

ULTRASTRUCTURE OF ACETYLCHOLINE RECEPTOR CLUSTERS ON CULTURED MUSCLE FIBERS

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The acetylcholine (ACh) receptors of the mature, innervated, vertebrate striated muscle fiber are restricted to the neuromuscular junction (8). Within this junction, receptors are restricted to areas of the folded plasma membrane close to the nerve ending (1, 2). The structural and molecular basis for this discrete distribution is unknown. ACh receptors on myotubes grown in culture from dissociated chick embryo skeletal muscle cells initially have an even distribution. However, as the myotubes mature, discrete regions with high receptor concentration (clusters) are formed (3, 9, 10). Analysis of these clusters may yield information on the mechanisms which serve to restrict the distribution of neurotransmitter receptors to (and within) the neuromuscular junction and other (5) synaptic connections. This report deals with the ultrastructure of cultured chick myotube ACh receptor clusters, as revealed by immunoperoxidase staining (1) of bound α -bungarotoxin (α BT), a specific ligand of the nicotinic ACh receptor.

MATERIALS AND METHODS

Muscle Cultures

The preparation and maintenance of the cultures has been previously described in detail (9, 10). In brief, 10–11-day old chick embryo thigh muscle was dissociated with collagenase. The cell suspension was enriched in myoblasts by preplating and then was plated in collagen-coated dishes. The culture medium consisted of 90% F14 medium (10), 6% horse serum, 2% fetal calf serum, and 2% chick embryo extract. 50% of the culture medium was conditioned (11) to promote myoblast fusion. Cytosine arabinoside (Ara C), 10^{-5} M, was added to some cultures on the 3rd day in order to stop

proliferation of fibroblasts (3). After the 5th day, Ara C as well as the conditioned medium and embryo extract were left out of the medium of these cultures.

α BT-Immunoperoxidase Staining

After 7–14 days of incubation, the cultures were incubated for 1 h with 2×10^{-8} M α BT under normal culture conditions. The α BT-treated cultures were washed four times with culture medium and then three times with F14 medium alone, followed by fixation for 1 h by one of the following procedures: (a) fixation at 0° – 4° C with 2% paraformaldehyde, 0.01 M NaIO₄, 0.075 M lysine in 0.037 M sodium phosphate buffer, final pH about 6.9 (7), followed by washes with 0.1 M sodium phosphate buffer, pH 7.1 (PB) and the same buffer with 0.1 mg/ml bovine serum albumin; or (b) fixation, beginning at 22° C, then at 0° – 4° C, with 4% paraformaldehyde, 0.1% glutaraldehyde, 0.075 M sucrose in 0.075 M sodium phosphate buffer, pH 7.1. This was followed successively by washes at 0° – 4° C with PB, 0.1 M sodium borate, pH 8.5, and 0.05 M NaBH₄ in 0.1 M sodium borate, pH 8.5 (30 min). The borohydride wash removes aldehyde groups (6), which could otherwise cause nonspecific binding of antibodies. The cultures were then washed with PB and PB with bovine serum albumin, as in (a). Method (b) gave slightly better cell preservation than method (a), but the results were otherwise the same. Higher concentrations of glutaraldehyde, e.g., 1.0–2.5%, destroyed the antigenicity of the bound α BT and thus could not be used.

Subsequent incubations with purified rabbit anti- α BT and horseradish peroxidase-conjugated IgG from goat antirabbit IgG, washing, refixation, and diaminobenzidine detection of peroxidase activity were performed as previously described (1). The stained cultures were postfixed for 45 min in 1% OsO₄ in PB, dehydrated in an ethanol series, and embedded, *in situ*, in Epon. Some areas of the cultures were marked and photographed

before sectioning. Thin sections were cut either parallel to the cell substrate or perpendicular to the long axis of the myotubes selected. Sections were stained with uranyl acetate and lead citrate, except where stated otherwise in Results. The immunoperoxidase reaction product appears as a dense black precipitate.

RESULTS

Light Microscopy

The distribution of α BT-binding sites visualized by immunoperoxidase staining confirmed and extended the results of autoradiography with ^{125}I -labeled α BT (3, 9, 10). Mature myotubes had a light brown stain over most of their surface, causing them to stand out in contrast to the substrate and fibroblasts (Figs. 1 *a* and 2). Superimposed on this overall stain was an irregular pattern of dense brown regions (clusters) which were usually oblong in shape and of various lengths, up to about 10 μm in length (Figs. 1 *a* and 2). The formation of these clusters was accelerated by treatment with Ara C. For example, most

myotubes in 7-day old Ara C-treated cultures had several ACh receptor clusters, while few clusters were found in untreated cultures of the same age (see also reference 9). The clusters were located at both the edges and the central areas of the myotubes, most frequently on small projections of the edges. Clusters on the edges appeared more dense by light microscopy, probably because the light impinging on the stained edge of a myotube would pass through a greater volume of stained plasma membrane and thus would be absorbed more than light passing through the center of the myotube, where it would pass through the stained plasma membrane perpendicularly.

The specificity of the α BT-immunoperoxidase staining of myotubes was tested by omission of α BT treatment or by inclusion of 10^{-3} M decamethonium (a specific ligand of the ACh receptor which competes with α BT) in the incubation medium shortly before and during α BT treatment. Both of these treatments eliminated the staining of clusters and markedly reduced the overall staining (Fig. 1 *b*). We tested the possibility that binding of

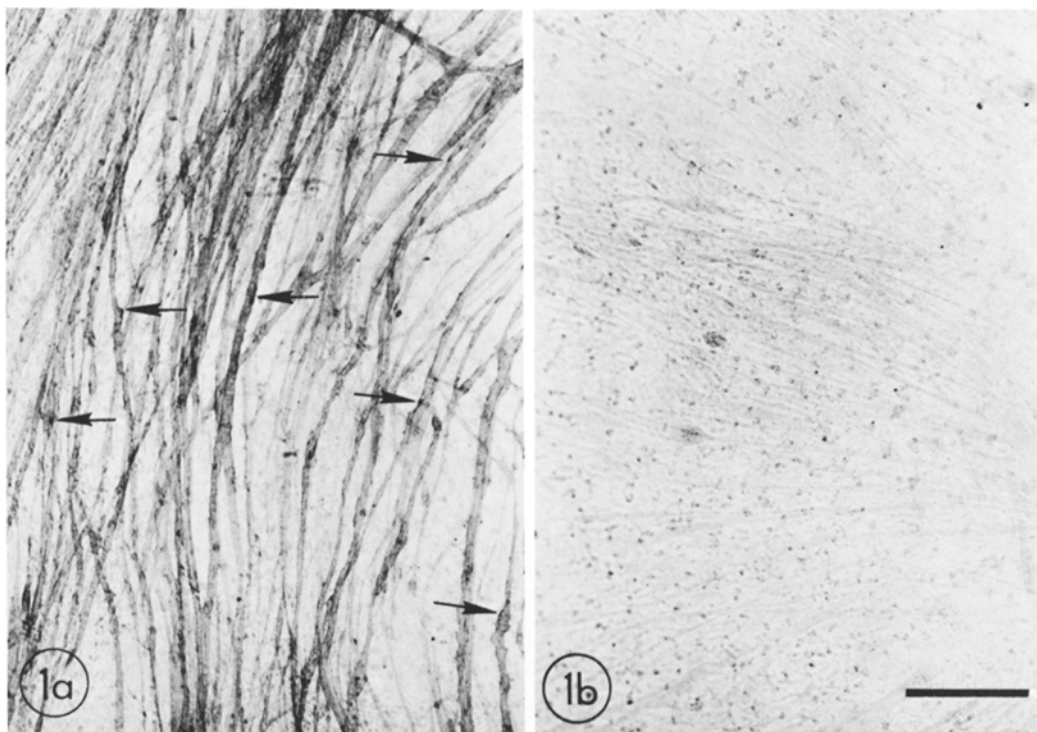


FIGURE 1 (a) Bright-field photomicrograph of a 14-day old culture stained by the α BT-immunoperoxidase method and embedded in Epon. The myotubes show both overall staining and densely stained clusters (arrows). (b) A duplicate culture treated identically except for the presence of 10^{-3} M decamethonium during the incubation with α BT. There is little or no staining of the myotubes. $\times 80$. Bar = 200 μm .

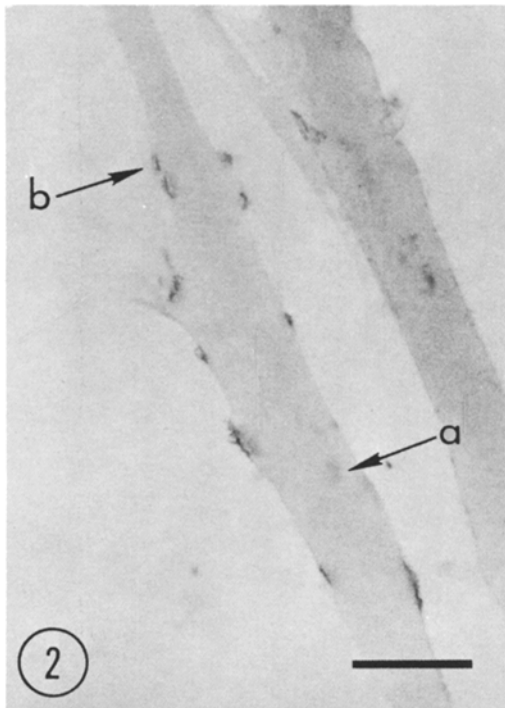


FIGURE 2 Bright-field photomicrograph of two myotubes in an Ara C-treated 11-day old culture stained by the α BT-immunoperoxidase method and embedded in Epon. Both overall staining and clusters can be seen on the myotubes. Arrows indicate the level of sectioning and the particular clusters shown in the electron micrographs of cross sections in Fig. 4 *a* and *b*. $\times 640$. Bar = 25 μ m.

α BT might in itself cause clustering of ACh receptors by fixing some cultures before α BT treatment. This prefixation was carried out in 4% paraformaldehyde, 0.075 M sucrose, and 0.075 M sodium phosphate buffer, pH 7.1, for 1 h at 0°–4°C, followed by PB washes. Subsequent treatment was as described in Materials and Methods, using fixation method (*b*). Prefixation caused a moderate reduction in the intensity of staining, but the specificity and distribution of stain on the myotubes were the same as in the cultures fixed only after α BT treatment. Therefore, the clustering of ACh receptors is not a result of their exposure to α BT. A similar conclusion may be drawn from the results of ACh sensitivity studies on living cultured muscle cells (3).

Electron Microscopy

In thin sections of α BT-immunoperoxidase-stained myotubes with receptor clusters, the intensity of staining on an average segment of section

through cluster plasma membrane (Figs. 3 *a–b*, 4 *a–b*, and 5) was at least seven times greater than on noncluster membrane (Figs. 3 *a*, 4 *c*, and 5), as determined by densitometry on electron micrographs. The density of osmium-impregnated reaction product seen in the electron micrographs may not be linearly proportional to the amount of α BT bound. However, it is reasonable to assume that the large difference in density of reaction product between cluster and noncluster regions corresponds to a large difference in the amount of bound α BT. There was considerable variation in intensity within a given cluster (Figs. 4 *a–b* and 5). In some sections the boundaries of the clusters appeared quite sharp (Fig. 3 *a*), while in others there was a gradual decrease in staining intensity at the cluster's edge, extending for 1 μ m or more (Fig. 5). Examination of 12 clusters previously located by light microscopy and of about the same number of clusters not prelocated revealed no distinctions, with respect to the location on the myotubes and the topography of membranes, between the clusters and the noncluster regions. Examination of cross sections of myotubes through selected clusters revealed that the "centrally" located clusters could be found either on the "bottom" (Fig. 4 *a*) of the cell (near the culture substrate) or the "top" (Fig. 5). The cluster membranes could be either smooth (Fig. 3 *b*) or ruffled (Fig. 4 *b*), with or without deep invaginations and microvilli, and all these variations in membrane topography were likewise observed in the noncluster regions. Invaginations, when present, were stained with an intensity similar to or somewhat less than that of the remainder of the cluster plasma membrane. Basement membrane or cell coat material was stained over the greater portion of the intensely stained cluster plasma membranes (Fig. 3 *b*), but not in the other regions. However, it is likely that areas of the cell coat directly overlying ACh receptor clusters became visible by trapping some of the diaminobenzidine reaction product produced specifically at the clusters. This is in agreement with the distribution of stained basement membrane observed in α BT-immunoperoxidase-stained mouse endplates (1). One relationship between membrane topography and intensity of staining was noticed: within clusters on ruffled membranes, the stain generally appeared more intense on the rounded projections than in between them. This was most clearly seen in cross sections of myotubes (Fig. 4 *b*). Also, in cross sections, the submembrane regions were

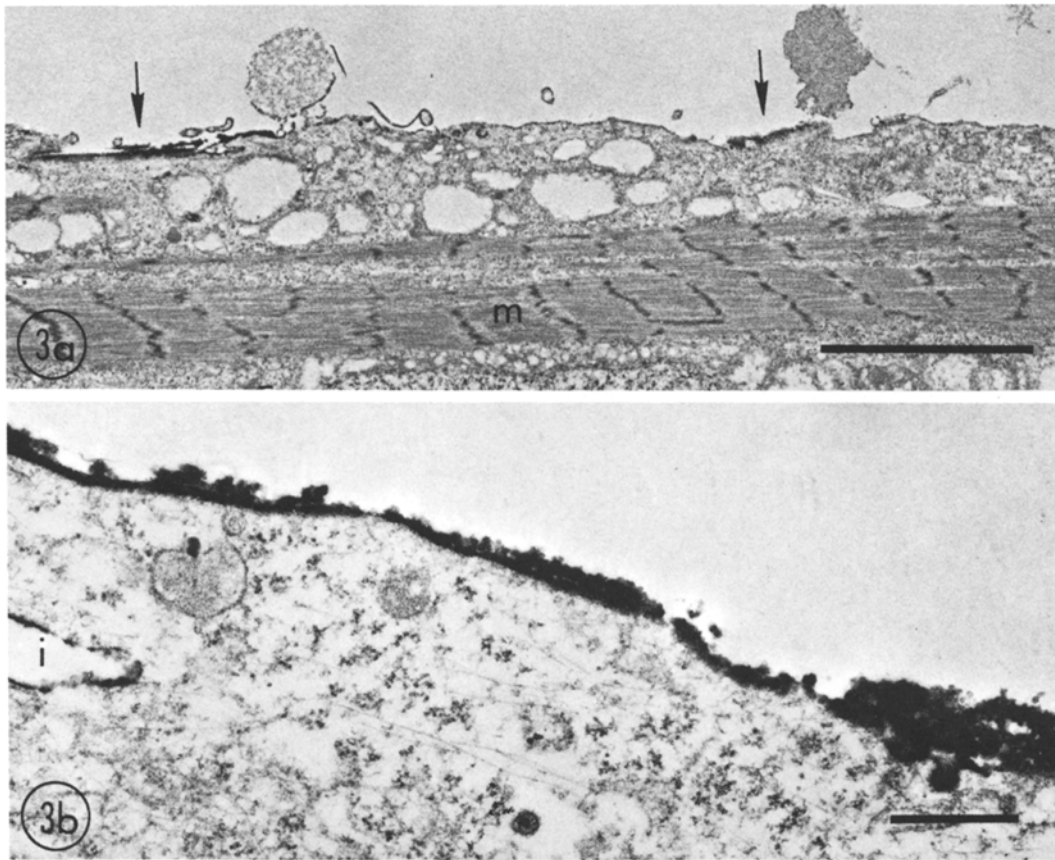


FIGURE 3 Electron micrographs of an α BT-immunoperoxidase-stained myotube from a 14-day old culture, sectioned parallel to the plane of the culture substrate. (a) Two heavily stained clusters are seen here (arrows) with alternate lightly stained regions. Note myofibrils (*m*). $\times 5,600$. Bar = $5.0 \mu\text{m}$. (b) Part of a parallel section through the cluster shown on the left of (a) is seen at higher magnification. Most of the plasma membrane is smooth and is coated with a layer of densely stained material (basement membrane or cell coat). Part of a presumed plasma membrane invagination (*i*) can be seen. $\times 33,000$. Bar = $0.5 \mu\text{m}$.

examined. Again, no consistent differences were found between clusters and noncluster regions.

DISCUSSION

Electrophysiological measurement of ACh sensitivity and light microscope autoradiography with $[^{125}\text{I}]\alpha\text{BT}$ have revealed the presence of nicotinic ACh receptor clusters on cultured skeletal myotubes (3, 9, 10). Since these techniques could not resolve the ultrastructural distribution of the receptors, two distinct models could be used to interpret those results: (a) there were more receptors per unit area membrane in the clusters; and (b) the concentration per unit area was the same both within and outside of clusters, but folding of the plasma membrane within the clusters made the concentration appear higher. Studies with ferritin-

labeled αBT (4) revealed small, unevenly distributed clusters of αBT -binding sites, but the relationship of these clusters to those observed by other techniques (3, 9, 10) was not clear. Clusters identified with the light microscope after αBT -immunoperoxidase staining and then examined by electron microscopy invariably had a heavy deposition of specific reaction product per unit area of membrane, regardless of membrane topography, while noncluster regions had little reaction product. This strongly suggests that model (a) is correct.

Use of this same technique also permitted examination of structural characteristics of the clusters in addition to receptor distribution. No qualitative differences between cluster and noncluster regions were found. However, the possibil-

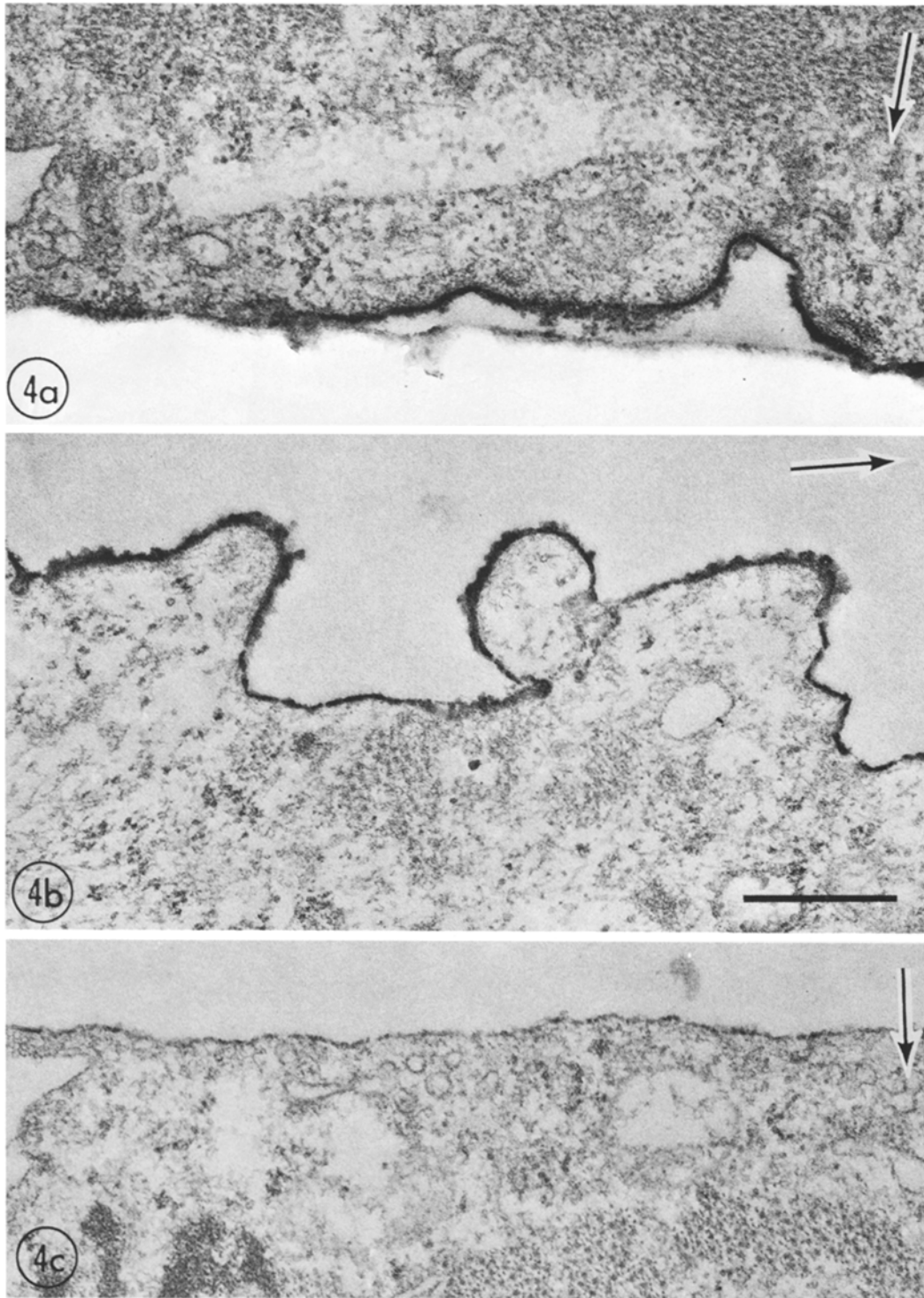


FIGURE 4 Electron micrographs of α BT-immunoperoxidase-stained myotubes from the same culture shown in Fig. 2, sectioned perpendicular to the long axis of the myotubes. The arrows point towards the location of the culture substrate. (a) Part of the lower cluster indicated in Fig. 2. This cluster is on the "bottom" of the myotube. (b) Part of the upper cluster indicated in Fig. 2. This cluster is on ruffled plasma membrane on the edge of the myotube. Note the more heavily stained convex regions of the cluster. (c) This is a noncluster region on the "top" of another myotube from the same culture. The plasma membrane is lightly stained. Magnification of (a-c), 45,000. Bar = 0.5 μ m.

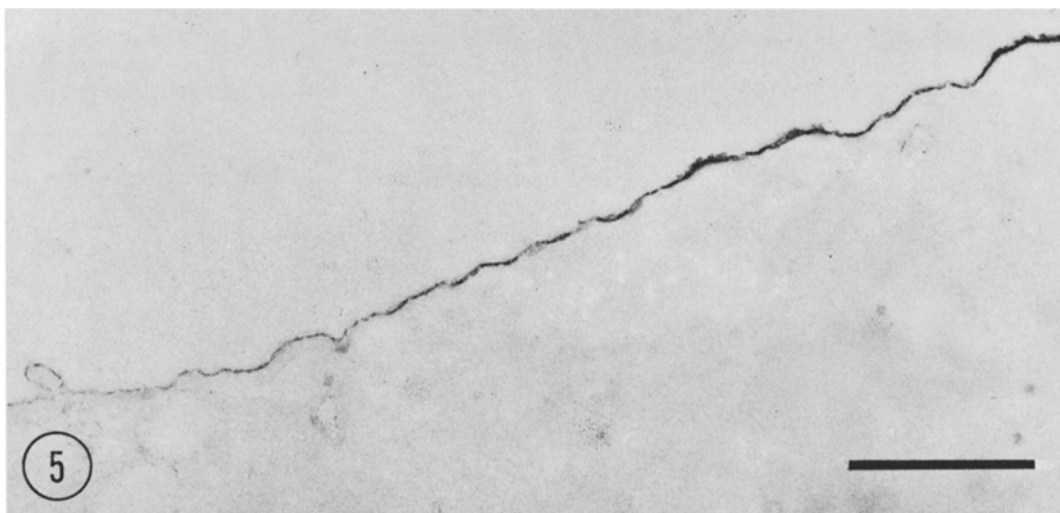


FIGURE 5 Electron micrograph of an α BT-immunoperoxidase-stained myotube from the same culture shown in Fig. 2, sectioned perpendicular to the long axis of the myotubes. The section was not stained with uranyl acetate or lead citrate. Part of a cluster (right) and a small part of a noncluster region (far left) are seen on the "upper" surface of the cell. Note the gradation in staining intensity between the two regions. $\times 24,000$. Bar = $1 \mu\text{m}$.

ity that the reaction product itself obscured some specializations in plasma membrane or submembrane structure cannot be excluded, although no such specializations were observed in unstained myotubes. As yet, no clues to the mechanism of restriction of receptors to specific regions of muscle plasma membrane have been found.

It remains to be determined whether the heterogeneity in the ACh receptor distribution described here has a basis similar to that of the heterogeneity of receptor distribution in innervated muscle.

SUMMARY

The structure of regions with a high concentration of ACh receptors (clusters) on cultured skeletal muscle myotubes was examined by immunoperoxidase staining of bound α BT. The clusters did not appear to differ from the other regions except in their higher concentration of receptor.

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