Distribution of Filipin-Sterol Complexes on Cultured Muscle Cells: Cell-Substratum Contact Areas Associated with Acetylcholine Receptor Clusters

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ABSTRACT Specialized areas within broad, close, cell-substratum contacts seen with reflection interference contrast microscopy in cultures of *Xenopus* embryonic muscle cells were studied. These areas usually contained a distinct pattern of light and dark spots suggesting that the closeness of apposition between the membrane and the substratum was irregular. They coincided with areas containing acetylcholine receptor clusters identified by fluorescence labeled α-bungarotoxin. Freeze-fracture of the cells confirmed these observations. The membrane in these areas was highly convoluted and contained aggregates of large P-face intramembrane particles (probably representing acetylcholine receptors). If cells were fixed and then treated with the sterol-specific antibiotic filipin before fracturing, the pattern of filipin-sterol complex distribution closely followed the pattern of cell-substratum contact. Filipin-sterol complexes were in low density in the regions where the membrane contained clustered intramembrane particles. These membrane regions were away from the substratum (bright white areas in reflection interference contrast; depressions of the P-face in freeze-fracture). Filipin-sterol complexes were also in reduced density where the membrane was very close to the substratum (dark areas in reflection interference contrast; bulges of the P-face in freeze-fracture). These areas were not associated with clustered acetylcholine receptors (aggregated particles). This result suggests that filipin treatment causes little or no artefact in either acetylcholine receptor distribution or membrane topography of fixed cells and that the distribution of filipin-sterol complexes may closely parallel the microheterogeneity of membranes that exist in living cells.

In recent years, there has been a substantial increase in the application of cytochemical agents that can be used for detecting the distribution of membrane lipids. In particular, the polyene antibiotic, filipin, has seen increasing use for the detection of membrane cholesterol in both artificial and natural systems. This is because filipin forms a specific complex with membrane 3-β-hydroxy sterols that is easily seen in freeze-fracture replicas as a distinct round protuberance or pit (3, 8, 15, 27, 36, 40, 42). This allows a simple method for viewing the regional distribution of membrane cholesterol. The use of this methodology in cell systems, however, is dependent upon several major assumptions: that the cytochemical agent is indeed highly specific in its interaction with membrane lipid, that it penetrates evenly into tissues, that it is not prevented from interacting or forming a complex with the lipid when present, and that there is little or no lateral movement of the lipid after fixation or after the formation of the complex with the cytochemical agent. If these agents are to be useful in describing the microheterogeneity of membrane lipid as it exists in the living cell, these assumptions must prove correct.

In some cases, the question of specificity has been extensively studied. This is particularly true of filipin (24, 26, 31), contributing to its growing application. Studies on specificity, however, have been primarily restricted to artificial systems because of the ease of interpreting the results.

In natural cell systems recent progress has been made using filipin; for instance, Elias et al. (15) have shown that variable penetration cannot explain the regional variation in the distribution of filipin-sterol complexes in sperm membranes, because barriers to filipin penetration can be eliminated in their prep-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 FEBRUARY 1983 363-372

aration. In addition, Friend and Bearer (17) have shown that the number of filipin-sterol complexes formed in *Drosophila* larval cells is proportional to the amount of sterol incorporated during culturing of the cells. However, several important problems remain. The influence of proteins or other lipids on the interaction of cholesterol with filipin is unknown; indeed, the absence of filipin-sterol complexes from particle aggregates or possible cell adhesion sites suggests a possible influence (3, 8, 15, 28, 36). Aldehyde fixation before or during filipin treatment is critical for the prevention of a filipin-induced redistribution of both intramembrane particles and filipin-sterol complexes (36), suggesting that the movement of proteins or the complex may not be a problem as long as fixation proceeds or accompanies treatment. Glutaraldehyde, however, does not prevent movement of lipid within the membrane bilayer (19, 23, 34), even though it can interact with phospholipids containing primary amines (11, 18, 21, 25, 30, 38, 47). Glutaraldehyde apparently does not interact with cholesterol (47) and obviously does not prevent the interaction of cholesterol with filipin. It is not known how far cholesterol will migrate to interact with filipin or how much freedom of movement the complex has in fixed cells. Therefore, lipid movement before or after formation of a lipid-specific complex, as well as the inhibition of complex formation by proteins, lipids, or regional restraints on membrane deformation, may be potential problems associated with using cytochemical agents such as filipin for specifically mapping the regional variation in cell membrane lipid.

To address some of the problems associated with cytochemical agents such as filipin, it is important to establish that a correlation does exist between specializations of the membrane observed on living cells and membrane areas defined by the extent of their reaction with filipin. One way to do this is to observe specializations on a single cell while it is living and then see if the same area is detectable following reaction with filipin and freeze-fracturing. This should allow precise identification of membrane areas that are not effected by filipin and, by assessing how closely the areas correlate, determination of whether migration of the complex or lipid is occurring. This may, in turn, allow insight into the problem of whether the lack of filipin-sterol complexes in a region results directly from a lack of sterol or indirectly through influences on the ability of filipin to form a complex.

We chose to do a correlation as described above using a combination of techniques. A close association between specialized areas of contact between the cell and the glass substrate and acetylcholine receptor clusters has been reported in cultures of rat myotubes (7). Both parameters may be observed in living cells by using reflection interference contrast (RIC) and fluorescence microscopy. There is evidence suggesting that both cell adhesion sites and acetylcholine receptor clusters influence the distribution of filipin-sterol complexes (8, 37). By freeze-fracturing cells that were first observed with fluorescence and RIC microscopy in the living state, we have been able to make precise observations on the influence of these cell surface specializations on the distribution of filipin-sterol complexes. Our results suggest that the distribution of filipin-sterol complexes is highly influenced by both the presence of acetylcholine receptor clusters and specialized very close contacts with the substratum. Filipin-sterol complexes usually do not form within these areas and do not appear able to migrate into them by lateral movement from surrounding regions. This supports the interpretation that the distribution of filipin-sterol complexes reflects the microheterogeneity of membranes in living cells. Whether this variation results from actual heterogeneity of membrane sterol, however, remains to be seen.

MATERIALS AND METHODS

Tissue Culture: Muscle cultures were prepared from stage 17–18 (29) *Xenopus laevis* embryos as described previously (8, 32). In order to view living cultures with a standard light microscope and then prepare the same cultures for freeze-fracture, we used a simple chamber. A single 4-mm circle was scored in the center of a square coverglass (No. 0; 22 mm × 22 mm). The coverglass was cleaned and placed into a plastic culture dish containing culture medium, with the scored surface facing up. Cells were plated within the 4-mm circle at low density. To view the cells under the microscope, we removed the coverglass from the culture dish and placed it face down onto a standard glass slide containing 1–2 drops of culture medium. Two thin spacers made from strips of No. 1 coverglass were glued to the slide surface to provide adequate separation between the glass surfaces. Culture medium was carefully absorbed from the top surface of the coverglass to allow layering of immersion oil. The edges of the chamber were not sealed. Small drops of culture medium were added from time to time to compensate for evaporation from the thin fluid layer.

Fluorescence Microscopy: Cultures were labeled with monotetramethylrhodamine-αbungarotoxin (R-αBGT) (35) for 30 min at room temperature. The concentration of R-αBGT used for staining was determined by titrating with increasing amounts until a maximum brightness of specific staining was achieved. Cultures were washed with eight rapid changes (~5 ml each) of culture medium before they were viewed.

Fluorescence microscopy was performed using a standard Zeiss microscope equipped with epillumination and a 150 W halogen light source. We used a Zeiss planapo 63/1.4 phase contrast oil immersion objective. Phase and fluorescent images were photographed on Kodak Tri X film and were developed in Diafine.

Reflection Interference Contrast Microscopy: Reflection interference contrast (interference reflection) microscopy was performed using basically the same system as for fluorescence microscopy (1). The Zeiss Antiflex-neofluar 63/1.25 oil immersion objective, designed specifically for RIC microscopy (5), was used. Photography was performed with Kodak Technical Pan film 2415, which was developed in D-19.

Freeze-Fracture: After cells were photographed in the light microscope, the immersion oil was carefully removed from the coverglass surface. The entire chamber was then placed into a large petri dish and the coverglass was floated off the slide by the slow addition of drops of fixative (0.5% glutaraldehyde plus 5 mM $CaCl_2$ in 0.05 M Na cacodylate buffer, pH 7.4). Control cultures were fixed for 1.5 h.

Cultures to be treated with filipin (gift of T. E. Grady, The Upjohn Co., Kalamazoo, MI) were initially fixed for 30 min. The solution was then exchanged for a fresh fixative solution containing 0.04% filipin and 1% DMSO (8). Cultures were treated with this solution for 1 h. After washing and equilibration with a 20% glycerol-buffer solution, the 4-mm disk was broken free from the rest of the coverglass. Freeze-fracture procedures with the double replica device by means of the gold-disk-coverglass sandwich technique have been described previously (32, 33, 48).

RESULTS

We will first describe the results obtained with light microscopy and then compare these with the results from freeze-fracture electron microscopy of both control and filipin-treated cells. All observations were made on 4-d-old muscle cell cultures.

Light Microscopy

When living cultured muscle cells were photographed under phase contrast optics, the most prominent features were the regular pattern of striations associated with myofibrils and the scattered, large, phase-bright yolk granules (Fig. 1*A*). When the same cells were photographed using RIC, they had a totally different appearance, related to the ability of RIC to detect the closeness of cell-substratum contact (1, 13, 22). Discrete dark gray to black areas in RIC photographs are thought to represent very close (focal) contacts (10–15 nm separation between the cell and the glass substratum), whereas broader medium-gray areas are thought to represent close contacts (~30 nm separation), and white areas to represent distinctly greater cell-sub-

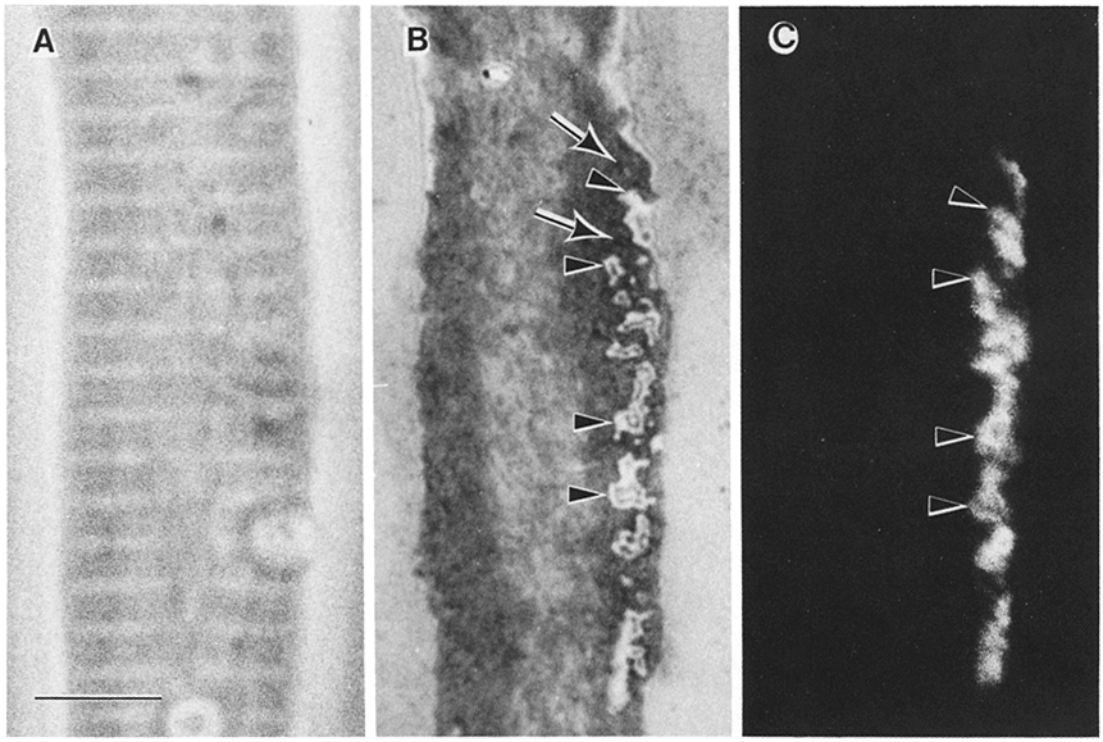

FIGURE 1 A portion of a muscle cell from a control culture as viewed by phase contrast (*A*), by RIC (*B*), and by monotetramethylrhodamine-α-bungarotoxin fluorescence (*C*). The α-bungarotoxin binding sites (arrowheads in *C*) are located in areas that appear white in RIC (arrowheads in *B*). These areas are in regions of broad, close, cell-substratum contact (darker grey areas). Arrows (*B*) indicate very dark gray to black spots that are often found around the irregular white patches. Bar, 5 μm. × 2,800.

stratum separation (<100 nm) (see Izzard and Lochner, reference 22). Most cells had broad areas of light gray that sometimes contained complicated patterns of irregularly shaped streaks. They also had darker gray areas that resembled the areas of broad, close contacts seen in fibroblasts (22). Muscle cells often made such close contacts over much of their surface (Fig. 1 *B*). Small very dark gray to black streaks or lines, indicative of focallike contacts (22) were occasionally seen near the periphery of cells, especially at the ends of extended processes. Although the dark appearance of these contact areas was similar to that of focal contacts seen in nonmuscle cells (probably fibroblasts) that sometimes contaminated the cultures, the shapes and orientations were far more heterogeneous.

Most muscle cells also had distinct areas of broad close contact with a fine structure of irregular white spots often surrounded by darker gray-to-black spots or patches (Figs. 1 *B* and 2 *C*). Sometimes, larger, irregularly shaped white spots were also seen in these areas. We interpreted these patterns to represent specialized broad close-contact areas. The fine pattern of white and dark spots would be where the membrane is especially convoluted, with the white spots indicating depressions in the membrane (away from the substratum) and the dark spots bulges (close to the substratum). The larger white spots would indicate larger areas of depressed membrane.

When acetylcholine (ACh) receptors were labeled with R-αBGT and then viewed under the fluorescence microscope, distinct patches of bright fluorescence were revealed as described by Anderson et al. (2) (Fig. 1 *C*). These patches of bright fluorescence represent ACh receptor clusters generally found on cultured embryonic muscle (2, 16, 39, 43). By comparing, on a single cell, the fluorescent image with the image obtained using RIC (Fig. 1 *B* and *C*), it was clear that there is a close association between the ACh receptor clusters found on the bottom of the cell and the specialized contact areas.

ACh receptor clusters usually consisted of fine speckles or lines of bright fluorescence (Fig. 1 *C*). Occasionally, more evenly stained patches were seen as described by Anderson et al. (2). To study in detail the spatial relationship between the specialized contact areas and ACh receptor clusters found on the bottom of the cell, we superimposed the two images. In confirmation of the results by Bloch and Geiger (7) on rat myotubes, the bright fluorescent speckles were almost always associated with the white spots or patches (depressions in the membrane) that interdigitated with grey areas (close contacts with the substratum). The fluorescent image, however, often extended slightly into neighboring dark-gray borders that usually surrounded the white spots. Not all white spots (depressions) were associated with fluorescence. On rare occasions fluorescent speckles seemed to coincide with dark spots, but it was difficult to decide whether this resulted from slight differences in focus or actual co-localization.

Comparison of Light Microscopy and Freeze-fracture Electron Microscopy

Cells that were photographed by the light microscope techniques were reidentified in freeze-fracture replicas. Under low magnifications, distinct areas of convoluted membrane were seen on control cells that coincided with the specialized contact areas seen with fluorescence microscopy and RIC (Fig. 2). At higher magnifications of the P-face, intramembrane particle aggregates characteristic of putative ACh receptor aggregates (12, 32, 48) could be seen within the specialized areas (Fig. 2 *D*). The fluorescent staining pattern closely followed the

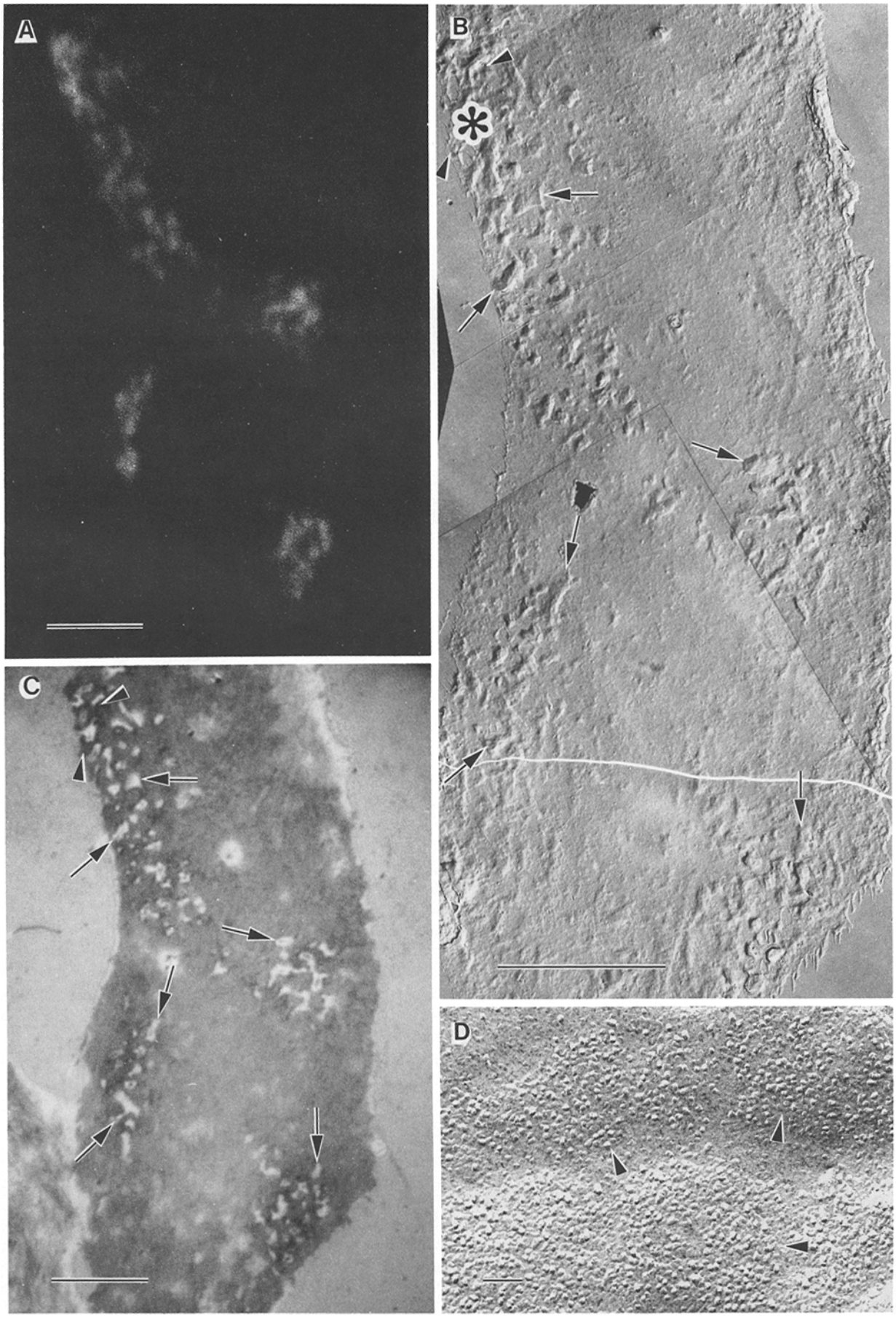

FIGURE 2 A portion of a muscle cell from a control culture as viewed by monotetramethylrhodamine–α-bungarotoxin fluorescence (*A*), by electron microscopy of the P-face following freeze-fracture (*B*), and by RIC (*C*). The α-bungarotoxin binding sites are located primarily in areas that appear as white spots in RIC (arrows) and as depressions of the membrane in freeze-fracture (arrows). Dark spots in RIC (arrowheads) appear as bulges in freeze-fracture (arrowheads). (*D*) A high magnification view of aggregated intramembrane particles (arrowheads) from the area indicated by * in *B*. *A* and *C*: Bars, 5 μm. × 2,800. *B*: Bar, 5 μ. × 5,000. *D*: Bar, 0.1 μm. × 96,000.

distribution of particle aggregates as previously reported by Cohen and Pumplin (12).

ACh receptor clusters on the membrane against the substratum seemed to be mainly of two types. In type 1, aggregates were clustered in close proximity to each other, and each aggregate was situated on a slight mound. The entire area of the cluster was slightly depressed and usually corresponded to a large white patch surrounded by medium gray in RIC. Fluorescent staining in a type-1 cluster appears fairly even throughout the cluster. Our previous descriptions pertained mainly to this type of cluster (8, 32) (see also Fig. 5). In type-2 clusters, aggregates are more scattered and usually associated with relatively deep but small scattered membrane depressions (Figs. 2 and 3). The aggregates were concentrated on the sides of the depression or on slight mounds rising from the floor of the depression (Fig. 3). These depressions were seen in RIC as a fine pattern of white spots and were usually surrounded by medium- to dark-gray areas as in Figs. 1*B* and 2*C*. The fluorescent staining pattern was seen as a group of fine speckles (Figs. 1*C* and 2*A*). Type-1 clusters seemed to predominate between day 1 and day 3 of culture and thereafter became less numerous. Type-2 clusters predominated after day 3 of culture. In all cases, the distribution of the particle aggregates coincided precisely with the distribution of bright fluorescence seen on cells stained with R-αBGT.

The pattern of white spots seen in areas of cell-substratum contact by RIC coincided closely with the pattern of membrane depressions seen on the P-face (Figs. 2*B* and *C*). However, on rare occasions discrepancies were found: small, round, deep invaginations observed in freeze-fractured membranes were sometimes seen as black spots in RIC. We do not know the reason for this discrepancy but believe that it may involve technical aspects of RIC (see Bech and Bereiter-Hahn, reference 5) rather than artefacts of freeze-fracture.

Although differences between membrane depressions (white areas in RIC) and close contacts (dark gray areas in RIC) were easily seen in replicas, the differences between close contacts and focallike contacts (darker gray to black in RIC) were more difficult to observe. The majority of cells did not have obvious bulging areas on the membrane surface against the glass. Possibly contributing to the problem was the fact that the small differences between close and focal contacts (20–30 nm) (22) may not be easily seen in freeze-fracture replicas without stereo images. Alternatively, the interpretations of RIC images suggested by Bereiter-Hahn et al. (6) and Beck and Bereiter-Hahn (5) might be more correct. They suggested that the differences between close and focal contacts result from an increased refractive index of the cytoplasm due to close associations between dense cytoplasmic filaments and the membrane at local contacts, not from variations in distance from the substratum. In some instances, however, areas of the fractured membrane did appear to bulge slightly and were associated with dark gray to black spots in RIC.

Filipin-treated Cultures

When fixed cultures were treated with filipin before freezing, most of the fractured membrane contained high densities of protuberances (average diameter, 28 nm) and complementary pits (average diameter, 23 nm), which are called filipin-sterol complexes (Fig. 4) (3, 8, 15, 27, 36, 37). To see how the distribution of filipin-sterol complexes was influenced by the pattern of substratum contacts and ACh receptor aggregates, we reidentified cells in platinum replicas that had been photographed under RIC and fluorescence microscopy before fixation and filipin treatment. This also allowed us to check whether filipin treatment could induce artifacts by influencing ACh receptor distribution or membrane topography.

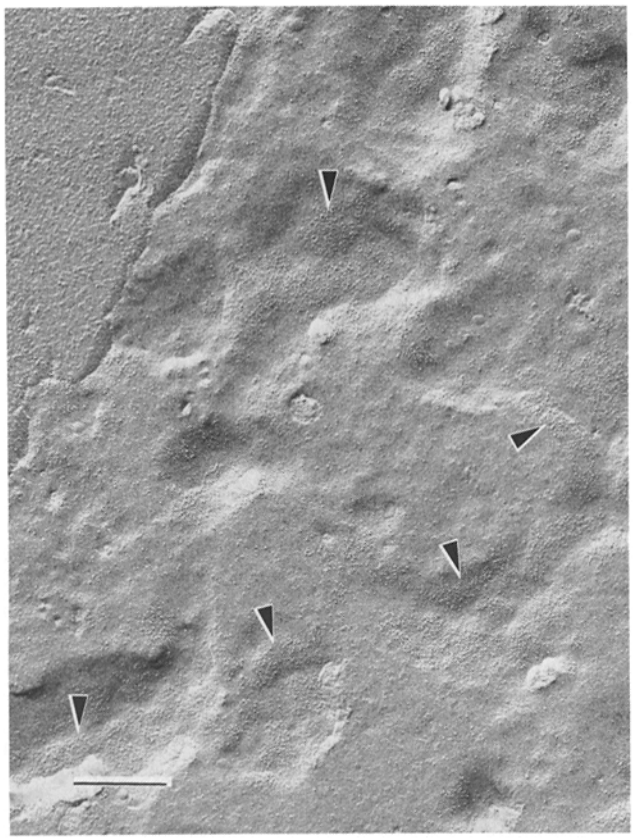

FIGURE 3 A view of the P-face from the same cell as in Fig. 2 showing the location of aggregated intramembrane particles. Aggregates (arrowheads) are located primarily in depressions of the membrane. Bar, 0.5 μm. × 26,000.

The specialized areas containing ACh receptor aggregates stood out as distinct filipin-sterol complex-deficient membrane regions when compared to the unspecialized membrane (reference 8 and Figs. 4 and 5*A*).

In spite of the filipin treatment, the distribution of ACh receptor particles coincided precisely with the distribution of bright fluorescence (Figs. 5*C* and *D*). This suggests that filipin treatment does not influence the distribution of ACh receptor particles (within the limits of resolution of our light microscope technique). In addition, membrane topography seen with RIC did not appear to be affected by filipin treatment. Depressions in the fractured membrane were almost always seen to coincide with white spots seen in RIC (Figs. 5*A* and *B*). The occasional exceptions were the same as those observed in control cells.

Filipin-sterol complexes were absent from the membrane containing particle aggregates that was located within depressions (white spots in RIC) (Figs. 5*E*). However, aggregate-free membrane within the depressions usually contained filipin-sterol complexes.

We previously reported that filipin-sterol complexes were absent from membrane areas containing particle aggregates presumed to represent ACh receptors (8). The correlation in the present study between the fluorescent area, as seen by R-αBGT labeling, and particle aggregates confirms the identity of these particles. We again found that filipin-sterol complexes

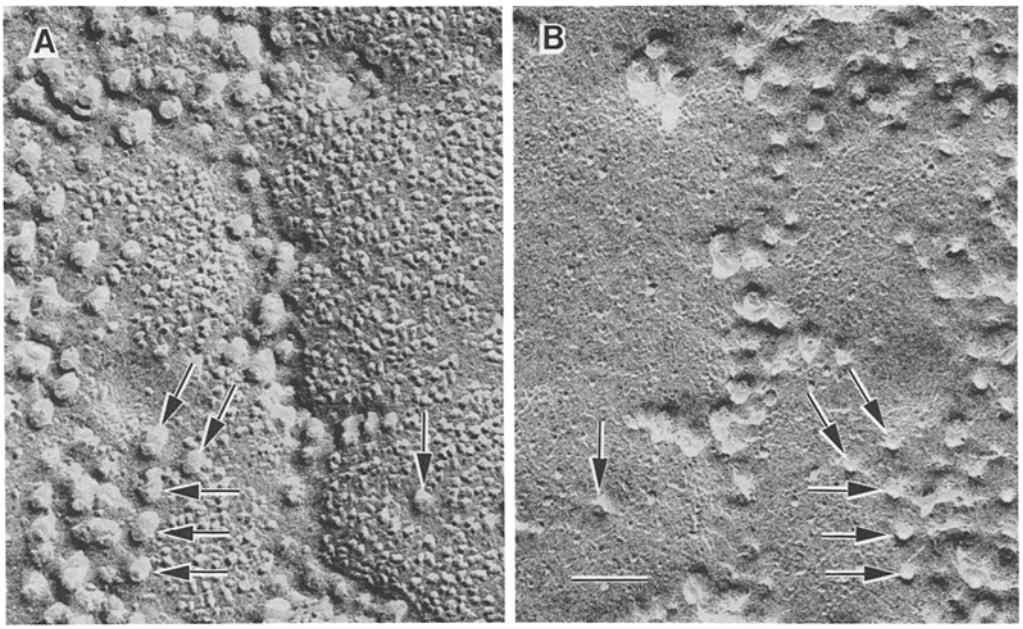

FIGURE 4 High-magnification mirror image views of filipin-sterol complexes surrounding particle aggregates. (*A*) P-face. (*B*) E-face. Complementary protuberances and pits are indicated by the arrows. Due to the geometry of fracturing and replication, the shadowing with platinum is in opposite directions when the images are aligned. The E-face (*B*) should be viewed upside-down to observe particles with the proper perspective. Bar, 0.1 μm. × 105,000.

were excluded from the membrane of particle aggregates (Figs. 4 and 5 *E*), supporting our initial finding.

In our previous work (8), we found that small, smooth membrane areas neighboring particle aggregates, as well as streaks of smooth membrane near the ends of cells, did not contain filipin-sterol complexes. In the present study, we investigated such specialized membrane areas with RIC, fluorescence microscopy and freeze-fracture. We found that dark gray to black patches or lines in the specialized contact areas seen with RIC occasionally appeared as bulges of the membrane in freeze-fracture replicas, and usually had few filipin-sterol complexes (Figs. 5 *A* and *B*). These membrane areas were not associated with R-αBGT fluorescence or particle aggregates (putative ACh receptors). This indicates that these areas free of both particle aggregates and filipin-sterol complexes are probably not artefacts of the treatment but may be related to specialized membrane contacts that are thought to be associated with dense filament bundles (6, 20). These areas were often as dark as focal contacts seen in thin nonmuscle cells (probably fibroblasts) that occasionally contaminated the culture (Fig. 6 *B*) although their shapes and orientations differed. The membrane of the nonmuscle cell focal contacts (Fig. 6 *A*), however, reacted with filipin to a greater extent than the membrane associated with dark spots or lines in RIC of muscle cells (Fig. 5 *A* and *E*). Broad areas of unreacted membrane were not seen in nonmuscle cells, although filipin-sterol complexes that did form in focal contacts seemed somewhat smaller than those found in the neighboring membrane (Fig. 6 *C*).

Streaks of smooth membrane found at the muscle cell periphery (mainly at the ends of the extended cells) on filipin-treated cells also coincided with dark gray to black lines or spots seen in RIC.

DISCUSSION

We have shown that, within certain muscle-cell, broad, close, substratum contacts, two different types of areas can be seen by RIC of living cultures, which represent regions of the membrane that do not show dense accumulations of filipin-sterol complexes when the cells are fixed, treated with filipin, and then freeze-fractured. The first type of area is usually seen as either a series of bright white spots in RIC photographs or larger white patches and is coincident with both types of fluorescent staining (type 1 and type 2) that are detected by R-αBGT. This area is seen either as a series of small depressions or, alternatively, as a larger single depression of the membrane containing particle aggregates in freeze-fracture. The second type of area is seen as a series of very dark spots or lines in RIC. This second type of membrane area can sometimes be seen to be correlated with slight bulges of the membrane in freeze-fracture, but is not associated with particle aggregates. It is also not associated with R-αBGT fluorescence. The former area is the membrane that contains aggregated ACh receptors, while the latter may be an area of the membrane that is involved in a specific adhesion to the substratum (22, 37).

Using RIC and fluorescent microscopy, Bloch and Geiger (7) observed that ACh receptor clusters of cultured rat myotubes appeared to interdigitate between points of substratum contact within broad, close-contact areas. Our observations confirm Bloch and Geiger's interpretation and further define this unique relationship by allowing the location of ACh receptor clusters to be mapped in terms of particle aggregates. Aggregated ACh receptors are primarily located in areas that appear white in RIC, suggesting that a separation of at least 100 nm exists between the cell membrane and the substratum. They appear to concentrate either on the sloping sides of the depression or on slight bulges of the membrane that arise from the floor of the depression.

These observations suggest that the membrane topography and distribution of ACh receptor particles seen on fractured cells are close representations of both the cell-substratum contact pattern (as seen in RIC) and the ACh receptor cluster distribution (as seen in fluorescence microscopy) found in living cells. To our knowledge, correlations between membrane topography in living cultured cells and that seen in freeze-fracture have not been previously reported.

When cells are treated with filipin, the relationship between the location of particle aggregates and the membrane topography is preserved, suggesting that filipin treatment does not cause changes in the membrane topography or particle aggregate location in fixed cells. More important, however, is the observation that the shape or outline of both the particle

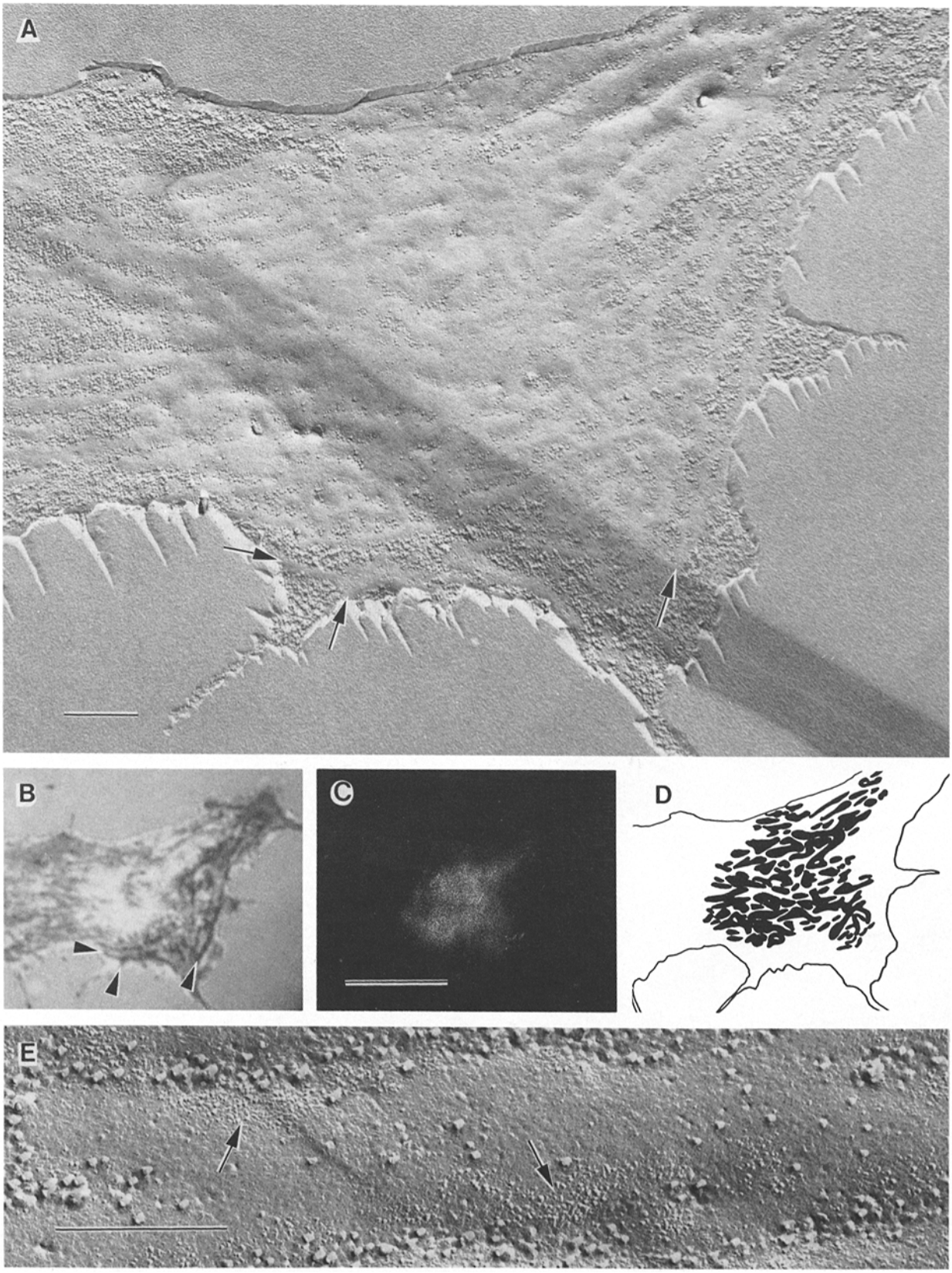

FIGURE 5 A portion of a muscle cell from a filipin-treated culture as viewed by freeze-fracture electron microscopy of the P-face (*A*), by RIC (*B*), and by monotetramethylrhodamine-α-bungarotoxin fluorescence microscopy (*C*). (*D*) Diagrammatically, the location of particle aggregates traced from (*A*). (*E*) Higher magnification of the P-face, showing the location of particle aggregates (arrows) and filipin-sterol complexes in relation to membrane bulges. Arrows in (*A*) show the location of aggregate-free membrane that does not react with filipin and appears as dark lines in RIC (arrowheads in *B*). *A*: Bar, 1 μm. × 12,000. *B* and *C*: Bar, 5 μm. × 2,800. *E*: Bar, 0.5 μm. × 57,000.

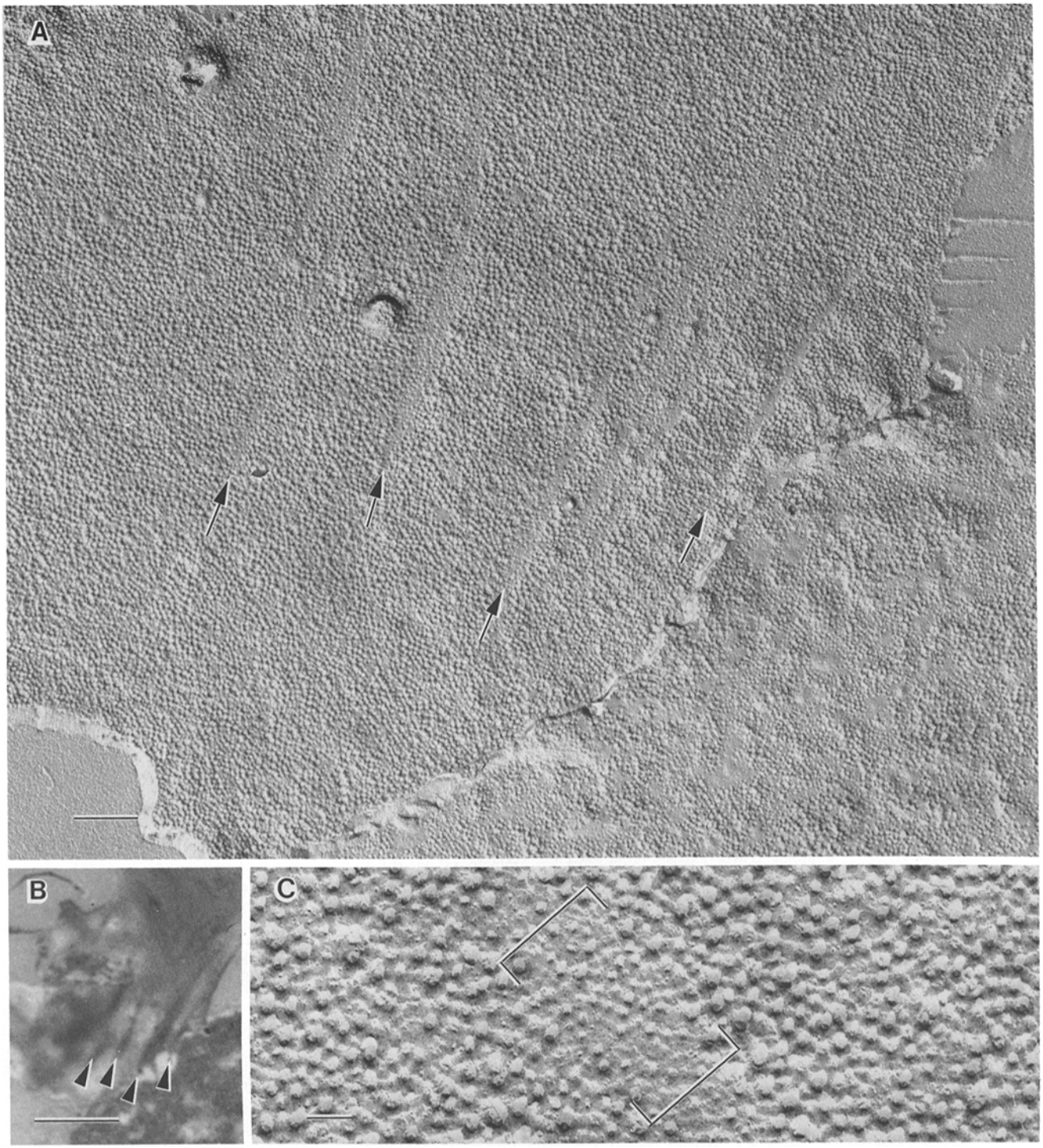

FIGURE 6 (*A*) A freeze-fracture view showing the P-face from a portion of a nonmuscle cell in a culture treated with filipin. Arrows indicate focal contacts that can be identified in the corresponding RIC image (arrowheads in *B*). (*C*) A higher magnification from (*A*). Brackets indicate the membrane of a part of a focal contact. *A*: Bar, 0.5 μm. × 21,000. *B* Bar, 5 μm. × 2,800. *C*: Bar, 0.1 μm. × 78,000.

aggregate and nonaggregate areas that do not react with filipin seem to remain true to the shape and size of the membrane specializations seen in living cells by either RIC or fluorescence microscopy. The result, that these regions retain the basic size and shape observed in the living state, probably means that filipin-sterol complexes are not free to migrate from neighboring areas into the filipin-unreactive domains. This suggests that membrane regions defined by the extent of their reaction with filipin may be precise representations of specialized membrane areas found on living cells.

Why filipin does not react with the membrane in these specialized areas is not easy to explain. Although cholesterol may very well be low in these areas, other explanations are also plausible. For instance, cholesterol could be in an esterified form that does not react with filipin (26) or, alternatively, physical barriers on or close to the membrane may prevent filipin and cholesterol from interacting. One simple explanation for the absence of complexes from the areas represented by

dark spots or lines in RIC could be that the very close contact between the cell membrane and the glass surface hinders penetration of filipin. However, this would not explain the absence of complexes from particle aggregate areas that are not close to the substratum, or the observation that nonmuscle cells, which occasionally contaminated our cultures, were affected by filipin to a considerably greater extent than muscle cells in areas of very close contact with the substratum (dark gray to black in RIC). Another candidate for a barrier directly on the membrane is the basal lamina. A prominent basal lamina is associated with the membrane of possible ACh receptor cluster areas (8, 10, 46). Inconsistent with this possibility, however, is the observation that prominent basal lamina material fills the synaptic gap at frog neuromuscular junctions, while only select parts of the membrane (ACh receptor aggregate and active zone membrane) do not react with filipin (28). Another, perhaps more likely, possibility is that membrane-associated cytoplasmic filaments or densities influence the ability of filipin to either penetrate or perturb the membrane. It would be very difficult to determine whether this possibility is correct, however, since manipulations that may remove, for instance, a possible barrier to filipin may actually remove a barrier to cholesterol, allowing it to enter a previously restricted membrane area. In addition, the observation that filipin does react in areas of focal contact of nonmuscle cells that are probably associated with dense filament bundles (6, 20, 45) suggests that cytoplasmic filaments or densities may contribute to the formation of filipin unreactive areas, but are not sufficient for identifying such areas.

It is also possible that a high density of membrane protein may influence the ability of filipin to either interact with cholesterol or deform the membrane. Proteins could conceivably bind tightly to cholesterol, thereby preventing interaction with filipin. Alternatively, glutaraldehyde may cross-link proteins, or proteins and phospholipids with amine groups, making the membrane rigid and unable to bend in response to filipin binding to cholesterol. There is no evidence, however, that cholesterol binds tightly to proteins. Indeed there is some evidence that cholesterol is excluded from close interaction with some proteins (44), that it is not affected by aldehyde fixation (47), and that it seems to move rather freely in lipid bilayers (41). Although membrane rigidity through glutaraldehyde cross-linking may seem reasonable for areas with high protein concentrations, it does not seem to be a feasible possibility for areas with average densities (dark areas in RIC) or particle-free areas that do not react with filipin (4). In addition, we have preliminary evidence that dispersal of particle aggregates, by blocking energy metabolism with sodium azide, does not influence the reactivity of filipin in these specialized areas (9).

A definitive answer to the question of whether the distribution of filipin-sterol complexes in cell membranes reflects actual heterogeneity in membrane cholesterol content awaits further experimentation. We believe this to be an important area to pursue since the lack of cholesterol in distinct regions of the membrane may also reflect phospholipid heterogeneity (14). Variations in cholesterol concentration or differences in phospholipid content between different regions of the membrane could have important effects on the function of membranes and membrane proteins. We are particularly interested in differences that may exist in the function of aggregated ACh receptors when compared to the diffusely distributed ACh receptors also present on the membrane of cultured muscle cells (16, 39, 43). These diffuse receptors lie in membrane areas that form filipin-sterol complexes and therefore may be exposed to a different lipid environment than aggregated ACh receptors.

We wish to thank Dr. Joann Otto for the use of the reflection interference contrast and fluorescence microscope and Dr. Shigehiro Nakajima for reading and commenting on the manuscript. We also wish to thank Sandra Cotter for processing the manuscript.

This work was supported by a NIH grant (NS-10457) and a grant from the Muscular Dystrophy Association.

Received for publication 10 June 1982, and in revised form 22 October 1982.

Note Added in Proof: A report (Pumplin, D. W., and R. J. Bloch. 1982, Effects of saponin on acetylcholine receptor clusters of cultured rat myotubes visualized by freeze fracture. *Soc. Neurosci. Abstr. 8:*696 (Abstr.).) has appeared on the use of the cholesterol-specific detergent, saponin, in cultures of rat myotubes. Saponin was found to react with membrane areas containing ACh receptor particle aggregates but not with the membrane of substrate contact domains. These effects were blocked by prefixation. This result suggests that rat myotubes may differ from cultured *Xenopus* muscle cells in their reaction with cholesterol-specific agents, and that this variation may be species dependent.

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