Mobility and Detergent Extractability of Acetylcholine Receptors on Cultured Rat Myotubes: A Correlation

M. STYA and D. AXELROD
Biophysics Research Division, The University of Michigan, Institute of Science and Technology, Ann Arbor, Michigan 48109

ABSTRACT On aneurally cultured rat primary myotubes, 10% of the acetylcholine receptors (AChR) are found aggregated and immobilized in endogenous clusters. The remaining receptors are diffusely distributed over the cell membrane and the majority of these are free to diffuse in the plane of the membrane. This study correlates the mobility of AChR (as measured with the fluorescence photobleaching recovery technique, FPR) with the detergent extractability of this receptor. Gentle detergent extraction of the cells removes the lipid membrane and the soluble cytoplasmic proteins but leaves an intact cytoskeletal framework on the substrate. Two studies indicate a correlation between mobility and extractability: (*a*) mobility of diffusely distributed AChR decreases as myotubes age in culture; previous work showed that extractability of AChR decreases as myotubes age in culture (Prives, J., C. Christian, S. Penman, and K. Olden, 1980, *In* Tissue Culture in Neurobiology, E. Giacobini, A. Vernadakis, and A. Shahar, editors, Raven Press, New York, 35–52); (*b*) mobility of clustered AChR increases when cells are treated with metabolic inhibitors such as sodium azide (NaN_3); extractability of clustered AChR also increases with this treatment. From these results we suggest the involvement of a cytoskeletal framework in the immobilization of AChR on the cell surface.

On mature rat myotubes maintained in primary cell culture, acetylcholine receptors (AChR)[1] are present in two states, differing in location on the membrane as well as in mobility within the plane of the membrane. About 90% of the AChR are diffusely distributed over the myotube surface, and the majority of these are free to diffuse in the plane of the membrane. The remaining 10% of the receptors are found in aggregates called endogenous clusters within which they are immobilized.

The mechanism underlying this immobilization is not yet clear. To elucidate the possible role of the cytoskeleton in the immobilization of AChR, we performed experiments designed to correlate the lateral mobility of AChR with their extractability from the cell membrane by gentle detergent treatment. With such treatment the plasma membrane lipids and soluble cytoplasmic proteins are removed rapidly, while the intact skeletal framework is retained on the substrate (4). These conditions do not increase the extremely slow rate at which bungarotoxin (BT, a highly specific ligand for AChR) dissociates from the receptors (7), and hence it is possible to observe the extent to which AChR are bound to the cytoskeleton by labeling the cells with fluorescent BT, subjecting them to detergent extraction, and measuring the loss of fluorescence.

We compare results of these extraction experiments with measurement of AChR lateral mobility by using the fluorescence photobleaching recovery technique (FPR; 3). Two types of studies indicate a correlation between the mobility of an AChR and its extractability with detergent buffer. First, we show that the mobility of diffusely distributed AChR decreases as myotubes age in culture. This result correlates with a previous observation (9) that, as myotubes age, fewer AChR can be extracted from their membranes with detergent treatment. Second, we show directly by FPR that treatment with sodium azide (NaN_3), which Bloch (5) has shown disperses clusters, causes the clustered AChR to become more mobile. In connection with this, we show that the detergent-extractable fraction of clustered AChR increases with NaN_3 treatment.

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor(s); BT, α-bungarotoxin; FRR, fluorescence photobleaching recovery technique; R-BT, tetramethylrhodamine labeled BT; TTX, tetrodotoxin.

MATERIALS AND METHODS

Culture Preparation and Treatment: **Primary rat myotube cultures were prepared as previously described (1). Medium was replaced every 2–**

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3 d. After myoblast fusion, which generally began on day 3, the medium was augmented with 0.6 μg/ml of tetrodotoxin (TTX, obtained from Sigma Chemical Co., St. Louis, MO) to prevent spontaneous contraction of the myotubes. Experiments were performed on myotubes in 6- or 7-d-old cultures for studies of NaN_3 treatment and in 5-, 6-, 7-, 10-, 11-, 12-, and 14-d-old cultures for lateral mobility studies at different ages of myotubes. In the latter experiments, care was taken that the different culture platings were treated identically throughout their lives.

Fluorescent Toxins: Tetramethylrhodamine α-bungarotoxin (R-BT) was prepared as previously described (10). Myotube cultures were exposed to R-BT in medium for 1 h at 37°C and then were washed several times in phosphate-buffered saline (PBS, Gibco Laboratories, Grand Island, NY) containing 0.6 μg/ml TTX (PBS/TTX). R-BT labeling is irreversible on the time scale of our experiments (6).

Lateral Mobility Measurements: Fluorescence of cells was viewed with an inverted epi-illumination microscope (Leitz Diavert) and an argon ion laser (Lexel 95-3) excitation source set at $\lambda = 514.5$ nm. The microscope objective was a × 50, NA = 1.00, water immersion (for studies of NaN_3 treatment), or a × 100, NA = 1.25, oil immersion (for studies of myotube age). Lateral motion of fluorescently labeled AChR was measured by the FPR (see reference 3). The fluorescence of labeled receptors in a small area on the membrane (2.0 μm^2 for NaN_3 studies and 0.7 μm^2 for aging studies) was bleached by a flash of focused laser light (50-msec duration and 4 mW of power for NaN_3 studies; 20-msec duration and 18-mW of power for aging studies). Subsequent lateral motion of unbleached fluorophore into the bleached region was measured by recovery of fluorescence excited by the same beam attenuated $\sim 2 \times 10^4$ times. Fluorescence recovery was recorded for 5–10 min after bleach.

Because occasional cell motion (≥0.2 μm over times of several minutes) affects the asymptotic level of the postbleach recovery in regions where AChR are diffusely distributed, it is not always possible to reliably measure the conventional "fractional mobility" (f) and diffusion coefficient (D) of the mobile fraction as separate parameters. Instead, we present results as the average $\bar{D}$ (= fD) of the mobile and immobile components. This product is relatively insensitive to errors in the estimate of the recovery asymptote. $\bar{D}$ is proportional to the ratio $f/\tau_{1/2}$ (as used for NaN_3 studies), where $\tau_{1/2}$ is the time for half-recovery of the mobile fraction (as used for NaN_3 studies), or to the initial slope of the recovery curve (as used for aging studies).

Measurement of Extraction Rates: Cells were labeled with R-BT (1 h at 37°C), washed twice with PBS/TTX, and washed twice with 2-ml volumes of 0.3 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES at pH 7.4 ("buffer A", see reference 9) before being placed in a 2-ml volume of buffer A on the microscope stage. The microscope was focused on a region of the myotube surface bearing a cluster of AChR, and fluorescence that was excited by a completely defocused laser beam was observed by a photomultiplier (RCA, C31034A). A diaphragm in the microscope image plane was used to limit the observed fluorescence to that emanating from a small field within the AChR cluster. Field size varied somewhat (depending on cluster size) but typically ranged from 9 to 25 μm^2.

The output of the photomultiplier tube was recorded with 1-s counting intervals before and after the addition of a 2-ml volume of 1% Triton X-100 in buffer A. This addition resulted in a 4-ml volume of 0.5% Triton X-100 buffer A, a solution in which membrane lipids and soluble proteins are rapidly extracted, leaving behind the cytoskeleton and its attached proteins. Fluorescence decay was recorded for 3 min after addition of the detergent.

RESULTS

Treatment with NaN_3 markedly increases the mobile fraction of clustered AChR (see Fig. 1 and 2). The effect is dependent on the duration of treatment (Fig. 1), dependent on the concentration of NaN_3 (Fig. 2*A*), and reversible except in cases of treatment with high concentrations of NaN_3 for prolonged periods of time. In cases where the mobile fraction is large enough to permit unambiguous determination of $\tau_{1/2}$ (i.e., $f \geq 0.25$), the diffusion coefficient of the mobile fraction (D) was calculated to be $\sim 1.5 \times 10^{-10}$ cm²/s. This D for NaN_3-treated clustered AChR is comparable with the normal D of diffusely distributed AChR as calculated from $\bar{D} = f/D$ and assuming a typical diffuse region fractional recovery f of 40–60%.

In contrast with the NaN_3 sensitivity of the mobility of clustered AChR, the mobility of the diffusely distributed receptors is not affected by treatment with NaN_3 (Fig. 3). For diffusely distributed AChR, mobility is described by an average diffusion coefficient $\bar{D}$ of both mobile and immobile fractions (see Materials and Methods). Oppositely directed effects of NaN_3 on f and $\tau_{1/2}$, leading to a constant ratio $f/\tau_{1/2}$, cannot be ruled out if these effects are small.

Exposure to NaN_3 also increases the fraction of clustered AChR susceptible to detergent extraction (Fig. 4). This effect

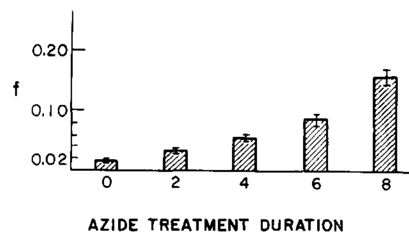

FIGURE 1 Effect of duration (in hours) of treatment with NaN_3 on clustered AChR. Cells were given 2.5 mM NaN_3 in medium and were examined after 2 ($n = 41$), 4 ($n = 35$), 6 ($n = 30$), and 8 h ($n = 22$) of exposure. Controls ($n = 48$) were kept in NaN_3-free medium throughout. The mobile fraction f increases as length of drug treatment increases. Only for the 8-h treatment was the mobile fraction large enough to measure the diffusion coefficient. For those samples, $D = (1.5 \pm 0.1) \times 10^{-10}$ cm²/s. Error bars are all SEM.

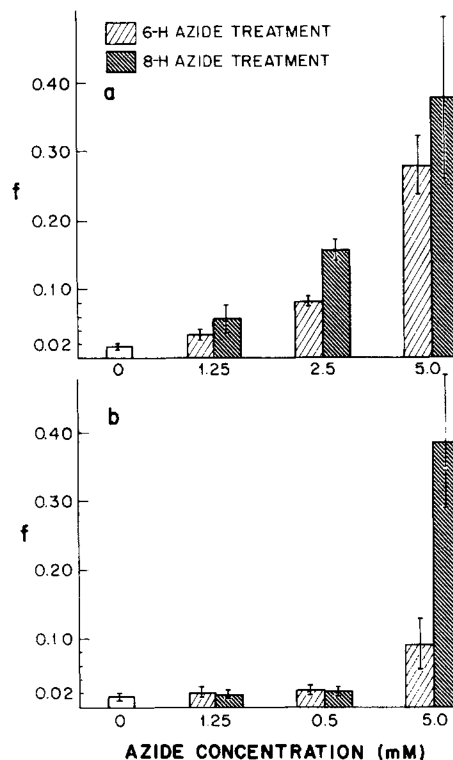

FIGURE 2 Effect of concentration of NaN_3 on clustered AChR. Controls ($n = 48$) were kept in NaN_3-free medium throughout. (*a*) Cells were exposed to NaN_3 in medium for 6 h (1.25 mM, $n = 7$; 2.5 mM, $n = 30$; 5.0 mM, $n = 3$) or 8 h (1.25 mM, $n = 7$; 2.5 mM, $n = 22$; 5.0 mM, $n = 3$) before FPR experiments. The mobile fraction increases as NaN_3 concentration increases. (*b*) Cells were allowed to recover in NaN_3-free medium for 18 h after exposure to NaN_3 in medium for 6 h (1.25 mM, $n = 7$; 2.5 mM, $n = 8$; 5.0 mM, $n = 4$) or 8 h (1.25 mM, $n = 7$; 2.5 mM, $n = 4$; 5.0 mM, $n = 3$). Mobile fraction decreases again except for harshest NaN_3 treatment (5 mM for 8 h). Only for these conditions was the mobile fraction large enough to measure the diffuse coefficients. $D = (1.6 \pm 0.1) \times 10^{-10}$ cm²/s immediately after NaN_3 treatment and $(1.9 \pm 0.3) \times 10^{-10}$ cm²/s after an 18-h recovery. Error bars are all SEM.

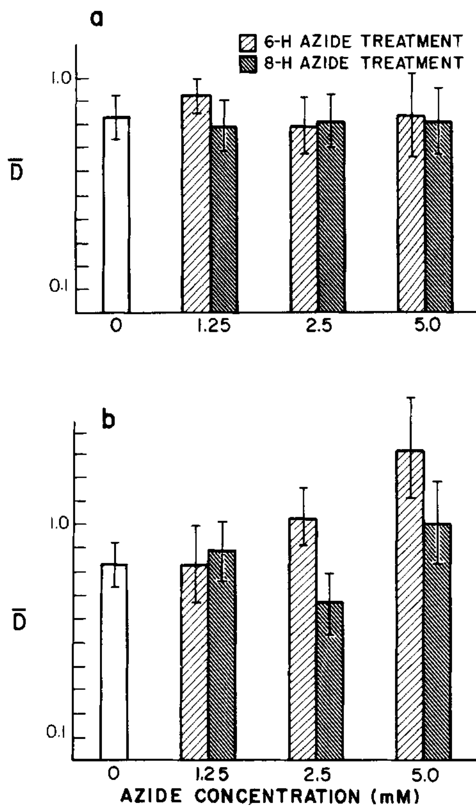

FIGURE 3 Effect of NaN_3 treatment on the average diffusion coefficient $\bar{D}$ (10^{-10} square centimeter per second) of diffusely distributed AChR. Controls (n = 48) were kept in NaN_3-free medium throughout. (*a*) Cells were exposed to NaN_3 in medium for 6 h (1.25 mM, n = 6; 2.5 mM, n = 9; 5.0 mM, n = 3) or 8 h (1.25 mM, n = 4; 2.5 mM, n = 10; 5.0 mM, n = 4) before FPR experiments. (*b*) Cells were allowed to recover in NaN_3-free medium for 18 h after exposure to NaN_3 in medium for 6 h (1.25 mM, n = 5; 2.5 mM, n = 8; 5.0 mM, n = 3) or 8 h (1.25 mM, n = 6; 2.5 mM, n = 9; 5.0 mM, n = 4) before FPR experiments. $\bar{D}$ remains relatively constant throughout. Error bars are all SEM.

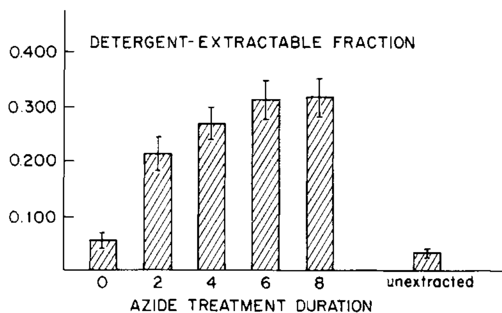

FIGURE 4 Effect of treatment with NaN_3 (duration in hours) on detergent extractability of clustered AChR. Cells were treated with 2.5 mM NaN_3 for 0 (controls, n = 7), 2 (n = 10), 4 (n = 13), 6 (n = 15), or 8 h (n = 10) before extraction with detergent. Fluorescence was observed before and for 3 min after addition of detergent buffer to cells [F(−) and F(3) respectively]. Nonspecific background fluorescence was subtracted from these values and the extractable fraction of receptors was calculated as [1 − F(3)/F(−)]. Decay of fluorescence due to the nonspecific bleaching of the probe during observation was measured by observing the fluorescence for 3 min in the absence of detergent, measuring the fluorescence at the beginning and end of this observation period [F(0) and F(3u) respectively], and calculating the decay as [1 − F(0)/F(3u)]. This loss of fluorescence is insignificant compared with the effects of extraction (unextracted, n = 18). Error bars are all SEM.

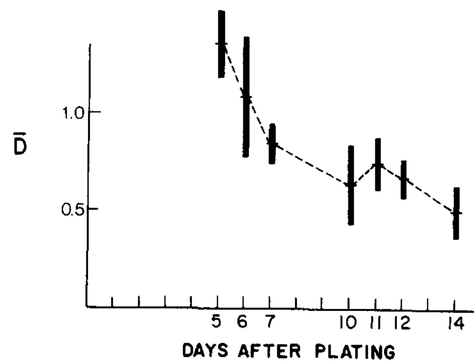

FIGURE 5 Effect of myotube age on average diffusion coefficient $\bar{D}$ (10^{-10} square centimeter per second) in area of diffuse AChR distribution. Myotube were tested at ages after plating of 5 (n = 10); 6 (n = 7); 7 (n = 29); 10 (n = 9); 11 (n = 8); 12 (n = 17); and 14 (n = 10). Errors bars are all SEM.

occurs earlier than the changes in receptor mobility that result from the same treatment. Although some photobleaching of the probe is unavoidable during measurement of fluorescence intensity, the decay in fluorescence caused by this bleaching is insignificant compared with the effect of extraction.

The average lateral diffusion coefficient $\bar{D}$ in areas of diffuse AChR decreases with myotube culture age (Fig. 5). $\bar{D}$ for the oldest culture examined (14 d) was only ~40% as large as $\bar{D}$ for the youngest cultures examined (5 d).

DISCUSSION

We have demonstrated a correlation between the average mobility of AChR in the plane of the membrane and the extractability of these receptors by a detergent-containing buffer.

As myotubes age in culture, the fraction of the total AChR (both clustered and diffusely distributed receptors) that is resistant to detergent extraction increases (9). On the basis of that result, it seems that, with age, an increasingly large proportion of the AChR become firmly attached to a cytoskeleton, which underlies the membrane. Such a firm attachment could retard the lateral mobility of the AChR, and indeed the results of our FPR experiments show a decrease in the average diffusion coefficient $\bar{D}$ of the diffusely distributed AChR with increasing age in culture. This decrease in $\bar{D}$ may be due to an increase in the fraction *f* of receptors that are immobilized, perhaps by microclustering around cytoplasmic anchors, as suggested by Prives et al. (9).

We also show that an increase in the average lateral mobility of clustered AChR, as induced by NaN_3, is accompanied by an increase in the rapidly detergent-extractable fraction of clustered AChR. The time courses of these two changes differ, the increase in detergent extractability occurring before the increase in average mobility. Although a molecular interpretation of this difference is not yet possible, it may indicate that the AChR response to NaN_3 treatment is not a simple one-step attachment-detachment reaction with the cytoskeleton. The FPR experiments also show directly that the NaN_3-induced dispersal of AChR on cultured myotubes, first described by Bloch (5), is due to an increase in the average mobility of the clustered AChR.

We note that agents that decrease the average mobility of AChR, such as neuron-conditioned medium (2), decrease the extractable fraction of receptors as well (9).

In a previous paper (11), we showed that diffusely distributed AChR can become part of clusters, thereby suggesting that the molecular difference, if any, between the two classes of receptors is unlikely to be fundamental. Here we find that the diffusely distributed receptors and the clustered ones do differ in their response to treatment with NaN_3. This observation suggests that the receptors differ in their connections to the cytoskeleton. Since NaN_3 increases the mobility of clustered AChR as well as their extractable fraction, it is reasonable to suggest that NaN_3 disconnects clustered AChR from a cytoskeletal framework which keeps them immobilized. Indeed, using electron microscopy, Peng (8) showed a codistribution of surface AChR clusters on cultured *Xenopus* muscle with a cytoplasmic mesh of filaments. The insensitivity of diffusely distributed AChR mobility to NaN_3 treatment could be explained by their lack of attachment to a cytoskeletal framework.

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REFERENCES

1. Axelrod, D. 1980. Crosslinkage and visualization of acetylcholine receptors on myotubes with biotinylated α-bungarotoxin and fluorescent avidin. *Proc. Natl. Acad. Sci. USA.* 77:4823–4827.
2. Axelrod, D., H. C. Bauer, M. Stya, and C. N. Christian. 1981. A factor from neurons induces partial immobilization of nonclustered acetylcholine receptors on cultured muscle cells. *J. Cell Biol.* 88:459–462.
3. Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.
4. Ben Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. *Cell.* 17:859–865.
5. Bloch, R. 1979. Dispersal and reformation of acetylcholine receptor clusters of cultured rat myotubes treated with inhibitors of energy metabolism. *J. Cell Biol.* 82:626–643.
6. Devreotes, P. N., and D. M. Fambrough. 1975. Acetylcholine receptor turnover in membranes of developing muscle fibers. *J. Cell Biol.* 65:335–338.
7. Heidman, T., and J. P. Changeux. 1978. Structural and functional properties of the acetylcholine receptor protein in its purified and membrane-bound states. *Annu. Rev. Biochem.* 47:317–352.
8. Peng, H. B. 1980. Correlation of surface acetylcholine receptor clusters and cytoplasmic structures in cultured muscle cells. *J. Cell Biol.* 87(2, Pt. 2): 83*a*. (Abstr.)
9. Prives, J., C. Christian, S. Penman, and K. Olden. 1980. Neuronal regulation of muscle acetylcholine receptors: role of muscle cytoskeleton and receptor carbohydrate. *In* Tissue Culture in Neurobiology. E. Giacobini, A. Vernadakis, and A. Shahar, editors. Raven Press, New York. 35–52.
10. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin:preparation, separation, and characterization. *Anal. Biochem.* 80:585–592 and erratum 83:336.
11. Stya, M., and D. Axelrod. Diffusely distributed acetylcholine receptors can participate in cluster formation on cultured rat myotubes. *Proc. Natl. Acad. Sci. USA.* 80:449–453.