DISPERAL AND REFORMATION OF ACETYLCHOLINE RECEPTOR CLUSTERS OF CULTURED RAT MYOTUBES TREATED WITH INHIBITORS OF ENERGY METABOLISM

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ABSTRACT

The effects of energy metabolism inhibitors on the distribution of acetylcholine receptors (AChRs) in the surface membranes of non-innervated, cultured rat myotubes were studied by visualizing the AChRs with monotetramethylrhodamine-α-bungarotoxin. Incubation of myotubes with inhibitors of energy metabolism causes a large decrease in the fraction of myotubes displaying clusters of AChR. This decrease is reversible, and is dependent on temperature, the concentration of inhibitor, and the duration of treatment. Cluster dispersal is probably not the result of secondary effects on Ca2+ or cyclic nucleotide metabolism, membrane potential, cytoskeletal elements, or protein synthesis. Sequential observations of identified cells treated with sodium azide showed that clusters appear to disperse by movement of receptors within the sarcolemma without accompanying changes in cell shape.

AChR clusters dispersed by pretreating cells with sodium azide rapidly reform upon removal of the inhibitor. Reclustering involves the formation of small aggregates of AChR, which act as foci for further aggregation and which appear to be precursors of large AChR clusters. Small AChR aggregates also appear to be precursors of clusters which form on myotubes never exposed to azide. Reclustering after azide treatment does not necessarily occur at the same sites occupied by clusters before dispersal, nor does it employ only receptors which had previously been in clusters. Cluster reformation can be blocked by cycloheximide, colchicine, and drugs which alter the intracellular cation composition.

KEY WORDS: acetylcholine receptor clusters, patching and capping, muscle membrane, energy metabolism inhibitors, fluorescent α-bungarotoxin, cultured rat muscle

Ever since capping was discovered in lymphocytes, clustering of plasmalemmal components has been extensively studied (for a review, see reference 36). Some researchers have tried to determine the physiological relevance of capping, but have encountered the difficulty that caps or clusters rarely form spontaneously or under physiological conditions. One of the few surface components known to do so is the acetylcholine receptor (AChR) of cul-

1 Abbreviations used in this paper: AChR, acetylcholine receptor; αBT, α-bungarotoxin; CCCP, carbonyl cyanide
tured (2-4, 20, 25, 39, 42) or denervated (24) skeletal muscle. Although they form in the absence of innervation, AChR clusters are reminiscent of the postsynaptic element at the neuromuscular junction, where receptors are also immobile (4) and densely packed (6, 17, 18, 32). To understand the principles involved in receptor clustering and their possible relationship to synaptic structures, I have studied the large AChR clusters which form on the surface of cultured rat myotubes.

Some clues to the mechanism of receptor aggregation may be gleaned from studies of lymphocyte cap formation. Upon exposure of lymphocytes to antibodies or lectins, aggregates of surface components, termed "patches," form and subsequently "cap" in one region of the plasma membrane. Patching, but not capping, occurs in the presence of energy metabolism inhibitors, suggesting that the latter, but not the former, is an active process (29, 37, 40). Further studies have implicated roles for Ca²⁺ (37, 41), cyclic nucleotides (16), and the cytoskeleton (12, 13, 35, 43), as well as metabolic energy, in the capping process.

AChR clusters may be studied as readily as lymphocyte caps by using fluorescent derivatives of the receptor-specific polypeptide, α-bungarotoxin (αBT) (1, 4, 33). I have found that, like some caps (12, 34), the AChR clusters of cultured rat myotubes disperse in the presence of inhibitors of energy metabolism. Upon withdrawal of inhibitors, dispersed AChRs can recluster. The mechanism, specificity, and drug sensitivities of cluster dispersal and reformation are considered here. A preliminary report has appeared (9).

**MATERIALS AND METHODS**

**Methods**

**Cell Culture:** Myotube cultures were prepared from hind limbs of neonate Sprague-Dawley rats, as outlined by Heinemann et al. (22). Usually, the dissociated muscle tissue from six hind limbs was added to 100 ml of culture medium, which consisted of 90% DulbeccoVogt modified Eagle's medium (DME) plus 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.; or Associated Biomedic Systems, Buffalo, N.Y.). The material eluting in a peak centered at 0.08 M NaCl was pooled and diluted in HEPES-buffered reaction medium to a final concentration of 5 μg/ml.

**Visualisation of AChR:** A fluorescent derivative of αBT was used. Monotetramethylrhodamine-αBT (R-αBT) was prepared as described (1, 33) and diluted to a final concentration of 5 μg/ml in reaction medium (95% DME plus 5% fetal calf serum) buffered with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) to pH 7.0. For study of fixed cultures, samples were usually stained with this solution for 20 min at 37°C, washed free of unbound stain, immersed in cold (~20°C) 95% ethanol, then rehydrated and mounted in glycerine. For study of living cells, cultures were stained with fresh R-αBT solution for 30 min at room temperature, then mounted on a chamber (3) containing reaction medium and maintained at 37°C except when under observation. All samples were observed with a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York, N.Y.) equipped with epi-illumination (3). Total magnification was 396.9. Ilford HP5 film (ASA 400; Ilford Ltd., Basildon, Essex, England) processed to an ASA of 1,200 was used for photomicrography.

In some experiments a fluorescein derivative of αBT was used. Conjugation of fluorescein isothiocyanate to αBT was performed as described for preparation of the tetramethylrhodamine-αBT conjugate (1). The protein-dye conjugate eluted from a Sephadex G-25 column was further purified by chromatography on SP-Sephadex using gradient elution with 0–0.5 M NaCl in 0.01 M Na acetate, pH 5.4. The material eluting in a peak centered at 0.08 M NaCl was pooled and diluted in HEPES-buffered reaction medium to a final concentration of 5 × 10⁻⁸ g/ml.

**Quantitation of Fluorescence Observations:** Samples were examined to determine what fraction of the myotubes had large AChR clusters (size range, 40–1,200 μm² in area). Cells without clusters were not further characterized except to note whether AChR was distributed uniformly or in small patches (<5 μm² in area) which appear as speckles in fluorescence microscopy (see Fig. 1).

The following criteria were set to allow quantitation. A cell was considered to be a myotube if it was multinucleated and had detectable R-αBT stain anywhere along its length. Where large syncytia formed, only one syncytial process was scored. AChR clusters were defined if the localization of R-αBT stain was (a) discrete, ≥40
RESULTS
canavalin A from Miles-Yeda (Rehovoth, Israel), theo-
dissolution of the residue in reaction medium. D600 was
m-chlorophenyl hydrazone (CCCP) and oligomycin were
data as the percent of the total which had AChR clusters.

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RESULTS
Cluster Dispersal by Energy
Metabolism Inhibitors
LOOS OF ACHR CLUSTERS: Most myotubes in
cultures prepared from dissociated rat skeletal
muscles have large clusters of AChR (4). When
stained with a fluorescent derivative of aBT, these
clusters appear as very bright, discrete patches
within a single plane of focus on the substratum-
attached face of the cell (Fig. 1A). When cultures
are treated with inhibitors of energy metabolism,
clusters of AChR are lost. Poisoned myotubes may
show little or diffuse staining with R-aBT (Fig.
1B), or may have a mottled or speckled appear-
ance in fluorescence optics (Fig. 1C and D). Even
when R-aBT-AChR complexes remain densely
packed after poisoning, however, the discrete
boundaries typical of a cluster are no longer pres-
ent (Fig. 1C). Thus, energy metabolism inhibitors
cause the loss or blurring of AChR clusters. This
effect was dependent upon the concentration of
the energy metabolism inhibitor used (Fig. 2), and
required 4–6 h to reach completion (Fig. 3).

OBSERVATION OF DISPERSAL: To learn
more about the process of AChR cluster disap-
ppearance, I followed the fate of individual clusters
on identified cells. To do so, I stained cultures
with R-aBT and then observed them by phase and
fluorescence microscopy. In azide-treated cultures,
I found that only 7 of 121 clusters remained after
6 h of exposure to azide. In contrast, 34 of 57
clusters were still intact after control incubations.
The results obtained for all cells observed are
summarized in Fig. 4.

Intermediate stages in cluster loss may be seen
at times earlier than 6 h. Fig. 5B shows a pair of
AChR clusters flanking sarcolemma with little R-
aBT stain (arrow). After 3 h of azide treatment,
the originally discrete boundaries of these clusters
have broken down, and, despite the overall reduc-
tion of stain intensity, the membrane region be-
tween them has become enriched in R-aBT-AChR
complexes (Fig. 5D, arrow). This constitutes evi-
dence for the movement of R-aBT-AChR com-
plexes from clusters to other sarcolemmal regions.

I never observed shedding of AChR clusters in
this series of experiments. I did, however, see an
increase in background fluorescence in both con-
trol and azide-treated cultures, probably because
of internalization and degradation of AChR bound
with R-aBT, which continues for some time in the presence of azide. The decrease in
cluster staining and the accompanying increase in
background fluorescence were not the primary

2 R. J. Bloch, unpublished observations.
FIGURE 1 Control and azide-treated myotubes after staining with fluorescent αBT and fixation in cold ethanol. Cultures of rat myotubes on collagen-coated glass cover slips were washed and then placed into 1 ml of reaction medium with or without 5 × 10⁻³ M sodium azide. After incubation for 6 h at 37°C, cultures were washed and then stained for 20 min at 37°C with R-αBT (5 μg/ml in HEPES-buffered reaction medium). After removal of unbound R-αBT, cultures were fixed in 95% ethanol precooled to −20°C, glycerinated, and observed and photographed as described in Materials and Methods. (A) Controls, showing three AChR clusters on two untreated cells; (B–D) azide-treated cultures showing seven representative cells without clusters. Speckles and mottling may be seen on the cells in Fig. 1C and D. All photographs were exposed and printed under identical conditions. White lines indicate the borders of cells not otherwise visible. Bar, 50 μm.

cause of cluster disappearance in poisoned cultures. Energy metabolism inhibitors decrease AChR turnover (7, 14, 22; see also Table 1). If turnover alone were responsible for cluster loss, and if azide does not specifically enhance the rate of turnover of clustered AChR, then control cultures prelabeled with R-αBT should lose receptor clusters faster than poisoned ones. That this was not the case is evident in the data of Fig. 4 and illustrated in Fig. 6, in which a control cell observed 6 h after labeling still displays a clearly defined, though faded, AChR cluster (Fig. 6H).

To examine the possibility that azide-induced enhancement of dissociation of fluorescent label was responsible for the loss of clusters, I restained and photographed control and poisoned cells, as illustrated in Fig. 6. Although restaining led to a marked increase in fluorescence (Fig. 6D and I), perhaps because of turnover of labeled surface receptor, it did not cause AChR clusters to reappear in poisoned cells (Fig. 6D). Decreases in R-αBT label due to dissociation are therefore not relevant to the azide-induced cluster loss.

Because control clusters were still clearly visible after initial fluorescence exposures, bleaching of tetramethylrhodamine fluorescence was also not a factor in disappearance of clusters.

GENERAL EFFECTS OF METABOLISM INHIBITORS: To assess the damage caused to myotubes by 6-h exposure to energy metabolism inhibitors, I examined several properties of treated cultures. (a) The number of myotubes per culture was only slightly decreased by azide treatment (15 ± 8% [n = 3]; see also Fig. 4). (b) Neither the size nor the shape of myotubes was altered by azide treatment. In one experiment, mean myotube dimensions were (length × width at widest point, in μm): controls, 440 (range, 90–1,440) × 30 (range, 16–55), n = 100 cells; azide-treated, 400 (range, 120–980) × 29 (range 16–71), n = 88 cells (see also Figs. 5 and 6). (c) Azide-treated cultures excluded radiolabeled Na⁺ as well as did controls. (d) Up-
FIGURE 2  Dose dependence of the effect of energy metabolism inhibitors on AChR clusters. The protocol was as described in Fig. 1, except that the concentrations of the inhibitors used were varied. Frequency of AChR clusters was quantitated as described in Materials and Methods. ×, CCCP (scale of lower axis applies); O, oligomycin (scale of lower axis × 10⁻²); Δ, sodium fluoride (scale of upper axis applies); ●, sodium azide (scale of upper axis applies).

FIGURE 3  Time course of azide and CCCP effects on AChR clusters. Cultures were treated with CCCP or sodium azide, as described in the legend to Fig. 1, except that the duration of exposure was varied. At the times indicated, duplicate cultures were washed free of drug, stained with R-aBT, fixed in cold ethanol, and quantitated (see Materials and Methods). ×, 10⁻³ M CCCP; ▲, 5 x 10⁻³ M sodium azide; ●, controls.

take of radiolabeled Na⁺ in the presence of carbachol (38) was nearly equivalent in azide-treated and control cultures (data not shown). (c) Electrophysiological measurements, kindly performed by Dr. Y. Kidokoro, showed no decrease in the resting potential of azide-treated myotubes (−58 ± 5 mV, n = 12) compared to controls (−54 ± 5 mV, n = 7). (f) Diodo-aBT (¹²⁵I-aBT) binding by poisoned cultures was only slightly reduced (Table I). (g) Appearance at the cell surface of newly synthesized AChR continued, although to a lesser extent, in the presence of energy metabolism inhibitors (Table I). These results suggest that the damage caused to cultured myotubes by exposure to energy metabolism inhibitors is not severe.

Cluster Reformation

Reversibility of Cluster Dispersal: The effects of azide, fluoride, and CCCP, but not of oligomycin, were reversible. Within 18 h after
Figure 5  Azide-induced dispersal of AChR clusters on identified cells. The procedure described in the legend to Fig. 4 was used. The cells shown here were treated with 5 mM sodium azide. (A, B) Phase and fluorescence images of cells immediately after azide addition; (C, D) same cells 3 h later. The arrows indicate a region of sarcolemma which increases in fluorescence intensity as clusters disperse. Note also that the phase appearance of cells does not change during azide treatment. Fluorescence photographic processing was identical for all images. Bar, 50 µm.

withdrawal of these drugs, clusters reformed. This was true whether cells were stained with R-aBT before dispersal and recovery, or afterwards (Table II), thus suggesting that clusters present at the end of the recovery period were formed of AChR present before dispersal. Thus, new myotube formation does not account for the reappearance of AChR clusters. Further characterization of cluster reformation could therefore be undertaken without the difficulties caused by this sort of artifact.

The time course of reappearance of AChR clusters after removal of energy metabolism inhibitors is shown in Fig. 7. Recovery was always preceded by a lag period, the duration of which depends upon the duration of the initial exposure of the cultures to the drugs. Exposure of cultures to CCCP for 2 or 6 h gave lags in cluster reappearence of 2 and 5–6 h, respectively. On the other hand, the rate of recovery from the effects of CCCP seemed to be independent of the duration of exposure, with a rate of 5–6% myotubes with clusters/h. Recovery from sodium azide poisoning showed only a short lag period and was more rapid (15%/h) than recovery from CCCP (Fig. 7). For these reasons, azide-treated cultures were used to examine the process of reformation of clusters.

INTERMEDIATES IN CLUSTER REFORMATION: Between 2 and 6 h after withdrawal of azide, myotubes displayed aggregates of AChR much smaller than control AChR clusters. To examine the possibility that these might be intermediates in cluster reformation, I investigated the time course with which they appeared in relation to larger receptor aggregations. To do so, I stained and fixed cultures at different times after azide removal, and classified each display of AChR into one of three categories: (a) small AChR aggregates; (b) pairs of AChR clusters; and (c) single clusters, as in controls. Schematic renditions of these configurations are presented in Fig. 8, where the changes in each are shown as a function of time after removal of azide. These results indicate
TABLE I
Effects of Energy Metabolism Inhibitors on Titer and Synthesis of AChR

| Treatment          | Total/culture | Synthesis/culture |
|--------------------|---------------|-------------------|
|                    | Unfixed | Fixed |                  |
| Control            | 100     | 100   | 100              |
| + CCCP (10⁻⁵ M)    | 81      | 103   | 27               |
| + Oligomycin (10⁻⁶ M) | 97      | 92    | 56               |
| + Sodium azide (5 x 10⁻⁴ M) | 92     | 92    | 42               |

To determine effects on total titers, myotube cultures on collagen-coated 35-mm tissue culture dishes were treated for 6 h at 37°C in 2 ml of reaction medium (control), or in reaction medium containing energy metabolism inhibitors. Cultures were then incubated with 2 x 10⁻⁷ M ¹²⁵I-oBT in the presence or in the absence of 10⁻⁴ M d-tubocurarine, as described (31). Samples were collected by Millipore filtration (Millipore Corp., Bedford, Mass.). Cold ethanol fixation, when applied, was performed after cultures were washed free of unbound ¹²⁵I-oBT. These samples were then rehydrated in phosphate-buffered saline containing 1% (vol/vol) fetal calf serum, and collected by filtration. Curare-protectable titers for control cultures (100% values) were 2.2 x 10⁻¹ pmol ¹²⁵I-oBT/dish for unfixed cultures, and 1.4 x 10⁻¹ pmol/dish for cultures fixed in ethanol (cultures prepared on different days). To determine the amount of newly synthesized AChR appearing at the cell surface during treatment, the same procedure was followed except that all samples were preincubated with 10⁻⁴ M unlabeled oBT before exposure to energy poisons (31). Alcohol fixation was not employed. The curare-protectable titer of newly appearing receptor in controls was 5.2 x 10⁻² pmol ¹²⁵I-oBT bound/dish (100% value), the equivalent of 31% of the total receptor titer per culture. Titers are reliable to SD of ± 10%.

that small aggregates rather abruptly decrease in relation to the total number of receptor aggregates present, whereas cluster pairs briefly, and single, large AChR clusters continually, increase in their relative frequencies.

To determine whether similar changes in AChR configurations could be seen on individual living myotubes, I first treated cultures for 6 h with 5 mM sodium azide, and then stained them with R-oBT and observed them periodically thereafter by phase and fluorescence microscopy. Initially, most myotubes displayed randomly distributed AChRs. AChR displays of class 1 began to appear after 3 h of incubation at 37°C and became more common in the next 2 h. During this time, clusters (class 3) and cluster pairs (class 2) began to appear. Eventually, some of the pairs merged to form single, large AChR clusters. In 90 cells studied in four experiments, I observed 15 transitions from class 1 to class 3, 13 from class 1 to class 2, and 10 from class 2 to class 3. Of the latter, six had also made the transition from class 1 to class 2. An additional 12 cells showed increases in the size of aggregates present, without changing from one class to another. These data indicate that an average of 49% (range, 19–84%) of cells chosen because they showed small AChR aggregates or pairs of AChR clusters made the transition to a more extensively aggregated AChR configuration. Examples are presented in Fig. 9.

From the examples presented in Fig. 9, and others, I determined that the rate of growth of AChR clusters during reformation is between 0.7 and 2 x 10⁻⁷ cm/s⁻¹.

AChR SPECIFICITY IN RECLUSTERING: I studied the clustering process further to answer the following question: are AChRs that were originally in clusters the only receptors capable of reforming clusters? As I knew of no way to label clustered but not diffuse receptors, I chose to examine two populations of AChRs which I could readily distinguish: those already on the cell surface, and those in an intracellular pool not yet incorporated into the surface (14, 21, 31). If only previously clustered AChRs are capable of reforming clusters upon removal of azide, then none of the AChRs inserted into the sarcolemma from intracellular pools during azide treatment (Table I) and after azide removal should appear in reforming clusters.

To test this prediction, I saturated the AChRs of myotube cultures with unlabeled oBT and then incubated in the presence of azide to disperse clusters. After removal of azide and brief incubation in inhibitor-free medium to allow reclustering, cultures were stained with R-oBT. They revealed the same small AChR aggregates, cluster pairs, and some large AChR clusters, as described above.

3 The reason for this wide range of results is not yet clear. It may be that myotubes maintained in sealed chambers become slightly anoxic. This would be expected to prevent cluster reformation in cells previously exposed to sodium azide. Other factors may be involved, however. Structures which stain with R-oBT do occasionally disappear from cells which otherwise appear to be healthy and to be capable of further aggregating their AChRs (see Figs. 9 I and J, and 11 B and C).
FIGURE 6 Azide-induced dispersal of AChR clusters on identified cells. As in Fig. 5, except that a control cell (Fig. 6F-J) as well as an azide-treated cell (Fig. 6A-E) is shown. (A, F) Phase images of cells at the start of the experiment. (B, G) Fluorescence images at the start of the experiment. (C, H) Fluorescence images after 6 h at 37°C; although fluorescence intensity is less in both images, the edges of the cluster in Fig. 6H are more clearly demarcated than those in Fig. 6C, which would be classified as “highly faded” (see Fig. 4). (D, I) Fluorescence images upon restaining cultures with R-αBT (30 min at room temperature) after the 6-h incubation; the control cluster, in Fig. 6I, is again very bright and retains its original structure, but the azide-treated cluster regains neither its original brightness nor its organization. (E, J) Phase images after the 6-h incubation and restaining; note that neither cell undergoes large shape changes during treatment. All fluorescence images were exposed, developed, and printed identically. Bar, 50 μm.
A. Myotube cultures were treated as described in the legend to Fig. 1. Samples were taken after 6 h of incubation. Parallel samples were washed three times in 1.5 ml of HEPES-buffered DME, then replaced in 1 ml of reaction medium and incubated for a further 18 h before staining, fixation, and observation.

B. As described for A, but cultures were stained before exposure to energy metabolism inhibitors and were not stained again at the termination of the experiment.

### Table II

Reversibility of AChR Cluster Dispersion

| Treatment                  | Control | +CCCP (10^-5 M) | +Oligomycin (10^-7 M) | +Sodium azide (5 x 10^-3 M) | +Sodium fluoride (5 x 10^-1 M) |
|----------------------------|---------|----------------|-----------------------|-----------------------------|--------------------------------|
| Myotubes with clusters     |         |                |                       |                             |                                |
| A                          | 86       | 7              | 9                     | 12                           | 4                               |
| Reversed                   | 90       | 72             | 22                    | 92                           | 72                             |
| B                          | 88       | 87             | 7                     | 17                           | 20                             |

A. Myotube cultures were treated as described in the legend to Fig. 1. Samples were taken after 6 h of incubation. Parallel samples were washed three times in 1.5 ml of HEPES-buffered DME, then replaced in 1 ml of reaction medium and incubated for a further 18 h before staining, fixation, and observation.

B. As described for A, but cultures were stained before exposure to energy metabolism inhibitors and were not stained again at the termination of the experiment.

### Figure 7

Time course of reformation of AChR clusters after removal of energy metabolism inhibitors. Cultures were treated as described in the legend to Table II, experiment A, but samples were collected at different times after removal of azide. ▲ Cultures pretreated with 5 x 10^-3 M sodium azide; ×, cultures pretreated with 10^-5 M CCCP; ○, controls, pretreated in reaction medium without inhibitor. Cultures pretreated for 6 h with 5 x 10^-4 M sodium fluoride recovered receptor clusters with approximately the same time course as shown for CCCP pretreated cultures (not shown).

And single clusters as described in Fig. 8. As αBT does not appreciably dissociate from AChRs in the time course of this experiment (21), all the sites which bound R-αBT at the termination of the experiment must be those inserted into the sarcolemma from the intracellular pool. Unless the bulk of newly inserted AChRs are specifically associated with dispersing clusters, a possibility considered below, this result indicates that receptors not previously clustered can reform clusters after reversal of azide poisoning. These observations suggest that the diffuse AChR present on the surface of myotubes before the addition of metabolic inhibitors can also be clustered during receptor reaggregation.

### Figure 8

Time course of appearance of intermediates in the reformation of AChR clusters. The protocol presented in the legend to Fig. 7 was followed for treatment with and recovery from azide, but quantitation was different. In this experiment, every R-αBT-AChR display observed which was not simply a diffuse staining pattern was classified into one of three categories: Class 1, an AChR aggregate less than ~20 μm² in area, or a group of such aggregates; Class 2, AChR cluster pairs which were within one cluster diameter of one another, such pairs which appeared to be merging, or an AChR cluster near a smaller aggregate, which, if alone, would be placed in Class 1; and Class 3, clusters which are located more than one cluster diameter from the nearest cluster or small aggregate, and which do not appear to be a pair of merging clusters. Schematic representations of these classes of AChR configurations are presented in the figure. Each of these configurations observed, i.e., a cluster of small aggregates, a pair of nearby clusters, or a lone cluster, was counted as one display. If, in Class 2, more than two clusters were nearby, each “pair” was counted once. For example, four clusters near one another were counted as three cluster “pairs.” 80 or more configurations were scored per sample, and the data were expressed as the percent of the total scored per culture which fell into one of the three categories. ○, Class 1; ×, Class 2; ▲, Class 3. In a separate experiment in which quintuplicate samples were analyzed, the changes between 4 and 6 h after azide removal were found to be statistically significant (P < 0.025–P < 0.001). Changes in control samples never exposed to azide were not significant (P < 0.2–P < 0.5).
Clustering of AChR observed on living cells recovering from azide. Cultures were first treated with $5 \times 10^{-3}$ M sodium azide and then washed free of the reagent, as described in the legend to Table II. They were stained for 30 min at 22°C with 5-µg/ml R-αBT in HEPES-buffered reaction medium and then washed and mounted onto chambers (3) filled with freshly prepared reaction medium. After incubation for 3 h at 37°C and periodically thereafter, cultures were observed under phase and fluorescence optics. Cells which showed AChR displays of Class 1 or Class 2 (Fig. 8) were photographed and sketched, and their grid positions were noted. After further incubation at 37°C, cells were relocated and any changes in AChR display were noted and photographed. Exposure, development, and printing of fluorescence images were constant. (A, B) Small aggregates growing to form a single cluster between 4.5 and 6 h after azide withdrawal. (C, D) Pair of clusters merging between 4.5 and 8 h after azide withdrawal. (E, F) Growth of a cluster between 4.5 and 8 h after azide withdrawal. (G–J) Appearance of small aggregates 4.7 h after azide withdrawal (Fig. 9 G), which grow over the next 1.6 h (Fig. 9 H); between 6.3 and 8.8 h after azide withdrawal, the bright structures to the right merge to form one large cluster, while the dimmer cluster to the left grows in size (Fig. 9 I); over the following 11.5 h, the dimmer cluster disappears as the brighter cluster, still present, fades, but extends its domain farther to the right (Fig. 9 J). The arrows in Fig. 9 G–I indicate an autofluorescent cell process which retracts during the period under consideration. Within each series, fluorescence images were exposed, processed, and printed identically. Bar, 50 µm.
I next determined whether receptors in the sarcolemma at the start of the experiment and receptors inserted into the sarcolemma subsequently are both incorporated into the same reforming AChR clusters. At various times after initial blockade of AChRs with R-αBT and un-derivatized αBT (which was complete; see Fig. 10 A and B), cultures were stained with F-αBT. After azide treatment, regions of high R-αBT intensities remaining on some cells could not be further stained with F-αBT (Fig. 10 C and D). This indicates that AChRs inserted into the sarcolemma during cluster dispersal did not associate preferentially with dispersing AChR clusters. At 4.5 and 7.5 h after reversal of azide inhibition, F-αBT and R-αBT staining coincided in most (>80%) cells, regardless of the size of the aggregate stained (Fig. 10 E–J). Co-clustering was not due to R-αBT dissociation from AChR in the course of the experiment (see Materials and Methods). I conclude that newly inserted AChRs and AChRs previously on the cell surface may aggregate together to form AChR clusters.

SITE SPECIFICITY OF RECLUSTERING: To examine the site specificity of reclustering, I observed cells to see whether clusters reformed at the same spot on the cell that they had originally occupied. To do so, I followed cluster dispersal on identified cells, and then allowed cultures to recover from azide poisoning for 18 h. Cells were finally restained with R-αBT and observed once more. In 14 of 33 cells, AChR clusters reformed at approximately the same location that clusters were found before being dispersed. In the remaining 19 cells, clusters reformed at sites distant from the original ones. These results suggest that cluster reformation does not entail strict site specificity. However, such experiments do not distinguish between the possibilities that there are a limited number of specific clustering sites which are mobile, and that clustering can occur randomly at a few of many spots on the cell surface.

De Novo Cluster Formation

It might be argued that the intermediates of AChR clustering described above occur only in myotubes recovering from effects of energy metabolism inhibitors. To examine this possibility, I studied de novo AChR cluster formation in myotubes never exposed to inhibitors.

I first examined clustering in 6- and 7-d control myotube cultures. In two cultures studied, several cells were observed in which small receptor aggregates grew to form clusters and in which cluster pairs merged to form single, large clusters (Fig. 11). The low frequency with which small AChR

Figure 10 Both old and new AChR are incorporated into reforming AChR clusters. Myotube cultures were incubated with 5-μg/ml R-αBT in HEPES-buffered reaction medium for 5 min at 22°C, washed, and then incubated for 1 h at 37°C with un-derivatized αBT in reaction medium containing 5 mM sodium azide. This procedure resulted in the saturation of ~70% of the measurable AChR per culture with R-αBT, and of >97% with either labeled or unlabeled toxin. Cultures were then washed at 37°C with reaction medium containing 5 mM sodium azide, and incubated for 5 h at 37°C in 1 ml of the same medium. Azide was then removed and cultures were reincubated in azide-free reaction medium. During incubation with azide and subsequent reversal, 15–20% of the total surface AChR turned over and became accessible to a second label. Immediately after reacting with un-derivatized αBT, at the termination of the incubation in the presence of azide, and at 4.5 and 7.5 h after azide removal, cultures were stained with 5-μg/ml F-αBT in HEPES-buffered reaction medium for 30 min at 22°C. Cultures were then washed free of excess label with buffered saline, pH 7.4, and fixed in ethanol precooled to −20°C. Photographs of fluorescein fluorescence (Fig. 10 B, D, F, H, and J) were exposed for 30 s, and of tetramethylrhodamine fluorescence (Fig. 10 A, C, E, G, and I) for 15 s. (A, B) Cluster labeled with F-αBT immediately after saturation with R-αBT and un-derivatized αBT. Note the absence of fluorescein fluorescence despite the high tetramethylrhodamine intensities, indicating complete blockage of sarcolemmal AChRs. (C, D) Receptor-rich region labeled with F-αBT after a total of 6 h of incubation in the presence of sodium azide. Note the continued failure of fluorescein fluorescence to associate with regions of high tetramethylrhodamine fluorescence. This indicates that AChRs inserted into the sarcolemma during incubation with azide do not associate preferentially with dispersing AChR clusters. (E, F) Cell labeled with R-αBT 4.5 h after reversal of azide poisoning. Note the small aggregates showing coincident fluorescence and tetramethylrhodamine stains. (G, H) Cell labeled with F-αBT 7.5 h after reversal of azide poisoning, showing coincident labeling of nearby pair of AChR clusters. (I, J) As in Fig. 10 G and H, showing a lone AChR cluster labeled with both fluorescent markers. Bar, 50 μm.
Figure 11. De novo formation of AChR clusters in control myotubes. 6-d-old myotube cultures were stained with R-αBT, mounted onto chambers containing inhibitor-free reaction medium, and scanned for cells displaying small AChR aggregates or pairs of AChR clusters, as described in the legends to Figs. 8 and 9. Such cells were photographed and sketched and their grid positions were noted. Cultures were then incubated at 37°C and observed intermittently thereafter. Any changes in AChR configuration on identified cells were noted and rephotographed. (A–D) Cell showing a transition from several small AChR aggregates at the beginning of the experiment (Fig. 11 A and B) to a larger, single AChR cluster 4 h later (Fig. 11 C and D). The aggregate to the right in Fig. 11 B disappeared, while the central aggregates, and perhaps the one to the left, merged to form the cluster in Fig. 11 C. (E–H) Cell showing the change from three small AChR clusters at the beginning of the experiment (Fig. 11 E and F) to a single, large cluster 6 h later (Fig. 11 G and H). Bar, 50 μm.

aggregates occur in control cells (see Fig. 8) makes this process difficult to study further.

I also studied receptor clustering in control cultures by examining de novo cluster formation in young myotubes. I stained myotube cultures at various times after the onset of fusion and then subjected them to an analysis like that used in Fig. 8. This study had two defects. First, staining intensity of myotubes before the fifth day after plating (1 to 2 d after onset of fusion) was too low to permit reliable detection of small receptor aggregates (class 1 of Fig. 8). Second, de novo cluster formation was not as synchronous as was recovery of clusters after azide removal. Despite these difficulties, the data in Fig. 12 show that small cluster pairs, present on myotubes 1 and 2 d after myoblast fusion begins, precede the larger, single clusters found typically on older myotubes.

Other Drugs

To examine the possibility that specific cellular functions might be involved in cluster stability and reformation, I studied the effects of other drugs on cluster integrity, on dispersal, and on recovery from azide poisoning. Results are presented in Table III. Several salient points may be made. (a) Only carbachol had an effect on cluster stability comparable to that of energy metabolism inhibitors (column 1). (b) Concanavalin A, prefixation with paraformaldehyde, and lowering the temperature effectively blocked cluster dispersal by CCCP (column 2) and cluster reformation after
azide poisoning (column 3). If these treatments have a common mode of action, it may be that they decrease the mobility of AChR in the sarcolemma. (c) Several drugs which had no effect on cluster stability or on cluster dispersal by CCCP did inhibit reaggregation of AChR after azide treatment. These were colchicine, cycloheximide, nicotine, ouabain, MnCl₂ and CoCl₂ (column 3). The following drugs had no effect on any of the processes studied: BaCl₂ (1 mM); SrCl₂ (1 mM); KCl (30 mM); tetrodotoxin (10⁻⁶ g/ml); D600 (2 × 10⁻⁹ g/ml); d-tubocurarine (0.1 mM); theophylline (1 mM); and 3',5'-cyclic GMP, 3',5'-cyclic AMP, and its dibutyryl derivative, all assayed at 1 mM with and without theophylline. In addition, the following drugs had no effect on cluster stability or dispersal: insulin (2.5 × 10⁻⁵ g/ml), ATP (2 mM), 2 mM extra CaCl₂, 4 mM extra MgCl₂, ethanol (1%), dimethylsulfoxide (1%), and 2-mercaptoethanol (0.05%).

DISCUSSION

This report addresses two basic questions regarding the AChR clusters of cultured rat myotubes: what keeps them together? and, how do they form? To approach these questions, I used energy metabolism inhibitors to disrupt clusters and then studied cluster reformation upon withdrawal of the drugs.

Cluster Dispersal

It is clear that energy metabolism inhibitors cause rat skeletal myotubes to lose their AChR clusters. Inhibitor-treated cells do not detach from the substratum or undergo large shape changes, nor do they lose a significant proportion of their AChR (Table I). They also retain membrane potentials and permeability barriers to Na⁺ comparable to control myotubes. Thus, severe damage is not incurred during inhibition of energy metabolism. These criteria indicate that clusters—not cells or parts of cells—are lost during treatment with inhibitors.

The loss of clusters seems to occur by receptor spreading through the plasma membrane, an example of which is presented in Fig. 5. More direct evidence that cluster spreading occurs within the plane of the membrane is lacking. The course of cluster loss (Fig. 3), however, is consistent with such a mechanism, assuming a diffusion coefficient comparable to that reported for mobile AChRs. Both shedding and internalization of AChRs are unlikely to account for cluster loss, as such mechanisms would be hard to reconcile with the spreading of clusters actually observed (Fig. 5). Furthermore, neither process should be stimulated by inhibitors of energy metabolism.

The way in which energy metabolism inhibitors disperse AChR clusters is still not clear. The data in Table III suggest that these compounds do not act by altering membrane potential, protein synthesis, cytoskeletal elements, cyclic nucleotide levels, or Ca²⁺ entry into sarcoplasm from extracellular or reticular stores. As oligomycin does not inhibit Ca⁺⁺ sequestration by mitochondria (10, 11, 27) but does disperse clusters, it also seems unlikely that inhibitor-induced Ca⁺⁺ leakage from mitochondria is necessary for cluster loss. The most likely alternative remains that the inhibitors act by blocking ATP synthesis. This would be consistent with the fact that the concentrations of inhibitors which effectively disperse clusters are known to poison ATP production (23, 26, 28, 30). Decreased ATP levels might also explain the partial blockade of insertion of intracellular pools of AChR into the sarcolemma (Table I), reported to

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1 This argument may require that clusters disperse by movement of individual receptors rather than of larger aggregates, or "speckles." "Speckles" would then form after cluster dispersal. At present, the role of "speckles" in cluster dispersal or in cluster reformation is not clear.

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be energy dependent (21). Such arguments do not rule out the possibility that changes in cellular factors other than ATP mediate cluster dispersal by energy metabolism inhibitors, nor do they explain the loss of clusters induced by carbachol. Nevertheless, they do support the idea that metabolic energy is used either directly or indirectly to hold clusters together.

Axelrod et al. (5) have reported that neither inhibitors of energy metabolism nor carbachol have any effect on the integrity of AChR clusters of rat myotubes. Their failure to see cluster loss in the presence of azide was probably because their experiment was performed at 22°C. The data of Table III indicate that lowering the temperature is sufficient to block cluster loss caused by CCCP. If the intracellular effects of the energy metabolism inhibitors are as sensitive to temperature as is the diffusion of mobile AChR within the sarcolemma (4), one might expect a 10°C reduction in temperature to cause a 5- to 10-fold decrease in the rate of cluster dispersal. I would probably fail to detect such a decrease in the rate of cluster dispersal in observations of fixed cell populations. Explanation of the disparate results with carbachol, which cannot be attributed to temperature differences, will be presented elsewhere.  

Cluster Reformation

Reformation of large AChR clusters after rever-

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R. J. Bloch, manuscript in preparation.
sal of azide poisoning occurs by a process of focal aggregation of receptors in a limited area of the sarcolemma. Schematically, this process can be rendered:

That is, small aggregates of AChR can grow by accretion of receptors around individual foci to become clusters, or to become cluster pairs which may then merge to yield single, large clusters. This scheme accounts not only for AChR reclustering after withdrawal of azide (Figs. 8 and 9), but also for de novo cluster formation in control myotubes (Figs. 11 and 12).

Three aspects of this focal aggregation process deserve mention. (a) It appears capable of recruiting diffuse AChRs regardless of whether they were previously in clusters, suggesting that diffuse and clustered AChRs are readily interconvertible by a mechanism dependent on metabolic energy. (b) There seems to be no discontinuity in the aggregation process. Receptor densities, as judged from the brightness of fluorescence images, seem to be similar in small aggregates and in larger clusters (Fig. 9). Drugs which inhibit reclustering (Table III) do not arrest aggregation at any particular step, but appear to prevent initiation of clustering by blocking the formation of the foci. Thus, once clustering begins with focus formation, the rest of the process usually follows. (c) In cells recovering from azide treatment, there does not appear to be any movement of foci towards one another that would more resemble a "contraction" of receptors into one region. Each focus appears to act independently, growing by the "filling in" of surrounding membrane with AChR, until its domain merges with that of a focus growing nearby (Fig. 9 C–D, and G–I). This aspect of AChR clustering is different from cap formation by lymphocytes, in which membrane components first aggregate and then move towards a common region of the cell surface.

At present, little else is known about the requirements of the clustering process. The data in Table III suggest that, in addition to a requirement for metabolic energy, clustering may also depend on protein synthesis and on the ionic composition and cytoskeletal integrity of the sarcoplasm. It is not yet clear whether these factors act directly at the site of cluster formation, or whether they prevent recovery after azide poisoning of ATP synthesis or of other functions. Nevertheless, it seems reasonable to postulate that AChR clusters are stabilized by cytoskeletal elements dependent on the sarcoplasmic levels of ATP and cations. While such elements need not actively transport receptors into clusters, they may bind to and immobilize receptors which diffuse into the region where clustering takes place.

It may be significant that the sites at which clusters form are nearly always on the substratum-attached side of cultured rat myotubes. Under certain conditions, clusters can even be left on the substratum when cells are removed by strong shear forces. While this suggests that clusters are part of the cell-substratum attachment site, attachment is probably not sufficient to induce clustering. Many cells attached to the substratum show no clusters or show clusters at only one of several attachment sites. Furthermore, clusters dispersed by treatment with azide do not always reform at the same site on the cell surface that they had originally occupied, though that site still seems firmly attached. Attachment may, however, be necessary for cluster formation in rat myotubes. Clusters reforming after azide poisoning are often found near the flattened ends of bipolar myotubes, apparently in close apposition to the substratum. Further studies will be required to learn whether adhesive factors are involved in specifying sites of AChR clustering.

Comparison of Clusters and Caps

Receptor clusters are similar to lymphocyte caps in several respects. Like caps formed by treatment with concanavalin A (12, 34), receptor clusters disperse upon exposure to energy metabolism inhibitors to yield mottled or speckled cells. Cluster reformation, like capping (29, 37, 40), is also sensitive to energy poisons. Clusters differ from caps, however, in their susceptibility to reagents acting on the cytoskeleton (12, 43): colchicine, for example, promotes concanavalin A-induced capping (43), but blocks cluster reformation (Table III). Also, as mentioned above, clusters form by a "filling in" rather than by a "contractile" process. Thus, studies of capping are of only limited relevance to the mechanism of receptor clustering.

Significance

The mechanism of AChR clustering, relevant to the general question of membrane organization, assumes further significance because clustering of
receptors in the region of neuromuscular contact is one of the early events in synapse formation (8, 15). Synapse formation in vitro seems also to proceed by a process of focal aggregation of AChRs within the muscle membrane along the path of the nerve (2, 3). If the AChR clusters of cultured rat myotubes are organized along the same principles as postsynaptic receptor aggregates, then the latter should also disperse in the presence of energy metabolism inhibitors.

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Note Added in Proof: I have recently found that clusters may be dispersed in Ca"+-free medium, and that recovery of clusters after reintroduction of Ca"+ may be blocked by the same drugs which inhibit cluster reformation upon removal of azide (manuscript in preparation). Ca"+-free conditions do not appear to inhibit energy metabolism. It seems, therefore, that these drugs prevent cluster reformation by acting at sites distinct from those governing recovery of energy metabolism.

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