

Insertion and Internalization of Acetylcholine Receptors at Clustered and Diffuse Domains on Cultured Myotubes

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ABSTRACT Two populations of acetylcholine receptors (AChRs) are present in cultured myotubes. One forms large aggregates or clusters and the other has a much lower density of AChRs, which are diffusely distributed. Both clustered and diffuse AChRs are inserted and removed (internalized) from the sarcolemma. To determine the insertion and removal rates of AChRs in these two plasma membrane domains, we used a double label technique to distinguish and quantitate newly inserted and "old" AChRs. Application of our method revealed that the rate of AChR internalization is the same at the clustered and diffuse regions of the plasma membrane, whereas the rate of insertion is threefold greater at the clusters than elsewhere in the plasma membrane. Thus, the increase in AChR number at the clusters is not due to an increase in their half-life, but to an increase in their rate of insertion.

During development acetylcholine receptors (AChRs)¹ are initially diffusely spread along the muscle fibers (21). Later in development, after the onset of synaptic transmission, AChRs aggregate into clusters beneath the nerve-muscle contact (6, 8, 19). The mechanism by which the neuron exerts its effect on the distribution of AChR is not known. However, recent evidence obtained using cDNA probes indicates that when there is an increase in the rate of insertion of new AChRs, the levels of mRNA encoding for AChRs also increase (37). In tissue culture the formation of AChR clusters is not limited to nerve-muscle synaptic contacts and is not dependent upon synaptic transmission (3, 42).

Cultured myotubes contain AChR clusters whose receptor density after incubation with brain extract is similar to that of the adult neuromuscular junction (43). AChR clusters have been observed in vitro on chick myotubes (18), uninervated rat (4) and mouse (17) myotubes, and *Xenopus* myocytes (2). The mechanism by which AChR clusters form is not known. AChR clusters have been shown to form in response to a variety of exogenous stimuli such as extracts from neuronal tissue (10, 17) or basal lamina (10, 42) and the application of silk thread (31) and latex beads (39). It has been shown that

the diffusely distributed receptors can participate in AChR cluster formation (48, 50). However, these diffusely distributed AChRs are not the only pool that contributes to receptor clustering. Newly inserted AChRs also participate in the formation of clusters. In this study, using a computerized image analysis system and a double-label technique that combines autoradiography and fluorescence microscopy, we determined the rates at which new AChRs are inserted and removed at clustered and diffuse AChR regions of the plasma membrane. This approach allowed us, for the first time, to distinguish between newly inserted and "existing" AChRs. Our findings show that the rate of appearance of new AChRs is faster at clusters than at the diffuse receptor regions, whereas the rate of the AChR degradation is the same at the two regions of the membrane.

MATERIALS AND METHODS

Cell Culture: We prepared primary cultures from hind limb of neonate Sprague-Dawley rats as described previously (15). Dissociated myoblasts were cultured in Dulbecco-Voigt modified Eagle's medium containing 10% (vol/vol) horse serum (Gibco Laboratories, Grand Island, NY), 0.5% chick embryo extract (vol/vol), 200 mM glutamine, and 40 mg/ml gentamicin. Cultured rat myotubes were plated in Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) containing collagenized glass coverslips as previously described (14, 15).

Scanning Electron Microscopy of Myotubes: The surface morphology of cultured rat myotubes was examined with the scanning electron

¹ *Abbreviations used in this paper:* AChR, acetylcholine receptor; α BTX, α -bungarotoxin; HRP, horseradish peroxidase; α BTX-TMR, tetramethylrhodamine-conjugated α BTX.

microscope to determine whether the surface of these cells is flat enough to give an accurate measurement for membrane area. Cells grown on plastic coverslips were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer as previously described (11, 12). Cells were dehydrated in graded series of alcohol-water mixtures, dried in a Denton "critical point" apparatus, and coated with gold-palladium in a Denton vacuum evaporator (Denton Vacuum Inc., Cherry Hill, NJ). Coverslips were mounted on metal specimen disks and examined in a JEOL 100CX electron microscope operating at 20 kV.

Electron Microscopy of AChR Clusters: To determine whether the surface membrane where AChRs cluster has membrane infoldings, we stained cells with α -bungarotoxin (α BTX) conjugated to horseradish peroxidase (HRP) as previously described (13). Cells were fixed in 2% paraformaldehyde solution (36) for 1 h, washed several times for a total of 1 h in phosphate-buffered saline (PBS)-containing (36) bovine serum albumin (BSA), and incubated for 2 h in the α BTX-HRP conjugate. Cultures were washed for 1 h in PBS-BSA solution and the reaction product was visualized with 3,3'-diaminobenzidine mixture (13). Control cultures were incubated first with unlabeled α BTX then with α BTX-HRP conjugate. Cells were postfixed in 2% OsO₄, dehydrated in alcohol and embedded in Epon 812. Under the light microscope we located cells containing the AChR clusters (as indicated by the dark brown reaction product) and then sectioned those areas for electron microscopy.

Quantitation of AChRs with ¹²⁵I- α BTX: We obtained purified α BTX from the Miami Serpentarium and ¹²⁵I- α BTX from New England Nuclear (Boston, MA). We determined the AChR surface binding sites and the rates of insertion and internalization using ¹²⁵I- α BTX (2×10^{-8} M, specific activity 17–25 Ci \times mmol) as previously described (14). Background counts were determined by incubation of cells with unlabeled α BTX (5×10^{-7} M) followed by exposure of cells to ¹²⁵I- α BTX for 1 h. All incubations were carried out at 37°C in a 5% CO₂ and 95% air incubator. Specific binding was taken as the difference between total ¹²⁵I- α BTX bound and the amount bound in the presence of unlabeled α BTX, and the background labeling was found to be 10–15% of total counts. The appearance of new AChRs was determined by incubation of cells for 1 h with unlabeled α BTX (5×10^{-7} M) to block existing surface receptors. The presence of α BTX does not effect the production of new AChRs (29). Cells were washed with Dulbecco-Voegt modified Eagle's medium to remove the unbound α BTX and return to the incubator in culture medium for 2–24 h. At each of the time points cells were pulsed with ¹²⁵I- α BTX for 1 h, washed with Hanks' balanced salt solution containing 0.1% BSA, and extracted with 50 mM Tris, pH 7.4, 50 mM NaCl, and 1% Triton X-100. Cells were scraped with a rubber policeman, and samples were centrifuged at 15,000 g for 15 min to remove debris, and counted in a gamma counter.

The AChR degradation rate was determined by incubation with ¹²⁵I- α BTX for 1 h. We washed cells to remove the unbound ¹²⁵I- α BTX and returned them to the incubator in culture media for 0–24 h. At selected times we removed cells from culture dishes as described above, and determined in a gamma counter the number of AChRs remaining.

Determination of ¹²⁵I- α BTX Binding Sites by Autoradiography: Cells were incubated with ¹²⁵I- α BTX (2×10^{-8} M, specific activity 17–25 Ci \times mmol) for 1 h, washed with Hank's balanced salt solution to remove the unbound toxin, and fixed in 2% formaldehyde (36). The concentration of the ¹²⁵I- α BTX used was sufficient to inactivate >98% of AChRs to iontophoretically applied acetylcholine (26, 32). Cells were washed with phosphate buffer, and coverslips on which cells were grown were attached to glass slides. The slides were air dried, dipped in Kodak NTB-2 emulsion diluted 1:1 with water at 42°C, and exposed in the dark at 4°C in dessicator boxes for 3 d. The autoradiograms were developed at 20°C in Dektol (Eastman Kodak Co., Rochester, NY) for 2.5 min, rinsed in distilled water, and then fixed in rapid fixer for 2 min. Cells were air dried, and the distribution of grains on myotubes was determined under a magnification of 1,250 using dark field illumination of a Zeiss microscope. The distribution of grains in a given area of the myotube and in the AChR clusters was determined with a camera lucida and a digitizing tablet connected to an Apple II computer (Apple Computer Inc., Cupertino, CA). The areas of myotubes were determined (a) by encircling each myotube segment with a pen on a digitizing tablet and (b) at the same time from stipling in the silver grains present on the myotube segments. By adjusting the plane of focus, we obtained the number of grains per calibrated surface area of the myotube. We used a similar procedure to determine the distribution of silver grains within an AChR cluster that was visualized with α BTX-TMR (see below). Background binding was determined on areas of slide away from myotubes and subtracted from the total. The background binding was $9 \pm 2\%$ of the total counts. The efficiency of grain production for ¹²⁵I using Kodak NTB-2 emulsion was determined to be ~ 0.5 grain per disintegration (1). The number of α BTX binding sites (M) was determined using the following formula (32): $M = (Gd/ E)/(SD/A)$, where G is the number of grains per unit area; d is the number of

decays correlated with one developed grain; E is exposure time in minutes; S is specific activity; D is decays per minute per Curie (2.22×10^{12}); and A is Avogadro's number.

Quantitative Double-labeled Studies: To determine the location of existing AChRs and to map the insertion of new receptors at clustered and diffuse regions of the plasma membrane, we performed double-labeled experiments using α BTX conjugated with tetramethylrhodamine (α BTX-TMR). α BTX-TMR was prepared as previously described (14), and we tested the specificity of α BTX-TMR conjugate binding to the AChRs by incubating cells for 1 h first with unlabeled α BTX (5×10^{-7} M) then with α BTX-TMR. Under those conditions, we observed no specific binding of α BTX-TMR to myotubes.

We mapped the insertion of "new" AChRs by blocking the "existing" AChRs with α BTX-TMR (5×10^{-8} M), which also served to mark existing clusters (9, 10). After washing out the unbound toxin, we placed cultures in the incubation medium for 0–12 h. At each of the time points examined cultures were incubated with ¹²⁵I- α BTX (2×10^{-8} M, specific activity 17–25 Ci mmol) and processed for autoradiography as described above. We examined the distribution of grains (newly inserted receptors) with a Zeiss photomicroscope II under dark-field illumination and the distribution of existing AChRs were examined with fluorescent optics (BP 546/12) excitation filter and LP 590 barrier filter. This procedure allowed us to determine the distribution of silver grains on the same fluorescent area of the cell. The distribution of the silver grains in the clustered and diffuse segments of myotube membrane was quantitated with the aid of a drawing tube and a digitizing tablet attached to a computer using the Bioquant program (Bioquant, Nashville, TN). This technique offers good resolution in determining the distribution of silver grains inside and outside of the cluster, since the silver grains are small ($>0.5 \mu\text{m}^2$) and the clusters are large ($500\text{--}800 \mu\text{m}^2$). The fluorescence optics used sharply outlined the boundary of the cluster, and this image was stored on the computer. By switching to phase-contrast or dark-field optics, we determined the distribution of grains, which allowed us to calculate the distribution of grains in these two areas of the myotube. This procedure for marking "old" clusters with α BTX-TMR is reliable, since the conjugate has been shown to bind essentially irreversibly to AChRs, and AChR clusters on myotubes are known to remain stable, moving $<1 \mu\text{m}$ in 19 h (24). On the other hand, it takes only $\sim 2\text{--}3$ h for new AChRs to be inserted into the surface membrane from the time of synthesis (20, 23). Thus, by blockage of all surface AChRs with α BTX-TMR, only the newly inserted AChRs are detected by autoradiography after removal of the fluorescent toxin.

RESULTS

Surface Morphology of Myotubes and AChR Clusters

Scanning electron microscopy was carried out to determine whether there were any membrane foldings on the myotube surface. This was important to know, because if membrane folding was present, our measurements on the light microscopic level of the cell's surface area would not give us an accurate value of grain distribution per micrometer squared of membrane surface. Fig. 1A shows a scanning micrograph of a cultured rat myotube. Examination of 60 myotubes from three coverslips showed that cells grown under normal culture conditions (see Materials and Methods) have a smooth surface. This agrees with published reports on cultured muscle cells from other species (13, 26, 32), and, therefore, our assumption is valid that the surface area of these cells measured by light microscopy was a close approximation of the true membrane area. However, scanning microscopy cannot identify regions of myotube membrane that contain a high AChR density. For this purpose, we used α BTX-HRP conjugate (11, 13) to label the AChRs. Regions of myotube membrane that contain AChR clusters are easily identified as a dark brown reaction product under the light microscope, and these, including the regions from which AChR clusters are absent, were sectioned for visualization with the electron microscope. Fig. 1B shows a region of myotube sectioned through an AChR cluster. The α BTX-HRP reaction product

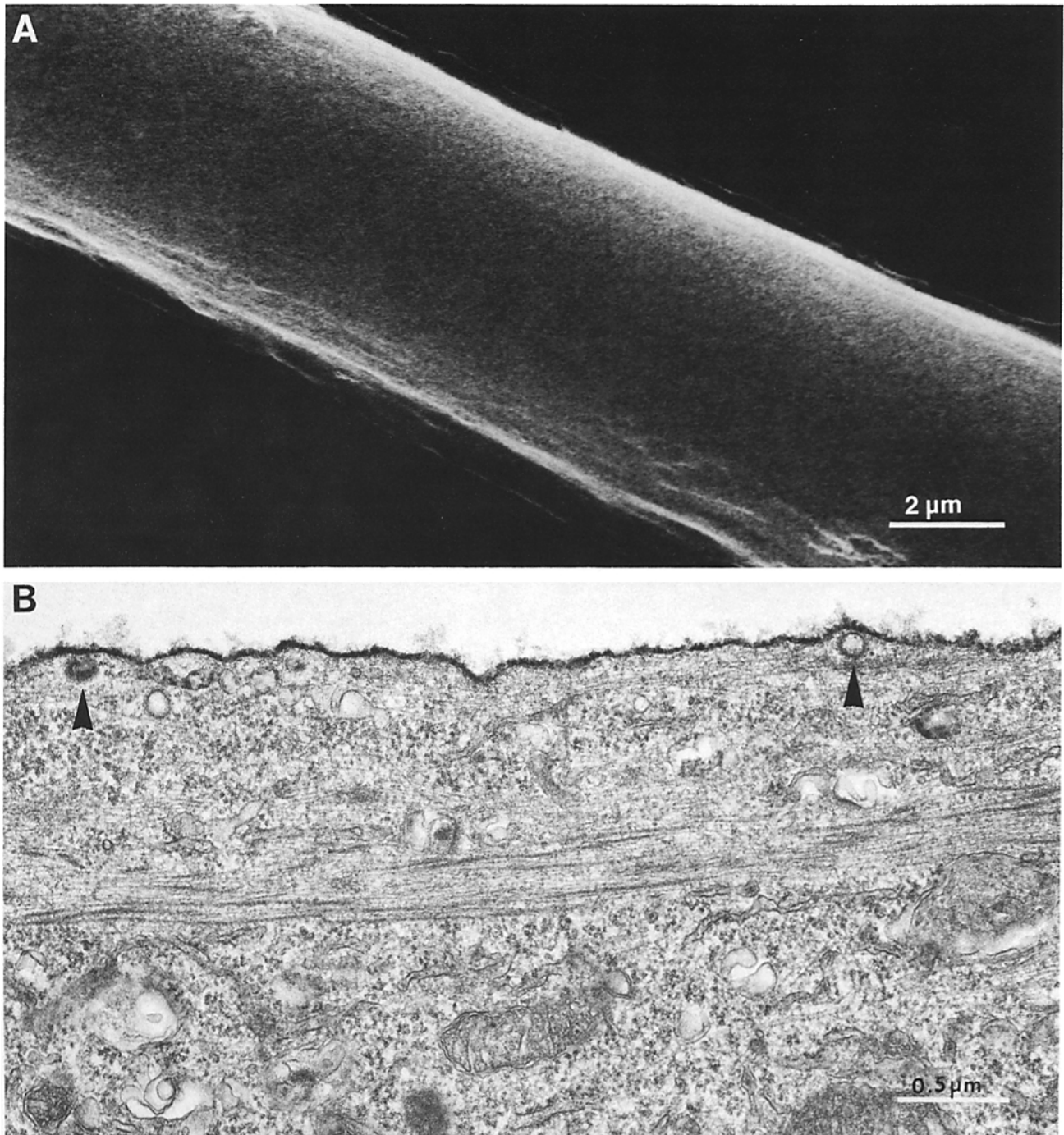


FIGURE 1 (A) Scanning electron micrograph of a cultured rat myotube. The myotube surface appears smooth and the cell is surrounded by an amorphous material. $\times 9,000$. (B) Transmission electron micrograph of a myotube sectioned through an AChR cluster. The cell surface containing α BTX-HRP reaction product is smooth, and occasionally membrane invaginations $0.2 \mu\text{m}$ deep are present at both clustered and receptor diffuse regions. Coated vesicles (arrowheads), some of which contain α BTX-HRP binding sites, are found near the membrane surface. $\times 36,400$.

was confined to the surface of the plasma membrane and no infoldings were observed (Fig. 1 B). These results agree with previous observations carried out on chick myotubes using immunoperoxidase technique (49). In addition, we have observed occasional invagination of the plasma membrane, but their presence was not limited to the area of the AChR cluster. Coated vesicles and coated pits, some of which contain α BTX binding sites, were present at the clustered regions (Fig. 1 B), and these structures are also present in the diffuse AChR

regions of the myotube. These results indicate that the formation of AChR clusters is not caused by increase in membrane area brought upon membrane folding.

AChR Distribution and Insertion Rates

Clustered and diffuse AChRs are present in cultured rat myotubes and these were quantitated with ^{125}I - α BTX autoradiography. Clusters were also visualized with α BTX-TMR and fluorescent microscopy. We have defined AChR clusters

as regions of membrane that contain intensely fluorescent patches (13, 14), and/or a region of membrane that contains a high grain density, giving the impression of clumping (10, 32). To quantify the distribution of grains in the clustered and diffuse region of the myotube, double-label techniques using autoradiography and fluorescent microscopy on the same cells were carried out. Computer-assisted grain counts made on cells using a camera lucida and a digitizing tablet demonstrated that the distribution of AChRs was always higher within a cluster than in the diffuse region of the muscle cell surface. However, despite the low densities of these diffuse AChRs, they account for most of the total number of receptors. Based on the percentage of myotubes in the culture dish, the percentage of myotubes containing clusters and the size of clusters, the percentage area occupied by clusters was determined. We found that 51.4% of the area of each 35-mm culture dish is covered by myotubes, and the rest is occupied by fibroblasts or open spaces devoid of cells. Approximately 30% of myotubes have clusters, and ~10% of the myotube area is occupied by clusters. By multiplying these values we determined the area of myotubes occupied by AChR clusters. Our calculations show that the AChR clusters occupy ~3.1%

of myotube surface area. After obtaining the distribution of grains per micrometer squared and the equation given in Materials and Methods, we calculated the average number of α BTX binding sites found in clusters and diffuse regions. In agreement with previous reports (32, 43), cultures grown under normal conditions have an average number of α BTX binding sites of $825 \pm 46.8/\mu\text{m}^2$ (\pm SE) in clustered regions and 210 ± 16.9 per μm^2 (\pm SE) in diffuse regions. We occasionally saw clusters with α BTX binding site density of 1,500–2,000 sites/ μm^2 . These data indicate that clustered receptors account for ~12% of total receptors.

Total counts of AChRs as obtained by gamma counting cannot differentiate between those receptors found in the clusters and those found in the diffuse region of the plasma membrane. To distinguish between these two populations of receptors and to determine their rates of insertion at the two membrane domains, we used a double-label technique employing fluorescent microscopy and autoradiography.

Fig. 2 shows double-label images of myotubes incubated with α BTX-TMR washed, left in culture medium for 0 to 12 h, incubated with ^{125}I - α BTX, and processed for autoradiography. By alternating between the fluorescent filters and dark-

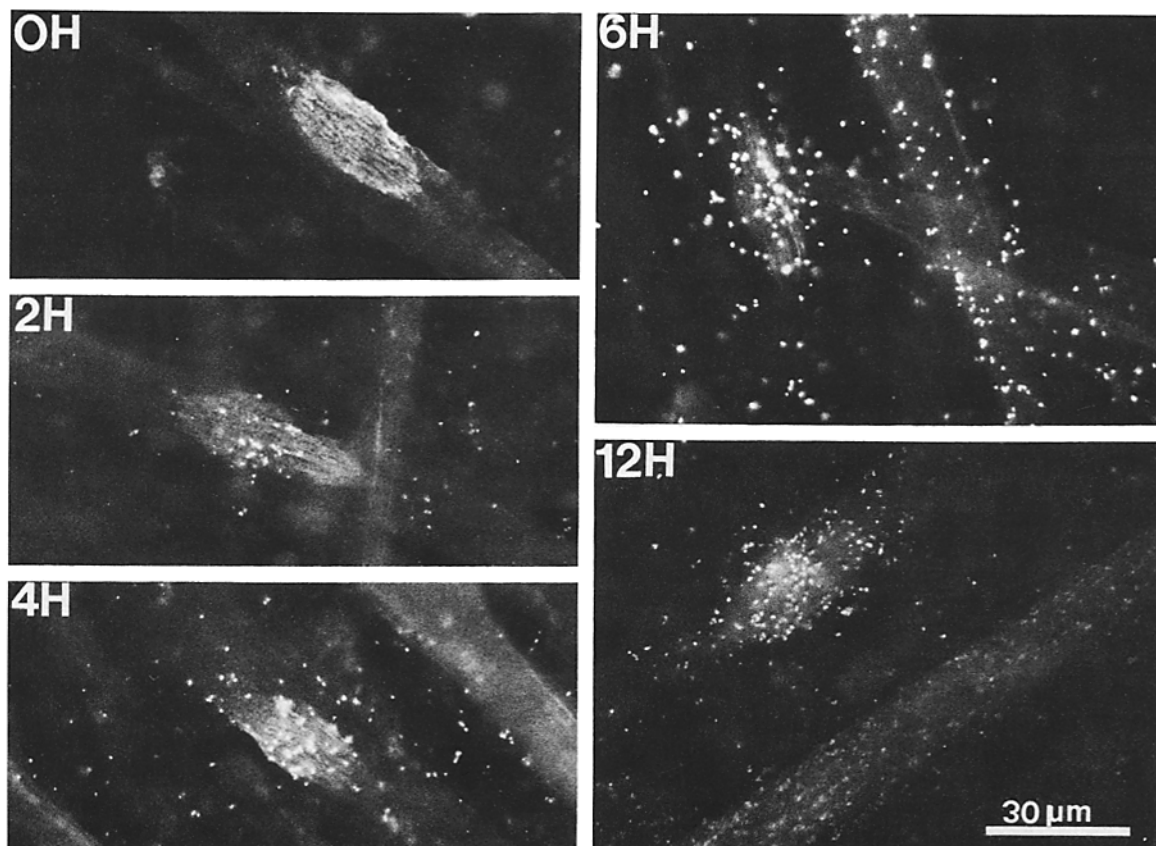


FIGURE 2 Double-exposure images of new AChRs in existing clusters and diffuse region of the plasma membrane. Rat myotubes were grown on collagen-coated glass coverslips and incubated for 1 h at 37°C with α BTX-TMR (7×10^{-8} M). After the unbound toxin-conjugate was washed out, cultures were returned to the incubation medium (Dulbecco-Voigt modified Eagle's Medium at 37°C) for 0–1, 2, 4, 6, and 12 h. After each of these times, cells were labeled with 20 nM of ^{125}I - α BTX for 1 h. The unbound ^{125}I - α BTX was washed out, and cultures were fixed in 2% phosphate-buffered formaldehyde. Coverslips were attached to slides, air-dried, and processed for autoradiography. The distribution of grains (newly inserted receptors) was examined with Zeiss photomicroscope II under dark-field illumination, and the distribution of the existing AChRs was examined with fluorescent optics. This procedure allowed us to determine the distribution of silver grains on the same fluorescent area of the cell. Some grains are out of focus because not all of them lie in the same plane of the cell surface. Cells were incubated with ^{125}I - α BTX at 0, 2, 4, 6, and 12 h after removal of α BTX-TMR. The newly inserted AChRs are present at both the existing clusters and the diffuse region of the myotube. All photographs were exposed and printed identically. $\times 480$.

field optics we could distinguish and quantitate the existing AChR clusters (as visualized with α BTX-TMR), and the newly inserted AChRs (as visualized by the silver grains). At 0 h after incubation first with α BTX-TMR then with ^{125}I - α BTX, no silver grains beyond background are present (Fig. 1). However, at later times after removal of α BTX-TMR, newly inserted receptors appear at both AChR clusters and in the non-clustered region of the myotube (Fig. 2). We quantitated these results using a computerized image analysis system. The number of silver grains on myotubes per unit area was determined at several points between 0 and 12 h (Fig. 3). We determined the net change in the appearance of new silver grains per hour after correcting for background by subtracting the silver grains appearing at a given time interval from total grains at the end of a time interval and dividing by the elapsed time. At time 0 after removal of α BTX-TMR we washed cultures extensively with Hanks' balanced salt solution to remove the unbound toxin before returning them to the incubator. This extensive washing and the change in temperature (cells were washed in a sterile hood at room temperature) appear to affect the synthesis of new AChRs. Thus, the rate of synthesis 2 h after removal of the α BTX-TMR is diminished as compared with the later incubation time points (Fig. 3). Data obtained from 600 myotubes show that the rate at which new AChRs appear at the clusters is two- to threefold faster than at the diffuse region of the myotube (Fig. 3).

Degradation of AChRs

The rate of degradation of total AChRs was determined by incubation of cells with ^{125}I - α BTX (2×10^{-8} M) for 1 h at 37°C and at 0–24 h after the unbound toxin was washed out, the radioactivity remaining in the cells was counted in a gamma counter. The rate at which total AChRs are degraded is described as a first-order process, and, as shown in Table I,

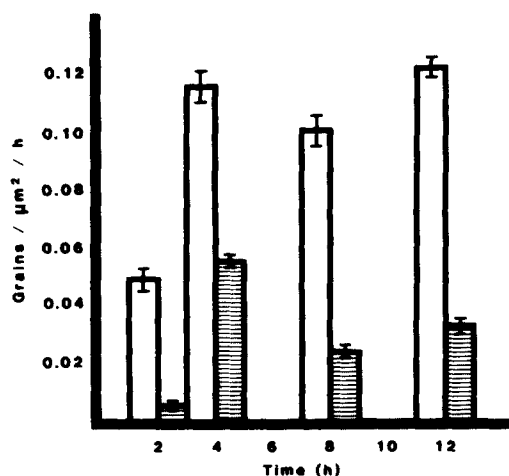


FIGURE 3 Change in the rate of accumulation of clustered and nonclustered AChRs. The distribution of grains was mapped with the aid of a drawing tube on a digitizing tablet connected to a microcomputer. This procedure allowed us to map the distribution of silver grains and at the same time adjust the plane of focus so that most of the grains per unit area of clusters and diffuse receptor region were counted. The rate of AChR insertion was obtained by subtracting the difference in density between each time point and dividing by the elapsed time. New AChRs appear in the clusters faster than they appear in the diffuse region of the plasma membrane. Each bar represents $80 \pm \text{SD}$ AChR clusters or $80 \pm \text{SD}$ myotube areas containing diffuse AChRs. Open bars, grains in cluster. Striped bars, diffuse grains.

they are removed at a rate of 0.58 h ($t_{1/2} = 11.9 \text{ h}$). The rate at which AChRs are removed at clustered and diffuse regions of the myotube was determined after processing for autoradiography by counting the distribution of silver grains. For this purpose, cells were labeled with ^{125}I - α BTX for 1 h, the unbound toxin was washed out, and cultures were returned to the incubator for 0–12 h. Newly inserted AChRs were identified with the fluorescent toxin and the “old” AChRs were identified by the distribution of silver grains (Fig. 4). A decrease in the number of silver grains after removal of ^{125}I - α BTX and subsequent incubation in culture medium was observed in both clustered and non-clustered regions of the myotubes. Our computerized analysis showed that the rate of AChRs internalization at clusters is approximately equal to the rate of internalization of diffuse AChRs (Table I). Furthermore, the rates at which receptors were removed from clustered and diffuse regions were not significantly different from those obtained for total AChRs using gamma counting (Table I), thus confirming that our microscopic determinations of AChRs are as accurate as gamma counting. Fig. 5 shows an example of a computer printout of the distribution of AChRs (silver grains) within the clusters and the diffuse region of the myotube during insertion (A) and internalization (B). The computer-assisted analysis of these changes is shown in Fig. 3 and Table I. Using this technique, we found that the rate at which AChRs are inserted into clusters is two to three times higher at the clusters than at the diffuse region of the membrane, whereas the rate of receptor internalization at the two membrane domains remains the same.

DISCUSSION

The AChRs at the neuromuscular junction are stable as compared with the extrajunctional receptors of denervated muscle (5, 16). The turnover rate of these extrajunctional AChRs is rapid with a $t_{1/2}$ of 15 to 30 h (22), whereas the junctional AChRs are degraded much more slowly with a $t_{1/2}$ of 6 to 13 d (5, 33, 35). Further studies showed that the rate at which AChRs are degraded at the neuromuscular junction is not uniform. At least two populations of AChRs, one with a rapid turnover rate (18 h) and one with a slow turnover rate (12 d) have been described (47).

In cultured embryonic myotubes only AChRs with a rapid turnover rate (11–18 h) have been identified (14, 29, 32, 46). These studies determined the rate of AChR degradation by monitoring the loss of ^{125}I - α BTX that specifically bound to AChRs. Previous observations showed that cultured myotubes contain both diffuse and clustered AChRs; however, no detailed studies were carried out that would allow one to determine whether the rates at which AChRs are inserted are the same or different in the two regions of the plasma membrane. We used double-label techniques and computerized analysis to answer these questions.

We expressed the distribution of the AChRs as grains per micrometer squared of myotube surface. Therefore, it was important to determine whether the myotube surface of the

TABLE I. AChR Degradation Rates

	Clusters	Diffuse	^{125}I - α BTX
Rate of loss (h^{-1})	0.066	0.070	0.058
$t_{1/2}$	10.4	9.7	11.9
Correlation coefficient	0.996	0.995	0.995

This table compares AChR degradation rates determined by autoradiography and gamma counting.

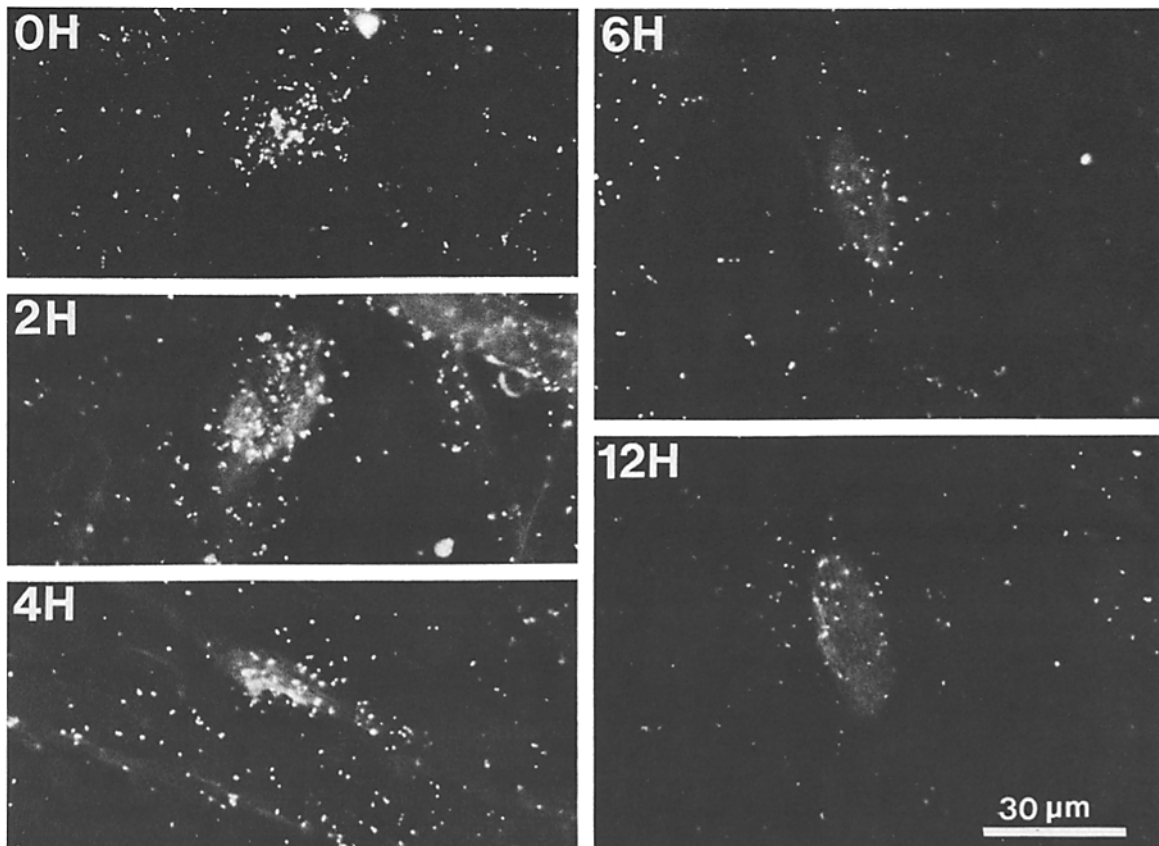
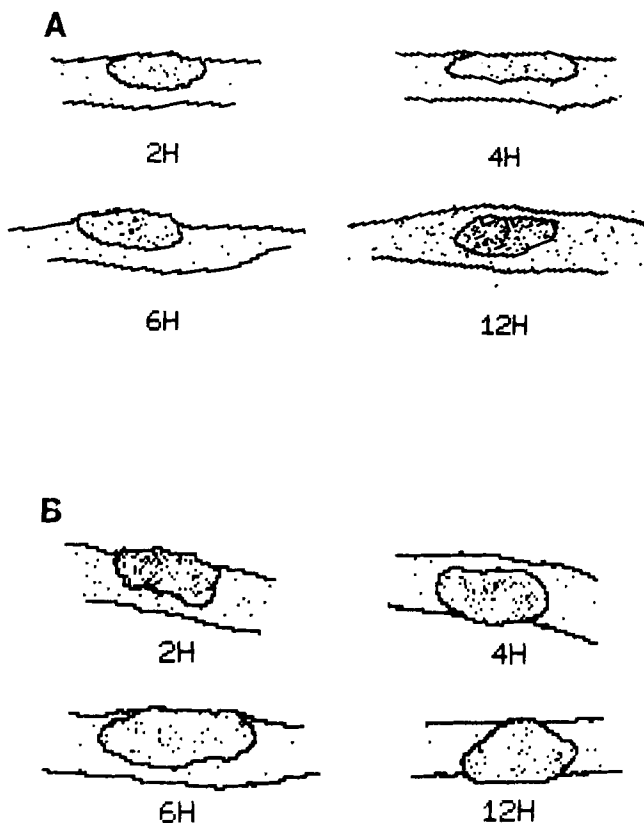


FIGURE 4 Double exposure of the distribution of silver grains and AChR clusters during internalization. Cells were treated as in Fig. 1 except for the order in which the toxin conjugates were used. Cells were incubated with ^{125}I - αBTX , washed, and left in culture medium at 37°C for 0–12 h. At each time (0–12 h) cells were stained with $\alpha\text{BTX-TMR}$. The number of grains remaining in clusters and the diffuse region of the sarcolemma decreases with time in culture medium. Cells were incubated with $\alpha\text{BTX-TMR}$ at 0, 2, 4, 6, and 12 h after the removal of ^{125}I - αBTX . All photographs were exposed and printed identically. $\times 480$.



two plasma membrane domains were identical under our culture conditions. Scanning electron microscopy of the myotubes revealed a smooth surface of the cell. Furthermore, transmission electron microscopy of cells stained with $\alpha\text{BTX-HRP}$ or by the immunoperoxidase technique (49) to locate AChR clusters revealed no increase in the degree of membrane folding at the AChR clusters as compared with elsewhere in the membrane. Similar results were obtained by electron microscopy of electrophysiologically mapped high density sites (32, 38). Sanes et al. recently reported an indication of membrane folding in myotubes exposed to brain extract, but even after exposure to brain extract most myotubes that they examined were free of membrane foldings (44). The results that we and others have obtained indicate that the surface area measured by light microscopy was a close approximation of the one seen under higher resolution, and the areas measured at the clusters are not greater than the area measured at the diffuse receptor region. Thus, membrane folding does not appear to contribute to AChR aggregation.

FIGURE 5 The distribution of silver grains in diffuse and clustered regions of plasma membrane during insertion (A) and internalization (B) as determined by computer analysis. The boundaries of AChR clusters as visualized by $\alpha\text{BTX-TMR}$ are circumscribed by the solid lines, and the dots represent the region where the silver grains are found.

The high AChR density domain can be accounted for by the following mechanisms: an increased rate of insertion; a decreased rate of turnover of AChRs in the clusters; or lateral diffusion of AChRs into clusters. AChRs are known to migrate in the plane of the membrane (4), and newly inserted AChRs do not stay precisely at their site of insertion. In view of the stability of the AChR clusters and the lack of membrane folding, which would contribute to an increase of surface area, the increase in the number of AChRs can be attributed to an increased insertion rate. This conclusion is supported by our data, which show that the rate at which AChRs are inserted is two- or threefold higher at clusters than in the diffuse region of the membrane. Furthermore, the rate at which AChRs are removed from the clustered and diffused regions is the same, and thus the increase in the number of the AChRs is not due to their longer half-life at the cluster. Since the half-life values were not significantly different for clustered and diffuse receptors quantitated by light microscopic autoradiography or gamma counting, our technique of quantitating AChRs was subject to the same experimental errors. In addition, the values for α BTX binding sites within a cluster and diffuse region of the membrane are comparable to those reported by others (10, 32, 43).

Recent studies by Ziskind-Conhaim et al. (50) indicate that the developing neuromuscular junctions of rat embryos arise at least in part from the diffusely distributed AChRs. Photobleaching recovery experiments indicated that the diffuse AChRs contribute to the formation of clusters; however, some clusters do not fill in at all with AChRs but can be recognized if stained with α BTX-TMR (48). Experiments using cDNA probes to the AChR show that mRNA encoding AChR is present at a much higher concentration at the junctional region of the mouse diaphragms than at the extrajunctional areas in the muscle (45). These data suggest that newly inserted AChRs also contribute to the formation and perhaps the maintenance of the AChR clusters. Although our approach did not allow us to determine where within the cluster, or outside of it, new AChRs are inserted or removed, our data analysis shows that the rate of insertion of receptors is greater within the clusters than in the diffuse receptor regions. We do not know whether there are preferential sites within the plasma membrane where newly synthesized AChRs are inserted or removed. If such preferential sites exist, it will be important to determine whether the AChRs at those sites are the products of the same or different genes.

The AChRs are removed from clusters and diffuse regions with a half-time of 11 h, but clusters do not completely disappear during this time. In addition, myotubes that have been incubated with unlabeled α BTX, washed, and within 2 h after washing stained with α BTX-TMR show the reappearance of AChR clusters. These observations indicate that these clusters must also contain newly synthesized AChRs. More direct evidence that a portion of AChRs within clusters was newly synthesized was recently obtained by Fischbach et al. (25) using computerized image analysis and fluorescent techniques. An increase in the rate of insertion of AChRs into clusters would allow the cluster to be recognized for a longer time than the diffuse receptors. Since the rate of internalization appears to be essentially the same at the clusters and the diffuse region of plasma membrane, an increase in the rate of insertion of newly synthesized AChRs would allow for the maintenance of the cluster stability and contribute to the increase in AChR density in this region.

Our results demonstrate that the high AChR density seen at the cluster is due to an increase in the rate of AChR insertion rather than to an increased half-life of receptors within the cluster. We do not know if the rate of insertion and internalization at clusters changes with innervation. However, in innervated chick embryonic myotubes, there does not appear to be any difference in the metabolic stability of clustered or diffuse AChRs (46). Changes in cytoskeletal proteins (7, 27, 28, 41) membrane fluidity (4), extracellular matrix (10, 42), or other yet to be identified factors may contribute to this increased rate of AChR insertion at the clusters. Our previous results indicate that AChRs are inserted and internalized by the coated vesicle-mediated pathway (13, 34). In this respect, it is interesting that the brain extract increases the number of AChR clusters (30, 40) as well as their size and density (43). These cells in the cluster region not only have α BTX binding site density comparable to the mature neuromuscular junction, but also an increase in number of coated vesicles containing toxin binding sites (13). Thus, AChR clusters may represent regions of the plasma membrane that may be metabolically specialized for efficient insertion and removal of AChRs.

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