Absence of Filipin-Sterol Complexes from the Membranes of Active Zones and Acetylcholine Receptor Aggregates at Frog Neuromuscular Junctions

YASUKO NAKAJIMA and PAUL C. BRIDGMAN
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT The polyene antibiotic filipin reacts specifically with membrane cholesterol and produces distinctive membrane lesions. We treated frog cutaneous pectoris and sartorius muscles with 0.04% filipin in a glutaraldehyde solution with or without prefixation with glutaraldehyde. Freeze-fracture of these muscles revealed numerous 19 to 38-nm protuberances and depressions (filipin-sterol complexes) in most areas of muscle, axon, and Schwann cell membranes. In the presynaptic membrane, however, these filipin-sterol complexes were absent from active zones consisting of ridges bordered with double rows of particles. In the postsynaptic membrane, filipin-sterol complexes were also virtually absent from the areas occupied by aggregates of large particles representing acetylcholine receptors. These results suggest that the membrane regions of active zones and acetylcholine receptor aggregates have a low cholesterol content.

Freeze-fracture studies of the frog neuromuscular junction have revealed membrane specializations that include presynaptic active zone ridges with double rows of particles and postsynaptic dense particle aggregates (16, 22, 36). These membrane specializations have been correlated with various synaptic functions (8, 9, 21, 22). However, little is known about the mechanism of formation and preservation of such membrane specializations, except for some suggestion that the postsynaptic membrane density and cytoplasmic filaments might have a role in stabilizing postsynaptic particle aggregates (3, 5, 23, 35).

Recently it has been reported that, in other systems, the cholesterol content of the membrane influences the fluidity of the cell membrane (10, 14, 24). Cholesterol appears to be absent from dense particle aggregates of rod outer segments (1, 2) and has been linked to the formation of protein particle aggregates in artificial membranes (11). We have also found in cultured *Xenopus* embryonic muscle cells that extrajunctional acetylcholine receptor clusters (or hot spots) are located in membrane regions that seem to be low in cholesterol (6, 7). Thus, we have examined the distribution of cholesterol in synaptic membranes of the frog neuromuscular junction, using a recently developed freeze-fracture cytochemical method (1, 2, 18, 29, 30, 38). We chose the polyene antibiotic filipin from among several cytochemical agents that bind membrane cholesterol. This antibiotic reacts with membrane cholesterol specifically (13, 26, 32) and produces small distinctive membrane lesions, filipin-sterol complexes, that are easily recognized in freeze-fractured membranes (1, 2, 18, 27, 30, 31, 38, 40, 41). By treating frog muscles with filipin we found that although most regions of nerve and muscle membranes contained filipin-sterol complexes, these complexes were virtually absent from the presynaptic active zone membrane and the postsynaptic membrane where aggregates of acetylcholine receptors were located.

MATERIALS AND METHODS

Filipin Treatment

Cutaneous pectoris or sartorius muscles with short stumps of nerve were dissected out from *Rana pipiens* and pinned down in chambers containing normal Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 4 mM HEPES buffer, pH 7.4) at room temperature (20°–22°C). The muscles were treated with filipin (gift of J. E. Grady, The Upjohn Co., Kalamazoo, Mich.) in three ways. For treatment A (simultaneous filipin and glutaraldehyde fixation), after 1-h treatment with 0.04% filipin in a 1.5% glutaraldehyde solution containing 1% dimethyl sulfoxide, 0.1 M cacodylate buffer (pH 7.4), and 1.8 mM $CaCl_2$ at room temperature, neuromuscular junction regions were dissected and small pieces of muscles were further treated with a fresh solution of the same composition for 3 h at room temperature. For treatment B (glutaraldehyde and then filipin), after muscles were fixed with a 1.5% glutaraldehyde solution containing 0.1 M cacodylate buffer (pH 7.4) and 1.8 mM $CaCl_2$ for 15 min at room temperature, they were treated with the same glutaraldehyde and buffer solution containing 0.04% filipin and 1% dimethyl sulfoxide at room temperature for 45 min. Then, neuromuscular regions were dissected out and further treated with the filipin-glutaraldehyde solution of the same composition for 1 h at room temperature. For treatment C (short filipin treatment), after muscles were fixed with a 2% glutaraldehyde solution containing 0.1 M cacodylate buffer (pH 7.4) and 10 mM $CaCl_2$ for 30 min at room temperature, they were further treated with 0.04% filipin in a 2% glutaraldehyde solution containing 1% dimethyl

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sulfoxide, 0.1 M cacodylate buffer (pH 7.4), and 10 mM $CaCl_2$ for 30 min at room temperature. Neuromuscular regions were then dissected out. For treatments A and B, cutaneous pectoris muscles were used, and for treatment C both cutaneous pectoris muscles and sartorius muscles were used. Controls were treated in an identical manner, but the solutions did not contain filipin. Subsequent handling was identical in both treated and control materials.

Freeze-fracture

After filipin-glutaraldehyde treatment, specimens were gradually equilibrated with 20% glycerol in the same buffer. Then each specimen was sandwiched between a set of gold disks designed for the double replication method (Balzers High Vacuum, Santa Ana, Calif). A drop of 20% polyvinyl alcohol solution in 20% glycerol in buffer (33) was used as the mounting medium. After specimens were frozen, they were fractured by the double replication method with a Balzers 360M freeze-etch device at −130°C, and replicas were examined with a Philips 300 EM.

Thin Section

After filipin-glutaraldehyde treatment, specimens were postfixed with 1.0% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) containing 1.8 mM $CaCl_2$ for 1 h at room temperature, block-stained with 1.0% uranyl acetate in 5 mM Na acetate buffer (pH 5.5), dehydrated, and embedded in Epon. Thin sections were examined with a Philips 300 EM.

RESULTS

In cutaneous pectoris and sartorius muscles, the nonspecialized plasma membranes of muscle cells, nerve axons, and Schwann cells were all affected by filipin. Freeze-fracture of these membranes showed unique structural alterations consisting of numerous protuberances, 22–38 nm in diameter (average diameter, 27 nm), and depressions, 19–32 nm in diameter (average diameter, 23 nm). These protuberances and depressions appeared in both the protoplasmic face (P face) and external face (E face) of cell membranes (Fig. 1 *a* and *b*). These alterations correlated with membrane profiles in thin sections. The filipin-affected cell membranes had small bumps bulging either outward into the extracellular space or inward into the protoplasm (Fig. 1 *d*). The appearance of these filipin-induced lesions (filipin-sterol complexes) is similar to that reported in the cholesterol containing membranes of other systems (2, 18, 30). The freeze-fractured control materials did not have these complexes (Fig. 1 *c*; Fig. 2 *a* and *b*). The cell membrane of thin-sectioned control material did not have the small bumps that are seen in thin sections of filipin-treated material (Fig. 1 *d*); however, occasionally, wavy contours or invaginations that probably represent openings to the T-tubule system were seen.

The density of the filipin-sterol complexes was variable, ranging from 100 to 400 μm^2. The variability in filipin-sterol complex density was seen even within a single specimen. However, specimens prepared with treatment C tended to have a lower density of filipin-sterol complexes than those prepared with treatments A and B (compare Fig. 3 *d* [treatment C] to Fig. 3 *a* and *b* [treatment A] and *c* [treatment B]). The filipin-sterol complex density of specimens prepared with treatment A was similar to that with treatment B. The greater density of filipin-sterol complexes observed with treatments A and B could be attributed partially to a longer treatment with filipin (4 h with treatment A and 1 h and 45 min with treatment B) than with treatment C (30 min). With these three kinds of filipin treatment, however, the same pattern of filipin-sterol complex distribution was observed. In agreement with Robinson and Karnovsky (38), there was no noticeable differences in the intramembrane particle distribution among all specimens studied: the controls, the specimens simultaneously treated with filipin and glutaraldehyde (treatment A), and the specimens treated with filipin after glutaraldehyde fixation (treatments B and C).

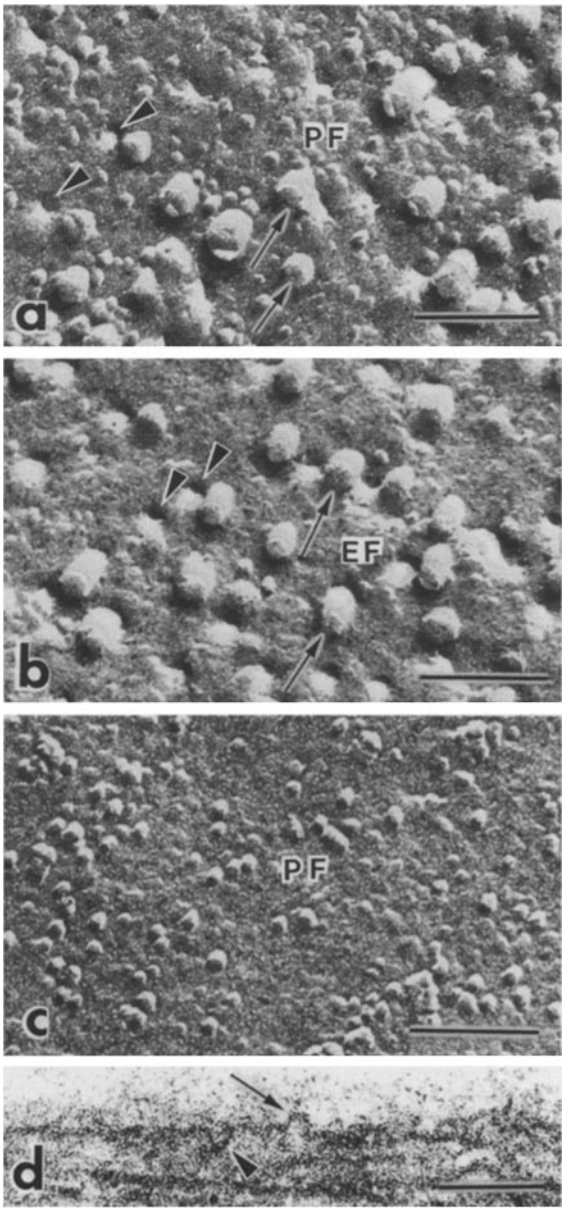

FIGURE 1 Muscle fibers treated with or without filipin. *a* and *b* were taken from specimens prepared with treatment B, *c* was taken from the control for treatment B, and *d* was taken from a specimen prepared with treatment A. *a* and *c* show the P face (*PF*), and *b* shows the E face (*EF*) of freeze-fractured muscle plasma membranes. Filipin-sterol complexes consisting of numerous protuberances (arrows) and small depressions (arrowheads) are seen in *a* and *b* but not in *c*. (*c*) A thin-sectioned muscle membrane shows bumps that project outward toward the extracellular space (arrow) or inward toward the protoplasm (arrowhead). Bars, 0.1 μm. *a* and *b*, × 170,000; *c*, × 140,000.

Fig. 2 *a* and *b*, which are freeze-fracture electron microscope pictures taken from a control, illustrate the normal presynaptic and postsynaptic membranes of the frog neuromuscular junction. As previously reported (16, 22, 36), the presynaptic membrane has membrane specializations at the active zones. The P face of the active zone membrane consists of a ridge and double rows of large active-zone particles bordering both sides of the

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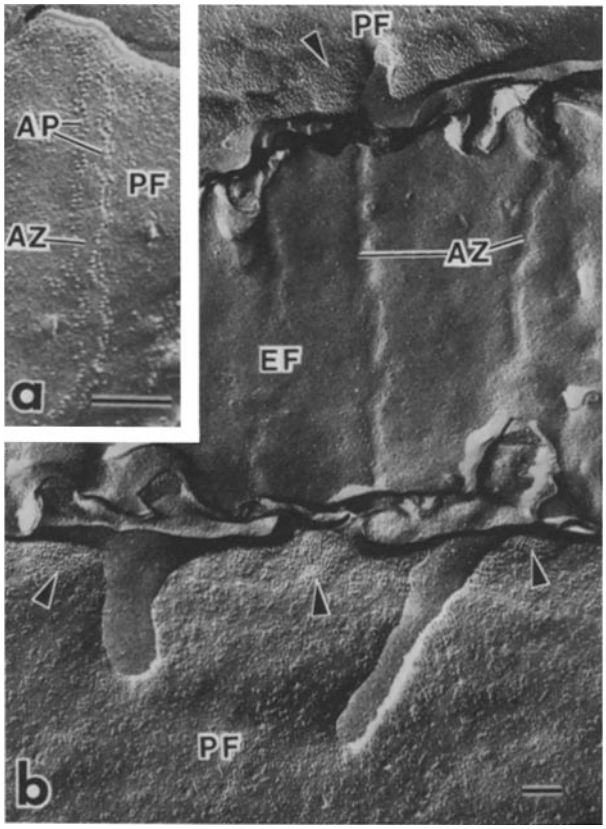

FIGURE 2 Freeze-fracture replicas of control neuromuscular junctions (control for treatment A). *a* shows the P face (*PF*) of the presynaptic membrane in which there is an active zone (*AZ*) consisting of a ridge bordered by double rows of active-zone particles (*AP*). *b* shows the E face (*EF*) of the presynaptic membrane and the P face (*PF*) of the postsynaptic membrane. In the presynaptic membrane, the active zones (*AZ*) appear as long narrow depressions. In the postsynaptic membrane, aggregates of large particles (putative acetylcholine receptors) (arrowheads) are seen on slightly bulged areas of the P-face membrane. Bars, 0.2 μm. *a*, × 53,000; *b*, × 24,000.

ridge (Fig. 2*a*). Fig. 2*b* shows an E-face view of the presynaptic membrane where several active zones are seen as long narrow depressions. In the postsynaptic membrane the slightly bulging P-face membrane has aggregates of large particles ~10–12 nm in diameter, which are considered to be acetylcholine receptors (Fig. 2*b*). The E-face membranes of these particle aggregates appeared as relatively particle-free depressions.

Freeze-fracture of filipin-treated neuromuscular junctions revealed that most areas of the presynaptic membrane were filled with the filipin-sterol complexes (Fig. 3*a* and *b*). These complexes were, however, absent from the active zone membrane (Fig. 3*a–d*). The filipin-sterol complexes were also absent from pits ~100 nm in diameter in the presynaptic membranes, which were seen only rarely in our preparation (Fig. 3*c* and *d*). These pits were seen in the presynaptic membrane areas away from active zones. The size and location of these pits are similar to large dimples that, according to Heuser et al. (22), represent coated pits.

In the freeze-fractured postsynaptic membrane, the membrane areas in which putative acetylcholine receptor particle aggregates are located virtually lacked filipin-sterol complexes (Fig. 3*a*, *b*, and *e*). However, these complexes were present in the membrane surrounding these particle aggregates, including slightly depressed, narrow membrane areas that are sandwiched between aggregates (Fig. 3*b* and *e*). The membrane of Schwann cell processes at the neuromuscular junction had dense filipin-sterol complexes (Fig. 3*a*) but nuclear membranes, which were often seen near the synaptic regions of the muscle, were devoid of filipin-sterol complexes.

In thin sections of filipin-treated neuromuscular junctions (Fig. 4), the majority of the presynaptic nerve membrane as well as the membrane of Schwann cell processes had a bumpy appearance representing filipin-sterol complexes. The same structural alterations were observed in the deeply infolded portion of the postsynaptic membrane (Fig. 4). The presynaptic membrane of the active zone, which is underlined by a cytoplasmic electron-dense material, however, did not have a bumpy appearance (Fig. 4). Similarly, the postsynaptic membrane, which is also underlined by a cytoplasmic electron-dense material, did not have an irregular bumpy appearance (Fig. 4). The synaptic vesicles in the nerve terminal appeared irregular and bumpy in shape (Fig. 4).

DISCUSSION

We have shown that, after treatment with filipin-glutaraldehyde solutions, active zones and pits ~100 nm in diameter in the presynaptic membrane and putative acetylcholine receptor aggregates in the postsynaptic membrane differ from the neighboring plasma membrane by the virtual absence of filipin-sterol complexes. Filipin is known to react specifically with cholesterol or other related 3-β-hydroxy sterols in cell membranes and to form specific membrane lesions, filipin-sterol complexes (2, 18, 27, 30, 40, 41). Certain cell membrane areas such as coated pits, which are known through a biochemical study (34), to be low in cholesterol, do not form filipin-sterol complexes (30). In addition, organelle membranes, such as nuclear membranes, that are also thought to be low in cholesterol, do not form filipin-sterol complexes (18). Therefore, the absence of filipin-sterol complexes in the above-mentioned synaptic regions of the membrane suggests that these regions may be low in cholesterol.

It is unlikely that the pattern of filipin-sterol complex distribution we observed is caused by the difference in the accessibility of filipin to various regions of the membrane. We see no reason why the active zone area should be less accessible to externally applied agents than the nonactive zone part of the presynaptic membrane. In addition, the postsynaptic membrane that is rich in receptor aggregates should be more accessible to filipin than the deep infoldings of the postsynaptic membrane. Yet, the infoldings have filipin-sterol complexes.

The lipid composition of the presynaptic active zone membrane and the postsynaptic membrane of neuromuscular junctions is not known. A few studies have reported the lipid composition of the electric organ postsynaptic membrane (25, 28, 37). These studies do not suggest a low cholesterol content. However, the membrane fractions used for these studies could contain some membrane parts devoid of acetylcholine receptor aggregates. Even a small amount of contamination by nonaggregate membranes could have a large effect on the results of a cholesterol content analysis. Thus, it is difficult to correlate these biochemical data with our morphological findings.

In other membrane systems, cholesterol has been implicated in the aggregation of proteins (11), and in certain cases aggregated protein particles were found in discrete membrane regions (2) of low cholesterol content (17). These observations

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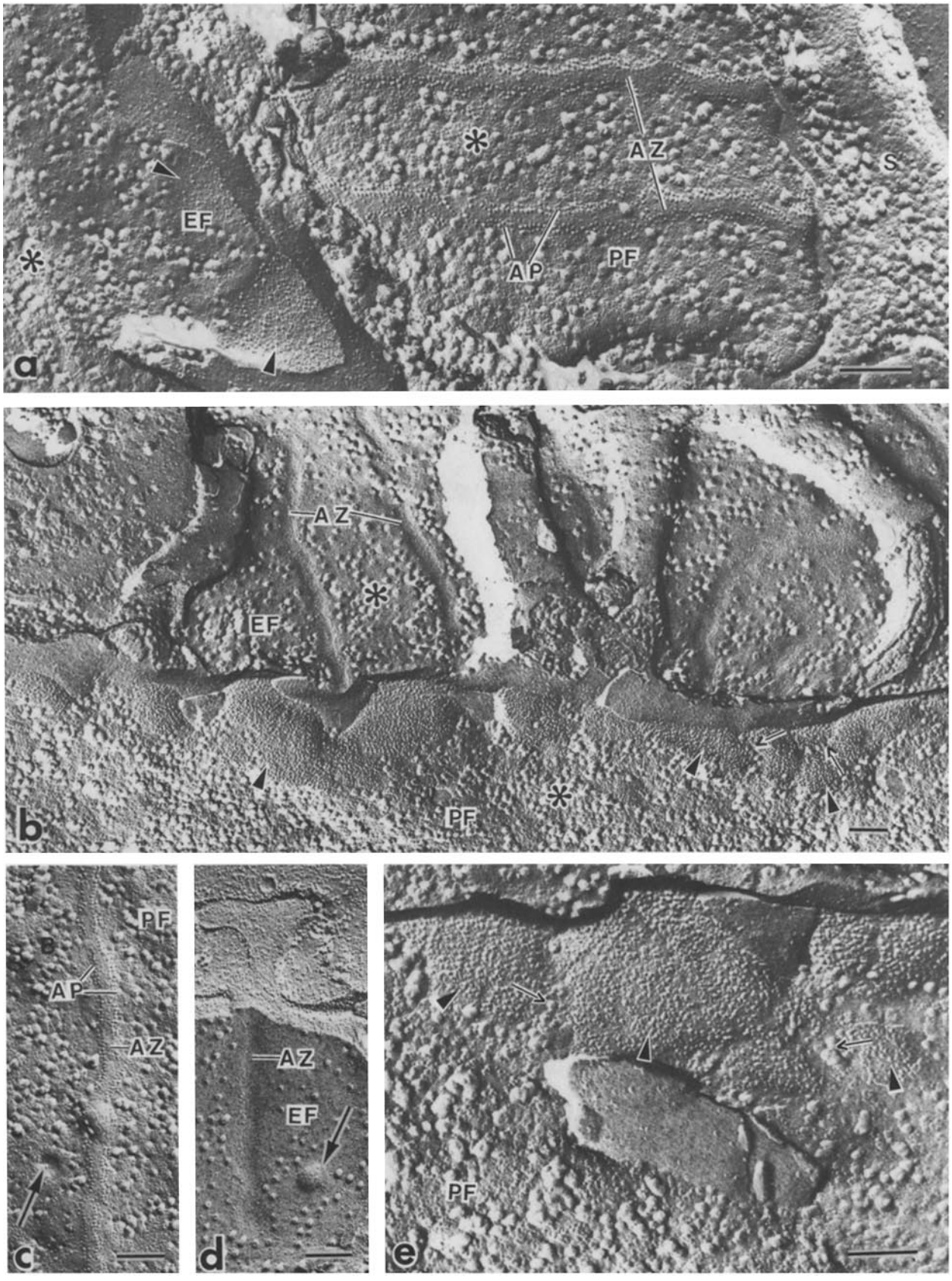

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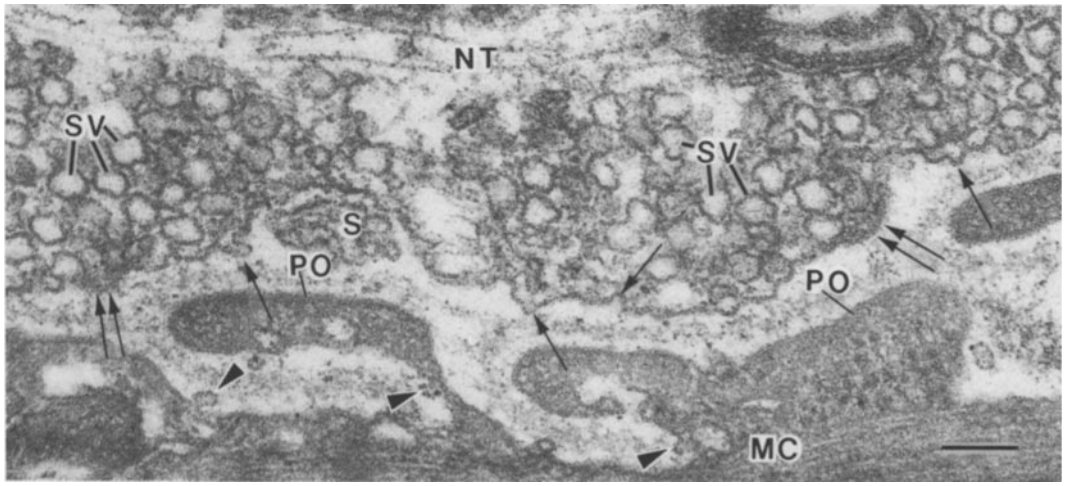

FIGURE 4 A thin section of a filipin-treated neuromuscular junction, taken from a specimen prepared with treatment A. The active-zone membranes (double arrows) of the nerve terminal (*NT*), which are underlined with cytoplasmic, electron-dense material, appear smooth, whereas other parts of the presynaptic membrane have an irregular appearance showing bumps (arrows). The membrane of a Schwann cell process (*S*) also has a bumpy appearance. In a muscle cell (*MC*), the postsynaptic membrane (*PO*), which is underlined with cytoplasmic electron-dense material, appears smooth. Parts of postsynaptic membrane infoldings show a few bumps (arrowheads). Synaptic vesicles (*SV*) in the nerve terminal appear irregular in shape. Bar, 0.1 μm. × 100,000.

and our finding that the acetylcholine receptor aggregates and active zone particles exist in low-cholesterol membrane regions suggest that cholesterol plays an important role in determining the distribution and function of membrane protein molecules. Although we do not know why discrete, low-cholesterol membrane regions are formed, there is a possibility that these regions might have different phospholipid compositions, because Demel et al. (15) reported that cholesterol has a preferential affinity for certain kinds of phospholipids such as sphingomyelin.

Under physiological conditions (above the lipid phase transition temperature), a low cholesterol content is associated with a high membrane fluidity (14). However, in this area of possible high membrane fluidity, active zone particles are not randomly distributed but are contained in double rows. Therefore, there is probably an agent other than regional cholesterol differences that is responsible for holding these particles in a fixed pattern within the plane of the membrane. Recently, the cytoplasmic electron-dense material and filaments, which underline the membrane of acetylcholine receptor aggregates, have been implicated in the formation of the aggregates (3, 5, 23, 35). It is also known that an electron-dense material underlies both the active zone membrane (4, 12) and the membrane of coated pits (19, 20, 39). Thus, all discrete regions of the synaptic membranes that lack filipin-sterol complexes correspond to the membrane regions underlined with some cytoplasmic electron-dense material or with cytoplasmic filaments. This suggests a possible relationship between regional membrane lipid composition and the anchoring of cytoplasmic filaments or electron-dense material.

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FIGURE 3 Freeze-fracture replicas of filipin-treated neuromuscular junctions. *a*, *b*, and *e* were taken from specimens prepared with treatment A, *c* was taken from a specimen prepared with treatment B, and *d* was taken from a specimen prepared with treatment C. Regions of the presynaptic and postsynaptic membrane showing filipin-sterol complexes are marked by asterisks in *a* and *b*. The P face and the E face of presynaptic and postsynaptic membranes are marked as *PF* and *EF*, respectively. *a* shows the virtual absence of filipin-sterol complexes in presynaptic active zones (*AZ*), which consist of ridges bordered by active-zone particles (*AP*). Filipin-sterol complexes are also absent from the E-face postsynaptic membrane of the putative acetylcholine receptor particle aggregates (arrowheads). The membrane of a Schwann cell process (*S*) has complexes. *b* shows the E face of the presynaptic membrane, which lacks filipin-sterol complexes at the active zones (*AZ*), which appear as long, narrow depressions. Bulging postsynaptic membrane areas, where putative acetylcholine receptor particle aggregates exist (arrowheads), also lack filipin-sterol complexes, but narrow depressed areas between particle aggregates have complexes (arrows). *c* shows the P face (*PF*) of the presynaptic membrane in which filipin-sterol complexes are absent from a pit ~100 nm in diameter (arrow) and from an active zone (*AZ*) that consists of a ridge and active zone particles (*AP*). *d* shows the E face (*EF*) of the presynaptic membrane in which a bulge ~100 nm in diameter (arrow) and an active zone (*AZ*), appearing as a long, narrow depression, lack filipin-sterol complexes. *e* shows that postsynaptic membrane areas where putative acetylcholine receptor particle aggregates (arrowheads) are located are devoid of filipin-sterol complexes. Arrows indicate narrow depressed areas between aggregates that have complexes. Bars, 0.2 μm. *a*, × 70,000; *b*, × 33,000; *c* and *d*, × 40,000; *e*, × 62,000.

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