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Membrane lipid heterogeneity associated with acetylcholine receptor particle aggregates in *Xenopus* embryonic muscle cells

(cholesterol/filipin/saponin/synaptogenesis/freeze-fracture)

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ABSTRACT **Filipin, digitonin, and saponin react with membrane cholesterol to produce unique membrane alterations (sterol-specific complexes) that are easily discernible in freeze-fracture replicas. We have treated both noninnervated and innervated *Xenopus* embryonic muscle cells in culture with these agents. Freeze-fracture of these treated muscle cells showed that most areas of the muscle plasma membrane contain sterol-specific complexes (19- to 40-nm protuberances and dimples with filipin, a scalloped appearance with digitonin, or an irregular, rough appearance with saponin). However, these complexes were virtually absent from membrane areas of junctional and nonjunctional aggregates of acetylcholine receptor particles. This result suggests that the membrane matrix of these aggregates is low in cholesterol and that this membrane lipid heterogeneity may be linked to the mechanisms involved in their formation and stabilization on muscle cells in culture.**

Embryonic skeletal muscle cells grown in culture show discrete areas of high acetylcholine receptor (AcChoR) density (1–4), which are commonly referred to as nonjunctional AcChoR clusters or hot spots. Freeze-fracture studies have shown that a single cluster is made up of numerous small aggregates of intramembrane particles representing AcChoRs (5–7). In response to innervation, a redistribution of the AcChoRs occurs that involves the disruption of preexisting clusters and the appearance of new clusters at the sites of neuromuscular contacts (8, 9). The causes of the formation and stabilization of these clusters are not known, although there is some circumstantial evidence that cytoskeletal elements may be involved (4, 10–13).

There have been a number of studies of the role of cholesterol in membrane fluidity and the interaction between cholesterol and membrane proteins (14–17). The possibility that differences in the lipid composition and fluidity of the plasma membrane may play a role in the formation and maintenance of AcChoR aggregates and the subsequent formation of AcChoR clusters has not been investigated. We have taken advantage of the recent development of cytochemical agents that can be used to specifically detect the presence of cholesterol in freeze-fractured membranes (18–20) to test whether heterogeneity of membrane lipid distribution exists. We have used three such agents—the polyene antibiotic filipin, the glycosylated sterol digitonin, and saponin. All three bind specifically to cholesterol in cell membranes (16, 21–25) and produce distinctive membrane structural alterations, "sterol-specific complexes," in freeze-fracture replicas (18–20). A preliminary report of this work is being presented elsewhere (26).

MATERIALS AND METHODS

Preparation of Cultures. Cultures were prepared as described (5, 27) with the following changes: Somites and neural tubes were isolated from either Nieuwkoop–Faber stage 17 or 20 *Xenopus laevis* embryos (28) after a 30- to 45-min incubation with collagenase (Worthington, Cls 3) in Steinberg's solution (1 mg/ml). For cocultures, neural tubes were kept in culture medium [Steinberg's solution supplemented with 10% L-15 medium/1% fetal calf serum/1% penicillin-streptomycin (GIBCO No. 514)] at 4°C until the following day. The neural tubes were then dissociated and added to the muscle cell cultures. For freeze-fracture, cells were cultured on 4-mm-diameter coverglasses.

Filipin, Digitonin, and Saponin Treatment of Cultures. All cultures were first fixed for 30 min at room temperature (20–22°C) in 0.5% glutaraldehyde buffered with 0.05 M Na cacodylate, pH 7.4/5.0 mM $CaCl_2$. The fixative was then exchanged for fresh fixative containing one of the following sterol-specific agents: 0.04% filipin (gift of J. E. Grady, Upjohn) plus 1.0% dimethyl sulfoxide, 0.2% digitonin (Sigma), or 0.1% saponin (Calbiochem). Fixation in the presence of these agents was carried out at room temperature for 15–30 min. Controls were fixed in fixative without the sterol-specific agents, with and without 1.0% dimethyl sulfoxide, for 15–30 min at room temperature and subsequently handled identically to the treated cultures. Cultures were washed several times with buffer and then prepared for freeze-fracture or thin-section electron microscopy.

Freeze-Fracture. After treatment with the sterol-specific cytochemical agents and glutaraldehyde, the cultures were equilibrated with 20% glycerol in buffer. A coverglass containing a culture was placed on a gold disc specimen carrier with a drop of 20% polyvinyl alcohol in 20% glycerol in buffer (29). After this gold disc-coverglass sandwich was frozen, it was fractured by the double replica method of Yee *et al.* (6) by using a Balzers 360 M freeze-etch device.

Thin Section. After treatment with the sterol-specific agents and glutaraldehyde, the cultures were postfixed with 1.0% osmium tetroxide in 0.05 M Na cacodylate buffer/5 mM $CaCl_2$ (pH 7.4) for 1 hr at room temperature and then block stained with 1.0% uranyl acetate in 50 mM NaOAc buffer (pH 5.5). Thin sections were cut perpendicular to the culture dish bottom. Both the thin sections and the freeze-fracture replicas were examined with a Philips 300 electron microscope.

RESULTS

Noninnervated Muscle Cells. For the study in which filipin was the sterol-specific agent, we used 8-hr- and 16- to 17-hr-old cultured muscle cells taken from stage 17 embryos, as well as 24-hr- and 7-day-old cultured cells taken from stage 20 embryos. In our previous freeze-fracture studies of similarly prepared muscle cells (5, 27, 30), we found that 8-hr-old cultures from

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Abbreviation: AcChoR, acetylcholine receptor.

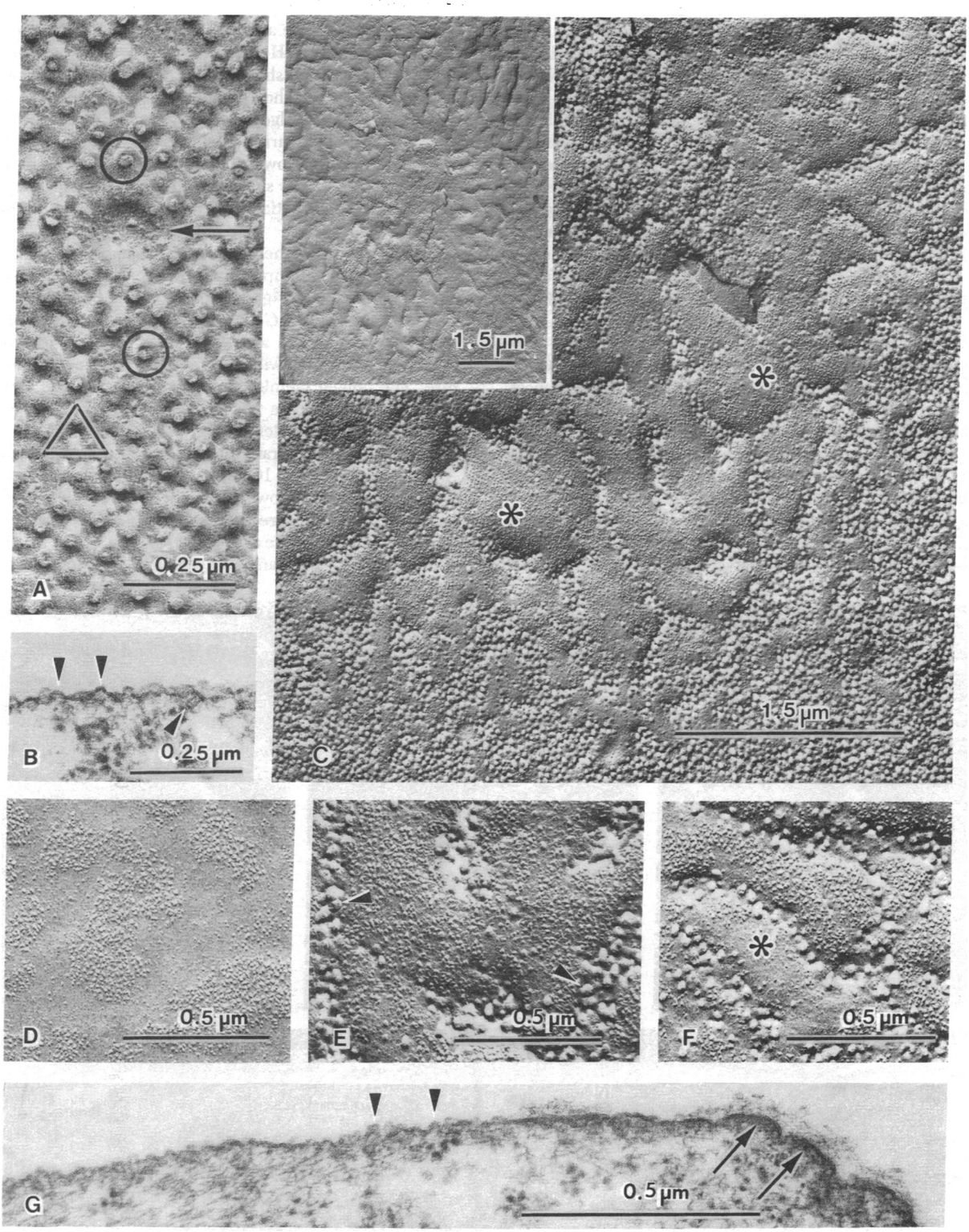

FIG. 1. Micrographs from 24-hr cultures. All freeze-fracture micrographs are of the P-face muscle cell membrane, and the shadowing direction runs roughly from the bottom of the picture to the top. (*A*) The 19- to 40-nm protuberances (circles) and dimples (triangle) characteristic of filipin–sterol complexes are distributed over the muscle cell membrane. Arrow points to a possible coated pit. (*B*) In thin section, the filipin-treated muscle cell membrane appears ruffled; small bumps bulging either outward into the extracellular space or inward into the protoplasm (arrowheads) may represent protuberances and dimples seen in freeze-fracture. (*C*) View of a cluster of particle aggregates from a filipin-treated cell. Protuberances and dimples are absent from membrane that contains particle aggregates (*). (*Inset*) Low-magnification view of the entire cluster area. (*D*) High-magnification view of a particle aggregate from a control culture. (*E*) High-magnification view of a particle aggregate from (*C*); 19- to 40-nm protuberances and dimples (arrowheads) surround the particle aggregate. (*F*) Same as (*E*), except from the periphery of a cluster. A slightly bulged membrane area (*) is devoid of protuberances or dimples and largely unoccupied by aggregates, although dispersed particles are present. (*G*) Thin section from a filipin-treated muscle cell. Small bumps (arrowheads) represent filipin–sterol complexes. Note bulged membrane areas (arrows) that are unaffected by filipin.

stage 17 embryos were sensitive to acetylcholine and had only diffusely distributed 11- to 19-nm intramembrane particles (putative AcChoRs). On the other hand, older cultures (16-hr to 7-day) also had AcChoR clusters (hot spots) consisting of 11- to 19-nm particle aggregates.

It has been reported that filipin treatment produces 25- to 30-nm protuberances or pits (filipin–sterol complexes) in membranes containing cholesterol (19, 20). We found that, after filipin treatment, most areas of the plasma membrane of cultured muscle cells of all ages had rather evenly distributed 20- to 40-nm (average, 28-nm) protuberances and 19- to 32-nm (average, 23-nm) dimples or pits. The density of these filipin–sterol complexes was 100–300/μm^2. A view of the P face of the muscle membrane in which protuberances and dimples can be seen is shown in Fig. 1*A*. The P face contained 6–7 times more protuberances than dimples; correspondingly, on the E face, dimples outnumbered protuberances by a factor of ≈6. Mirror image views of complementary replicas showed that protuberances on the P face corresponded exactly to dimples on the E face and vice versa. A micrograph of a thin section of the affected membrane shows a bumpy-rugged appearance (see Fig. 1*B*) and that the unit membrane structure is difficult to discern. Control materials did not show such filipin–sterol complexes.

When the muscle cells were cultured for at least 16 hr (16 hr to 7 days in these experiments), clusters of 11- to 19-nm particle aggregates appeared that probably represent assemblies of AcChoRs (hot spots). We found that the membrane regions that had particle aggregates were not affected by filipin treatment (Fig. 1*C*). Higher magnification pictures of these particle aggregates are shown in Fig. 1 *D–F*. Fig. 1*D*, which was taken from a control, shows that particle aggregates are usually situated on slightly bulged areas of plasma membrane and that these aggregates are surrounded by slightly depressed, narrow membrane areas of low particle density. The filipin-treated muscle cells (Fig. 1*E*) showed protuberances and dimples in these narrow, low-particle-density areas surrounding the particle aggregates.

In thin sections, the plasma membrane of filipin-treated muscle cells had a bumpy appearance (protuberances and dimples) (arrowheads in Fig. 1*G*) except at the bulged membrane areas (arrows in Fig. 1*G*). These bulged areas, which probably represent the particle aggregate areas in freeze-fracture pictures, looked normal, with an intact trilaminal membrane structure. The bulged membrane areas were associated with cytoplasmic electron-dense material and were often covered with extracellular fuzzy material (Fig. 1*G*) (27, 31).

The bulged membrane areas seen in a particle (putative AcChoR) cluster (Fig. 1*C*) were often almost totally filled with particle aggregates. However, inside some of the bulged membrane areas, there were spaces of variable size that were virtually devoid of particle aggregates, as seen in Fig. 1*F*. Yet, the whole bulged membrane area was free of filipin–sterol com-

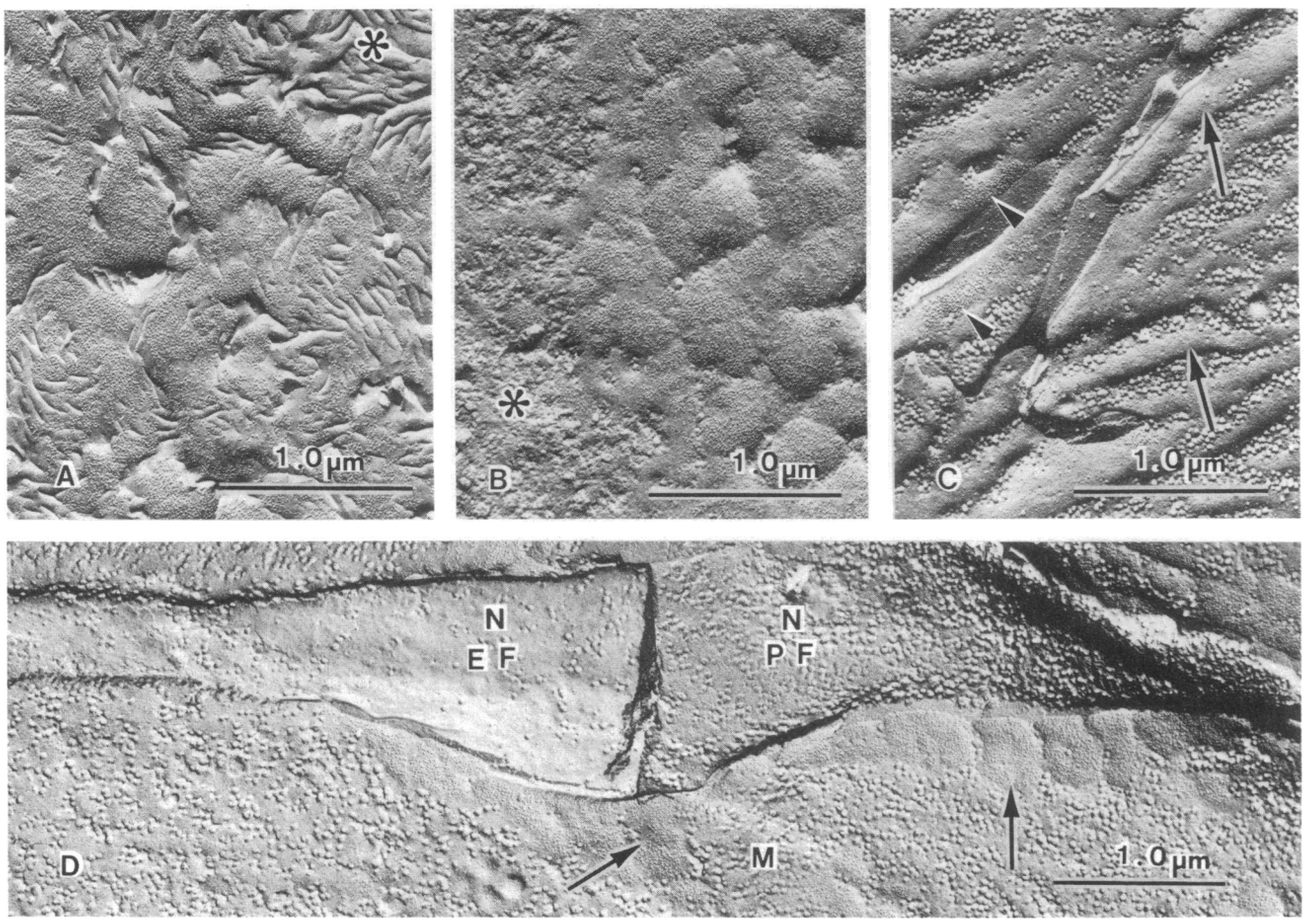

FIG. 2. (*A*) Portion of a cluster of particle aggregates from an 8-day-old muscle cell culture treated with digitonin. Scalloped digitonin-sterol complexes (*) are virtually absent from the particle aggregates. (*B*) Portion of a cluster of particle aggregates from an 8-day-old muscle cell culture treated with saponin. Rough membrane formed by saponin–sterol complexes (*) is absent from the particle aggregates. (*C*) Near an edge of a filipin-treated muscle cell. Streaks of membrane devoid of filipin–sterol complexes (arrows) contain small aggregates of particles (arrowheads). (*D*) Nerve (N)-muscle (M) contact from a 2-day coculture treated with filipin. Particle aggregates in the postsynaptic membrane (arrows) do not contain filipin–sterol complexes. EF, E face; PF, P face.

plexes. These results suggest that the absence of sterol-specific complexes is not necessarily caused by the presence of tight particle aggregates. In addition, a small number of bulged membrane areas, totally free of particle aggregates and without filipin–sterol complexes, were seen adjacent to some clusters.

Our youngest cultured muscle cells (the 8-hr cells from stage 17 embryos) had diffusely distributed 11- to 19-nm particles (possible AcChoRs) but no aggregates of such particles. After filipin treatment, the membrane containing the diffusely distributed particles had filipin–sterol complexes over most of its area.

We also treated 24-hr- and 8-day-old muscle cell cultures with digitonin and saponin, which are also known to interact specifically with sterols (22–25). In freeze-fracture, most areas of the plasma membranes of these treated cells showed distinct structural alterations—a scalloped appearance with digitonin (Fig. 2*A*) and an irregular, rough appearance with saponin (Fig. 2*B*). These membrane structural alterations resemble the sterol-specific complexes reported for other kinds of cells treated with the same cytochemical agents (18, 19). These sterol-specific complexes, however, did not exist in the membrane of 11- to 19-nm particle aggregates (Fig. 2 *A* and *B*), in agreement with the results with filipin-treated muscle cells.

Innervated Muscle Cells. Our previous study (5) showed that 1- to 2-day-old cocultures of nerve and muscle cells usually have clusters of 11- to 19-nm particle aggregates distributed along the area of nerve contact. In these clusters, particle aggregates are separated by small areas of low particle density membrane.

Freeze-fracture of filipin-treated 1- to 2-day-old cocultures showed that the areas of particle aggregates in the subsynaptic membrane were free of filipin–sterol complexes, whereas the muscle membrane surrounding the aggregates contained complexes (Fig. 2*D*). The nerve membrane also contained filipin–sterol complexes (Fig. 2*D*). In thin sections, nonjunctional muscle plasma membranes had a bumpy appearance (protuberances and dimples) and lacked a distinct trilaminar membrane structure. However, areas of muscle membrane at or just adjacent to the nerve contact often had a normal smooth appearance with an intact unit membrane structure.

Other Areas Free of Sterol Complexes. Several discrete areas of the muscle membrane were devoid of sterol–specific complexes in all cultures fractured, regardless of the agent used for treatment or the age of the culture. These areas included shallow membrane depressions (see Fig. 1*A*), similar to those reported by Montesano *et al.* (20), that may represent coated pits (20, 32, 33). Sterol-specific complexes were also absent from long streaks of membrane that were often found on thin extended processes of muscle cells. In 24-hr- to 8-day-old cultures, these streaks sometimes contained aggregates of 11- to 19-nm intramembrane particles (see Fig. 2*C*). Tight aggregates of intramembrane particles characteristic of gap junctions were also free of sterol complexes, confirming a report by Elias *et al.* (18).

DISCUSSION

By freeze-fracturing cultured muscle cells that had been fixed with solutions containing filipin, digitonin, or saponin, we have found that the plasma membrane areas containing putative AcChoR particle aggregates differ from the surrounding plasma membrane by the virtual absence of the distinctive membrane alterations (sterol–specific complexes) caused by these cytochemical agents. All three of these agents bind specifically to cholesterol or other related 3-β-hydroxysterols in cells membranes (16, 21–25, 34), to produce sterol-specific complexes. Certain membrane areas, such as coated vesicles, coated pits, and nuclear membranes, that have low cholesterol contents are known to not produce such sterol-specific complexes (18, 20). Thus, the absence of sterol-specific complexes in the region of AcChoR particle aggregates suggests that these areas of membrane are low in cholesterol.

An alternative interpretation is that the presence of a high density of particle aggregates, by some unknown mechanism, prevents the formation of the sterol-specific complexes. However, the AcChoR aggregates reside in well-defined bulged membrane areas and, as shown in Fig. 1*F*, inside this bulged portion of the membrane, there are sometimes small spaces where virtually no particle aggregates exist. Yet, filipin–sterol complexes are not formed in these small spaces. Similar unaffected areas were also seen on cells treated with digitonin or saponin. This observation suggests that the entire bulged membrane area is low in cholesterol and may serve as a matrix for the particle aggregates. Although we cannot rule out the possibility that either fixation or glycerol treatment may have induced the artificial formation of these areas, they do not closely resemble the particle-free patches reported in other systems (35). The particle-free patches or blisters reported to result from aldehyde-fixation artifacts usually have distinct boundaries and are heterogeneous in shape and size. The areas we observe are free of particle aggregates, but contain diffusely distributed particles and have fairly regular shapes and indistinct boundaries.

Biochemical analysis of lipid content has been reported for membranes of rat skeletal muscle (36, 37) and the electric organs of *Electrophorus* (38) and *Torpedo* (39, 40). The weight ratio cholesterol:phospholipid varies, depending on the method of membrane preparation (0.1–0.3 for rat sarcolemma and 0.3–0.4 for electric organs). It is difficult to relate these data to our results because the membrane area containing dense AcChoR aggregates could have a small total lipid content relative to nonaggregate areas and, in the biochemical studies, it is not known what percentage of the membrane area was occupied by AcChoR aggregates. Therefore, even moderate contamination by non-aggregate-containing membrane could greatly influence the lipid content analyses.

Frog plasma membrane is especially rich in polyunsaturated fatty acids (41), which lower the phospholipid phase-transition temperature. Our experimental temperature (22–20°C) can therefore be considered to be well above the phase-transition temperature. Demel and De Kruijff (16) have shown that, above the phospholipid phase-transition temperature, cholesterol causes a decrease in membrane fluidity. Therefore, it can be speculated that the low-cholesterol-content membrane in which the AcChoR aggregates reside may be in a more fluid state than the surrounding higher-cholesterol-content membrane. If regional differences in membrane fluidity or cholesterol content can cause the segregation of the AcChoRs into discrete areas, a high fluidity in areas containing aggregates seems inconsistent with the observation that the intramembrane particles are not always randomly distributed throughout the entire low-cholesterol-content membrane matrix. This seems to imply that aggregation may occur through other mechanisms, such as interaction with cytoskeletal elements or direct receptor-receptor interactions. However, even if such mechanisms do operate, the observation that aggregates are found only within a distinct low-cholesterol-content membrane matrix, which appears to be underlined with cytoplasmic densities, suggests an interaction between regional membrane lipid composition and cytoplasmic components.

The role of cholesterol in protein aggregation has recently been suggested in other systems. Cherry *et al.* (42) have reported that the addition of cholesterol to pure phospholipid membranes induces the segregation of proteins (bacteriorhodopsin) into discrete membrane areas. Andrews and Cohen (19)

have reported that filipin–sterol complexes are absent from densely particulate areas of frog rod outer segments, which are known biochemically to have a low cholesterol content (43). These reports, as well as ours, suggest an association between membrane cholesterol content and the distribution of membrane protein molecules. However, although we have focused on the possible importance of cholesterol, we do not want to deemphasize the possible role of the intracellular filamentous system in the formation of the particle aggregates (4, 10–13).

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1. Fischbach, G. D. & Cohen, S. A. (1973) *Dev. Biol.* **31,** 147–162.
2. Sytkowski, A. J., Vogel, Z. & Nirenberg, M. W. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 270–274.
3. Axelrod, D., Ravdin, P., Koppel, D., Schlessinger, J., Webb, W. W., Elson, E. L. & Podleski, T. R. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4594–4598.
4. Anderson, M. J., Cohen, M. W. & Zorychta, E. (1977) *J. Physiol. (London)* **268,** 731–756.
5. Peng, H. B. & Nakajima, Y. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 500–504.
6. Yee, A. G., Fischbach, G. D. & Karnovsky, M. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3004–3008.
7. Cohen, S. A. & Pumplin, D. W. (1979) *J. Cell Biol.* **82,** 494–615.
8. Anderson, M. J. & Cohen, M. W. (1977) *J. Physiol. (London)* **268,** 757–773.
9. Frank, E. & Fischbach, G. D. (1979) *J. Cell Biol.* **83,** 143–158.
10. Axelrod, D., Ravdin, P. M. & Podleski, T. R. (1978) *Biochim. Biophys. Acta* **511,** 23–38.
11. Block, R. J. (1979) *J. Cell Biol.* **82,** 626–643.
12. Heuser, J. E. & Salpeter, S. R. (1979) *J. Cell Biol.* **82,** 150–173.
13. Peng, H. B. & Wolosewick, J. J. (1979) *J. Cell Biol.* **83,** 135a (abstr.).
14. Chapman, D. (1973) in *Biological Membranes*, eds. Chapman, D. & Wallach, D. F. H. (Academic, New York), Vol. 2, pp. 91–144.
15. Jain, M. K. (1975) *Curr. Top. Membr. Transp.* **6,** 1–57.
16. Demel, R. A. & De Kruijff, B. (1976) *Biochim. Biophys. Acta* **457,** 109–132.
17. Warren, G. B., Houslay, M. D., Metcalfe, J. C. & Birdsall, N. J. M. (1975) *Nature (London)* **255,** 684–687.
18. Elias, P. M., Goerke, J. & Friend, D. S. (1978) *J. Cell Biol.* **78,** 577–596.
19. Andrews, L. D. & Cohen, A. I. (1979) *J. Cell Biol.* **81,** 215–228.
20. Montesano, R., Perrelet, A., Vassalli, P. & Orci, L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6391–6395.
21. Norman, A. W., Spielvogel, A. M. & Wong, R. G. (1976) *Adv. Lipid Res.* **14,** 127–170.
22. Sperry, W. M. (1963) *J. Lipid Res.* **4,** 221–225.
23. Williamson, J. R. (1969) *J. Ultrastruct. Res.* **27,** 118–133.
24. Kinsky, S. C. (1970) *Annu. Rev. Pharmacol.* **10,** 119–142.
25. Bangham, A. D. & Horne, R. W. (1964) *J. Mol. Biol.* **8,** 660–668.
26. Bridgman, P. C. (1980) *Soc. Neurosci. Abstr.* **6,** 752 (abstr.).
27. Peng, H. B., Nakajima, Y. & Bridgman, P. C. (1980) *Brain Res.* **196,** 11–31.
28. Nieuwkoop, P. D. & Faber, J. (1967) *Normal Table of Xenopus laevis (Daudin)* (North-Holland, Amsterdam).
29. Pauli, B. U., Weinstein, R. S., Soble, L. W. & Alroy, J. (1977) *J. Cell Biol.* **72,** 763–769.
30. Bridgman, P. C. & Greenberg, A. (1979) *Soc. Neurosci. Abstr.* **5,** 476 (abstr.).
31. Weldon, P. R. & Cohen, M. W. (1979) *J. Neurocytol.* **8,** 239–259.
32. Orci, L., Carpentier, J.-L., Perrelet, A., Anderson, R. G. W., Goldstein, J. L. & Brown, M. S. (1978) *Exp. Cell Res.* **113,** 1–13.
33. Röhlich, P. & Allison, A. C. (1976) *J. Ultrastruct. Res.* **57,** 94–103.
34. Verkleig, A. J., De Kruijff, B., Gerritsen, W. F., Demel, R. A., Van Deenen, L. L. M. & Ververgaett, P. M. J. (1973) *Biochim. Biophys. Acta* **291,** 577–581.
35. Hasty, D. L. & Hay, E. D. (1978) *J. Cell Biol.* **78,** 756–768.
36. Ashworth, L. A. E. & Green, C. (1966) *Science* **151,** 210–211.
37. Schapira, G., Dobocz, I., Piau, J. P. & Delain, E. (1974) *Biochim. Biophys. Acta* **345,** 348–358.
38. Kallai-Sanfacon, M.-A. & Reed, J. K. (1980) *J. Membr. Biol.* **54,** 173–181.
39. Popot, T.-L., Demel, R. A., Sobel, A., Van Deenen, L. L. M. & Changeux, J.-P. (1978) *Eur. J. Biochem.* **85,** 27–42.
40. Michaelson, D. M. & Raftery, M. A. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4768–4772.
41. Anderson, R. E. & Risk, M. (1974) *Vision Res.* **14,** 129–131.
42. Cherry, R. J., Müller, U., Holenstein, C. & Heyn, M. P. (1980) *Biochim. Biophys. Acta* **596,** 145–151.
43. Eichberg, J. & Hess, H. (1967) *Experientia* **23,** 93–94.