoplasm revealing bands of thick myofilaments (M). It then enters the plane of the plasma membrane revealing the P fracture face which exhibits randomly scattered particles. Particles tend to be concentrated over some of the large convexities. × 65,000.

FIGURE 4   In the P fracture face in the upper half of the picture there is a concavity containing several elongated particle aggregates in a stack. × 70,000.

FIGURE 5   The fracture plane passes through sarcoplasm and sarcolemma of adjacent muscle cells. Thick myofilaments (M) protrude above the background sarcoplasm. A shallow concavity (arrow) in the P fracture face reveals several diagonally oriented rows of particles. E, external leaflet of sarcolemma. × 65,000.

they represent a mixture of dyads, hemidesmosomes, and desmosomes, all of which are convex outwards and of about the same dimensions. Most are probably dyads which far outnumber hemidesmosomes and desmosomes in thin sections. Like the protoplasmic leaflet of most plasma membranes, that of the earthworm muscle cell contains many large intramembranous particles. These tend to be larger and somewhat more concentrated over the convexities, but otherwise appear to be randomly scattered over the surface (Fig. 3).

The most distinctive feature of this fracture face consists of shallow trough-like concavities which occur very infrequently in comparison with the convexities. These are sometimes seen individually (Figs. 4 and 5), but more often occur in clusters either on the same cell (Fig. 9) or on contiguous cells (Fig. 6). Such troughs have been identified over myofilament-containing portions of the muscle cell as well as over attenuated processes. They measure ~0.03–0.15 μm across and vary in length from 0.15 μm to more than a micron (Fig. 14). The long axis of the trough frequently shifts producing a sinuous shape (Fig. 9). These troughs are unique in that they contain conspicuous, large particles aligned in rows which are oriented diagonally with respect to the long axis of the trough (Figs. 4–9, 12–14). Particles vary in size from ~70 to 150 Å, but most are closer to the high end of the range. They may appear as individual spherical globules (Fig. 8), but many appear to be elongated and to consist of two fused subunits lying side by side within a row (Figs. 13 and 14). Often multiple adjacent particles in a row appear joined together forming elongated ridges (Figs. 4 and 9). Irregularities appear frequently in the arrangement of the rows. Sometimes a row is incomplete (Fig. 13), resulting in an abrupt change in the orientation of an adjacent row. In other instances, the rows are rather loosely spaced and the angle between the rows and the axis of the trough is variable (Fig. 7). The rows are generally oriented at angles of 30°–60° (or 120°–150°) to the long axis of the trough.

Examples also occur of rows packed together forming paracrystalline two-dimensional nets. In Fig. 12, for example, particle alignments at angles of ~68° and ~118° to the primary rows can be detected, and the rows themselves form an angle of ~135° to the axis of the trough. In those regions where particles can be resolved individually, they are spaced at ~150 Å-intervals along the rows, and the rows themselves are spaced at ~170 Å-intervals in the perpendicular direction. The concentration of particles is ~4,000/μm^2 in such closely packed paracrystalline regions.

The outer leaflet of the muscle cell membrane (E fracture face) is relatively smooth and particle free, except for patches of particles located in rounded depressions (not shown) which presumably are complementary to some of the convexities seen in the P fracture face. Fig. 11 illustrates an elongated convexity in the E fracture face containing diagonally oriented grooves. This structure, which is complementary to the troughs in the P fracture face, contains very few particles along the grooves. In this respect, the earthworm postjunctional membrane resembles that of vertebrate myoneural junctions (5, 8) more than that of crustacean junctions (7) which exhibit particles either in both fracture faces or primarily in the E face.

Because of the unusually wide junctional cleft separating the pre- and postjunctional membranes in this animal, plus the small size of the junctional contacts, the fracture plane usually does not pass through both pre- and postjunctional membrane specializations in the same region, and consequently the postjunctional membrane is ordinarily

FIGURE 6 P fracture face of adjacent muscle cells. In the upper cell, three shallow troughs are visible containing diagonally oriented rows of particles. These arrays are regularly spaced at intervals of ~0.4 μm. Another one (arrow) appears at the lower left in the adjacent muscle cell. × 80,000.

FIGURE 7 Longer and deeper trough in muscle cell membrane. More than 30 particle rows can be counted in this case. × 115,000.

FIGURE 8 Wider and deeper trough in a muscle plasma membrane showing a somewhat more regular arrangement of particles than in the preceding figures. × 140,000.

FIGURE 9 Sinuous troughs in a muscle cell membrane. The particle rows are rather loosely packed and the axis of the trough shifts in a number of locations. × 70,000.
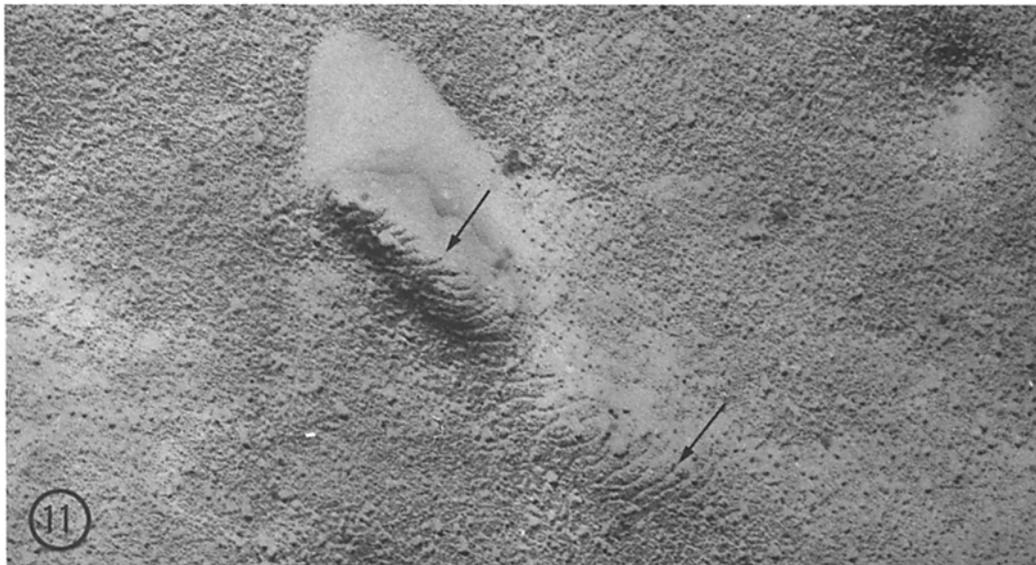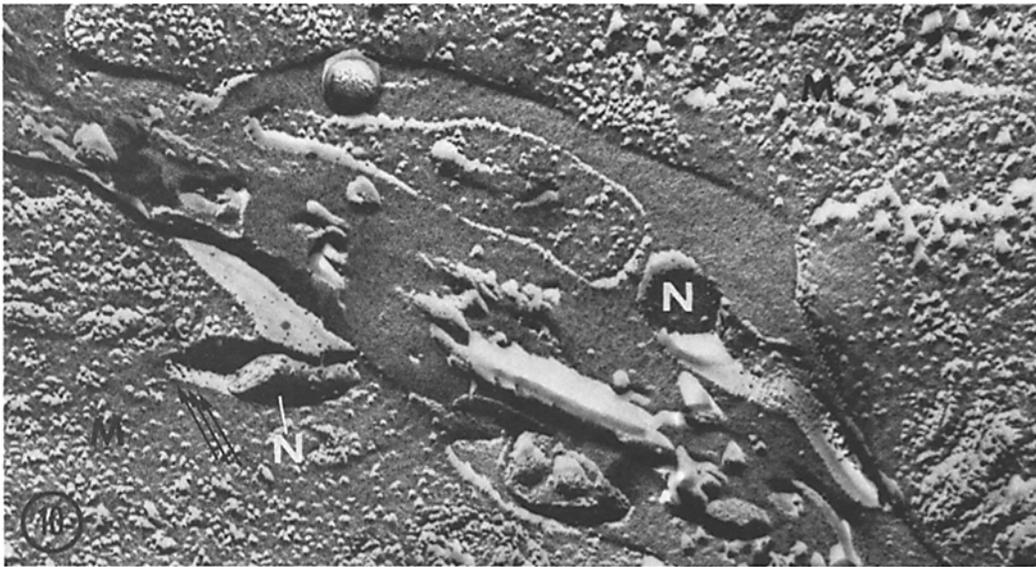
FIGURE 10 Two muscle cells (M) separated by a connective tissue septum containing nerve fibers (N). Where the nerve fiber at the left indents the muscle cell surface several diagonal rows of particles are visible in the P face of the sarcolemma (arrows). The plasma membrane (E face) of the nerve fiber at the right extends towards the membrane of the adjacent muscle fiber and exhibits an aggregate of large particles where the respective membranes are apposed. × 65,000.

FIGURE 11 E face of muscle cell membrane showing postjunctional membrane specialization complementary to that seen in Fig. 7. The specialized membrane is convex as shown by the large white shadow extending toward the top of the figure. Diagonal grooves vary in orientation and may appear bent, due to the curvature of the membrane (upper arrow), or nearly straight (lower arrow). Very few particles occur along the grooves, indicating that nearly all of the large particles in the postjunctional membrane remain attached to the P face when the membrane is cleaved. × 105,000.

seen alone. Fig. 10, however, shows a rare example in which the fracture plane passes through a trough in a muscle cell membrane revealing diag- onal rows of P face particles, and also passes through a segment of a small caliber nerve fiber lying within the trough.

DISCUSSION

The trough-shaped regions described here in freeze-fracture replicas correspond in several respects to the postjunctional membrane specialization observed previously in thin sections of earthworm muscle. In the latter, the specialized region may appear as a concave patch measuring ~0.15 μm from edge to edge, or it may be flatter and more extensive. These respective images are consistent with sections passing approximately transversely and longitudinally through the elongated troughs seen in freeze-fracture images. Most random sections through an elongated structure of this sort would tend to be oblique to its long axis rather than precisely parallel or perpendicular to it, and would thus produce profiles that are generally broader than the structure itself but not so long. This probably accounts for the apparently greater average width of the specialized postjunctional patches seen in thin sections as compared with the troughs present in the freeze-fracture replicas. In addition, because the curvature of the troughs is along their transverse axis, those sections that are nearly parallel to the long axis of the trough and are of greatest apparent extent would tend to show little or no curvature of the plasma membrane (cf. Fig. 7 in reference 16).

Because of the rather regular arrangement of the particle rows in the troughs, it is easy to see how sections perpendicular to the rows could generate thin-section images containing regularly disposed intramembranous elements. Thus, as shown in Fig. 15a, a thin section at an angle of 45° to the long axis of the trough shown in Fig. 12 would cross the primary particle rows perpendicularly. Assuming a section thickness of ~500–1,000 Å, approximately three to six elements from each row would be seen superimposed on each other to produce a substructure with a repeat period of ~170 Å, representing the spacing of the particle rows in this instance. The apparent width of the specialized patch in thin sections would be ~1.4 times the true width of the trough.

In instances where the rows are closely packed into paracrystalline nets, sections at other angles could produce periodic patterns as well. For example, as shown in Fig. 15b, if the same trough were sectioned at an angle of 113° to its long axis, the elements along the line 68° to the primary rows would superimpose to produce an apparent period of ~140 Å in the thin-sectioned membrane. Because of the sectioning angle, which is nearly transverse, the apparent width of the spe-

cialized patch of the membrane would be increased only slightly (~1.1 times). Similarly, if this trough were sectioned at an angle of 163° to its long axis (Fig. 15c), the elements along the line 118° to the primary rows would superimpose to give an apparent period of ~130 Å in the membrane, and the apparent width of the specialized patch in this nearly longitudinal section would be increased considerably over the real width of the trough (~2.9 times). Finally, a section 139° to the trough's long axis would cut perpendicularly across a line 94° to the primary rows (Fig. 15d). In this case, particles from alternate rows would superimpose to give an apparent repeat period of ~70 Å in thin sections, and the apparent width of the specialized patch would be ~1.5 times that of the trough. Sections at orientations other than these would result in incongruous superimposition of the substructural elements and no clear periodicity. The spacings derived in this way correspond approximately to the spacings of 160, 140, and 80 Å observed previously in thin sections (16). Moreover, the particle concentration of ~4,000/μm^2 in such paracrystalline regions is comparable to the estimated concentration of granules in the postjunctional patches seen in thin sections (16), the difference probably reflecting some shrinkage in the dehydrated, embedded specimens.

On the basis of the size of the troughs, their distribution, frequency of occurrence, and in particular the fact that the troughs represent the only concave membrane specialization of these dimensions found in the P face of freeze-fracture replicas, it is concluded that these troughs correspond to the postjunctional specialized regions seen in thin sections. On the basis of the arrangement and concentration of the particles within the troughs, it appears likely that these intramembranous particles correspond to the granules and projections associated with the outer dense lamina of the membrane. The freeze-fracture data thus indicate that the granular substructure of the postjunctional patches extends into the membrane at least as far as the hydrophobic middle lamina, and it is therefore concluded that these granular elements are integral components of the postjunctional membrane.

These data are consistent with the view that the postjunctional granules represent acetylcholine receptors, which have been shown to be high molecular weight, integral membrane proteins (14). As in the case of the putative acetylcholine
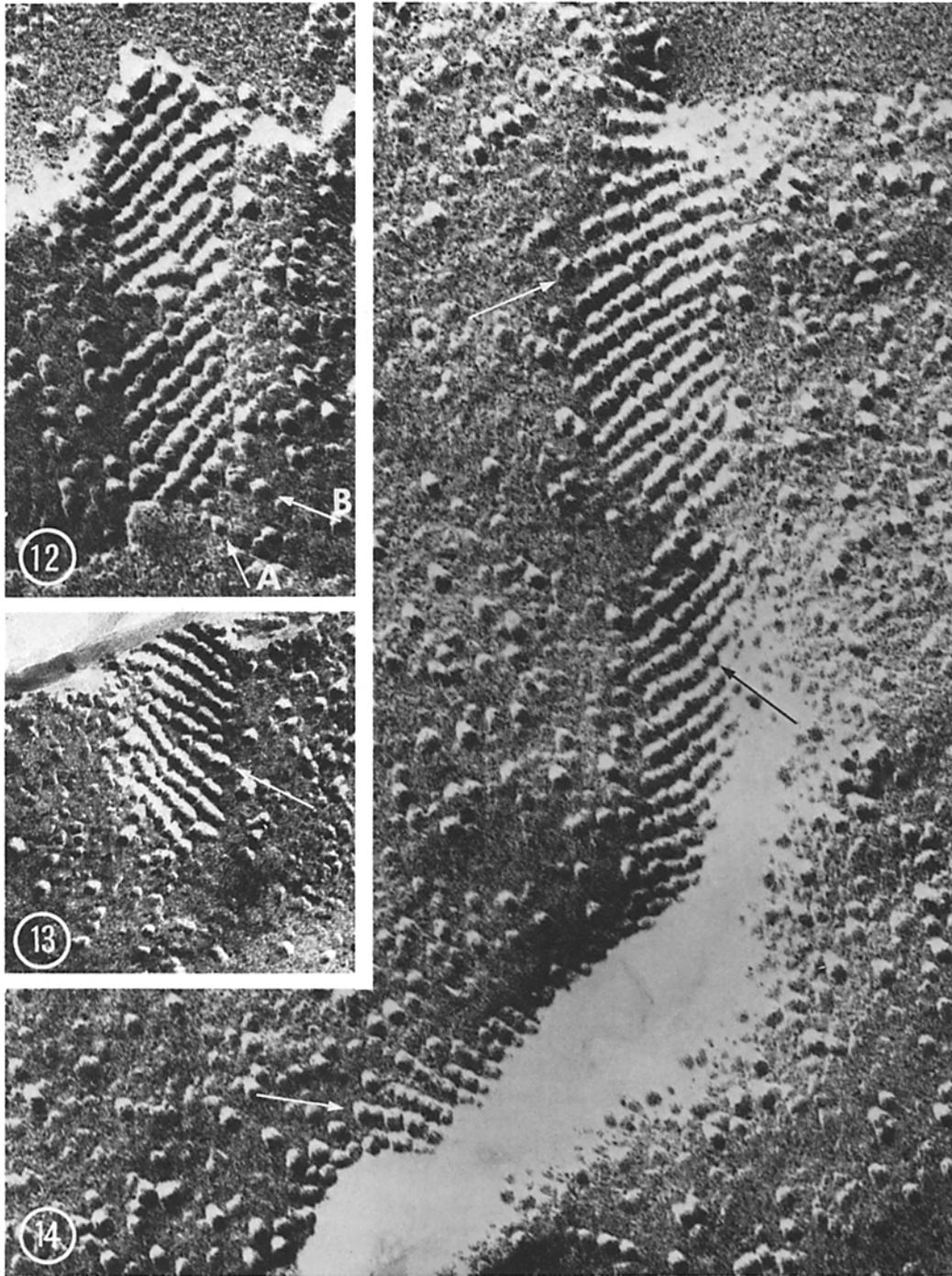
FIGURE 12 Paracrystalline array of particles in a shallow trough. The rows here are oriented at an angle of ~135° to the trough axis. Tails of particles at angles of 68° (A) and 118° (B) to the rows are visible at arrows. × 187,000.

FIGURE 13 Shallow trough in which the angle of the rows to the long axis of the trough changes abruptly due to incomplete row (arrow). × 177,000.

FIGURE 14 Elongated trough containing ~ 50 rows of particles. Within rows the particles are closely packed, but the rows themselves tend to splay out at the edges of the trough. The orientation of the rows changes along the length of the trough. In some of the rows particles appear to consist of doublets (white arrows). Particle alignment crossing rows is shown by black arrow. × 187,000.
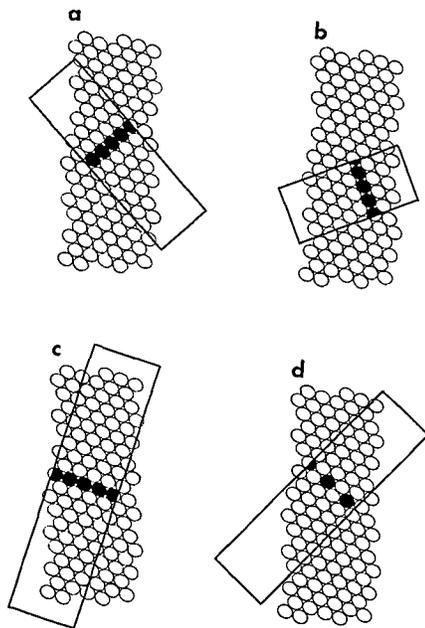
FIGURE 15 Diagram based on Fig. 12 showing *en face* view of freeze-fractured postjunctional trough, and indicating how particle alignments along different axes could give rise to periodic substructure in thin sections. The width of the rectangle shows the approximate thickness of a thin section, and the darkened particles indicate which rows of particles would be seen superimposed in the section. The angle between the plane of section and the long axis of the postjunctional trough is (*a*) 45°, (*b*) 113°, (*c*) 163°, and (*d*) 139°.

receptors of vertebrates (5, 8), the postjunctional intramembranous particles at earthworm cholinergic junctions also remain adherent to the protoplasmic leaflet of the postjunctional membrane during the cleaving process. However, to a much greater extent than their vertebrate counterparts, they associate to form rows and sometimes also form two-dimensional nets. Paracrystalline patterns are not ordinarily seen in the cholinergic postjunctional membranes of vertebrates (11), although regular arrays are occasionally visible in the E face of the postjunctional membrane in *Torpedo* electrocytes (12, 21), and rows of particles are sometimes visible in negatively stained preparations (11). Lattices have also been reported (3). "Reticular" patterns occur in tangentially cut amphibian motor endplate membranes (19), and "herringbone rows" of intramembranous particles have been described in mammalian postjunctional membranes (5, 15). Among inver-

tebrates, regular arrays of particles have been demonstrated in noncholinergic, crustacean, postjunctional membranes (7). There may be a real difference in the degree to which postjunctional elements from various sources form lattices, or merely a difference in the degree to which highly ordered arrangements survive the vicissitudes of specimen preparation. Diagonal arrays of intramembranous particles also occur at nonsynaptic locations and are therefore not peculiar to postjunctional membranes. The paranodal axoglial junction of vertebrates (9, 22, 23) is characterized by a prominent diagonal pattern in the apposed membranes, and similarly in a crustacean nerve (13), patches exhibiting such diagonal patterns also occur at the axoglial interface.

The conspicuous concavity of the postjunctional troughs in earthworm muscle, which contrasts with the shape of vertebrate postjunctional membranes, was initially attributed to tension exerted by the obliquely oriented intracellular filaments that insert into the dense cytoplasmic plaques underlying these specialized patches. However, on the basis of the freeze-fracture data, it now seems that the distinctive contour of the membrane in these regions could equally well result directly from the shape of the particles that form rows within the troughs, or from the manner in which the particles interact with each other. Thus, in a simple case, if these elements extended through the entire width of the membrane and were wedge-shaped, i.e. narrower at the level of the outer half of the plasma membrane than at the level of the inner half, then the particles would tend to form curved rows and the patch as a whole would be concave.

SUMMARY

(*a*). Elongated concavities can be seen in the P fracture face of earthworm muscle cell membranes. These contain diagonally oriented rows of particles which sometimes form paracrystalline arrays. (*b*). These regions correspond to specialized patches of muscle cell membrane found previously in thin sections of earthworm myoneural junctions with respect to shape, dimensions, particle concentration, and particle array. (*c*). It is concluded that the intramembranous particles in these concavities correspond to the putative acetylcholine receptors seen in the thin sections, and that these elements extend into the hydrophobic portion of the membrane.

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