A Factor from Neurons Induces Partial Immobilization of Nonclustered Acetylcholine Receptors on Cultured Muscle Cells

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ABSTRACT A factor or factors released by cultured NG108-15 neuroblastoma × glioma hybrid cells and added to the medium of rat myotube primary cultures was found to immobilize some of the previously mobile acetylcholine receptors in the myotube membrane. Partial receptor immobilization occurred within 3 h after the beginning of treatment with the NG108-15-conditioned medium factor and persisted for at least 24 h of continuous treatment. A similarly derived conditioned medium concentrate from the non-neuronal parent glioma cell line did not immobilize receptors, relative to untreated controls. Acetylcholine receptors were visualized by fluorescent α-bungarotoxin and their lateral motion was observed by the technique of fluorescence photobleaching recovery.

During synaptogenesis, contact between a nerve and muscle is correlated with the formation of acetylcholine receptor (AChR) aggregations on the muscle membrane (for a review, see reference 1). In vivo, the causative relationship between contact and receptor aggregation is not yet clear. But in vitro, a neuron can induce AChR to aggregate at the point of its contact with a muscle cell (2, 3, 4). It is possible that the induction process may be mediated by some chemical factor released locally by the impinging neuron. In support of this hypothesis, several laboratories have found that addition of factors from sciatic nerve extract (5), embryonic spinal cord or brain extracts (6, 7), or NG108-15 neuroblastoma × glioma hybrid cell conditioned medium (8) to the medium of myotube cultures can induce an increase in the number of AChR aggregations (2, 7, 8), the total number of AChR molecules (6, 7), or the apparent maturation of the myotubes (5). Some progress toward partial purification and identification of these factors has been made (6, 9, 10). The medium conditioned by NG108-15 cells (8) is the only preparation that shows its effect on myotubes within several hours, rather than days, of treatment.

Rat primary myotubes normally display aggregated AChR which are laterally immobile, and diffusely distributed AChR which are composed of both an immobile and mobile fraction (11). We show here that medium conditioned by NG108-15 cells, which increases the countable number of AChR aggregations, also immobilizes a significant portion of the otherwise laterally mobile AChR in areas of diffuse distribution.

MATERIALS AND METHODS

Culture Preparation and Treatment

Primary rat myotube cultures were prepared as previously described (12) in Dulbecco's modified Eagle medium plus 10% fetal calf serum (DMEM + FCS) in dishes with glass coverslip bottoms. Myoblasts began fusing into myotubes on day 3 or 4. Cultures were exposed to 1×10^{-5} M cytosine arabinoside on days 5–7. On the 7th or 8th d, DMEM + FCS was replaced by DMEM containing 2 mg/ml bovine serum albumin (BSA). On the next day, the medium was replaced by 1 ml of DMEM + BSA to which was added a lyophilized material obtained from 25 ml of conditioned media as follows. "Neuron conditioned medium" (NCM) was prepared by concentrating medium conditioned by NG108-15 cells approximately 100-fold by ultrafiltration against an Amicon PM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), followed by dialysis first against phosphate buffer and then against distilled water, followed by lyophilization (9, 10). A material lacking AChR aggregation activity (8) was obtained by an identical protocol starting with the non-neuronal C6 glioma cell line ("glioma conditioned medium," or GCM).

Tetrodotoxin (TTX), at 3 μg/ml, was usually added to the media on day 7 or 8 and thereafter, to prevent myotube twitching. TTX did not alter the effects of NCM reported here.

The myotube cultures were treated with conditioned medium for times ranging from 2 to 24 h. To visualize AChR, we exposed myotube cultures to tetramethylrhodamine-labeled α-bungarotoxin (R-Bgt; see reference 13) for 1 h at 10^{-7} M in medium at 37°C and then washed them either just before the 1st hour ("prelabeling") or during the last hour ("postlabeling") of the conditioned medium treatment.

Lateral Mobility Measurement

The fractions of AChR that were laterally mobile, and their diffusion coefficient, were determined by the fluorescence photobleaching recovery technique

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(FPR, also known as FRAP [11, 14]). Rat primary myotubes display AChR patches even in the absence of neuronal induction (11, 12, 15–17); these patches are almost all on the lower surface of the myotubes facing the solid substrate. To avoid these patch areas, we performed all photobleaching experiments at the upper surface in areas of diffuse fluorescence. In these experiments, the short depth of focus of the optics and a small aperture in a microscope image plane blocks most fluorescence originating from the lower surface of the myotube (18).

FPR experiments were performed on living cells at 22°C in Hanks' balanced salt solution + BSA + TTX. The FPR apparatus was built around an epi-illumination fluorescence microscope (Leitz Diavert with × 50, NA = 1.00 water immersion objective) and an argon laser set at $\lambda = 514.5$ nm. Fluorescence of R-Bgt/AChR was excited by a laser spot focused on the upper surface of myotubes, with an e^{-2} spot radius of 0.8 ± 0.1 μm (see method of measurement described in reference 19) and a power of 0.1 μW. Photobleaching was performed by a single 50-ms duration flash of this beam at 2 mW power. Fluorescence recovery was recorded for ~5–10 min. Characteristic half-recovery times averaged ~20 s.

RESULTS

NCM treatments of 3 or more hours of duration caused a 25–70% decrease in the fraction f of laterally mobile AChR in areas of diffuse AChR distribution, relative to GCM controls (Fig. 1). This decrease was observed in cultures that had been prelabeled with R-Bgt as well as those that had been postlabeled. The average diffusion coefficient D of these mobile AChR did not differ consistently between NCM- and GCM-treated myotubes: for NCM, $D = (1.0 \pm 0.2) \times 10^{-10}$ cm^2/s; and for GCM, $D = (0.9 \pm 0.1) \times 10^{-10}$ cm^2/s.

The average mobile fraction f_{GCM} of GCM-treated controls varied among separate groups of cultures prepared on different days, with a mean of 0.56 ± 0.08 (SD). Fraction f_{GCM} showed no significant dependence on GCM treatment duration and did not differ significantly from the mobile fraction observed on untreated myotubes. However, fraction f_{NCM} for NCM-treated cultures was always significantly less than the corresponding f_{GCM} measured on the same group of cultures at the same respective treatment duration. Fig. 1 displays the quantity $100 \times (1 - f_{NCM}/f_{GCM})$, which can be interpreted as the percentage of mobile AChR that are immobilized by NCM after each treatment duration.

Immobilization of AChR by NCM appears within 3 h after the start of exposure and persists for at least 24 h of continuous exposure. The apparent decrease in immobilization shown for the postlabeling experiments (Fig. 1 *b*) after long exposure times is not statistically significant. The significant population of immobile AChR that does persist through 24 h of NCM treatment was probably mainly comprised of AChR present on the surface at the start of exposure to NCM. By blocking these preexisting AChR with unlabeled α-Bgt before exposure to NCM and then postlabeling with R-Bgt, we found that only about one-third of the diffuse area AChR visualized at 24 h had been incorporated into the surface after the start of exposure to NCM. (The resulting fluorescence in this preblocking experiment was too low to determine the lateral mobility of these newly incorporated AChR.) We conclude that NCM causes a rapid and persistent net decrease in the fraction of mobile AChR in diffusely fluorescent areas by immobilizing some of the mobile AChR present in the membrane before exposure to NCM.

Previous work (20) has revealed a significant increase in the number of myotube AChR patches after as brief as 2 h of NCM treatment. In our culture system, we confirmed a greater than threefold increase in AChR patches per microscope field of view after 10 h of NCM treatment; a 2-hour treatment produced a much smaller effect. While the great majority of the preexisting patches are located on the bottom of the myo-

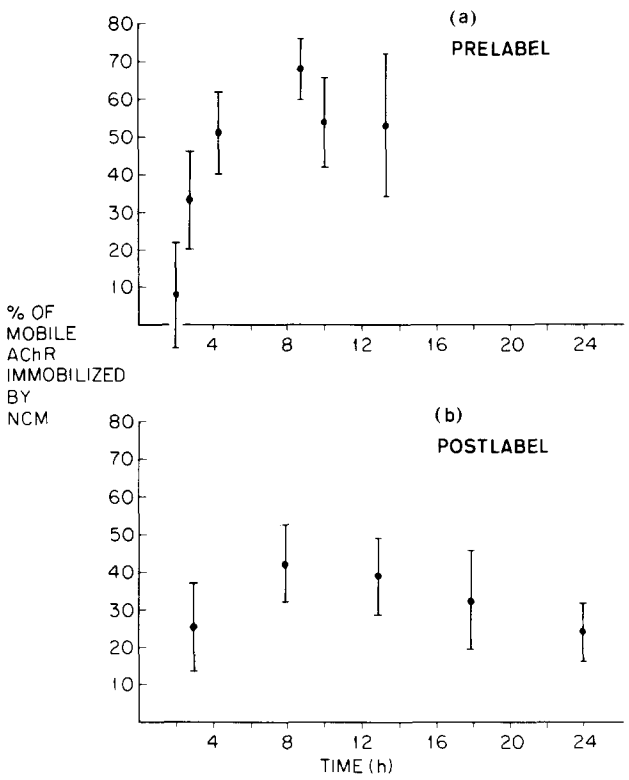

FIGURE 1 The percentage of mobile AChR in diffuse areas which are immobilized by a continuous NCM treatment for the indicated duration in hours. The percentage immobilization is $100 \times (1 - f_{NCM}/f_{GCM})$ for each time point. (*a*) Prelabeled by R-Bgt, showing the mobility of only those AChR on the surface after conditioned medium treatment which were also present on the surface before the treatment. (*b*) Postlabeled by R-Bgt, showing the mobility of all AChR on the surface after the conditioned medium treatment. In some cases, different time point measurements represent averaged data taken from different groups of cultures. All error bars represent standard errors. The following table shows the average fractional mobilities f_{NCM} and f_{GCM} upon which these graphs are based. n is the number of measurements (each on different myotubes) involved in each average. P is the probability (derived from the t distribution) that a mean based on an infinite number of measurements would indicate an NCM-induced percentage immobilization of mobile AChR of >0%.

Duration	Medium	f (± SE)	n	P
h				
Prelabel				
2	NCM	0.48 ± 0.05	12	0.71
	GCM	0.52 ± 0.06	10	
2.7	NCM	0.29 ± 0.04	9	0.983
	GCM	0.43 ± 0.06	9	
4.3	NCM	0.24 ± 0.05	8	>0.995
	GCM	0.49 ± 0.04	8	
8.7	NCM	0.18 ± 0.04	10	>0.995
	GCM	0.57 ± 0.05	9	
10	NCM	0.30 ± 0.08	7	>0.995
	GCM	0.65 ± 0.03	9	
13.3	NCM	0.21 ± 0.08	9	0.988
	GCM	0.45 ± 0.06	9	
Postlabel				
3	NCM	0.50 ± 0.06	8	0.963
	GCM	0.67 ± 0.07	8	
8	NCM	0.30 ± 0.05	13	>0.995
	GCM	0.52 ± 0.07	14	
13	NCM	0.35 ± 0.04	13	>0.995
	GCM	0.57 ± 0.07	10	
18	NCM	0.46 ± 0.08	4	0.946
	GCM	0.68 ± 0.07	7	
24	NCM	0.45 ± 0.02	15	0.995
	GCM	0.59 ± 0.06	14	

tubes (at regions of intimate cell/substrate contact [12, 17]), NCM-induced patches appeared mainly on the top and at the edges of the myotubes. These new patches were neither so dense nor so sharply defined as the preexisting ones, thus rendering the count less than precise. After NCM, diffuse areas on the top of myotubes often appeared more speckled than they do after GCM. Although FPR experiments were performed at the least speckled areas, the possible existence of submicroscope micropatches may account for the increased fraction of immobile AChR.

DISCUSSION

Rapidly mobile AChR (with a diffusion coefficient ($D \simeq 1 \times 10^{-10}$ cm^2/s) and relatively immobile AChR ($D < 10^{-12}$ cm^2/s) coexist on rat myotube membrane in areas of diffuse AChR distribution. We have demonstrated here that some of the rapidly mobile component can be immobilized by a neuronally derived factor. This result raises questions both about the molecular mechanisms and about the biological relevance of NCM action.

We can rule out the possibility that NCM-induced AChR immobilization simply measures a selective depletion of mobile AChR from diffuse areas as they move into developing clusters, leaving behind an increased proportion of endogenously immobile diffuse area AChR. Considering that ~40% of diffuse area AChR are endogenously immobile, an NCM-induced immobilization of the magnitude we observe would then be accompanied by at least a 45% reduction in fluorescence intensity from R-Bgt-AChR in diffuse areas. However, we observe a fluorescence intensity reduction of only 15–20% at those NCM treatment durations yielding maximal immobilization. Therefore, NCM clearly immobilizes some diffuse area AChR which are not recruited into macroscopic clusters in the time scale of these experiments. FPR allows detection of this effect to which AChR cluster counting experiments are not directly sensitive.

Concerning the molecular mechanisms, one might propose that NCM contains some factor that directly cross-links acetylcholine receptors to one another, and that this cross-linking is solely responsible for the lateral immobility of AChR. However, this proposal does not account for some recent data. NCM decreases the rate of total AChR internalization (21) on rat myotubes. However, direct cross-linking and immobilization of AChR via multivalent biotinylated α-Bgt/avidin complexes (12) or by anti-AChR antibodies (22) greatly increases the rate of AChR internalization in both endogenous AChR patches and in diffuse areas (12). Therefore, the mechanism of AChR immobilization by NCM appears to differ from that achieved by multivalent ligand cross-linkage of AChR. We suggest that NCM-induced AChR immobilization is caused by AChR anchorage, perhaps by attachment to a cytoskeletal structure.

The molecular relationship between NCM-induced AChR immobilization in areas of diffuse distribution and NCM-induced AChR patch formation (21) is not clear; they may not be identical processes. Patching does not necessarily follow from AChR immobilization: concanavalin A (15, 23) and biotinylated α-Bgt/avidin complexes (12) immobilize AChR without inducing large patches on rat myotubes. The time-course of both NCM-induced AChR immobilization and patch formation is as short as 2 or 3 h. It is not clear which of these events happens first, or whether they are caused by the same factor in NCM (9, 10). In analogy with patching followed by capping on lymphocytes (24), perhaps immobilization of AChR in submicroscope patches in diffuse areas is followed by a lateral gathering of these patches into larger clusters.

NCM-induced AChR aggregation into clusters appears to be neuron specific. This activity is attributable to a protein of mol wt >150,000 daltons found in medium conditioned by neuronal cells and in extracts of embryonic brain (9, 10). No aggregation activity is found in a variety of non-neuronal materials (8–10). NCM-induced AChR immobilization is also caused by a factor released into the medium by neuronal cells. Dialyzed and lyophilized conditioned medium from NG108-15 neuroblastoma × glioma cells, when added to fresh chemically defined medium, produces an AChR immobilization, whereas medium conditioned by C6 glial cells produces no change in the proportion of mobile AChR.

One might speculate that NCM-induced AChR immobility is analogous to AChR immobility at synaptic endplates (11). The local release of an NCM-like substance by a neuron, along with physical contact (12, 17, 25) or basal lamina specializations (26), may be involved in synaptogenesis. AChR clusters on muscle can develop in the embryo even if direct neural contact is prevented, but the distribution of these clusters is clearly modulated by neural contact (27). The molecular mechanisms of NCM action and their possible relationship to the stabilization of AChR at developing synapses remain to be investigated.

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