Lipid Domains of Acetylcholine Receptor Clusters Detected with Saponin and Filipin

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ABSTRACT The acetylcholine receptor (AChR) clusters of cultured rat myotubes contain two distinct, interdigitating, membrane domains, one enriched in AChR, the other poor in AChR but associated with sites of myotube-substrate contact (Bloch, R. J., and B. Geiger, 1980, *Cell*, 21:25–35). We have used two cholesterol-specific cytochemical probes, saponin and filipin, to investigate the lipid nature of these membrane domains. When studied with freeze-fracture electron microscopy or fluorescence microscopy, these reagents reacted moderately and preferentially with the AChR-rich domains of AChR clusters. Little or no reaction with the membrane in "contact" domains was seen. In contrast, membrane regions surrounding the AChR clusters reacted extensively with filipin. These results suggest that, in rat myotubes, the composition or the state of the lipids differs between the two membrane domains of AChR clusters, and between clusters and surrounding membrane. In chick myotubes, AChR clusters do not appear to react with filipin or saponin, although surrounding membrane reacts extensively with these reagents.

The nicotinic acetylcholine receptor (AChR)[1] is distributed heterogeneously within the plasma membrane of vertebrate skeletal muscle. In normal adult muscle fibers, most of the AChR are localized at the postsynaptic membrane of the neuromuscular junction (13). In aneural cultures of vertebrate skeletal muscle cells, AChR also aggregate into limited regions of the plasma membrane (1, 3, 5, 12, 14, 24, 32, 43). Although the presence of nerve may influence the nature or extent of AChR aggregation, a muscle cell can distribute its AChR heterogeneously within its surface membrane even in the absence of nerve. Muscle cells may generate or stabilize these specialized surface structures by both intracellular (6, 16, 18–20, 36, 41, 42) and extracellular (2, 6, 9, 11, 21, 34) means. The relative importance of these influences, their relationship to AChR self-aggregation (e.g., reference 10), and the role of the lipid environment in AChR aggregation are still in question.

The large clusters of AChR that form in the plasma membranes of cultured rat myotubes are well suited to studies of AChR aggregation. These clusters form almost exclusively where the myotube membrane comes into close contact with the tissue culture substrate (2, 6). As a result, the clusters, visualized with a fluorescent derivative of α-bungarotoxin (α-Btx), almost always lie in a single plane of focus, allowing the fine structure to be more closely examined. As AChR clusters are localized at the cell-substrate interface, they may also be readily examined by complementary replica freeze-fracture (12, 30, 46) allowing ultrastructural studies of AChR distribution.

Largely as a result of light microscopic studies (but see Results, below), the following picture has emerged of the AChR clusters of cultured rat myotubes. (*a*) Clusters can be 20–30 μm across and as much as 50–100 μm long (3, 5). They often assume a rectilinear organization, in which strips of membrane ($\leq$1 μm in width) enriched in AChR alternate with membrane strips of similar size depleted of AChR. (*b*) Where they are enriched, AChR are organized as apparently regularly spaced smaller aggregates or "speckles" (e.g., Fig. 2 of reference 6). We refer to these areas of the membrane and the linear arrays they generate as "AChR domains." Within AChR domains the intramembrane particles identified with AChR (12) are regularly spaced (37). (*c*) The membrane strips depleted of AChR are closer to the tissue culture substrate than are the AChR domains and are likely to be involved in cell-substrate contact (6). We refer to these regions as "contact domains." (*d*) The intracellular cytoskeletal protein, vinculin, is enriched near contact domains, but not near AChR do-

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; α-bungarotoxin, α-Btx; IMP, intramembrane particle.

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mains of clusters (6). Because of their organization into distinct domains, AChR clusters of rat myotubes can be used to distinguish between factors primarily associated with contact regions, such as vinculin, and those likely to be more directly associated with AChR aggregation. Here we consider the possibility that cholesterol is preferentially associated with one of the two domains of AChR clusters.

Cholesterol has been localized in membranes using saponin or polyene antibiotics such as filipin. Both reagents form large complexes with cholesterol that are visible as distinct pits or protrusions in thin sections or freeze-fracture replicas (e.g., references 7 and 39; reviewed in reference 15). It has been generally presumed that cholesterol is localized in membrane regions where pits or protrusions form, and that it is depleted in membrane regions where they are absent. Using this technique, it has been argued that coated pits (27), sperm-head membrane (15), substrate-apposed membrane (8, 40) and smooth muscle membrane apposed to cytoskeletal structures (26) are devoid of cholesterol. Recently, the presence of cholesterol in AChR aggregates has been investigated in two laboratories. Perrelet et al. (33) showed that filipin-cholesterol complexes were absent from AChR aggregates but were present at the base of folds of the postsynaptic membrane of *Torpedo* electroplax. Nakajima and Bridgman (7, 28) showed that filipin or saponin complexes were absent from AChR aggregates in cultured *Xenopus* myotubes and at the postsynaptic membrane of neuromuscular junctions of *Rana*. In contrast to these results, we have found that saponin and filipin do deform the membrane of AChR clusters in rat myotubes, although deformation due to filipin was less extensive within AChR clusters than elsewhere in muscle membrane. We did not find deformations within AChR aggregates in chick myotubes, a result similar to that seen in *Torpedo* and *Xenopus*.

MATERIALS AND METHODS

Cultures of rat myotubes were prepared as described (5, 6). Medium consisted of Dulbecco-Vogt modified Eagle's medium supplemented with 10% (vol/vol) cadet calf serum (Biocell Laboratories, Carson, CA). Most experiments presented here were performed on myotube cultures used 7 d after plating. Although rich in myotubes, these cultures also contained fibroblasts, which we also examined. Chick myotube cultures were prepared from pectoral muscle of 12-d-old White Leghorn embryos as previously described (25, 38) and grown on coverslips coated with calf-skin collagen.

For saponin treatment, cultures were washed once with PBS and then once with buffered saline supplemented with 1% (wt/vol) bovine serum albumin, 10 mM $MgCl_2$, and 1 mM EGTA, pH 7.2 (Solution A). Cultures were then placed into saponin solution, which consisted of 0.2% (wt/vol) saponin, in Solution A. At times ranging from 15 s to 8 min after the saponin solution was introduced, the detergent solution was removed by aspiration and replaced with fixative. In early experiments, this consisted of 2% paraformaldehyde in buffered saline, followed by 2% glutaraldehyde (PolyScience Corp., Niles, IL) in 0.12 M cacodylate buffer, pH 7.2. In later experiments, samples were fixed directly in the glutaraldehyde solution. We found that the two fixation procedures were equally effective.

In other experiments, we examined the effects of filipin on myotube cultures. The procedure we followed was identical to that outlined above, but, instead of saponin, 0.06% filipin (Dr. Joseph Grady, Upjohn Co. Kalamazoo, MI) in a solution containing 1% dimethyl sulfoxide was used. In experiments using prefixed cells, identical protocols were followed, but cells were treated first with 2% glutaraldehyde in cacodylate buffer for 5 min at 22°C.

After exposure to experimental or control solutions and fixation, circles (4 mm diameter) were cut from the coverslips. Myotubes on the circles were cryoprotected by exposure to 33% (vol/vol) glycerol for 30 min to 2 h and frozen in barely melted Freon 22. They were freeze fractured at −119°C and < 10^{-6} torr with the complementary-replica method (31, 46). Replicas were cleaned in household bleach followed by dichromate-sulfuric acid cleaning solution and examined and photographed at 10–20,000× in a Philips 201 electron microscope. Replicas associated with the specimen carrier are highly enriched in cytoplasmic leaflets (P faces) of plasma membrane apposed to the tissue culture substrate. This membrane contains all the large clusters of AChRs made by rat myotubes. Cytoplasmic leaflets of fibroblast membranes are also found in the replicas.

Diameters of pits or protrusions were measured with a Zeiss MOP-30 digitizer. Magnifications were calibrated with a 2160 line/mm grating.

Calf skin collagen, albumin, and saponin were obtained from Sigma Chemical Co. (St. Louis, MO).

Fluorescence Studies: To visualize filipin-cholesterol complexes and AChR by fluorescence, we first labeled myotube cultures with monotetramethylrhodamine-α-Btx (R-α-Btx), as described previously (5). They were then gently sheared with a stream of ice-cold buffered saline containing 2% (wt/vol) paraformaldehyde. This treatment removed most of the myotubes and fibroblasts but left some large fragments of myotubes attached to the glass (6). These fragments often contained AChR clusters, in which both AChR domains and contact domains are recovered intact (6). "Sheared" preparations were fixed for an additional 15 min in the paraformaldehyde solution, then treated for 15 min in 0.1 M glycine in buffered saline, pH 7.2. Samples were then washed and treated with filipin, as described above, for 3 min, postfixed with 2% paraformaldehyde, mounted in 9 parts glycerol to one part 1 M Tris-HCl, pH 8.0, and observed in a Zeiss IM35 microscope equipped for fluorescence.

Filipin-cholesterol complexes have major absorption and emission bands at 357 and 480 nm, respectively (4). To observe these complexes in the fluorescence microscope, we selected the Zeiss filters (cat no. 487701) which allow excitation with the 365 nm Hg line and observation of emission between 450 and 490 nm. Observation was made with a 63×/N.A. 1.25 Plan Neofluar objective. Filipin-cholesterol complexes visualized with this apparatus appeared icy-blue in color. No crossover was evident in double-label experiments using filipin and R-α-Btx. Exposures were made for 30 s. HP5 film (Ilford, Basildon, Essex, Great Britain), used for fluorescence photomicrography, was processed to an ASA of 1200.

RESULTS

AChR Clusters of Rat Myotubes

When studied by freeze-fracture electron microscopy, rat myotube AChR clusters reveal a distinctive organization (Fig. 1). In agreement with light microscopic studies of these structures (6), clusters are rectilinear: regions enriched in irregular 10-nm particles (Fig. 1, *A*) interdigitate with regions depleted of such particles (Fig. 1, *C*). As the 10-nm particle contains AChR (12), the particle-enriched regions can be considered to be AChR domains (D. W. Pumplin and R. J. Bloch, manuscript in preparation). We assume that regions depleted of 10-nm particles represent contact domains. Both AChR and contact domains contain smaller intramembrane particles which, within contact domains, are randomly distributed. AChR particles within AChR domains are not randomly distributed, however. Instead, they are evenly spaced within the membrane at a concentration of 800–1,000/μm^2 (37).

To learn whether cholesterol is enriched in either AChR domains or contact domains, we incubated rat myotube cultures with saponin. Incubation was terminated by removal of the saponin solution and addition of a solution containing glutaraldehyde (see Materials and Methods). The following changes were observed in the AChR domains. After 15 to 30 s, membranes were altered only slightly. However, after 1 to 2.5 min, large-scale deformations appeared throughout the AChR domains and AChR particles aggregated (Fig. 2). The large-scale deformations consisted of irregularly outlined patches of membrane lying farther from the substrate than the surrounding membrane. In addition, we observed some areas which appeared to be membrane folded upward into the cell. Infoldings and large-scale deformations were seen only very occasionally in control or prefixed myotubes. Small pits or protrusions typically found in other cell types treated with saponin (7) were not seen in rat myotubes.

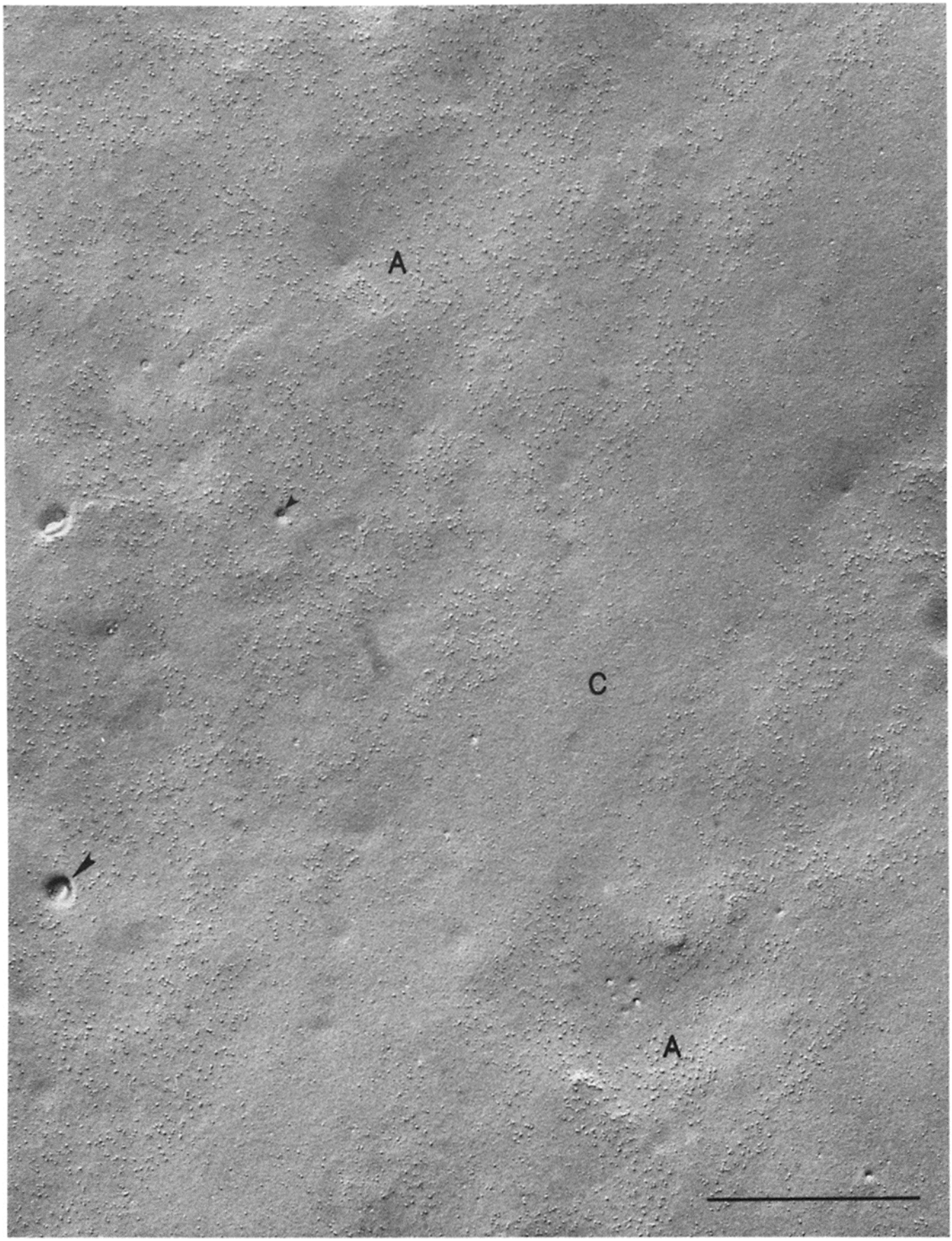

FIGURE 1 Portions of an AChR cluster on an untreated myotube. Areas identified as containing a high concentration of AChR, using a fluorescent derivative of α-bungarotoxin, are coincident with areas highly enriched in large angular intramembrane particles (12). These AChR domains (*A*) interdigitate with areas that lie close to the substrate, as identified by interference reflection microscopy (6). These contact domains (*C*) contain few large IMP. IMP within AChR domains are evenly distributed. Caveolae (small arrowheads) and larger depressions having the diameter of coated pits (large arrowhead) are occasionally seen. Bar, 1 μm. × 40,500.

In contrast to these changes in AChR domains, contact domains showed little or no evidence of pits, depressions or large-scale deformation. Likewise, no intramembrane particle (IMP) aggregation was seen within contact domains (Fig. 3, *A*). Some membrane regions distant from AChR clusters showed deformations attributable to saponin (not shown), but there was little IMP aggregation associated with these. All effects of saponin seen within AChR domains were prevented by prefixation with glutaraldehyde (Fig. 3, *B*).

Treating rat myotubes with filipin induced filipin-sterol complexes, visible as circular pits or protrusions 23.4 ± 3.6 nm (mean ± SD, $n = 69$) in diameter, similar to those reported in other membranes (7, 8, 15, 26–28, 30, 33, 39, 40). Complexes attained the highest density outside AChR clusters (Fig. 4). A lower density of complexes occurred in AChR clusters. Complexes were found more frequently, but not exclusively, within the AChR domains, and were associated with some aggregation of AChR particles (Fig. 4, *A*). Nonetheless, AChR particles are excluded from the filipin-sterol complexes themselves. Filipin-induced complexes were seen in myotubes fixed both before and after treatment. However, complexes formed in prefixed myotubes were almost solely protrusions spaced randomly within AChR domains (a few pits can be seen), while complexes in myotubes fixed after filipin treatment occurred as approximately equal numbers of pits and protrusions in close-packed arrays (Fig. 4, *A*).

We further investigated the correspondence of filipin-cholesterol complexes with AChR-rich membrane using fluorescence techniques. Friend (15) has suggested that membrane-bound filipin may be observed using the fluorescence microscope. Given the absorption and emission maxima of filipin-cholesterol complexes (4), we were able to select the appropriate filters to observe filipin fluorescence in intact myotubes (Fig. 5, *A*). The intensity of this fluorescence was too great to permit more detailed study of AChR clusters. To reduce the levels of background fluorescence, we removed the bulk of the cellular material from the myotube cultures with a stream of buffer containing 2% paraformaldehyde, and then reacted the material which remained attached to the substrate with filipin (see Materials and Methods). These sheared preparations, which contain fragments of AChR clusters (6), stain with filipin to a lesser extent than do intact cells. Nevertheless, background fluorescence, presumably due to binding of filipin to internal structures exposed by shearing, may still be significant. When a clear view is obtained of the filipin fluorescence originating in the plane of the membrane containing AChR clusters, there is a rough correspondence between AChR-rich regions (AChR domains) and regions showing filipin fluorescence (Fig. 5, *C* and *D*, arrows). Contact domains are usually poorly labeled with filipin (e.g., Fig. 5, *C* and *D*, arrowheads). If sheared, fixed clusters were first extracted with ethanol at −20° or with a solution containing 0.5% Triton X-100, subsequent staining with filipin was greatly reduced (not shown). AChR prestained with R-α-Btx were not affected by these extractions. This suggests that filipin fluorescence from sheared but unextracted receptor clusters results from the interaction of filipin with structures or materials resembling lipids. These results are in agreement with the observations, made with freeze-fracture electron microscopy, that filipin interaction with AChR clusters occurs preferentially in the AChR domains.

Rat myotubes treated with the metabolic-energy inhibitor sodium azide lose AChR from clusters (5). This treatment also reduces particle density in AChR domains and causes the particles to shift from an even to a random distribution within AChR domains; nonetheless, boundaries between AChR and contact domains are preserved (D. W. Pumplin and R. J. Bloch, manuscript in preparation). Myotubes treated with 5 mM NaN_3 for 4 h were fixed, exposed to filipin, and freeze-fractured. Clusters partially disrupted by azide, as shown by the microclusters of particles within AChR domains (Fig. 6, arrowhead) retained sharp boundaries between AChR and contact domains. Filipin-induced complexes were much more prevalent in AChR domains than in adjacent contact domains, a distribution pattern similar to that in control myotubes. These results suggest that conditions which alter the distribution of AChR within clusters do not alter the heterogeneous pattern of protrusions caused by filipin.

The results reported above suggest that saponin and filipin react preferentially with the AChR-rich regions of AChR clusters. This is in sharp contrast to the results obtained in studies of AChR aggregates in *Xenopus*, *Rana*, and *Torpedo* (7, 8, 28, 33). To check the reliability of our observations, we examined the effects of saponin and filipin on the plasma membranes of two other cells, rat fibroblasts, and chick myotubes.

Rat Fibroblasts

Fibroblasts contaminate the cultures of rat myotubes we normally use, and thus could be studied in the same replicas as those containing AChR clusters. The effects of saponin and filipin on fibroblasts are very different from their effects on myotubes, however. (*a*) Both reagents cause extensive formation of protrusions, but do not induce large-scale deformations (Fig. 3). Saponin-induced protrusions are 30.4 ± 3.2 nm (mean ± SD; $n = 70$) in diameter, while filipin-induced protrusions are 23.9 ± 3.2 nm in diameter ($n = 74$). (*b*) Protrusions induced by saponin (Fig. 7, *C*) or filipin (not shown) aggregate at later times (1–3 min). (*c*) Pitting of fibroblast membrane induced by saponin (Fig. 7, *A*), but not by filipin (Fig. 7, *D*), is prevented by prefixation with glutaraldehyde. Prefixation does prevent aggregation of IMP and of filipin-induced protrusions, however (Fig. 7, *D*). Our results indicate that cholesterol-specific reagents do not readily reveal heterogeneous membrane domains in fibroblast cells.

Chick Myotubes

Myotubes prepared in culture from embryonic chick muscle also display aggregates of AChR. These AChR aggregates differ from those seen in rat myotubes, as they consist of densely packed AChR IMP (12) and appear on both the upper and lower surfaces of myotubes. In these respects, they resemble the AChR aggregates seen in cultures of *Xenopus* myocytes (7, 32). To learn whether chick myotubes more closely resemble those of *Xenopus* or rat in their response to cytochemical probes for cholesterol, we treated chick myotubes with saponin or filipin and examined them with freeze-fracture electron microscopy. We found the following. (*a*) Both filipin (Fig. 8) and saponin (not shown) caused extensive pitting of chick myotube membrane, but pitting never occurred within regions of the membrane containing AChR aggregates. (*b*) Pit formation by saponin (data not shown), but not by filipin, was prevented by prefixation with glutaraldehyde (Fig. 8, *A*). Filipin applied after fixation induced protrusions almost exclusively (Fig. 8, *A*), while filipin applied before fixation re-

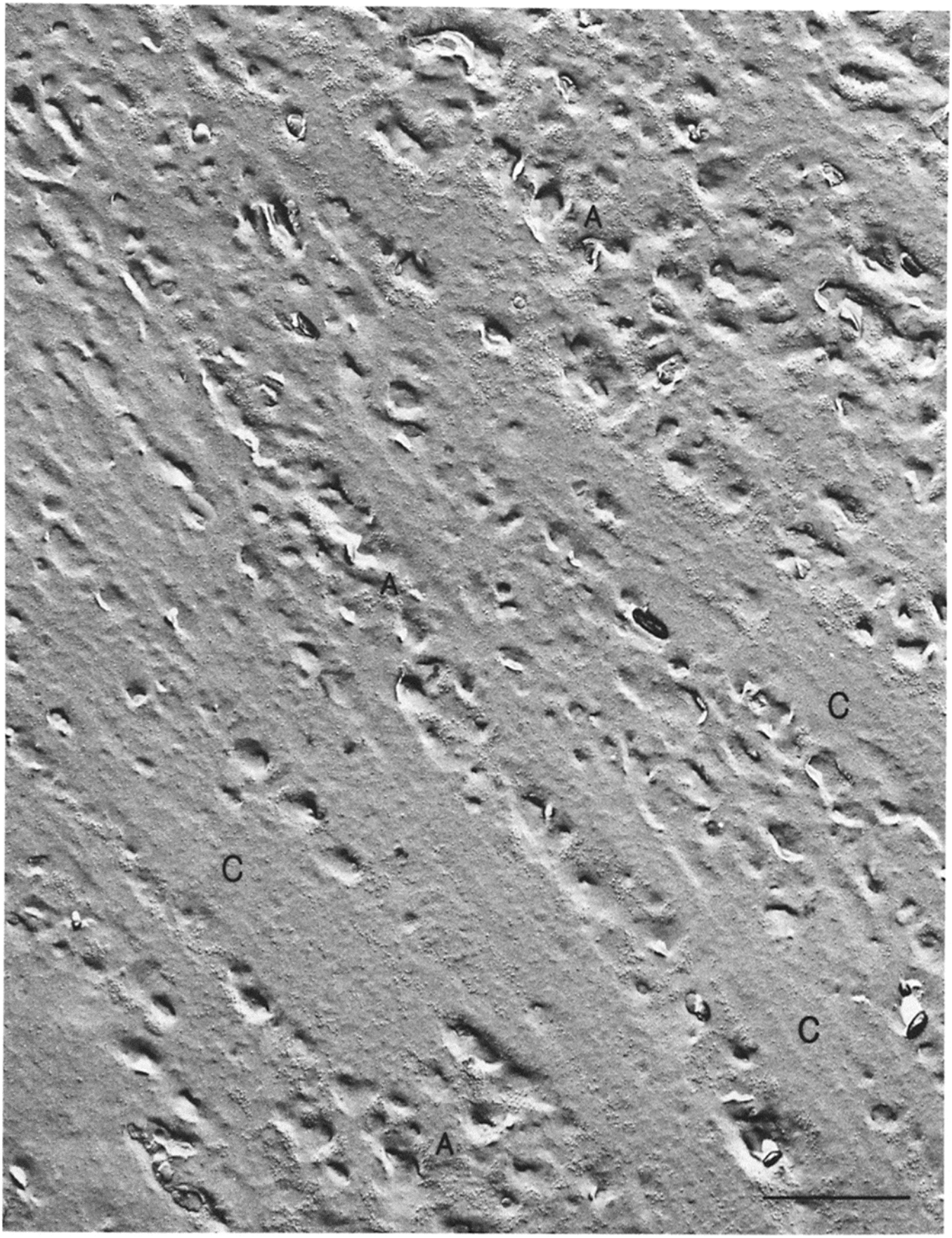

FIGURE 2 Rat myotube treated with saponin. Rat myotube cultures were treated with 0.2% saponin in a modified buffered saline (see Materials and Methods) for 3 min at 22°C. Cultures were then fixed and processed for freeze-fracture. The AChR cluster pictured here shows large-scale deformations in AChR domains (*A*) and aggregation of the large IMP representing AChR. Contact domains (C) appear unaffected. This low magnification micrograph shows that deformations occur within most or all of an extensive AChR cluster. Note the linearity of both AChR and contact domains. Bar, 1 μm. × 28,000.

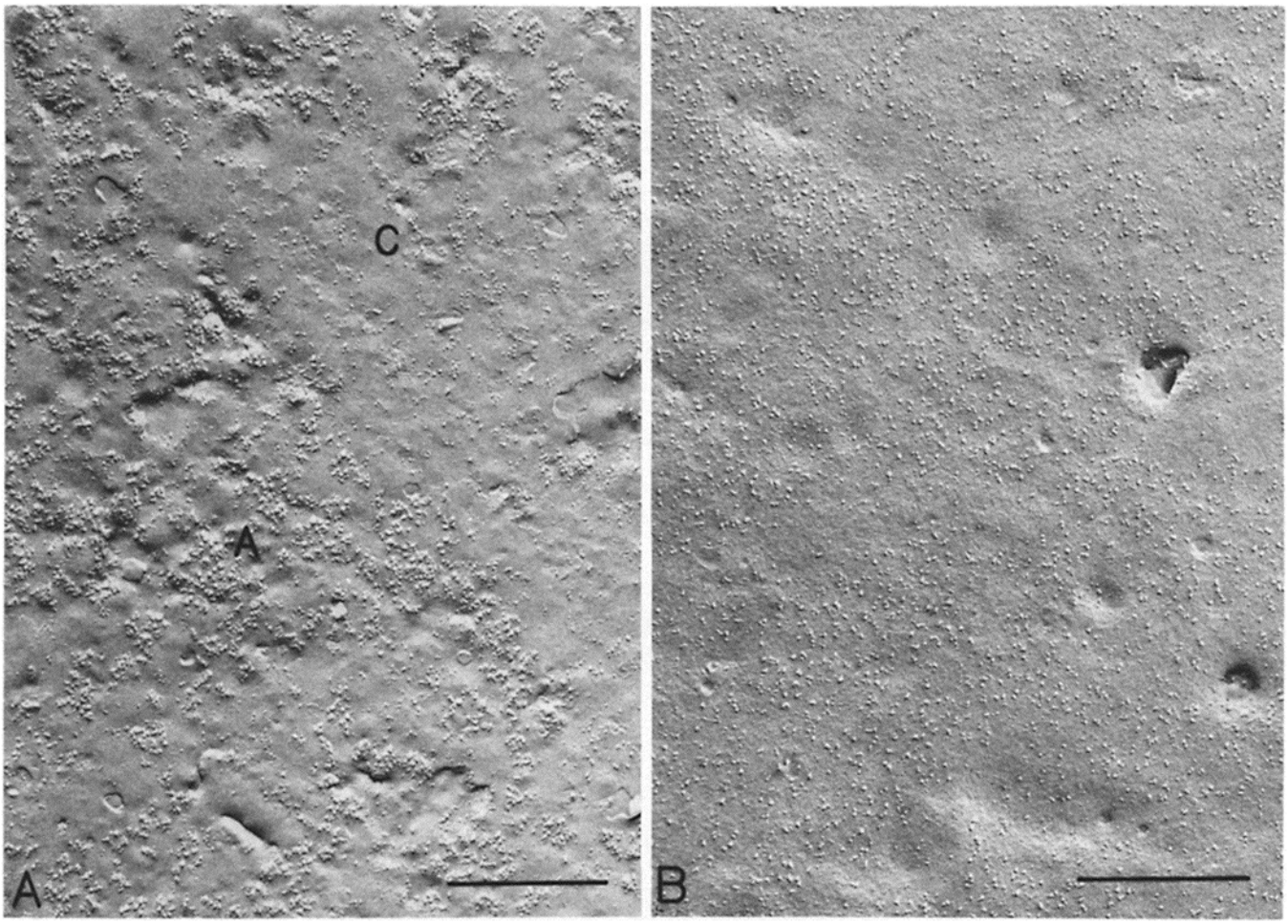

FIGURE 3 (Panel *A*) Rat myotube treated with saponin. Conditions are those of Fig. 2. The higher magnification illustrates aggregation of large particles in AChR domains (label *A*) while particles in contact domains (*C*) are apparently unaffected. Bar, 0.5 μm. × 44,000. (*B*) Rat myotube treated with saponin after fixation. Cells were fixed, then treated as in panel *A*. Saponin has no apparent effect on the AChR clusters of fixed cells. Bar, 0.5 μm. × 47,500.

sulted in approximately equal numbers of pits and protrusions (Fig. 8, *B*). Saponin applied before fixation also induced pits and protrusions in approximately equal numbers, while saponin applied after fixation had no apparent effect on the membrane. In their resistance to saponin- or filipin-induced membrane deformation, the AChR aggregates of cultured chick myotubes resemble the AChR aggregates of *Xenopus* myocytes and are distinct from the AChR domains within AChR clusters of cultured rat myotubes.

DISCUSSION

We initiated these experiments to learn whether membrane domains in AChR clusters of cultured rat myotubes differed in cholesterol. By reacting myotube cultures with the cholesterol-specific cytochemical reagents, saponin and filipin, and then examining the cultures by freeze-fracture electron microscopy, we were able to visualize membrane changes resulting from the interaction of cholesterol with the probes. We found that changes in AChR clusters induced by the probes were limited to membrane domains enriched in AChR (AChR domains). The contact domains, located between AChR domains, were relatively unaffected. Comparable results were obtained when filipin distribution was observed with the light microscope: fluorescence of filipin correlated reasonably well with membrane regions that labeled with α-Btx. It thus appears that, in rat myotubes, filipin and saponin react preferentially with cluster domains containing AChR.

We were concerned about the reliability of our results, for two reasons. First, only filipin caused extensive formation of clearly defined pits or protrusions in rat myotube membrane; saponin did not. Second, our observation that AChR clusters display filipin-cholesterol pits and protrusions differs from that reported by two other groups (7, 8, 28, 33). To determine whether our results with these reagents were consistent with those obtained by others, we examined two additional types of cells, rat fibroblasts and chick myotubes.

Complexes formed in rat fibroblast membranes within 1 min after addition of either filipin or saponin to cultures. At later times, the complexes were grouped and IMP aggregated at the edge of the groups. After prefixation with glutaraldehyde, filipin-induced protrusions continued to form, but neither protrusions nor particles aggregated. Our results with filipin are essentially identical to those that Robinson and Karnovsky (39) obtained with baby hamster kidney and LM cells and human polymorphonuclear leukocytes. The apparent alteration of protrusions into pits was not discussed by these workers but is evident in their micrographs. Because both filipin and saponin deformed membranes of rat fibroblasts, we conclude that their failure to deform contact do-

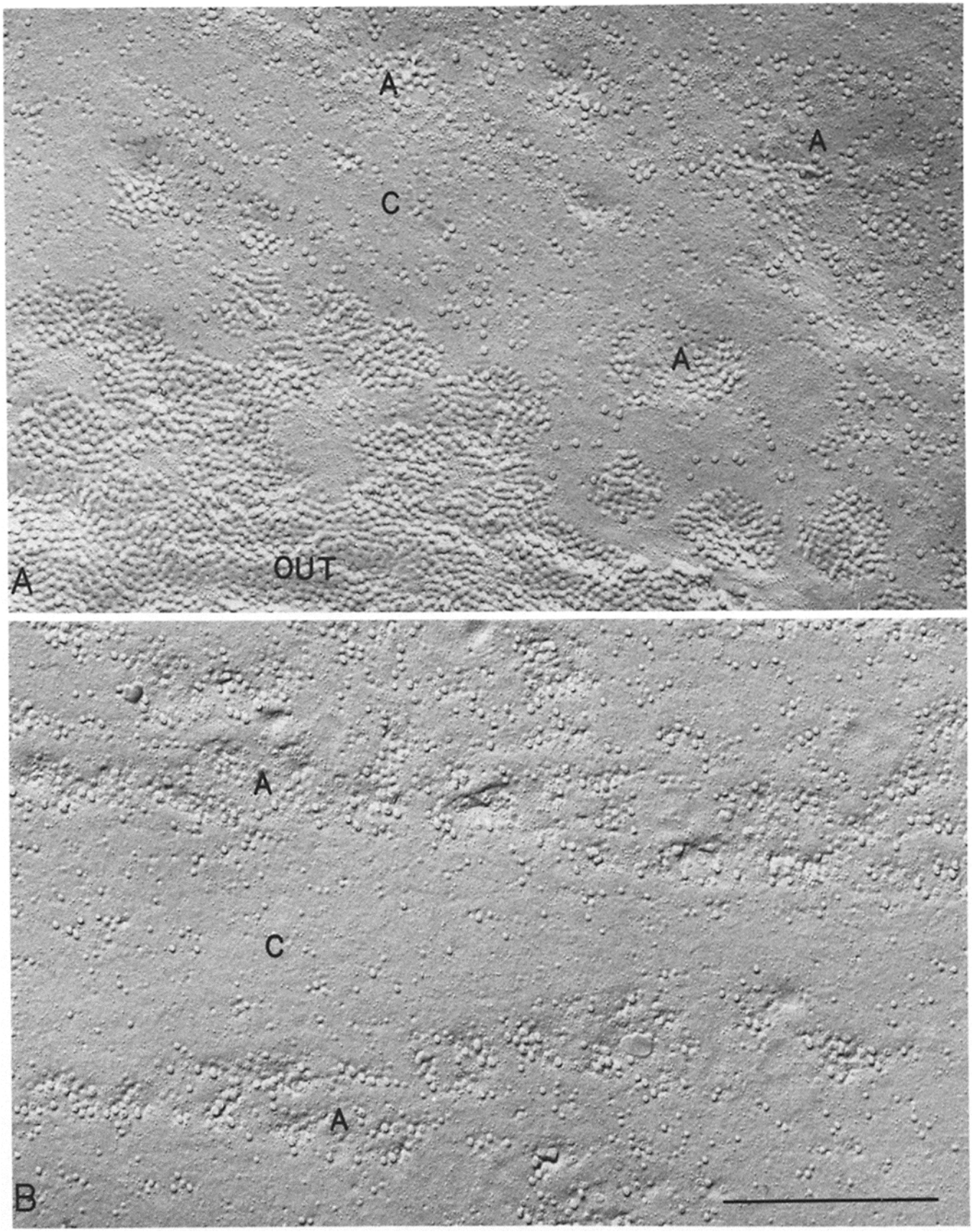

FIGURE 4 (*A*) Rat myotube treated with filipin prior to fixation. Cultures were reacted for 3 min at 22°C (see Materials and Methods), then fixed. Filipin-sterol complexes appear as pits or protrusions. They are closely packed at the lower left corner of the micrograph, in a membrane area (*OUT*) outside the AChR cluster. Within the cluster, complexes are much more prevalent in AChR domains (*A*), identified by their large IMP. Both filipin-sterol complexes and AChR are aggregated. The contact domains (*C*) have a much lower density of complexes. (*B*) Rat myotubes treated with filipin after fixation. Cells were fixed, then treated with filipin as in *A*. Filipin-sterol complexes are much more prevalent in AChR domains (*A*) than in contact domains, but they appear to be randomly distributed, and no IMP aggregation occurs. In prefixed myotubes, almost all complexes protrude from the P face of the membrane. Bar, 1 μm. × 40,000.

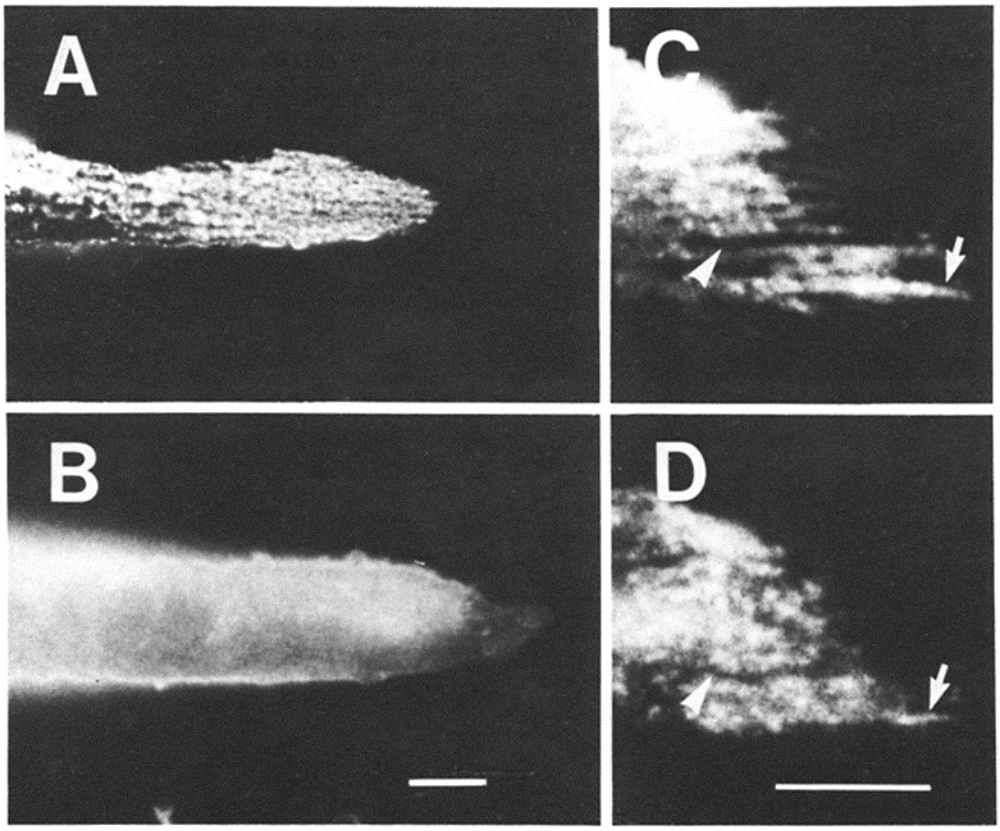

FIGURE 5 Fluorescence of membrane-bound filipin at AChR clusters. Rat myotube cultures were labeled with R-α-Btx, then sheared and fixed with 2% paraformaldehyde, and labeled with filipin (see Materials and Methods). Samples were mounted in a buffered glycerol solution and observed with the appropriate filters to visualize tetramethylrhodamine (*A* and *C*) and filipin (*B* and *D*) fluorescence. (*A* and *B*) A portion of an intact myotube, in which filipin fluorescence is high over large areas of the myotube surface, and perhaps intracellularly. (*C* and *D*) A fragment of a myotube, prepared by shearing, which displays clustered AChR arranged in a linear pattern (*C*). A similar, but not identical, pattern of filipin fluorescence is seen in the same fragment (*D*): AChR domains stain with filipin (arrows) while contact domains do not (arrowheads). Bars, 10 μm. (*A* and *B*) × 1,050. (*C* and *D*) × 2,100.

mains of AChR clusters in rat myotubes is probably due to chemical or structural properties of the myotube membrane in these regions. It is possible that the large-scale deformations observed in AChR domains exposed to saponin may be the result of additional strain of the lipid bilayer that results from the failure to form pits despite incorporation of the reagent into the membrane.

Filipin and saponin also induced membrane protrusions in chick myotubes, but these protrusions were excluded from the small areas of chick myotube membrane containing high concentrations of AChR. AChR clusters in chick myotubes, like those found in *Xenopus* myocytes (7, 8, 32), *Torpedo* electroplax (33) and at the neuromuscular junction (28), appear as paracrystalline arrays of membrane particles containing little, if any, particle-free membrane. Our finding that AChR aggregates of chick myotubes are not altered by reaction with filipin or saponin is therefore consistent with the results reported by Bridgman and Nakajima (7, 8, 28) and Perrelet et al. (33) for similar structures.

It should be noted that filipin-cholesterol complexes are less abundant within AChR clusters than elsewhere in rat myotube membranes, and that, where they are present, AChR IMP are excluded. In these qualitative ways, then, rat myotube receptor clusters resemble the AChR-rich membrane seen in muscle from other species. Nevertheless, the AChR clusters of rat myotubes display many more filipin-cholesterol complexes than do those of other muscle cells. It is not yet clear whether rat and chick AChR clusters differ in their cholesterol content, in their reactivity to cytochemical probes, or in their ability to deform. Other authors have pointed out that dense membrane particle arrays may obscure the pits or deformations formed by reaction of cholesterol with filipin (15). However, membrane deformations of similar size, such as caveolae and putative coated pits, are readily recognizable within AChR aggregates of chick myotubes (37).

There are several ways to interpret our observations on the preferential deformation of rat myotube AChR domains by saponin or filipin. The most straightforward explanation is that the distribution of filipin-cholesterol complexes truly reflects the distribution of cholesterol in the membrane. If this is correct, cholesterol in rat myotubes is reduced within AChR clusters relative to the surrounding membrane and is absent from contact domains of AChR clusters. The presence of cholesterol within AChR-rich membrane has also been suggested by biochemical analyses of preparations from electroplax tissue (17, 22, 35). If, indeed, cholesterol is present within the AChR domains of AChR clusters, it may be distributed asymmetrically between the two leaflets of the lipid bilayer (15, 30). This is suggested by the observation (Fig. 4, *B*) that in prefixed myotubes nearly all the protrusions induced by filipin point outward, towards the extracellular space. When applied to unfixed rat myotubes, filipin may induce movement of cholesterol between the lipid leaflets, indicated by reversal of curvature of some the complexes, and the lateral movement of complexes, indicated by a change from random to closest-packed arrangements of the complexes in membrane areas outside the clusters. Despite these rearrangements, complexes remain nearly absent from contact areas.

An alternative explanation for our observations is that, although cholesterol may be distributed equally between AChR and contact domains, only AChR domains can deform sufficiently to reveal the complexes formed between cholesterol and filipin or saponin. Coated pits (27), and smooth

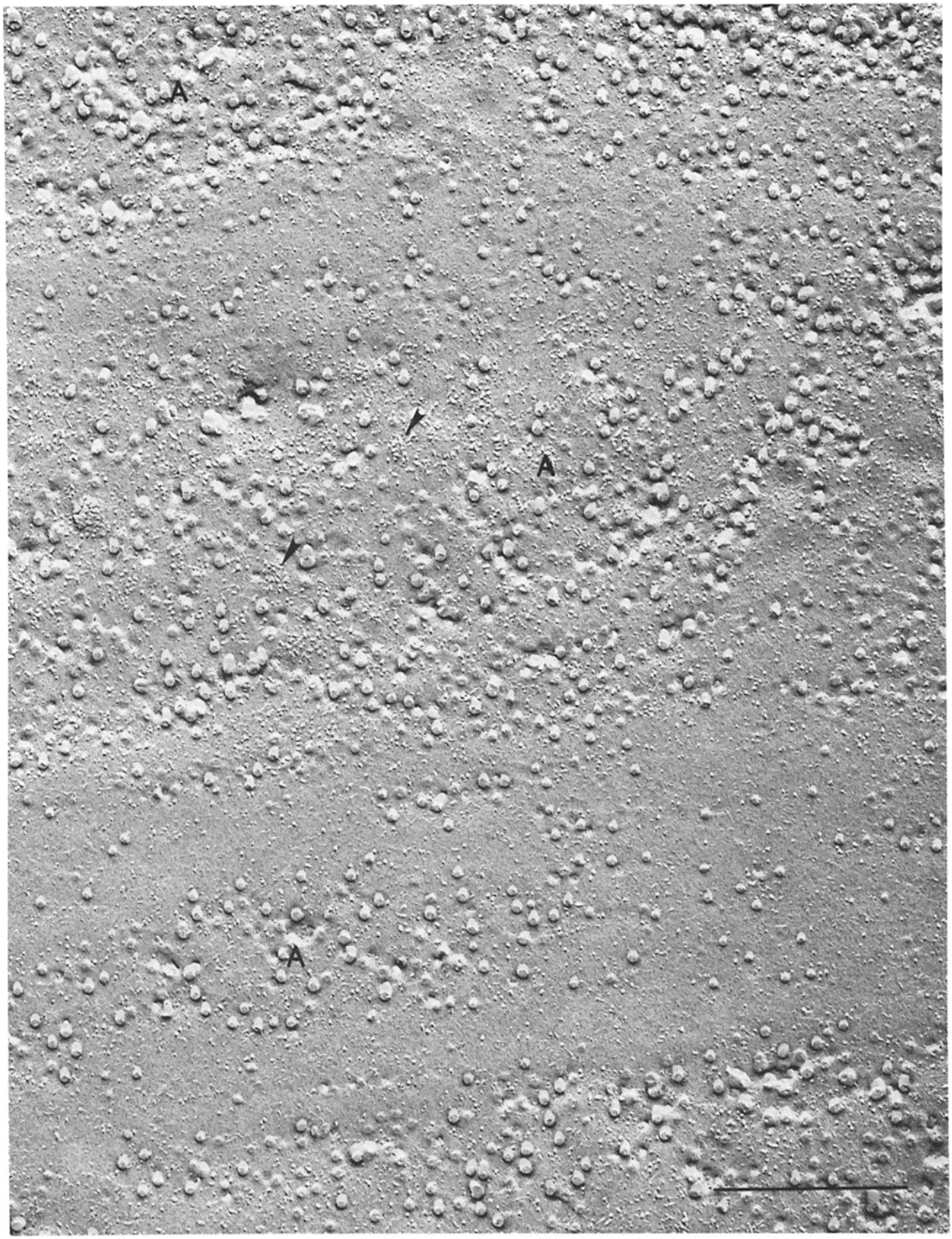

FIGURE 6 Effect of pretreatment with sodium azide on heterogeneous membrane distribution of filipin-induced protrusions. Myotube cultures were treated for 4 h with 5 mM sodium azide, to disperse AChR clusters partially (5). Samples were then fixed with glutaraldehyde and reacted with filipin, as described in Materials and Methods. Large particles within AChR domains (*A*) are randomly distributed with some resulting microclusters (arrowheads), indicating a partial disruption of the clusters by the azide. Protrusions due to filipin occur mainly within the AChR domains, whose boundaries are marked by a sharp decrease in the concentration of large particles. The distribution of filipin deformations, and the predominance of protrusions over pits, is similar to that in prefixed and control myotubes. Bar, 0.5 μm. × 73,000.

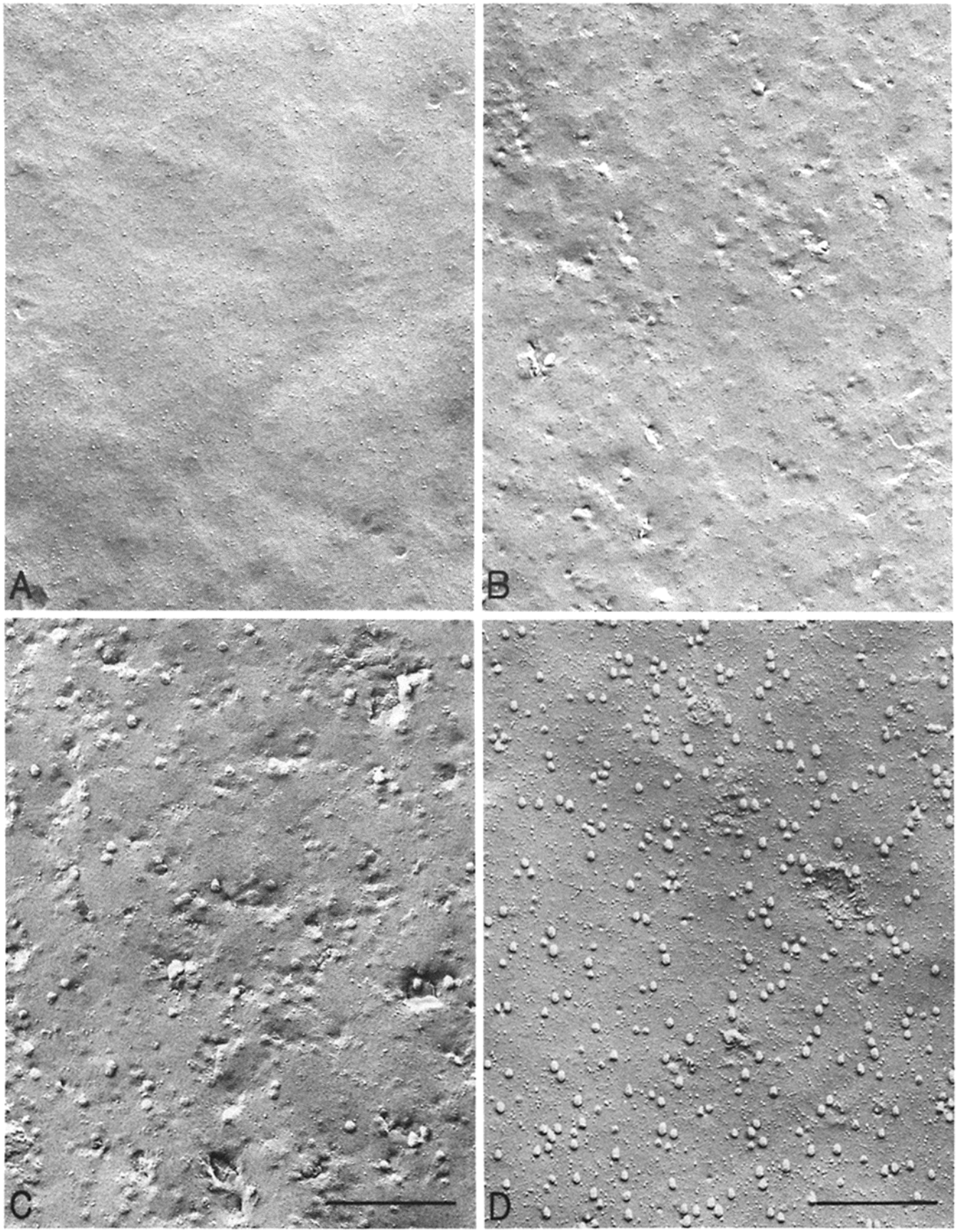

FIGURE 7 Rat fibroblasts treated with saponin or filipin. Fibroblasts present in muscle cultures were treated as described in Fig. 3 and 4. (*A*) Saponin-cholesterol complexes are not visible in prefixed fibroblasts. (*B*) Unfixed fibroblasts begin to show complexes after exposure to saponin for 15 s. (*C*) Complexes are well formed and partially aggregated after a 3-min exposure to saponin. (*D*) Prefixed fibroblasts exposed to filipin develop filipin-sterol complexes, which are distributed relatively evenly and consist almost entirely of protrusions from the P face. All micrographs are of P faces. Bars, 0.5 μm. (*A*, *B*, and *D*) × 44,000; (C) × 47,500.

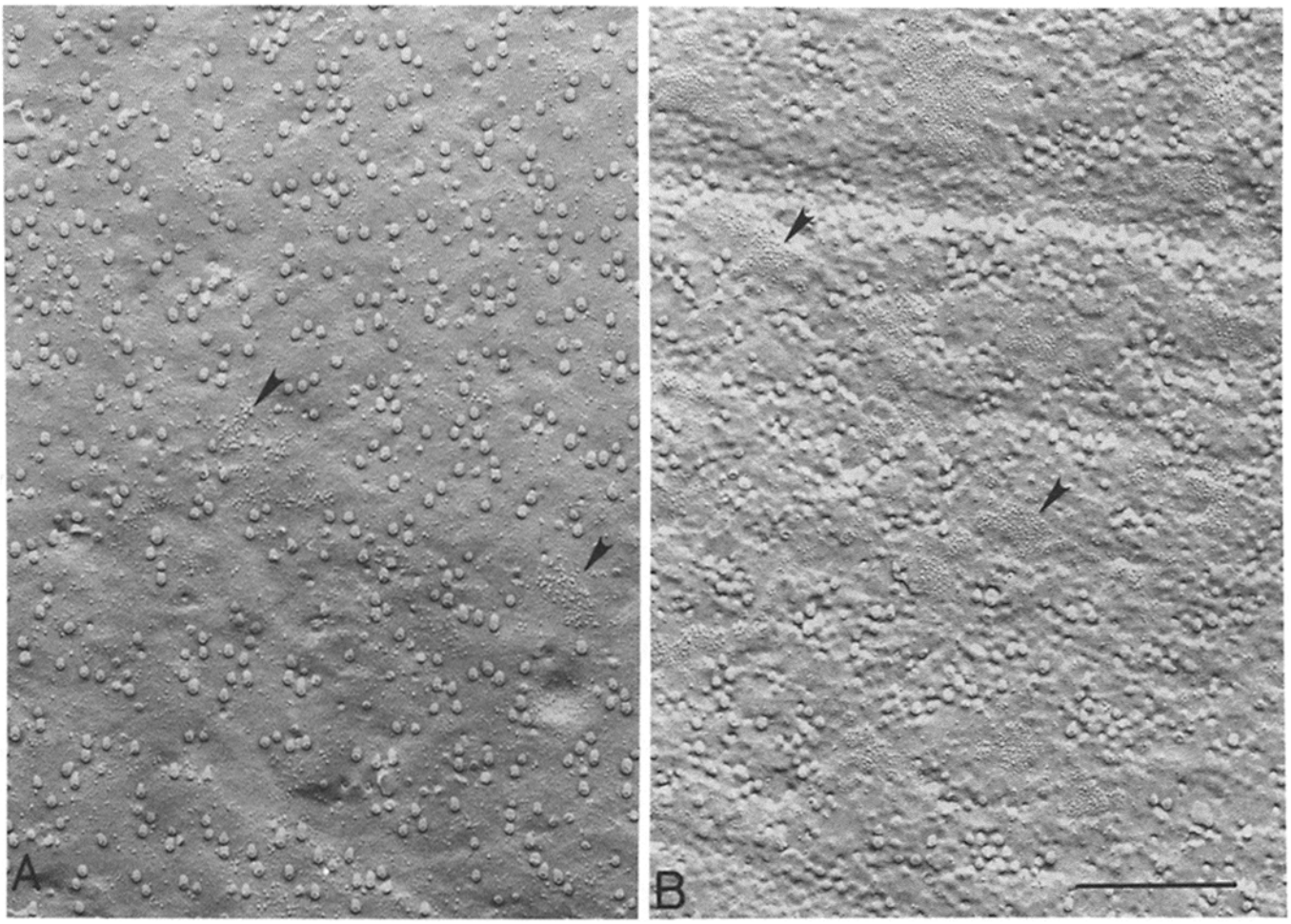

FIGURE 8 Chick myotubes treated with filipin after (*A*) and before (*B*) fixation. Treatments were as described in Figs. 3 and 4. In both cases filipin-sterol complexes are excluded from clusters of AChR, visible as tightly packed groups of large IMP (arrowheads). Complexes appear to be evenly distributed through the remaining membrane. In prefixed myotubes, complexes appear almost always as protrusions from the P face (*A*), while in myotubes fixed after filipin treatment (*B*), the complexes form pits and protrusions in approximately equal numbers. Bar, 0.5 μm. × 44,000.

muscle membrane apposed to cytoskeleton (26), are specialized membrane regions that are also resistant to deformation or pitting by cholesterol-specific probes. The contact domains of rat myotubes are probably constrained extracellularly by proximity to the tissue culture substrate and intracellularly by extensive cytoskeletal structures (2, 6, 36). In addition, membranes mediating substrate contact in other cells do not show significant pitting in response to filipin (8, 40). However, the observation that filipin-induced pits occasionally form within contact domains, even in prefixed myotubes, suggests that the membrane in that region is neither completely free of cholesterol, nor completely resistant to deformation. Another argument against this possibility is the fact that fluorescence signals resulting from filipin incorporation into AChR clusters are intense in AChR domains but faint in contact domains (Fig. 5). If the intensity of this signal accurately reflects filipin concentration, then there is probably little filipin within contact domains. It therefore seems unlikely that the membrane fails to deform despite high levels of filipin incorporation.

A third explanation for the observed heterogeneity is that the two domains within AChR clusters contain similar amounts of cholesterol, but that addition of filipin or saponin induces the movement of cholesterol from contact domains into AChR domains. For this to occur, one of several conditions must be met. (*a*) The probes may be preferentially taken up by AChR domains by virtue of the unique phospholipid or protein composition of these domains. The great specificity of filipin for cholesterol (23, 29) makes this unlikely. (*b*) Alternatively, filipin or saponin may bind to cholesterol in contact domains and the bound sterol may then migrate into AChR domains. The reaction time (>30 s) we used is sufficient for diffusion of cholesterol, or of cholesterol bound to filipin or saponin, across the ~1-μm distances separating AChR domains from contact domains, assuming diffusion coefficients on the order of 5×10^{-8} cm^2/s (45). We think it unlikely that the fully formed complexes of cholesterol with the probes (seen as pits or protrusions) can diffuse from contact domains to AChR domains, as prefixation prevents the movement of complexes required for their aggregation.

A fourth possibility is that, although the two domains may contain equal amounts of cholesterol, filipin may have physical access only to the cholesterol within AChR domains (8). For example, aqueous filipin may not be able to penetrate the small distances between the contact domain membrane and the glass substrate. This seems an unlikely possibility, especially as a heterogeneous filipin distribution was also observed when filipin was applied from the cytoplasmic membrane face (Fig. 5). Alternatively, the bulk of the cholesterol in contact

domains may be clustered (44) and thus inaccessible to filipin. The latter alternative would imply a significant difference in the state of the membrane lipids of AChR and contact domains.

We may be able to evaluate the relative importance of these possible mechanisms by using other experimental approaches. First, if cholesterol is indeed organized heterogeneously, other lipids may also differ in their distribution between AChR and contact domains. To study this, experiments with fluorescent lipid probes are now in progress. Second, if constraints imposed by intracellular structural elements prevent deformation of the membrane by filipin or saponin, drugs that disrupt the cytoskeleton may alter the ultrastructural effects of these probes on AChR and contact domains. Finally, an understanding of the lipid structures within cluster domains would be significantly advanced if the lipid composition of the AChR cluster could be determined. This should soon be possible. Extensive treatment of rat myotube cultures with saponin, under conditions similar to those described here, causes shedding of the bulk of cellular material into the detergent solution but leaves most of the AChR cluster attached to the tissue culture substrate (R. J. Bloch, manuscript in preparation). Chemical and ultrastructural analyses of such isolated AChR clusters are now in progress.

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