Disruption and Reformation of the Acetylcholine Receptor Clusters of Cultured Rat Myotubes Occur in Two Distinct Stages

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Abstract. We have examined the redistribution of acetylcholine receptor (AChR) intramembrane particles (IMPs) when AChR clusters of cultured rat myotubes are experimentally disrupted and allowed to reform. In control myotubes, the AChR IMPs are evenly distributed within the AChR domains of cluster membrane. Shortly after addition of azide to disrupt clusters, IMPs become unevenly scattered, with some microaggregation. After longer treatment, IMPs are depleted from AChR domains with no further change in IMP distribution. Contact domains of clusters are relatively poor in IMPs both before and after cluster dispersal. Upon visualization with fluorescent α-bungarotoxin, some AChR in azide-treated samples appear as small, bright spots. These spots do not correspond to microaggregates seen in freeze-fracture replicas, and probably represent receptors that have been internalized. The internalization rate is insufficient to account completely for the loss of IMPs from clusters, however.

During reformation of AChR clusters upon removal of azide, IMP concentration in receptor domains increases. At early stages of reformation, IMPs appear in small groups containing compact microaggregates. At later times, AChR domains enlarge and IMPs within them assume the evenly spaced distribution characteristic of control clusters.

These observations suggest that the disruption of clusters is accompanied by mobilization of AChR from a fixed array, allowing AChR IMPs to diffuse away from the clusters, to form microaggregates, and to become internalized. Cluster reformation appears to be the reverse of this process. Our results are thus consistent with a two-step model for AChR clustering, in which the concentration of IMPs into a small membrane region precedes their rearrangement into evenly spaced sites.

LARGE aggregates of membrane proteins are found in a wide variety of cellular membranes, but the mechanisms controlling aggregate formation are still poorly understood. Some aggregates are assembled in intracellular membranes and then inserted into the plasma membrane (25, 42). Others form in response to extracellular stimuli by micropatching of plasmalemmal proteins and subsequent agglomeration into limited regions, or "caps" (35, 40). In the nervous system, aggregates of neurotransmitter receptors in the membrane of the postsynaptic cell underlying the site of innervation have been described at several synapses (18, 21, 41). The aggregation of postsynaptic acetylcholine receptors (AChRs)[1] during synaptogenesis in vertebrate skeletal muscle has been extensively studied (1, 2, 5, 12, 13, 16, 17, 19, 23, 32, 36, 37, 44; for reviews see references 18 and 38). Similar investigations have been made of AChR aggregates which form in aneural cultures of vertebrate muscle (e.g., 1, 2, 4, 6, 15, 16, 19, 22, 33, 43). In the rat, these aggregates are very similar to those that appear at the developing neuromuscular junction (for review see reference 38).

A variety of light and electron microscopic techniques have been applied to the study of the large AChR aggregates, or "clusters," which form at the sites of myotube–substrate contact in cultures of rat myotubes (3, 11, 22). These clusters are composed of two interdigitating membrane domains: AChR domains, which are rich in receptor, and contact domains, which are poor in receptor, lie closer to the tissue culture substrate, and are believed to be involved in attaching the myotube to the substrate (11). Within the AChR domains, large, irregular intramembrane particles (IMPs) characteristic of AChRs (16) are distributed evenly within the plane of the membrane (29). The average distance between particles, 30–35 nm (29), is too great to be spanned by the AChR polypeptides themselves, so the regularity of interparticle spacing must be due to extrinsic factors. These factors can be altered by exposing myotubes to sodium azide, to carbachol, or to medium lacking Ca^{2+}, all of which disrupt AChR clusters in a reversible fashion (6, 7, 9).

We have used a combination of fluorescence microscopy

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; IMP, intramembrane particle; R-BT, monotetramethylrhodamine-α-bungarotoxin.

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and freeze-fracture electron microscopy to study the distribution of IMPs within AChR domains during cluster disruption and reformation. This approach has allowed us to visualize changes in receptor distribution at the molecular level, a level of resolution which was not possible in earlier studies. We have found that cluster disruption by azide proceeds by release of most AChRs from the evenly spaced array, resulting in random or microaggregated distributions of IMPs, and a net loss of IMPs from receptor domains. Cluster reformation proceeds by enrichment of IMPs into randomly scattered or microaggregated arrays within AChR domains, followed by rearrangement into more evenly spaced sites. Our results are consistent with the idea that clustered AChRs in control cells are bound to a lattice which is altered or destroyed when clusters are dispersed by azide.

Materials and Methods

Primary cultures of myotubes were prepared from minced lower leg muscles of neonatal rats. Cells were grown on glass coverslips in Dulbecco-Vogt modified Eagle's medium (medium) supplemented with 10% (vol/vol) calf serum, or 10% calf serum and 5% FCS, as previously described (6, 11). Myotubes were used after 6–8 d in culture. For some experiments, myotubes were removed from coverslips by treatment with pancreatin, replated, and used 1–2 d later (11). Replated cultures were nearly devoid of fibroblasts and contained myotubes with large AChR clusters at their sites of attachment to the substrate. The use of such cultures facilitated experiments in which replicas of areas mapped for fluorescence were examined. In these experiments, myotubes were labeled (6) with monotetramethylrhodamine-α-bungarotoxin (R-BT), prepared as described (31). Clusters were identified under the fluorescence optics and photographed. After treatment with azide (see below), the same clusters were rephotographed and the myotubes were fixed for freeze-fracture.

To disrupt AChR clusters partially, myotubes were treated for 1–4.5 h with 5 mM sodium azide (6), added to medium containing 5% calf serum or FCS. For studies of cluster reformation, cultures were incubated with 5 mM sodium azide for 6–6.5 h to induce almost complete cluster loss. The azide was then washed out, and clusters were allowed to reform for 4.5, 6, or 18 h before fixation and processing for freeze-fracture.

Cultures were fixed with 5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2, and freeze-fractured by the complementary replica technique (43), described previously (16, 28, 29). Replicas containing cytoplasmic leaflets (P faces) were floated off the specimen carriers with 1:2 (vol/vol) glycerol/water, cleaned with household bleach followed by dichromate-sulfuric acid cleaning solution, and picked up on slot grids coated with Formvar and carbon.

As previously noted (16), the pattern of myotubes in the original culture is visible in the replicas, allowing relocation of areas which in some samples had previously been identified by fluorescence. Fluorescence and replica features were matched with the aid of phase-contrast images of the original myotubes and extensive montages of replicated myotube membrane. Light and electron microscope magnifications were calibrated with standards (100 and 2,160 lines/mm, respectively).

The concentration and distribution of particles were determined by counting IMPs in micrographs enlarged to 79,000×, using an overlay grid ruled in squares (40.3 mm^2), as described previously (29). The size of the square was chosen to be compatible with the short-range order which was visually apparent from the micrographs. This sized square contained a mean of 5–6 particles. If larger squares were used which contained a mean of 12–13 particles, deviations from the Poisson were not statistically significant because a single AChR domain contained too few squares. For each micrograph, 1,000–2,000 particles were counted in a group of contiguous squares. Particles were not counted in squares containing caveolae or other membrane irregularities. The number of particles in each square was recorded and a histogram was constructed of the number of squares containing 0, 1, 2, . . . , n, particles. Such a histogram will have a Poisson distribution if the particles are distributed randomly. A more sharply peaked histogram indicates an even distribution of particles, while a broader histogram indicates microaggregation. The observed histograms were compared with a Poisson distribution having the same mean, using the chi^2 test on the cumulative form of the histogram.

To measure the diameters of particles perpendicular to the direction of shadowing, micrographs were taken at 70,000×, enlarged to 175,000×, and examined using a digitizing tablet and appropriate software (Bioquant; R & M Biometrics, Inc., Nashville, TN).

Quantitative fluorescence measurements were performed as follows. Myotube cultures were treated with 5 mM sodium azide for different periods of time, and were then labeled with R-BT for 15 min at ambient temperature. Substrate-attached material was then prepared by extraction with saponin for 5–10 min (reference 8, method I, as modified in reference 10). This material contains most of the AChR clusters of control myotube cultures and the remains of clusters seen in azide-treated cultures (8, 10). The resulting preparations were fixed in fresh, ice cold 2% paraformaldehyde in buffered saline, mounted in 90% glycerol, 10% 1 M Tris-HCl, pH 8, and observed with a Zeiss IM35 fluorescence microscope under rhodamine optics. The fluorescence from regions of AChR-rich membrane 5 μm in diameter was recorded using a photomultiplier and an I-V convertor (10).

Results

The IMPs representing AChR molecules in the large, substrate-apposed AChR clusters of control rat myotubes are evenly spaced: that is, the concentration of IMPs across an AChR domain is more constant than would occur if the particles were distributed randomly (29). If this spacing of IMPs is the result of interactions of the receptors with a lattice-like structure in or next to the membrane, then disruption of AChR clusters could occur by one of several mechanisms: (*a*) movement of sections of the lattice with their bound IMPs to other parts of the cell; (*b*) gradual release of IMPs from the lattice without affecting its basic structure; (*c*) loss of organization of the lattice, with simultaneous release of most of the IMPs. We used freeze-fracture electron microscopy to examine rat myotubes that had been treated with sodium azide to disrupt their AChR clusters and found that the third alternative best accounted for the changes we observed in IMP distribution.

Qualitative Evaluation of Cluster Disruption

Controls. Some of the characteristics of the AChR clusters of rat myotubes have been reported in previous studies from our laboratories (28, 29). In this study, four representative clusters on control myotubes labeled with R-BT were identified and mapped completely to the corresponding freeze-fracture replica. The close match confirmed that receptor-rich AChR domains accurately coincided with areas having a high concentration of large, irregular IMPs. The interdigitating contact domains were low in fluorescence and particle concentration (Fig. 1). Domain boundaries were sharply defined both by fluorescence and, in replicas, by a sudden change in particle concentration. In some myotubes, the membrane of contact domains was distinctly smoother than that of AChR domains. When this was observed, the boundaries between the undulating and smooth membrane coincided with the changes in particle concentration.

Azide. We studied freeze-fracture replicas of 12 clusters from four myotube cultures exposed to 5 mM sodium azide for 4–4.5 h. Nine additional clusters from three cultures were observed by fluorescence microscopy before and after azide treatment, then mapped to replicas. In all cases, treatment of myotubes with azide for 4–4.5 h induced a loss of fluorescence from AChR domains (Fig. 2), as previously described (6). The corresponding freeze-fracture replicas showed that cluster disruption by azide also involved loss of IMPs from AChR domains (Fig. 3).

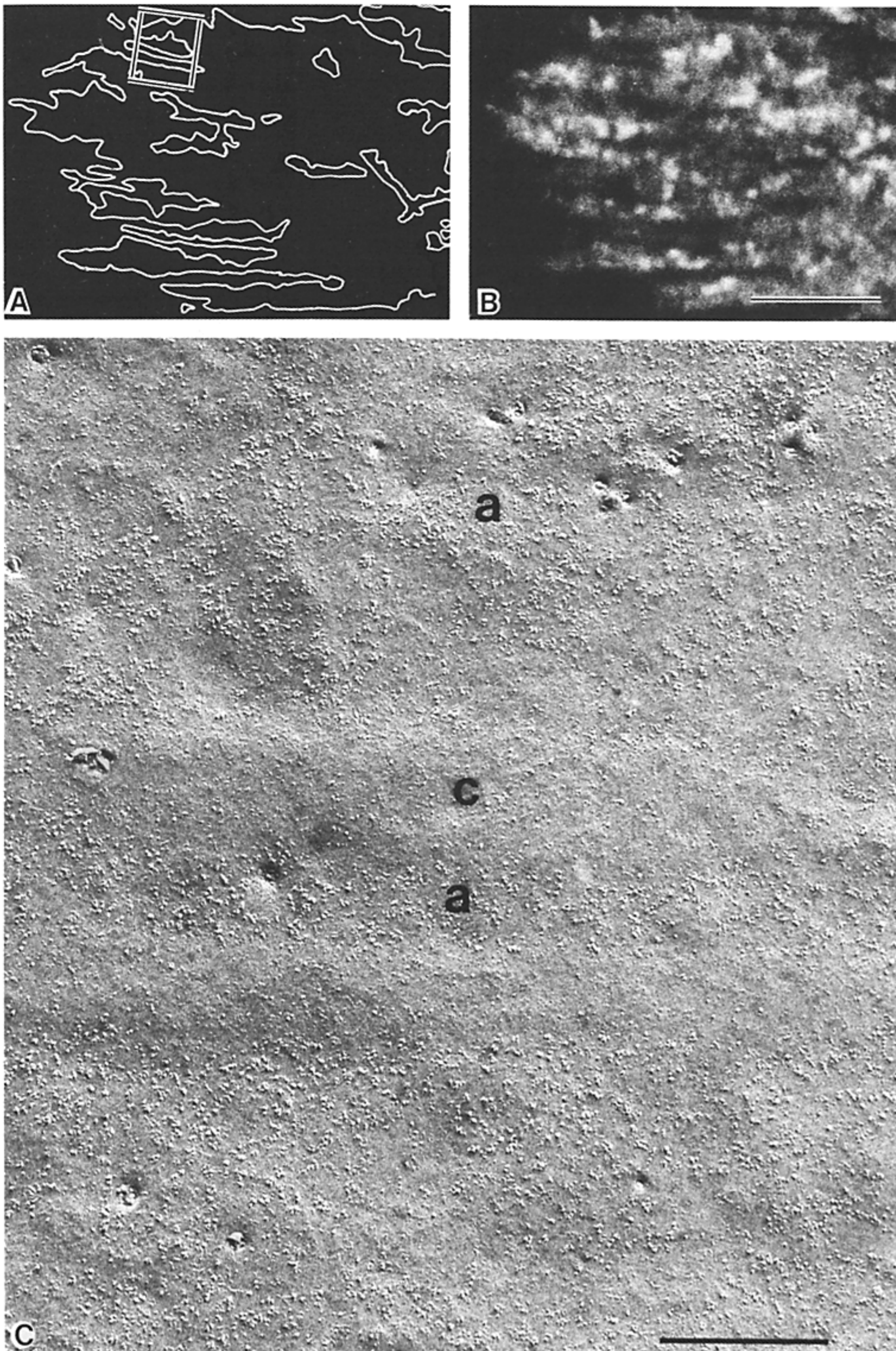

Figure 1. AChR cluster of a control rat myotube. Rat myotubes were stained with R-BT, fixed in formaldehyde, and observed by fluorescence microscopy. An AChR cluster was photographed under rhodamine illumination (*B*). The cluster was then frozen, fractured, and replicated. Differences in the fluorescence and IMP content indicate contact domains and AChR domains (*c* and *a* in *C*). Boundaries between domains were traced from a montage of electron micrographs and photographically reduced to the size of the fluorescent image (*A*). The boundaries seen by fluorescence and freeze-fracture accurately correspond. The area of the replica shown in *C* represents the square in *A*. Bar, (*B*) 5 μm; (*C*) 0.5 μm.

Both the loss of fluorescence and the loss of IMPs reduced the contrast between adjacent AChR and contact domains, making it more difficult to determine the exact boundaries between these domains. Nonetheless, these boundaries could often be distinguished in azide-treated myotubes. For the myotube shown in Figs. 2 and 3, transitions between undulating and smooth membrane, together with differences in particle concentrations, demarcated the domain boundaries. The presence of these boundaries in both fluorescence images and replicas indicated that contact domains were preserved during this treatment, despite the loss of fluorescence and IMPs from AChR domains. Moreover, the locations of contact domains were preserved, as seen by the match of boundaries seen in freeze-fracture replicas after azide treatment to the same boundaries seen by fluorescence before treatment (Fig. 2). The same preservation of contact domains was also seen in four other clusters by comparison of fluorescence before and after azide treatment.

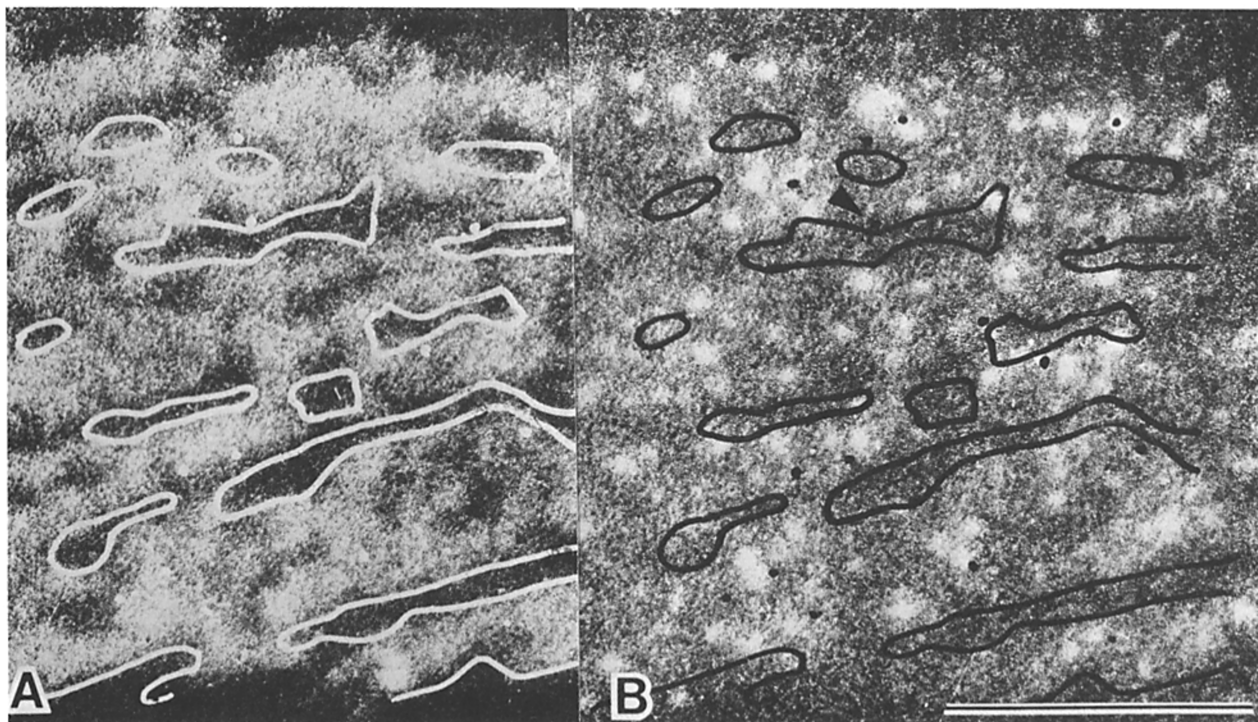

Figure 2. AChR cluster labeled with R-BT and photographed before and after azide treatment. Myotube cultures were labeled with R-BT and observed under rhodamine optics. Cells were chosen which showed large, clear AChR clusters; these were photographed under phase-contrast and fluorescence illumination. Cultures were then incubated in 5 mM sodium azide for 4.5 h. Cells were relocated and photographed again; then frozen, fractured, and replicated. (*A*) Before treatment with azide, bright AChR domains interdigitate with dark contact domains. (*B*) After treatment with azide, contrast between the domains decreases due to loss of fluorescence from AChR domains. Despite this, contact domains remain darker than adjacent AChR domains and the positions of contact domains are largely unchanged. Small bright spots of fluorescence appear within the AChR domains. Fluorescence boundaries agree with domain boundaries (lines in *A* and *B*) derived from the corresponding freeze-fracture replica, a portion of which is shown in Fig. 3. Dots indicate locations of large depressions (coated pits) in the corresponding replica. Depressions do not coincide with fluorescent spots. The dot indicated by the arrowhead in *B* represents the large depression shown in Fig. 3. Bar, 5 μm.

The distribution of IMPs in the E faces of AChR clusters resembled that of complementary P faces of the same clusters. The ratio of P face to E face particles in the same membrane was ~2:1 in both control and azide-treated samples. IMP concentrations in complementary membrane faces therefore decreased in parallel. This rules out the possibility that particles from the P face of AChR domains were "lost" by transfer to the complementary E face during azide treatment.

Quantitation of IMP Redistribution

AChR Domains. IMP loss from AChR domains was quantified in sister cultures exposed to azide for 0, 1, 2 or 4.5 h. Control and azide-treated cultures were fixed immediately in glutaraldehyde. Clusters were located by searching the replicas, and one or two micrographs per cluster were taken of representative areas having well-defined AChR domains and contact domains for subsequent determination of IMP concentration. AChR domains of control clusters had similar IMP concentrations to AChR domains of clusters previously identified by fluorescence of bound toxin. However, the even spacing of controls was disturbed by some step in the process of examining clusters under fluorescence optics (compare, e.g., Fig. 5 *A* with Fig. 1)[2] and so this step was omitted from the experiments which follow.

During the initial hour of treatment with sodium azide, we found little loss of IMPs from AChR domains (~3%, not significant by *t* test: Fig. 4, triangles). During this initial period, however, the distribution of IMPs across AChR domains was significantly altered by azide. In control AChR domains, IMPs were evenly distributed (Fig. 5 *A*); a histogram of particle counts per unit area was narrower than the comparable Poisson distribution having the same mean (Fig. 6). After 1 h in azide, IMPs became partially microaggregated (Fig. 5 *B*); the histogram of particle counts in equal areas was broader than the corresponding Poisson distribution (Fig. 6). These deviations from the Poisson distribution were significant in both cases ($P < 0.01$ by chi^2 test). The particle distribution was therefore nonrandom both in control and in samples treated for 1 h in azide.

After longer treatments with azide, we observed a significant loss of IMPs from AChR domains (Fig. 4, triangles). This loss of IMPs was paralleled by a loss in R-BT binding sites in AChR clusters (Fig. 4, open circles), observed by semi-quantitative fluorescence techniques (see Materials and Methods). The decrease in the number of IMPs and in the amount of R-BT bound to cluster membrane are both consistent with the idea that AChRs are lost from AChR domains during cluster dispersal by azide.

Contact Domains. The IMP concentration in contact domains increased during the first 2 h of azide treatment, from $197 \pm 40/\mu m^2$ ($n = 12$) in controls, to $306 \pm 61/\mu m^2$ ($n = 11$) and $324 \pm 49/\mu m^2$ ($n = 8$) at 1 and 2 h, respectively.

2. We are currently trying to learn if the disturbance is caused by observation of the samples under fluorescence optics, or by the labeling and mounting of the samples preparative to observation.

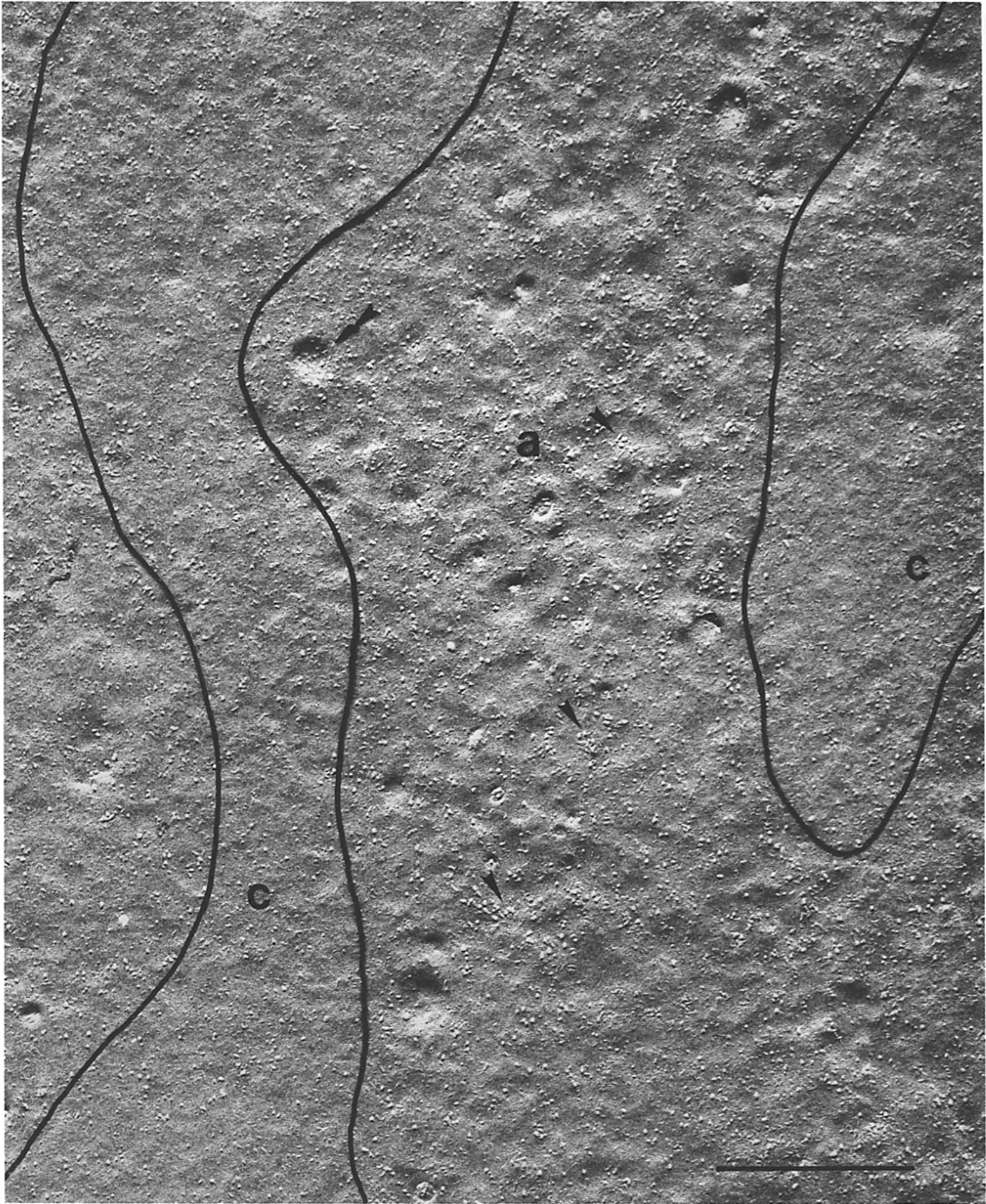

Figure 3. A portion of an AChR cluster from a myotube treated with azide. Myotubes were treated as described in the legend to Fig. 4; then fixed, frozen, fractured, and replicated. IMPs are scattered unevenly across an AChR domain (*a*) and are included in microaggregates (*arrowheads*). Both the change in concentration of IMPs and the change in membrane appearance from smooth to slightly undulating were used as indicators of boundaries (*thick lines*) between AChR and contact domains. Large depressions (coated pits: *double arrowhead*) are a feature of AChR domains. Bar, 0.5 μm.

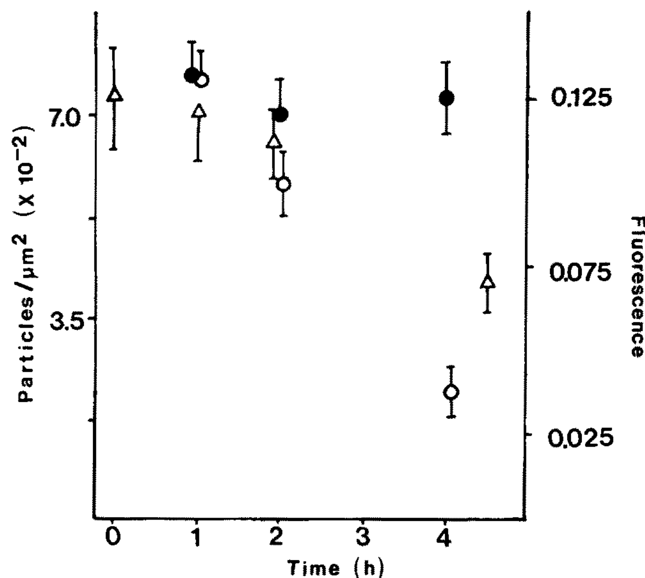

Figure 4. Time course of loss of IMPs from AChR domains, and R-BT label from AChR clusters. Myotubes were treated with 5 mM sodium azide for various times. Some samples were then fixed in glutaraldehyde, fractured, and replicated. Particles in the AChR domains of these samples were counted (*open triangles*: 0, 1, and 2 h). Other samples were labeled with R-BT, fixed with paraformaldehyde, and observed under fluorescence before processing for freeze-fracture and particle counting (*open triangle*: 4.5 h). Still other samples were labeled with R-BT and extracted with saponin to isolate AChR clusters. The fluorescence signal emitted by bound R-BT was measured using a photomultiplier attached to the fluorescence microscope (*open circles*). The results show that AChR IMPs or AChR-bound R-BT are lost from cluster regions as azide treatment is extended. Fluorescence measured in controls did not change over this time period (*closed circles*).

Some of this increase may have been due to difficulty in accurately distinguishing borders between domains at later times, but presumably also reflected migration of IMP into contact domains during this period. Over the initial 2 h of incubation with azide, AChR domains lost an average of 70 IMPs per μm^2, while contact domains gained an average of 127 IMPs per μm^2. Given the scatter of the data, the two results are reasonably comparable. Despite this migration, the contact domains retained a much lower concentration of IMPs than adjacent AChR domains. This was true even at longer times of azide treatment, when the IMP concentration of AChR domains decreased to 300–400 per μm^2, because at this time the IMP content of contact domains returned to the lower levels typical of controls (not shown).

Particle Diameter. The distributions of particle diameters in AChR domains and contact domains are compared in Fig. 7. Although the distributions overlapped extensively, they were significantly different ($P < 0.01$, Kolmogorov-Smirnov test) due to the presence of larger particles in AChR domains. For the same reason, the mean and variance of particle diameters differed significantly between AChR domains and contact domains, both in control myotubes and in myotubes treated for 2 h with azide (Table I). However, mean particle size did not appear to change within either domain during exposure to azide (Table I).

As the particle concentration in contact domains increased during the initial 2 h of azide treatment (see above), we considered the effect of migration of particles from AChR domains into contact domains. A hypothetical contact domain containing 1,000 IMPs was increased in IMP number by addition of 645 particles having the same size distribution as particles in AChR domains. This increase corresponds to the percentage increase in particle number seen in contact domains observed after 1 h of azide treatment. The resulting size distribution histogram was not significantly different from the original size distribution in the domain ($P > 0.05$, Kolmogorov-Smirnov test). Thus, migration of IMP into contact domains was not detectable as a change in particle size in these domains.

AChR Internalization

A prominent feature seen in some clusters labeled with R-BT and treated with sodium azide was a series of fluorescent spots ~0.3 μm in diameter (Fig. 2). Comparison of fluorescence micrographs of the same cluster before and after azide incubation showed that the spots were localized almost exclusively at AChR domains (Fig. 2). Spots were randomly distributed along the domains, as determined from a histogram of the distribution of spacing between 84 adjacent spots. Two lines of evidence indicated that these spots were due to labeled AChR which had been internalized into regions subjacent to, rather than in the plane of, the plasma membrane. (*a*) Spots were much less prominent, or were not seen at all, in clusters treated with azide, then labeled with R-BT at low temperature, and fixed immediately. (*b*) Spots had no correlate in corresponding freeze-fracture replicas of clusters which had been labeled and incubated with azide. Replicated membrane did contain microaggregates of 10–20 large IMPs and circular depressions ≥100 nm in diameter, recognized as coated pits in recent thin-section studies (Pumplin, D. W., manuscript in preparation). However, neither coincided in number or position with the fluorescent spots (Fig. 2). The spots therefore represent structures which are localized out of the fracture plane, probably internal to the P face of the plasma membrane. These results suggest that internalization of some AChR occurs during disruption of clusters by azide. However, this internalization is only sufficient to account for the loss of ~15–20% of the AChR IMPs during cluster disruption (see Discussion).

Cluster Reformation

The studies described above showed that AChR IMPs became unevenly scattered and partially microaggregated as clusters were disrupted. Previous results had indicated that, after azide-induced disruption, clusters reformed and that reformation began at distinct spots, called "foci" (6). If cluster reformation were simply the reverse of disruption, we would expect random and microaggregated arrays of AChR IMPs to appear at foci before rearranging into evenly spaced sites.

To test this possibility, we treated myotube cultures for 6–6.5 h with 5 mM sodium azide, to disperse clusters completely (6). Cultures were then washed extensively and the myotubes were allowed to recover in normal medium for 4.5 h. AChRs were labeled with R-BT and fixed, and the reforming clusters were photographed before fracture. Additional cultures were allowed to recover for 6 or 18 h, then fixed directly in glutaraldehyde. We confirmed that cluster

Figure 5. Redistribution of IMPs in azide-treated clusters. Samples were fixed directly in glutaraldehyde before processing for freeze-fracture. (*A*) In control clusters, IMPs are evenly distributed across AChR domains. (*B*) In myotubes treated with azide (5 mM, 1 h), IMPs in AChR domains are partially microaggregated (*arrowheads*). Bar, 1 μm.

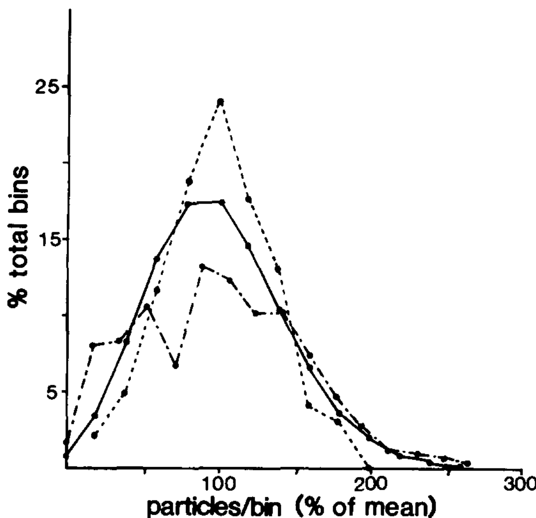

Figure 6. Distribution of IMPs in control and azide-treated myotubes. The distribution of IMPs in control clusters and in clusters that had been exposed for 1 h to 5 mM sodium azide was determined by counting particles in a number of equal-sized areas. If IMPs are randomly distributed, the density of IMPs in the areas should have a Poisson distribution (*solid line*). Deviations from the Poisson indicate an even distribution of IMPs in AChR domains of control myotubes (*dashed line*), and a microaggregated distribution in AChR domains of myotubes treated with azide for 1 h (*dashed and dotted line*). The redistribution occurs with only a small net loss of IMPs from AChR domains during this period (e.g., Fig. 4). Both deviations from the Poisson are significant ($P < 0.01$, chi^2 test). Portions of the domains which were counted are shown in Fig. 5. For the control cluster, 2,028 particles were counted in 403 squares (5.03 particles/square). For the azide-treated cluster, 1,491 particles were counted in 263 squares (5.67 particles/square).

loss was complete after the initial 6–6.5-h incubation by staining some cultures with R-BT and observing them by fluorescence after fixation. Any experiments in which ≥5% of the myotubes retained AChR clusters were discarded.

At 4.5 h after removal of azide, it was possible to locate myotubes with AChR clusters which appeared to be reforming, as judged from fluorescence observations of R-BT distribution. It was difficult, however, to assess the particle distribution in these clusters due to the disturbance associated with fluorescence observation (see above).

More reliable assessments of particle distribution during cluster reformation were obtained 6 h after removal of azide, by fixing samples directly in glutaraldehyde. Our observations on these clusters agreed with earlier studies: clusters tended to reform towards the ends of myotubes, where the cells were relatively broad and flat; i.e., at sites of estab-

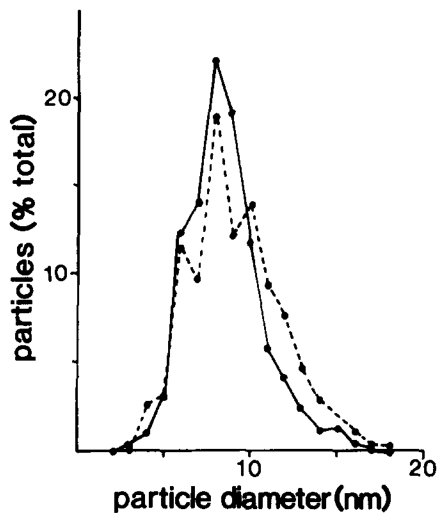

Figure 7. IMP diameter in AChR domains and contact domains. The size of IMPs in the AChR (*dashed line*) and contact (*solid line*) domains was determined for myotubes treated with azide for 2 h. The range of diameters is similar in both domains, but particles with a larger diameter are relatively more abundant in AChR domains. The difference is significant ($P < 0.01$, Kolmogorov-Smirnov test). Similar size distributions were seen in control myotubes (see Table I).

lished cell–substrate attachment (11). The smallest distinguishable AChR-rich membrane appeared as foci in slightly undulating membrane lying between strips of smoother membrane, thus resembling AChR domains in position and appearance (Fig. 8 *A*). IMPs within the foci were unevenly distributed, and some were microaggregated (Fig. 8, *A–C*). Similar small foci also appeared at the periphery of larger clusters showing several AChR and contact domains. AChR domains in these larger clusters had IMPs randomly to evenly arranged (Fig. 8, *D* and *E*) at concentrations of 500 $\pm$ 41/μm^2 (n = 5), ~70% of the concentration in control clusters. By 18 h after removal of azide, reformed clusters were indistinguishable from controls in concentration and distribution of IMPs (Fig. 6 *F*). These results suggest that, upon reformation of AChR clusters after dispersal with azide, the first step is the enrichment of AChR domains with AChR IMPs. These IMPs assume an uneven, partially microaggregated distribution before being reorganized into the even array typical of control AChR domains.

Discussion

The large, substrate-apposed AChR clusters of cultured rat myotubes are composed of two types of domains which alternate with one another across wide areas of the plasma membrane. The receptor-rich or AChR domains contain nearly all the AChR, recognizable either after labeling with derivatives of α-bungarotoxin or in freeze-fracture replicas as large, angular IMPs. An unusual but consistent feature of these domains is that the AChR IMPs are regularly spaced at distances that are too great to be spanned by the polypeptides which make up the receptor. This suggests that the IMPs are anchored to a scaffolding, or lattice, which dictates the spacing between particles.[3]

Table I. Particle Sizes in AChR Domains and Contact Domains of Control and Azide-treated Myotubes

Treatment	Domain	Particle size
		nm
Control	AChR	8.6 ± 2.7 (415)*
	Contact	8.0 ± 2.0 (439)*
Azide (2 h)	AChR	8.6 ± 2.6 (356)‡
	Contact	8.1 ± 2.1 (492)‡

*‡ Means ($P < 0.01$, t test) and variances ($P < 0.005$, F ratio) were significantly different between corresponding AChR domains and contact domains.

We tested the possible role of such a lattice in AChR distribution by exposing myotubes to different conditions which destabilize AChR clusters and observing the distribution of AChR IMPs that resulted. We postulated that such treatments could have one of three effects. (*a*) They could break up the lattice into smaller units which, together with the AChR IMP still bound, could diffuse into other areas of the membrane. (*b*) They could leave the lattice intact but cause the slow dissociation of bound AChR IMPs, which would then be free to redistribute into random arrays, to aggregate, or to diffuse away. (*c*) They could disrupt the lattice, simultaneously releasing many AChR IMPs, which would then be free to redistribute, aggregate, or diffuse away. Our results using sodium azide to disrupt AChR clusters appear to rule out the first alternative. Our studies of cluster reformation after the removal of azide appear to rule out the second possibility, and to support the third.

The possibility that intact lattices with their bound IMPs move through the membrane during disruption of AChR clusters is inconsistent with all our observations. If entire AChR domains became mobile as a unit, then AChR and contact domains, and the boundaries between them, would move during azide treatment. However, boundary locations appeared to be unaltered by azide (Fig. 2). If smaller portions of AChR domains became independently mobile, we would expect to see small "islands" of membrane with evenly spaced IMPs. These never appeared in our replicas. Instead, we saw the random or microaggregated IMPs which are consistent with independent movement of individual particles. It therefore seems highly unlikely that AChR clusters are disrupted by movement of organized bits of membrane to other parts of the cell. Such a mechanism would also be improbable on energetic grounds.

The possibility that AChR IMPs are released from a lattice that remains intact during azide-induced cluster disruption also seems unlikely. This model predicts that disrupted clusters would continue to have some IMPs that were regularly spaced, but the spacing between IMPs would be increased in discrete steps. This organization might be difficult to observe, as it would be obscured by the randomly distributed IMPs that had been previously released. In a more easily observed consequence of this model, cluster reformation would involve gradual replenishment of the IMPs into binding sites of an already organized lattice, and microaggregates should not be observed. Neither of these predictions

3. Electrostatic repulsion between neighboring AChR IMPs could also account for a spacing that is more even than that predicted by the Poisson distribution. For this to occur, each AChR IMP must be in an energy well deeper than its thermal energy, kT. However, each receptor IMP would need a net charge of at least 20–25 electronic units for such a configuration to be stable.

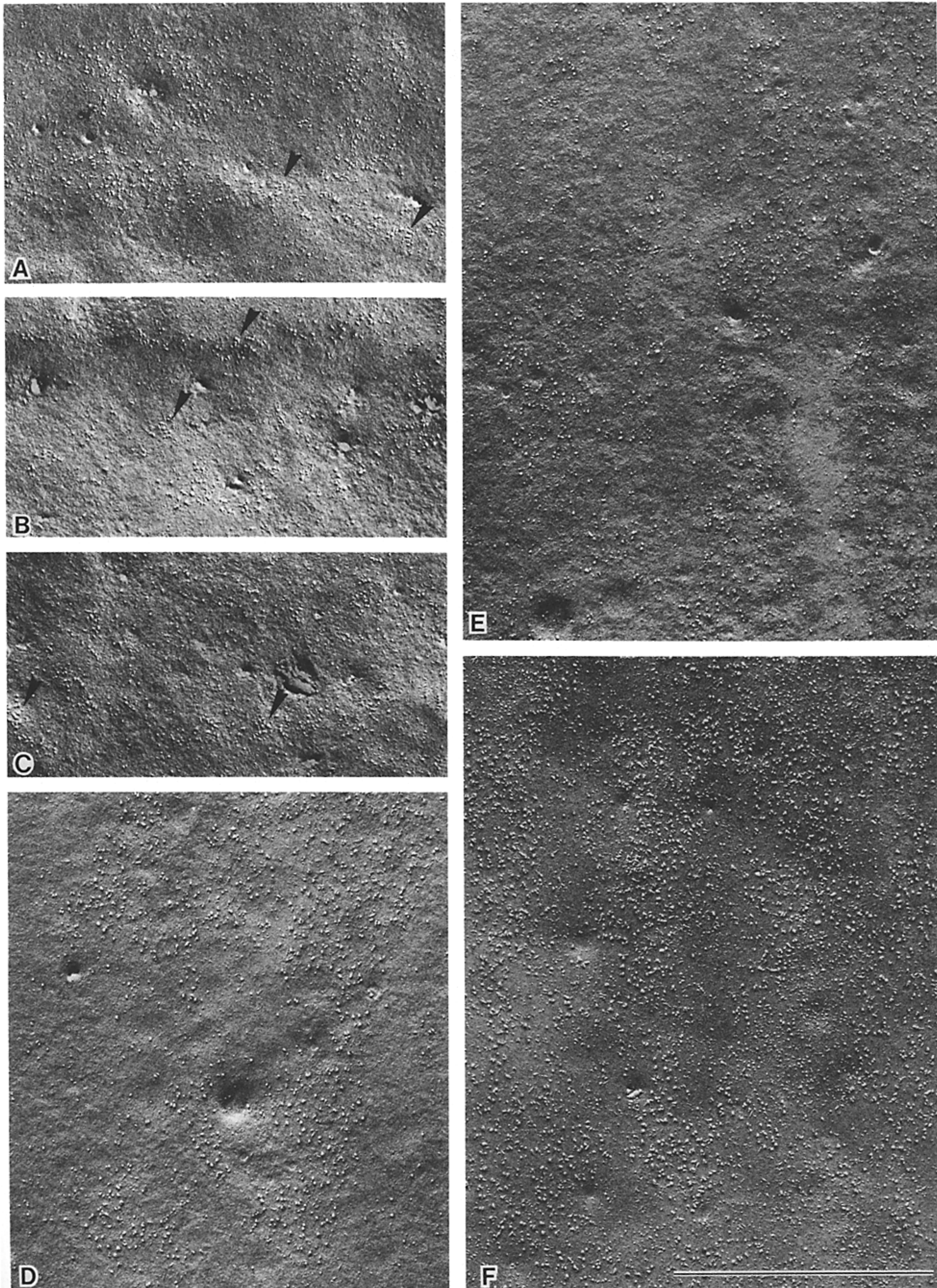

Figure 8. AChR clusters during recovery from azide. Myotube cultures were treated with 5 mM azide for 6 h, to disperse clusters completely. Azide was then washed away, and the cultures were allowed to recover in the incubator for 6 h (*A–E*) or 18 h (*F*) before fixation with glutaraldehyde and processing for freeze-fracture. The first recognizable portions of a reforming cluster consisted of scattered IMPs often occurring in microaggregates (arrowheads in *A–C*). Clusters with more extensive AChR domains contained randomly scattered IMPs at ~70% of the particle concentration of domains in control myotubes (*D* and *E*). The AChR domains shown in *A–E* were found on different myotubes. By 18 h after removal of azide (*F*), AChR domains were indistinguishable from controls. Bar, 1 μm.

were confirmed by our experimental observations of IMP distribution during treatment with, or recovery from, azide. In preliminary experiments, however, we have found that ~50% of the AChR domains in clusters disrupted by treatment with carbachol (9) or by withdrawal of Ca^{2+} (7, 15) continued to show relatively evenly spaced IMPs. The remaining IMPs may be bound to a lattice which is essentially unaffected by these disruptive treatments. If these results are confirmed in future experiments, they would suggest that cluster disruption can proceed by two different mechanisms, one involving dissociation of AChR IMPs from an intact lattice; the other, dissociation of the lattice itself.

The possibility that cluster disruption can occur as a result of a breakdown in the lattice is consistent with all the experiments presented here. In azide-treated myotubes, there is no evidence for any IMPs remaining bound in regularly spaced arrays. Instead, the IMPs of cluster membrane all became randomly distributed and microaggregated. Similarly disorganized arrays were observed during cluster reformation after azide treatment. No evidence that IMPs were being replaced into regularly spaced binding sites was obtained until later times of recovery. The large structures we observed at this time could not have remained after termination of azide treatment. These reforming structures contained many AChR domains with randomly scattered and microaggregated IMPs, particularly at the edges of the clusters. 18 h later, such arrays were rare; nearly all AChR domains contained regularly spaced IMPs, indistinguishable from those in control cultures. These results suggest that cluster reformation proceeds first by enrichment of IMPs in AChR domains, which occurs in the absence of regularly spaced binding sites, followed by a sorting out of IMPs into these sites once they form. Thus, both cluster dispersal and cluster reformation appear to involve an intermediate step in which AChRs are present at high concentrations in disordered arrays.

The idea that AChR clustering might involve more than a "simple one-step" process was first suggested by Stya and Axelrod (39), on the basis of their observations of AChR mobility and detergent extractability. They found that the AChR of azide-treated myotubes became extractable in Triton X-100 before they became mobile in the plane of the membrane, as measured by fluorescence photobleaching recovery. Our results provide a possible explanation for their observations. Upon release of AChR IMPs from the array, microaggregation occurs, with some internalization. Microaggregated and internalized receptors should appear immobile by fluorescence photobleaching recovery, but should be readily extractable with neutral detergents.

The fluorescent spots of AChR–R-BT complexes which appear over the AChR domains during azide treatment do not have any correlate in freeze-fracture replicas and therefore probably represent internalized AChR. The preponderance of the fluorescent spots in azide-treated cells, in contrast to controls, where they are more difficult to recognize, suggests that receptor internalization occurs normally in the presence of azide, but that further processing of AChR in internalized compartments is inhibited. This is consistent with the effects in these cells of inhibitors of energy metabolism on AChR degradation and on the total amount of AChR present at the cell surface (6).

A rough calculation suggests that internalization cannot account for all the loss of AChR seen during cluster dispersal. Receptor internalization involves the accumulation of AChR IMPs into coated pits, which are converted to coated vesicles (20, 30). A hypothetical cluster generalized from those we observed may have 250 μm^2 of AChR domain with 700 particles/μm^2, for a total of 1.75×10^5 particles. After 4 h of incubation at 37°C, a cluster of this size would internalize ~100 coated vesicles containing AChR. IMPs in AChR domains have mean diameters of 8.6 nm (see above), so ~330 particles can be close-packed into a hemispherical coated pit 125 nm in diameter. If each fluorescent spot arises from internalization of a single coated pit, then the number of internalized particles should be 100×330, or 3.3×10^4. This is $3.3 \times 10^4/1.75 \times 10^5$, or 19% of the total IMPs of the cluster. The half-life of clustered AChRs on control rat myotubes has been estimated at between 13 and 22 h (33; Bloch, R. J., and W. G. Resneck, unpublished observations), implying that 12–19% of the surface receptor should be internalized in 4 h. Nevertheless, whether the internalization rate is taken from measured turnover times, or estimated from the number of spots, it is insufficient to account for a decrease of ~50% in the IMPs of AChR domains observed after 4.5 h of azide treatment. Furthermore, internalization alone could deplete clusters of AChR only if insertion of new AChRs were also completely blocked by azide. Previous results suggest that insertion of new AChRs into the surface membrane continues at ~42% of control levels during exposure to azide (6). Thus, internalization alone is insufficient to account for the loss of IMPs from AChR domains.

The microaggregates which reform upon reversal of azide poisoning may arise from two distinct processes that could be related to the mechanism of cluster reformation. On the one hand, the microaggregates may form as a result of random association of scattered particles which diffuse into nascent AChR domains, or they may represent the contents of coated vesicles which have fused with the plasma membrane, contributing new AChRs to the reforming cluster. Several laboratories have reported that some of the AChR in receptor clusters forming de novo appear from intracellular stores (14, 32, 44). The microaggregates that appear in reforming AChR domains, however, are very small compared to the expected IMP content of coated vesicles. It is also known that at least some of the receptors that reappear in reformed clusters were present on the cell surface when azide was first added to the cultures (6). These receptors presumably enter AChR domains by lateral diffusion from other parts of the plasma membrane. The relative importance of selective insertion and diffusion mechanisms for the reformation of AChR domains remains to be determined.

Cluster dispersal during treatment with azide probably also occurs largely by diffusion of AChR IMPs into neighboring membrane regions. Although the path of diffusion of IMPs into and out of AChR domains is not immediately evident, it appears from our data that AChR IMPs diffuse into neighboring contact domains, thereby transiently raising the concentration of IMPs in those areas. A second possibility is that the diffusion of IMPs is "channeled" through contiguous AChR domains. "Channeling" has been suggested by others to explain rapid diffusion in partially immobilized systems (27), and has been treated theoretically (26, 34). Both processes may be occurring during cluster dispersal.

We have interpreted our observations of AChR IMP redis-

tribution in terms of interactions of receptors with a lattice associated with the cytoplasmic face of the plasma membrane of the myotube. Alternative sites for the location of such a lattice, in the extracellular space or within the lipid bilayer, have not yet received any experimental support. There is growing evidence, however, that two independent intracellular structures can interact with AChR IMPs. One is probably composed of clathrin and other proteins associated with coated pits and vesicles (Pumplin, D. W., and R. J. Bloch, manuscript in preparation). Our evidence suggests that these structures are not significantly affected by treatments that disrupt AChR clusters, and other experiments, now in progress, indicate that clustered AChRs are not associated exclusively with clathrin-coated membrane. The second structure associated with clustered AChR, and the one more likely to generate the lattice to which AChR IMPs bind, is composed in part of actin (10) and spectrin (Bloch, R. J., and J. S. Morrow, manuscript in preparation). The association of actin with regions of receptor clustering is lost during the treatments we have used to disperse clusters and returns during cluster reformation (10). Our observation during cluster reformation that AChR enrichment precedes the development of an orderly array suggests that AChR IMPs may provide important anchoring sites for the submembraneous cytoskeleton. This may be an example of the "receptor-mediated assembly and stabilization hypothesis" proposed by Moon et al. (24). Experiments are now in progress to characterize the cytoskeleton further, and to understand its interactions with the plasma membrane at both the biochemical and ultrastructural levels.

In summary, we have found that clustering of AChR IMPs occurs in two distinct steps: receptor enrichment into particular membrane regions, followed by redistribution of the individual IMPs into an evenly spaced array. Similar steps have been recognized in the assembly of the human erythrocyte membrane (24), and may also be important in the formation of other membrane protein aggregates. Given the extensive similarity between the AChR clusters we have studied here and the AChR-rich membrane of embryonic skeletal muscle (38), it seems likely to us that the assembly of the postsynaptic element of the neuromuscular junction proceeds in a similar fashion.

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