

Extracellular Synaptic Factors Induce Clustering of Acetylcholine Receptors Stably Expressed in Fibroblasts

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Abstract. The clustering of nicotinic acetylcholine receptors (AChRs) is one of the first events observed during formation of the neuromuscular junction. To determine the mechanism involved in AChR clustering, we established a nonmuscle cell line (mouse fibroblast L cells) that stably expresses just one muscle-specific gene product, the AChR. We have shown that when *Torpedo californica* AChRs are expressed in fibroblasts, their immunological, biochemical, and electrophysiological properties all indicate that fully functional cell surface AChRs are produced. In the present study, the cell surface distribution and stability of *Torpedo* AChRs expressed in fibroblasts (AChR-fibroblasts) were analyzed and shown to be similar to nonclustered AChRs expressed in muscle cells. AChR-fibroblasts incubated with antibodies directed against the AChR induced the formation of small AChR microclusters ($<0.5 \mu\text{m}^2$) and caused an

increase in the internalization rate and degradation of surface AChRs (antigenic modulation) in a manner similar to that observed in muscle cells. Two disparate sources of AChR clustering factors, extracellular matrix isolated from *Torpedo* electric organ and conditioned media from a rodent neuroblastoma-glioma hybrid cell line, each induced large ($1-3 \mu\text{m}^2$), stable AChR clusters with no change in the level of surface AChR expression. By exploiting the temperature-sensitive nature of *Torpedo* AChR assembly, we were able to demonstrate that factor-induced clusters were produced by mobilization of preexisting surface AChRs, not by directed insertion of newly synthesized AChRs. AChR clusters were never observed in the absence of extracellular synaptic factors. Our results suggest that these factors can interact directly with the AChR.

SYNAPSE formation at the neuromuscular junction is characterized by an accumulation of synapse-specific membrane proteins, extracellular matrix components, and cytoskeletal elements in both the developing muscle cell and nerve growth cone. An early morphological specialization observed during synaptogenesis is clustering of acetylcholine receptors (AChRs)¹ at the point of nerve-muscle contact. Subsequent organization of other extracellular and intracellular components occurs around this AChR accumulation (reviewed in Salpeter, 1987; Bloch and Pumplin, 1988). Several factors have been identified that induce clustering of AChRs in cultured muscle cells: extracts of *Torpedo* extracellular matrix (ECM), chick, rat, and pig brain extracts, media conditioned by neuroblastoma-glioma hybrid NG108-15 cells, and positively charged latex beads (Rubin and Barald, 1983; Burden, 1987; Scheutze and Role, 1987; Bloch and Pumplin, 1988). One AChR clustering factor (termed agrin) has been identified from matrix-enriched fractions of *Torpedo* electric organ and antibodies directed against it show that agrin-like molecules are concentrated at neuromuscular junctions in vivo (Fallon et al., 1985; Reist

et al., 1987; Godfrey et al., 1988). Clustering factors such as agrin may cause clustering through direct interactions with the AChR molecule or they may trigger AChR clustering through unidentified muscle cell receptors.

In addition to presynaptic factors that appear to be involved in AChR clustering, several postsynaptic cytoskeletal proteins have also been implicated in this process (Froehner, 1986; Burden, 1987; Bloch and Pumplin, 1988). Of the several potential proteins involved in AChR clustering, anchoring, or stabilization, a protein that has received considerable notice is one of $M_r \sim 43,000$ (43K protein). This protein is distinct from actin; it copurifies with the AChR through many purification steps (Sobel et al., 1978; Hamilton et al., 1979; Neubig et al., 1979; Elliott et al., 1980; Gysin et al., 1981; Porter and Froehner, 1983); it has been cross-linked to the β -subunit of the AChR (Burden et al., 1983); and it colocalizes with the AChR in electric organ (Sealock et al., 1984; Bridgman et al., 1987; Toyoshima and Unwin, 1988; Mitra et al., 1989), at developing *Xenopus* nerve-muscle synapses in culture (Burden, 1985), at mammalian neuromuscular junctions (Froehner et al., 1981; Bennett, 1983; Flucher and Daniels, 1989), and at spontaneous AChR clusters on cultured rat myotubes (Bloch and Froehner, 1987). The 43K protein is highly concentrated at the ver-

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1. *Abbreviation used in this paper:* AChR, acetylcholine receptor.

tebrate neuromuscular junction and is usually thought to be muscle specific (Froehner, 1986; Burden 1987). However, the 43K protein has been detected in some nonmuscle cells (Musil et al., 1989) and although it is present in stoichiometric amounts with AChR in the mouse muscle cell line, BC3H-1, AChRs cannot be induced to cluster in this line (LaRoche and Froehner, 1987). Observations such as these indicate that the 43K protein may have a role other than clustering of AChRs or that other proteins are involved in the process.

To simplify the muscle cell and further define the role of the AChR in clustering, we first constructed a mouse fibroblast L cell line that stably expresses the *Torpedo* AChR (All-11 cells; Claudio et al., 1987). AChRs in All-11 cells, without added factors, appear uniformly distributed over the surface of the fibroblast (Claudio et al., 1989a). Each cell expresses ~40,000 AChRs giving a surface density of ~50/ μm^2 (Hartman et al., 1990). This value is only twofold lower than the surface density of AChR on embryonic myotubes which range from 75–125 AChRs/ μm^2 (Bevan and Steinbach, 1977; Ziskind-Conhaim et al., 1984). The half-lives of surface AChRs in fibroblasts (Paulson and Claudio, 1990) and in primary rat skeletal muscle cells (Salpeter et al., 1982) are virtually identical (~13 h at 37°C). Surface AChRs in All-11 cells are composed exclusively of $\alpha_2\beta\gamma\delta$ pentamers (Hartman et al., 1990) and they display all of the proper pharmacological, electrophysiological, and immunological properties expected of *Torpedo* AChRs (Claudio et al., 1987). When they are cocultured with 1-d-old *Xenopus laevis* embryonic neurons, functional contact is established between the neuron and the fibroblast (Hartman et al., 1990). Thus AChR fibroblasts appear to be an ideal system for investigating the role of the AChR in clustering, since a uniform population of fully functional AChRs is expressed on the cell surface.

In the present report, we demonstrate that *Torpedo* AChRs expressed in fibroblasts are uniformly distributed in the cell surface membrane until challenged with extracellular synaptic clustering factors. Two very dissimilar sources of these factors induce AChR clusters that are different from antibody-induced clusters but quite similar to each other and to clusters induced in cultured muscle cell lines. We were not able to detect 43K protein in All-11 cells by either immunoblotting or immunofluorescence microscopy. We were able to detect 43K mRNA, however, but the ratio of 43K to AChR mRNA was ~150-fold less in All-11 cells than it was in C2 muscle cells. We conclude from the results presented in this report that *Torpedo* AChRs can cluster in response to extracellular synaptic factors and that they can do so without muscle-specific cytoskeletal components.

Materials and Methods

Cell Culture

The establishment and maintenance of All-11 cells (a clonal isolate of mouse fibroblast L cells cotransfected with pSV2-*Torpedo californica* α , β , γ , δ and thymidine kinase) has been described previously (Claudio et al., 1987). 3T3-43K, a stable fibroblast cell line expressing *Torpedo* 43K protein was established by inserting the *Torpedo* 43K cDNA (Baldwin et al., 1988) into the EcoRI cloning site of pDOJ (also referred to as pDORneo; Penn et al., 1990) 3' to the Moloney murine leukemia virus long terminal repeat. An internal neomycin-resistance gene provides the drug resistance. 10 μg

pDOJ-43K DNA was transfected onto 10^6 ψ 2 cells, supernatant was harvested 18 h after the glycerol shock, and 5×10^5 NIH3T3 cells were infected with 1 ml of filtered culture supernatant (Mann et al., 1983; Claudio et al., 1989b). Cells were selected in DME containing 10% calf serum and 0.6 mg/ml G418 (Gibco Laboratories, Grand Island, NY) and maintained in an atmosphere of 5% CO_2 at 37°C. Several hundred colonies were pooled and grown into a stable cell line. Mouse C2 muscle cells were maintained in DME supplemented with 20% FBS, and 0.5% chick embryo extract. To induce myocytes to differentiate and fuse to form multinucleated myotubes, 75% confluent cells were changed to DME containing 5% FBS. Approximately 50% of the cells fused in 3–5 d at 37°C. NG108-15 neuroblastoma-glioma cells were obtained from Marshall Nirenberg and maintained in DME supplemented with 10% FBS 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine in an atmosphere of 10% CO_2 at 37°C.

Temperature-Shift Protocol and Quantitation of Surface AChRs

For clustering experiments, 20 mM sodium butyrate was added to the medium of All-11 cells for 36 h at 37°C to induce expression of the four AChR cDNAs (Claudio et al., 1987). Cells were then shifted to 20°C for 36 h in media without sodium butyrate, then placed in serum-free DME containing 0.1% BSA for 30 min at 37°C before the addition of clustering factors. Cells were maintained at 37°C in the presence of clustering factors for an additional 2–4 h. The number of surface AChRs was determined using [^{125}I] α -bungarotoxin (BuTx), an ~8-kD polypeptide which binds the AChR with a K_D ~ 8×10^{-11} M (Claudio et al., 1987). All-11 cells were washed with PBS, incubated with 5 nM [^{125}I]BuTx (New England Nuclear Research Products, Wilmington, DE) diluted in PBS containing 0.005% CaCl_2 , 0.005% MgCl_2 (PBS/Ca/Mg), and 0.1% BSA for 2 h at room temperature, washed three times (2 ml each) with PBS/Ca/Mg, solubilized in 1N NaOH containing 0.5% Triton, and counted in a γ counter.

Preparation of Clustering Factors

Torpedo ECM was made according to the procedures of Godfrey et al. (1984). 7 g of frozen *Torpedo* electric organ were homogenized in 80 ml of 150 mM sodium chloride, 20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.1 mM NEM, 1 mM PMSF, and 50 $\mu\text{g}/\text{ml}$ Trasylol in an 8-stroke Brendlar 2000. The homogenate was centrifuged at 30,000 g for 90 min in an SW28 rotor (Beckman Instruments, Fullerton, CA). The pellet was resuspended in 80 ml of 2 M MgCl_2 , 20 mM Tris, pH 7.4, 0.1 mM NEM, 1 mM PMSF, and 50 $\mu\text{g}/\text{ml}$ Trasylol, and stirred for 30 min at 4°C. The suspension was centrifuged at 30,000 g for 90 min and the supernatant dialyzed overnight against DME. Dialyzed material was spun again at 30,000 g for 60 min, the supernatant was collected and stored in aliquots at -70°C. The protein concentration was 0.28 mg/ml as determined by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, CA) measured at 595 nm. A matrix-enriched (purified ~1,000-fold for cluster-inducing activity; ~1% agrin) Cibacron pool of *Torpedo* ECM (Nitkin et al., 1987) was generously provided by Justin Fallon (Worcester Foundation for Experimental Biology, Shrewsbury, MA).

Conditioned media was prepared according to Bauer et al. (1985) and either used fresh or after it had been filtered and frozen at -70°C. The protein concentration varied from 50–85 $\mu\text{g}/\text{ml}$ of media. Filtered media was prepared by collecting concentrated material from the outer chamber of a Centrifrep-10 filter unit (Amicon Corp., Danvers, MA) after centrifugation, then reconstituting it to its original volume in serum-free media. Material (<10 kD) collected in the central chamber of the unit was frozen at -70°C and used as control media.

Fluorescent Labeling of Cell Surface AChRs

Cells grown directly on 11 \times 22 mm no. 1 glass coverslips (Scientific Products, MacGaw Park, IL) were washed in PBS/Ca/Mg and 0.1% Fraction V BSA (Sigma Chemical Co., St. Louis, MO). Cells were blocked in PBS containing 1% BSA, and incubated in a 1/30 dilution of mAb 35 hybridoma supernatant (American Type Culture Collection, Rockville, MD) in PBS/Ca/Mg/BSA for 1 h at room temperature. mAb 35 was raised in rat against the AChR α subunit (Tzartos and Lindstrom, 1980). After five washes in PBS/Ca/Mg (~20 ml per wash), cells were fixed in 2% paraformaldehyde containing 75 mM lysine and 10 mM NaIO_4 (PLP) (McLean and Nakane, 1974) for 10 min at room temperature. Coverslips were dipped three times in PBS to remove PLP, blocked in 100 mM sodium phosphate containing 5 mg/ml BSA, and incubated for 30 min at room temperature

in a 1/40 dilution of phycoerythrin-conjugated goat anti-rat IgG (Calbiochem-Behring Corp., La Jolla, CA) in 100 mM sodium phosphate, pH 7.0 with 5 mg/ml BSA. Cells were washed five times in PBS/Ca/Mg, and incubated in 20 μ g/ml fluorescein-conjugated WGA (Vector Laboratories, Burlingame, CA) for 10 min at room temperature. Cells were washed five times in PBS/Ca/Mg, once in distilled water, and mounted on glass slides in Fluor-Save mounting media (Calbiochem-Behring Corp.).

Fluorescence was visualized using a Zeiss IM35 microscope at 570 nm with a 40X Plan NeoFluar objective. Cells were photographed directly using an Olympus OM-4T 35-mm camera on Kodak Tri-X Pan 400 ASA film. Approximately 500 cells were analyzed for surface acetylcholine clusters using a Macintosh IIcx computer and the Image 1.27 software program available from the National Institutes of Health. 35 mm photographs were printed on Agfa Rapitone PI-4 high-contrast photographic paper and digitized using an Applescan eight-bit Flatbed Scanner in the greyscale mode at 75 dots per inch resolution, contrast setting of 4.0, and brightness of 8.0. Fluorescent spots were counted and measured for integrated density and area. Fluorescent spots measuring $<0.1 \mu\text{m}^2$ are below our limits of resolution and have been subtracted from cluster counts.

Fluorescent Labeling of Intracellular Proteins

All-11 and 3T3-43K cells were grown on no. 1 glass coverslips until 75% confluent, and temperature shifted as described. C2 cells were grown on collagen-coated glass coverslips, and fused in 5% FBS for 3–5 d. Cells were washed with PBS/Ca/Mg and either fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in PBS for 10 min at room temperature and permeabilized using rapid dehydration/rehydration in ethanol, or fixed and permeabilized in PLP containing 0.1% saponin. Nonspecific binding was blocked by incubating for 10 min in PBS/Ca/Mg containing 1.0% BSA. Cells were then labeled with a 1:30 dilution of mAb 35, or 10 nM mAb 1234A (an antibody raised against the *Torpedo* 43K protein; LaRochelle and Froehner, 1987), for 2 h at room temperature, and washed three times for 10 min with PBS/Ca/Mg containing 0.1% BSA. AChR was then labeled with phycoerythrin-conjugated goat anti-rat IgG secondary antibody (Calbiochem-Behring Corp.), diluted 1:40 in 0.1 M sodium phosphate, pH 7.0 containing 5 mg/ml BSA, and incubated with cells for 1 h at room temperature. 43K protein was labeled with fluorescein-conjugated goat anti-mouse IgG second antibody (Calbiochem-Behring Corp.), diluted 1:150 in PBS/Ca/Mg containing 1% BSA and incubated with cells for 1 h at room temperature. Cells were washed twice with PBS/Ca/Mg, and rinsed briefly in distilled water before mounting in Fluor-Save (Calbiochem-Behring Corp.) on glass slides. Immunofluorescence was visualized and recorded as described above.

Northern Blots

Total RNA was isolated from fibroblasts and C2 muscle cells using standard procedures (Chirgwin et al., 1979). 10 μ g of total RNA were electrophoresed in each lane of a 1% agarose formaldehyde gel, and transferred to Zetabind filters (AMF Cuno, Wallingford, CT). Filters were hybridized with $\sim 8 \times 10^8$ cpm of ^{32}P -labeled probe (Multiprime Kit; Amersham Chemical Co., Arlington Heights, IL) in 50% formamide hybridization buffer for 20 h at 42°C, as recommended by the manufacturer. Membranes were washed at a final stringency of $0.5 \times \text{SSC}$, 0.1% SDS at 68°C. cDNA probes included a 1,090-bp PvuII-PstI fragment of the *Torpedo* α subunit (Claudio, 1987), a 1,487-bp EcoRI-PstI fragment of the mouse δ subunit (LaPolla et al., 1984), and a 1,500-bp EcoRI fragment of the mouse 43K cDNA (Frail et al., 1988).

Immunoblots

Confluent 60-mm dishes of fibroblasts and C2 muscle cells were lysed directly in 100 μ l SDS-PAGE sample buffer containing 2% SDS and 10 mM DTT. Samples were boiled for 5 min and sonicated full force in a Branson sonifier 450 (Branson Ultrasonic, Danbury, CT) for 30 s with a duty cycle at 50%. Lysates were centrifuged at 10,000 g for 10 min, and one-half of the total sample was electrophoresed on 7.5% SDS-polyacrylamide gels. Protein was transferred to nitrocellulose transfer membrane (Micron Separations, Inc., Westboro, MA) using semi-dry electrotransfer (Hoefer Scientific Instruments, San Francisco, CA). Membranes were incubated for 30 min at room temperature in blocking solution which was 10 mM Tris, 150 mM sodium chloride, 0.05% Tween-20 (TBST) containing 5% dry milk. Membranes were then incubated in blocking solution containing mAb 1234A at a final concentration of 0.9 μ g/ml for 18 h at 4°C, then washed 1 h and incubated in blocking solution containing horseradish peroxidase-conjugated anti-mouse IgG (BioSys, Compiegne, France) at 1:5000 dilu-

tion for 1 h at room temperature. After washing in TBST, labeling was detected with Enhanced Chemi-Luminescence (Amersham Chemical Co.). Membranes were exposed for 15 min with Kodak XRP film.

Results

Surface Distribution of *Torpedo* AChRs Stably Expressed in Mouse Fibroblasts

A temperature-shift protocol was developed to induce rapid surface expression of *Torpedo* AChRs in fibroblasts. All-11 cells were first incubated in 20 mM sodium butyrate at 37°C for 36 h which increases *Torpedo* AChR subunit mRNA levels (40- to 100-fold) and protein levels (Claudio et al., 1987). Next, the cells were shifted to 20°C in the absence of sodium butyrate for 36 h to allow assembly of *Torpedo* AChR pentamers. The assembly of *Torpedo* AChR subunits is profoundly temperature sensitive, requiring that cells be incubated at temperatures below 37°C (Paulson and Claudio, 1990). At 20°C, 22–36% assembly efficiencies of each subunit are achieved, efficiencies similar to those of mouse AChR in both muscle and fibroblast cells (Ross et al., 1991). In all clustering experiments, cells were then shifted back to 37°C in serum-free DME for 30 min, followed by the addition of extracellular synaptic factors. Once assembled, *Torpedo* AChRs remain fully functional when shifted to 37°C. Factors are incubated with cells at 37°C for only 2–4 h before analysis of cell surface AChR distributions.

The surface distribution of AChRs expressed in fibroblasts was determined by indirect immunofluorescence, and compared to AChR distribution in cultured C2 muscle cells. Live cells were incubated briefly with mAb 35 (an antibody that preferentially recognizes assembled rather than unassembled α subunit; Ross et al., 1991), fixed and labeled with a second antibody conjugated to the fluorescent molecule phycoerythrin. As shown in Fig. 1 A, *Torpedo* AChR is distributed evenly on the fibroblast cell surface at nearly the same level as seen in C2 myotubes (Fig. 1 B). Fluorescent labeling is not detected on AChR fibroblasts that have not been shifted to temperatures permissive for assembly (Fig. 1 E), or in C2 cells that have not been fused (Fig. 1 F).

Antigenic Modulation

It is well documented in muscle that when many anti-AChR antibodies bind surface AChRs, they become cross-linked, then rapidly internalized and degraded (called antigenic modulation; Appel et al., 1977; Heinemann et al., 1977; Drachman et al., 1978; Prives et al., 1979). We wished to determine whether the surface AChRs expressed in All-11 cells could be modulated by anti-AChR antibodies in a fashion similar to AChRs expressed in muscle cells. When AChR fibroblasts (Fig. 1 C) or C2 myotubes (Fig. 1 D) were incubated with mAb 35 and fixed after incubation with phycoerythrin-conjugated anti-rat IgG, small punctate AChR microclusters with surface areas $<0.5 \mu\text{m}^2$ were formed on both cell types. In addition to producing surface AChR microclusters, anti-AChR antibodies also induced a rapid loss of surface AChRs in All-11 cells (Fig. 2). The half-life of surface AChRs in All-11 cells at 37°C was reduced from 14 h in the absence of antibody to only 1.2 h (the mAb 35-bound population; see legend to Fig. 2). Thus, both the surface distribution and stability of AChRs in fibroblasts can be modu-

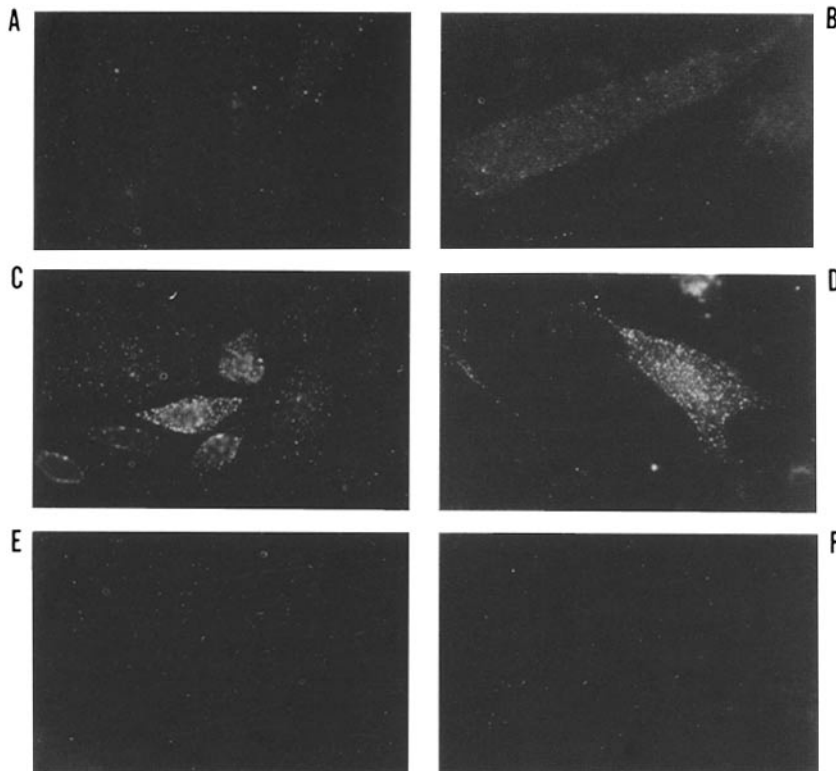


Figure 1. Cell surface distribution of AChRs in untreated and antibody-treated All-11 cells and mouse C2 myotubes. (A) All-11 cells (L fibroblasts stably expressing *Torpedo* AChRs) were temperature-shifted (to induce expression of surface AChRs), incubated in serum-free media at 37°C as described (see Materials and Methods), labeled for 1 h with mAb 35 (anti-AChR antibody), fixed in PLP, and labeled with phycoerythrin-conjugated anti-rat IgG. (B) C2 muscle cells were induced to differentiate and surface labeled for AChR as in A. When live cells were incubated with mAb 35 and fixed after incubation in secondary antibody, antibody-induced micro clusters of AChRs were seen on the surface of both All-11 cells (C) and C2 myotubes (D). The surface area of each microcluster is $<0.5 \mu\text{m}^2$. No labeling was detected on All-11 cells that were not temperature shifted (E) or on unfused C2 monocytes (F). Typically the number of AChRs/cell for All-11 fibroblasts is $\sim 40,000$, giving a surface density of $\sim 50/\mu\text{m}^2$. The surface density of receptor on embryonic myotubes is $\sim 75\text{--}125/\mu\text{m}^2$. As seen in A and B, the intensity of receptors in All-11 cells is approximately half that of C2 myotubes. All frames were photographed directly onto Kodak Tri-X Pan 300 film with identical exposure times. $\times 400$.

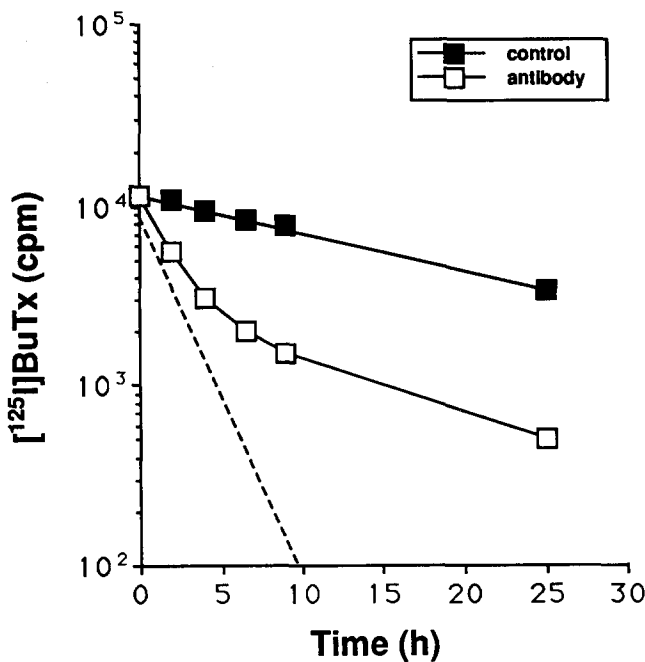


Figure 2. Anti-AChR antibody-induced antigenic modulation of surface AChRs in All-11 cells. mAb 35 induced a dramatic loss in surface AChR levels when added to culture media, decreasing the half-life of surface AChRs from 14 h in control serum-free media to only 1.2 h in media containing mAb 35. All-11 cells were temperature shifted to induce expression of AChRs, incubated with a 1/500 dilution of mAb 35 hybridoma supernatant, and surface AChR levels determined at different times at 37°C using [¹²⁵I]BuTx surface-labeling of live cells at room temperature (see Materials and Methods). In the absence of antibody the turnover of surface AChR can be fitted by a single exponential function. In the presence of

antibodies, and this modulation is similar to that of AChR expressed in muscle cells.

Induction of 1–3- μm^2 Surface Clusters

When All-11 cells were incubated at 37°C for 4 h with 10 units of a matrix-enriched Cibacron pool of *Torpedo* ECM (Nitkin et al., 1987), clusters significantly larger ($\sim 1\text{--}3 \mu\text{m}^2$) than antibody-induced clusters, were produced (Fig. 3 A). Matrix-enriched ECM also induced AChR clusters on the surface of C2 myotubes (Fig. 3 B). The very large cluster area seen on the upper of the two C2 myotubes is composed of >30 AChR clusters which are similar in size to the All-11 AChR clusters. Because ECM can induce similarly sized AChR clusters in both All-11 cells and C2 myotubes, it suggests that muscle-specific components other than the AChR are not required for this initial stage of clustering. It is possible that the clusters observed on All-11 cells are analogous to the microaggregates described by Olek et al. (1983) on primary rat myotubes. Olek et al. suggested that large AChR aggregates ($>10 \mu\text{m}^2$) assemble by the coalescence of smaller aggregates. The relatively small size of a fibroblast cell ($\sim 10 \mu\text{m}$ diameter) would preclude the formation of clusters of a size similar to the largest clusters observed on myotubes. It is also possible that a muscle-specific protein

antibody the data are better explained as comprising two populations; AChRs which turn over rapidly due to the binding of mAb 35 (76% of the total), and unbound AChRs which turn over at a similar rate to control data (24% of the total). The turnover rate of the mAb 35-bound population (broken line) was obtained by subtracting the unbound population from the total.

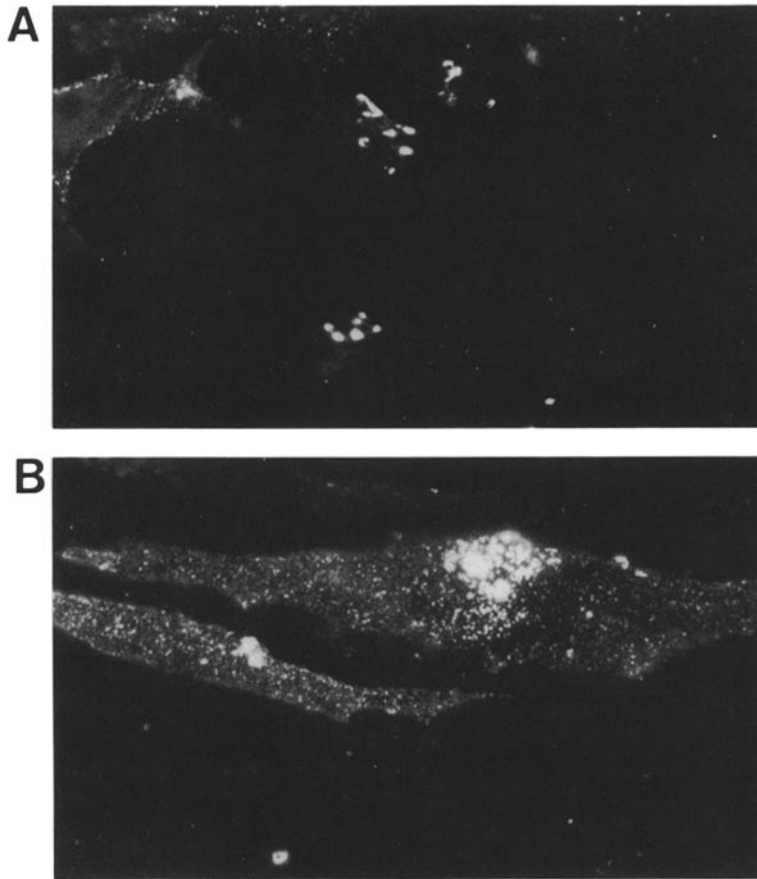


Figure 3. AChR clusters are induced on All-11 cells and C2 myotubes by an $\sim 1,000$ -fold purified preparation of *Torpedo* ECM. Temperature-shifted All-11 cells (A) and C2 myotubes (B) were incubated for 4 h at 37°C with 10 units of matrix-enriched Cibacron pool of *Torpedo* ECM. The cluster sizes on All-11 cells are $\sim 1\text{--}3\ \mu\text{m}^2$ with ~ 30 clusters of similar size appearing to constitute the large C2 cluster. $\times 600$.

may be required for coalescing of small clusters into one area of the cell.

After temperature shifting All-11 cells to 20°C for 36 h, 50–75% of the cells show AChR surface labeling. Unlabeled cells probably express levels of AChR that fall below our detection limits, since essentially all cells are labeled by extending the incubation at 20°C from 36 h to 3 d, which results in a threefold increase in total surface expression. *Torpedo* ECM induces AChR clusters on $\sim 50\%$ of cells showing AChR labeling, with as many as four to six $1\text{--}3\text{-}\mu\text{m}^2$ clusters per cell. Approximately 60 clusters with surface areas $>1\ \mu\text{m}^2$ were observed per 100 cells and they were estimated to cover 3–5% of the total cell surface. The ECM caused a 16-fold increase (over spontaneously occurring clusters) in the number of AChR microclusters with surface areas $<0.5\ \mu\text{m}^2$, and a 15-fold increase (over antibody-induced clusters) in the number of AChR clusters with areas of $0.5\text{--}1.0\ \mu\text{m}^2$. AChR clusters as large as $1\text{--}3\ \mu\text{m}^2$ were never observed in All-11 cells incubated in serum-free DME in the absence of clustering factors. To determine whether ECM treatment affected the distribution of cell surface proteins in general, or was specific to AChRs, All-11 cells were double-labeled with fluorescein-conjugated WGA and mAb 35-phycoerythrin-conjugated anti-rat IgG. Fig. 4 A shows the $1\text{--}3\text{-}\mu\text{m}^2$ clusters of AChR while Fig. 4 B shows an even distribution of cell surface proteins. The absence of WGA-labeled clusters indicates that AChRs have been specifically mobilized by the ECM. Godfrey et al. (1984) have shown

that the clustering activity of *Torpedo* ECM can be destroyed by heating to 100°C for 5 min or by treating with trypsin, and that the active component is $>50\ \text{kD}$. We find that clustering activity is retained in the high molecular weight fraction after dialysis by centrifuge filtration with a molecular weight cutoff of 10 kD and that when ECM is heated to 95°C for 10 min, it loses its ability to cluster *Torpedo* AChRs in All-11 cells (data not shown). Both results indicate that the active agent in our ECM preparation is similar to that described by Godfrey et al. (1984). Using a laser scanning confocal microscope and visual analysis of serial sections displayed in pseudocolors of fluorescently labeled ECM-treated All-11 cells, we were able to determine that AChR clusters were on the fibroblast cell surface (Fig. 5).

Incubation of All-11 cells in NG108-15 conditioned media (37°C for 2–4 h) also induced AChR clusters on the fibroblast cell surface which strongly resembled ECM-induced clusters in both size and frequency (Fig. 6). Similar to results obtained with ECM, $\sim 50\%$ of the All-11 cells with detectable AChR labeling showed surface AChR clusters after treatment with conditioned media. Conditioned media caused an eightfold increase in the number of AChR clusters $<0.5\ \mu\text{m}^2$, a 15-fold increase over antibody-induced levels in the cluster range of $0.5\text{--}1.0\ \mu\text{m}^2$, and produced ~ 60 AChR clusters $>1\ \mu\text{m}^2$ in area per 100 cells. Double labeling of All-11 cells with WGA confirmed that conditioned media, like *Torpedo* ECM, does not induce clustering of cell surface proteins in general (data not shown). The clustering

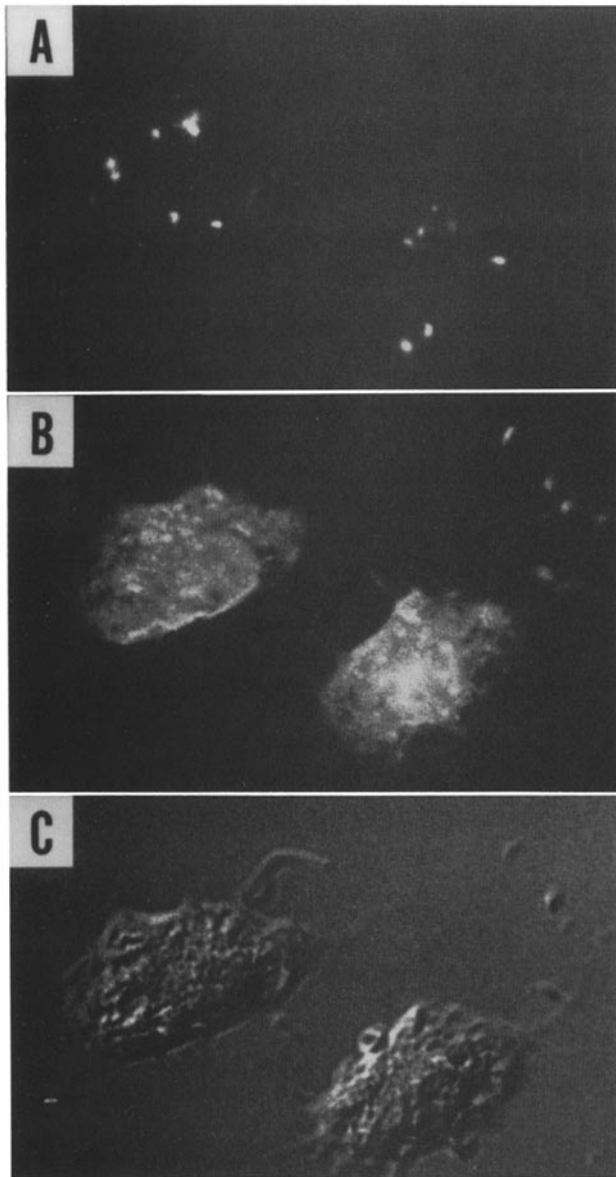


Figure 4. *Torpedo* ECM extract induced AChR cluster formation on All-11 cells without affecting the distribution of total cell surface protein. (A) AChR surface labeling of temperature-shifted All-11 cells after incubation in 2.8 µg/ml of *Torpedo* ECM extract at 37°C for 2.5 h, mAb 35 labeling, and visualization as described in the legend to Fig. 1. The surface areas of the AChR clusters ranged from 1 to 3 µm². (B) The cells shown in A were double-labeled with fluorescein-conjugated WGA, and a homogeneous distribution of total cell surface protein is seen after incubation with ECM. (C) The Zeiss differential interference contrast (DIC) image of cells shown in A and B. ×400.

activity of NG108-15 conditioned media is retained in the molecular weight fraction >10 kD after fractionation by centrifuge filtration. The >10 kD fraction, reconstituted in serum-free media to its original 1× concentration, shows clustering activity identical to that of complete NG108-15 conditioned media. Specifically, 95 AChR clusters <0.5 µm², 52 clusters 0.5–1.0 µm², and 58 clusters >1.0 µm²

were counted per 100 cells after a 3-h incubation in the >10-kD fraction of conditioned media. Fibroblasts treated with the low molecular weight fraction of NG108-15 conditioned media had on average, only 14 small clusters (<0.5 µm²) per 100 cells, which is indistinguishable from cells in control serum-free media (Fig. 7).

Factor-induced Clusters Are Different from Antibody-induced Clusters

The clustering activities of mAb 35, *Torpedo* ECM, and NG108-15 conditioned media are compared in Fig. 7. In the absence of any externally added factors, only small AChR micro-clusters <0.5 µm² were found on the fibroblast cell surface at a frequency of 12 clusters per 100 cells. Antibody cross-linking increased the number of these microclusters fivefold to 60 per 100 cells, and produced ~3 clusters per 100 cells with surface areas 0.5–1.0 µm². Both ECM and conditioned media increased the number of small AChR clusters (<1.0 µm²), but in addition, produced 55–60 clusters per 100 cells with surface areas of 1–3 µm². The different size distributions of antibody-induced and factor-induced AChR clusters suggest that ECM and NG108-15 conditioned media induce clustering through a mechanism different from the direct intermolecular cross-linking produced by divalent antibodies. Consistent with this hypothesis, we find that *Torpedo* ECM and NG108-15 conditioned media do not alter surface AChR levels in fibroblasts, in marked contrast to modulation of surface AChR by antibody. Whereas a 6-h treatment with mAb 35 at 37°C of [¹²⁵I]BuTx-labeled surface AChRs in All-11 cells results in a loss of 83% of the surface AChRs, similar treatments with ECM or NG108 conditioned media results in <10 and 5% (respectively) losses of surface AChRs (Fig. 8).

43K Protein

The 43K protein has been shown to be concentrated at sites of AChR accumulation in muscle cells, and may serve as a link between AChRs and the underlying cytoskeleton (Walker et al., 1984; Bloch and Morrow, 1989). To determine whether an endogenous 43K protein may be expressed in All-11 cells which might be influencing the ability of *Torpedo* AChR to respond to externally added clustering factors, we first attempted to visualize expression of 43K protein by indirect immunofluorescence. Using an antibody (mAb 1234A) directed against the 43K protein, we found diffuse labeling in permeabilized C2 myotubes (Fig. 9 A) but failed to detect any label in permeabilized temperature-shifted All-11 cells (Fig. 9 B). The All-11 cells shown in Fig. 9 B were double-labeled with anti-AChR antibodies (mAb 35) and as shown in Fig. 9 D, labeling of assembled intracellular AChRs is clearly visible. These results demonstrate that All-11 cells do not express an endogenous 43K protein at least at the level expressed in C2 myotubes. It has been shown that 43K protein isolated from *Torpedo* electrocytes is myristoylated (Carr et al., 1989) and that myristoylated proteins are often associated with the plasma membrane (Towler et al., 1988). NIH3T3 cells stably expressing *Torpedo* 43K (3T3-43K cells) were permeabilized and labeled with mAb 1234A to determine the pattern of expression of this protein in a fibroblast cell. As shown in Fig. 9 C, 43K protein is diffusely distributed throughout the cell

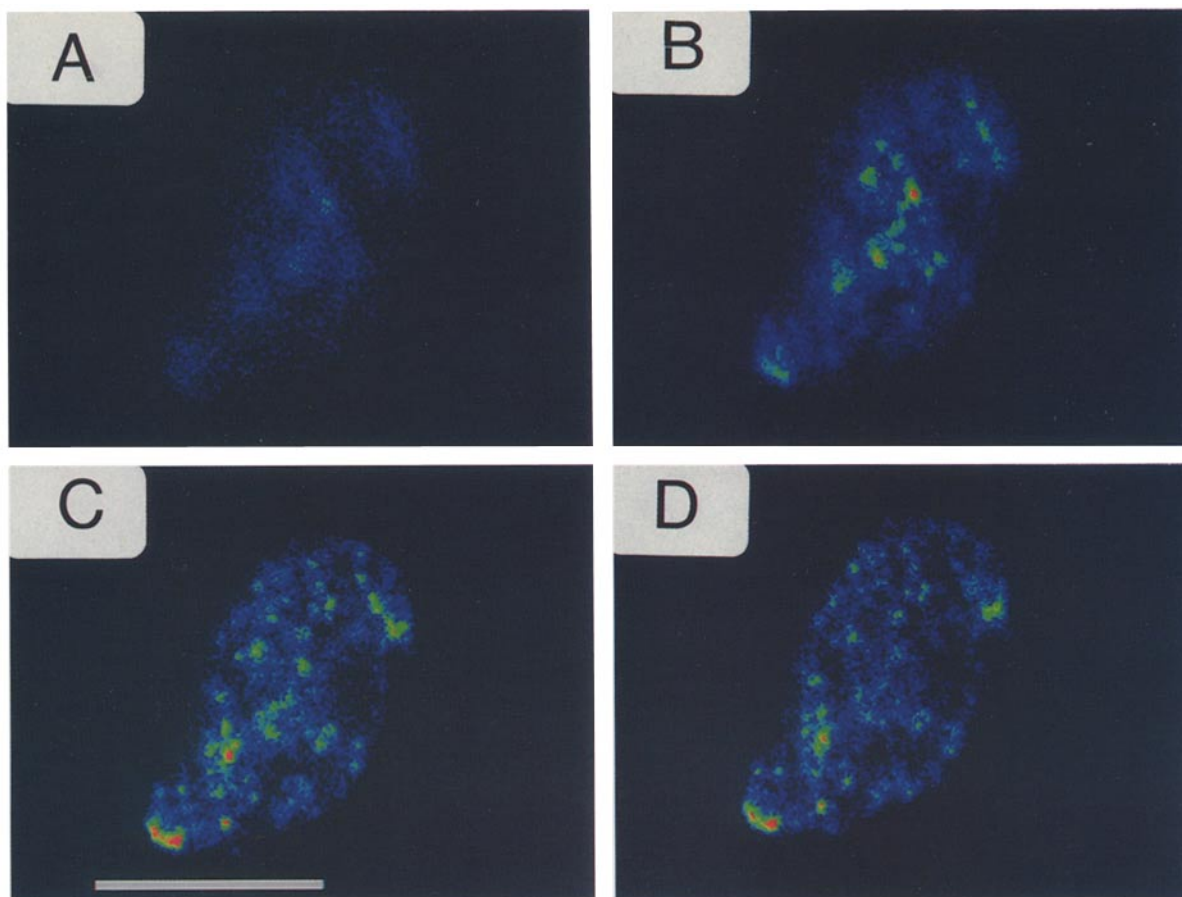


Figure 5. ECM-induced AChR clusters are expressed on the fibroblast cell surface. Laser scanning confocal microscopic analysis of All-11 cells (treated as in Fig. 4) revealed that AChR clusters were present on the fibroblast cell surface. For high resolution analysis, confocal images were collected on a Bio-Rad laser scanning confocal microscope (model MRC500) using a Lietz 50 \times water immersion objective with a numerical aperture of 1.4 and visual analysis of serial sections were displayed in pseudocolor. The confocal images had an apparent thickness of 700 nm, and a resolution of 200 nm. Individual confocal sections were made at 1 μ m intervals, beginning from the top of the cell. The intensity of the fluorescent labeling in this pseudocolor scale is increasing from blue-shaded areas (lowest intensity) to green, yellow, and red (highest intensity). (A) Two AChR clusters (pale blue) are just detectable in the first confocal section at the top of the cell. (B) The intensity of fluorescence increases as the confocal section passes through these clusters. The tops of several other clusters are now also visible. (C) Fluorescent intensity of the first two clusters is now decreased as the confocal section passes underneath the clusters. Three to four other clusters in the plane of the section are now quite intense. (D) The first two clusters are no longer visible, and the confocal plane is now passing underneath the three to four clusters seen in C. Bar, 10 μ m.

and appears to be associated with the plasma membrane as might be expected if *Torpedo* 43K is myristoylated in fibroblasts as it is in electrocytes.

Immunoblots of whole cell lysates of C2 myotubes, All-11 cells, and 3T3-43K cells gave results similar to those of the immunofluorescence experiments: strong labeling of *Torpedo* 43K in 3T3-43K cells, moderate labeling in C2 cells (fused or not), and no detectable labeling of 43K in All-11 or NIH3T3 cells (Fig. 10 A). We also quantified 43K mRNA levels in cells by Northern blotting. Total RNA was isolated from C2 cells, temperature-shifted All-11 cells, the All-11 parental L fibroblast cell line, and NIH3T3 cells. RNA analyses using a cDNA probe corresponding to the full-length mouse 43K cDNA are shown in Fig. 10 B. Low but detectable levels of 43K mRNA are seen in All-11 cells. Interestingly, the parental line, L fibroblasts, expresses levels comparable to those in C2 myoblasts and myotubes; all three of

which are greater than 43K mRNA levels in All-11 cells. Why 43K mRNA is expressed in an L fibroblast is unknown but as shown in Fig. 10 B, 43K mRNA is not expressed in another mouse fibroblast line, NIH3T3. Densitometric scanning of the bands indicated that the level of 43K mRNA in All-11 cells was \sim 10% of that expressed in C2 myotubes. Comparable 43K mRNA levels are expressed in C2 myotubes and myoblasts indicating that the 43K gene is not regulated as the AChR is by muscle cell differentiation. Parallel RNA blots (data not shown) were probed with *Torpedo* (All-11 and L cell RNAs) or mouse (C2 RNAs) subunit cDNAs. Quantitation of the bands after densitometric scanning and corrections for probe intensities revealed that C2 myotubes express approximately fourfold more 43K than AChR subunit mRNA, while All-11 cells express 30–50-fold less 43K than AChR subunit mRNA. The ratio of 43K to AChR subunit mRNA in C2 muscle cells is therefore \sim 150-

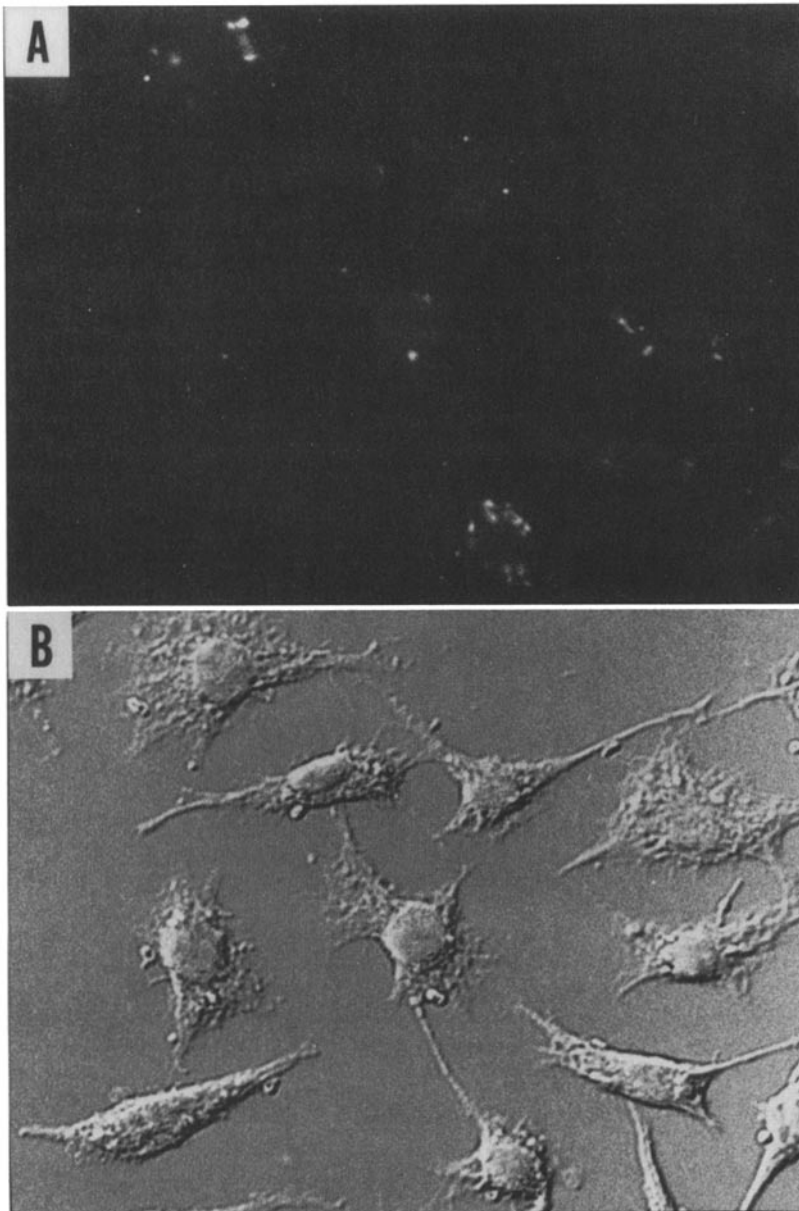


Figure 6. NG108-15 conditioned media induced AChR cluster formation on the surface of All-11 cells. (A) All-11 cells were incubated for 4 h at 37°C in NG108-15 conditioned media which had a protein concentration $\sim 60 \mu\text{g/ml}$. AChRs were labeled with mAb 35 and visualized using indirect immunofluorescence as described. AChR clusters with surface area $1\text{--}3 \mu\text{m}^2$, similar to clusters induced by ECM, were produced. (B) DIC image of cells in A. $\times 400$.

fold greater than the same ratio in temperature-shifted All-11 cells.

Differences have been reported concerning the amount, ratio, and regulation of AChR and 43K at the level of protein and mRNA, and it is not clear whether or not a particular ratio of protein or mRNA is required for a potential interaction between AChR and 43K. In adult *Torpedo* electric organ (Burden et al., 1983; LaRochelle and Froehner, 1986) the levels of 43K and AChR protein are approximately equimolar, however, early in development, AChR protein is expressed but 43K is not (Kordeli et al., 1989; LaRochelle et al., 1990). In two rodent muscle cell lines, the levels of 43K and AChR protein are approximately equimolar but in only one cell line do AChRs cluster (LaRochelle and Froehner, 1987). Transcripts encoding the 43K and AChR α subunit appear coordinately during *Xenopus* embryonal development, and upon denervation, both transcripts increase ~ 30 -fold (Baldwin et al., 1988). In contrast, in rat skeletal mus-

cle, the 43K transcript increases only ~ 3 -fold while that of the AChR α subunit increases ~ 30 -fold upon denervation (Froehner, 1989). In most, but not all cases where AChR clusters are apparent and where 43K was shown to colocalize with those clusters, the two proteins are present in similar quantities. In the present study, we show that although detectable levels of 43K mRNA are present in All-11 cells, the ratio of 43K to AChR is significantly lower than it is in C2 cells and in addition, 43K protein is not detectable at all. Even if low levels of 43K protein are being expressed in All-11 cells, the levels appear to be too low to play a role in AChR clustering (see Discussion).

Discussion

Torpedo AChR subunits are synthesized at 37°C but do not assemble into AChR complexes unless the temperature is lowered (Paulson and Claudio, 1990). At 20°C, 50–75% of

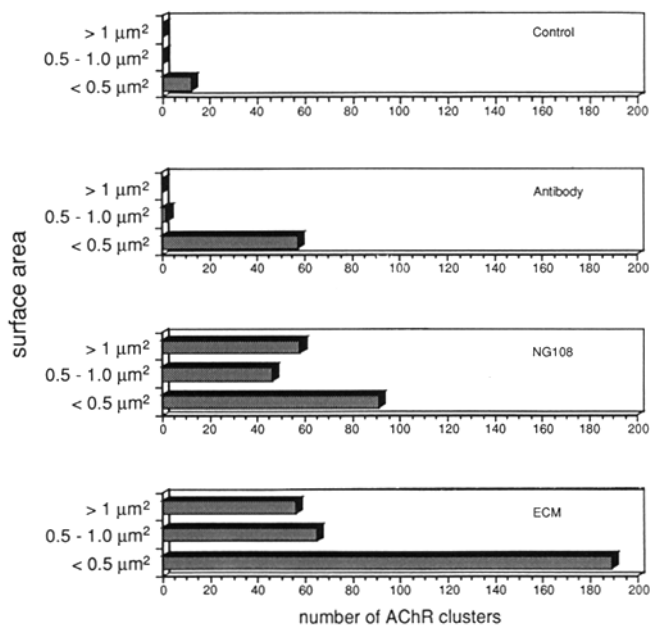


Figure 7. Factor-induced clusters are much larger than antibody-induced clusters. The surface areas of clusters and microclusters produced in factor-treated (ECM and NG108-15 conditioned media), antibody-treated, and control cells were quantitated (100 cells/treatment) and compared. Three sizes of AChR cluster surface area are indicated on the y-axis for the four cell treatments, with the number of clusters of each size observed per 100 cells quantitated on the x-axis. All-11 cells were temperature shifted, then incubated at 37°C in serum-free media (control), 1/400 dilution of mAb 35 (antibody), 60 μg/ml NG108-15 conditioned media (NG-108), or 2.8 μg/ml ECM (ECM). Fluorescently labeled clusters were quantitated using Image 2.1 software (NIH) on a Macintosh IIfx computer.

the cells express surface AChR within 36 h and 100% of the cells express surface AChRs by 3 d. These surface-expressed *Torpedo* AChRs are composed exclusively of $\alpha_2\beta\gamma\delta$ pentamers (Hartman et al., 1990) with binding and channel properties consistent with a single population of molecules (Claudio et al., 1987). In contrast, when the four mouse AChR subunit cRNAs are injected into *Xenopus* oocytes, a heterogeneous population of channels is expressed: proper $\alpha\beta\gamma\delta$ channels but also $\alpha\beta\gamma$ (Kullberg et al., 1990) and $\alpha\beta\delta$ (Charnet, P., C. Labarca, and H. A. Lester. 1991. *Biophys. J.* 59:527a) channels. Mouse AChR expressed in oocytes may therefore not be an ideal expression system for investigating properties of AChRs where a uniform population of molecules is desired. As shown in Fig. 1, *Torpedo* AChRs have surface expression levels and a cell surface distribution similar to those of mouse AChRs in C2 myotubes. These cell lines thus appear well suited for investigating one of the early events of synaptogenesis, AChR clustering. They have an advantage over muscle cells in that just one muscle-specific gene product is expressed, thereby allowing the response of the AChR to added factors to be directly monitored. Because assembly of *Torpedo* AChRs is acutely temperature sensitive, use of *Torpedo* AChR fibroblasts has the further advantage of allowing us to separate two mechanisms of cluster formation: directed insertion of new AChRs and movement of preexisting surface AChRs.

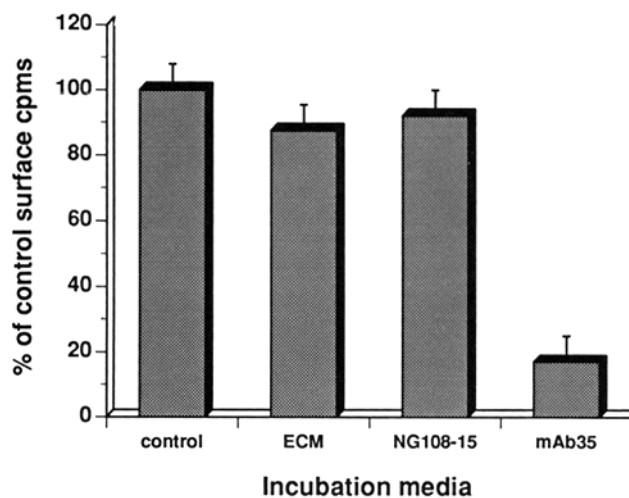


Figure 8. Factor-induced surface AChR clusters are as stable as control AChRs, whereas antibody-induced clusters are rapidly internalized. The number of AChRs (determined with [¹²⁵I]BuTx binding) on the surface of temperature-shifted All-11 cells was decreased by <5% when the cells were incubated in NG108-15 conditioned media, and by <10% when cells were incubated with ECM. Incubation with mAb 35, however, resulted in 83% loss of surface AChR. All incubations were performed at 37°C for 6 h in DME, with clustering agents added at the following concentrations: 2.8 μg/ml ECM; 60 μg/ml NG108-15 conditioned media; and 1/400 dilution mAb of 35 hybridoma supernatant.

When anti-AChR antibodies were incubated with All-11 cells, microclusters were formed with surface areas <0.5 μm² (Fig. 1), similar in size to antibody-induced clusters observed on cultured muscle cells (Lennon, 1978). These *Torpedo* AChRs were also rapidly internalized after binding and cross-linking by antibodies, as has been observed in muscle cells. When *Torpedo* ECM or NG108-15 conditioned media were incubated with All-11 cells, AChR clusters were formed that had surface areas (1–3 μm²) greater than those induced by antibodies and the clusters were stable, unlike antibody-induced clusters. These results suggest that the mechanism by which extracellular synaptic factors produce AChR clusters is different from that of antibody-induced clusters, the latter being thought to occur by direct intermolecular cross-linking of AChRs.

Clusters induced by ECM and NG108-15 conditioned media were similar in size, distribution, number, and stability, suggesting that the active agent in each might be the same. The physical properties of the clustering factor in NG108-15 conditioned media are similar to those of agrin (Bauer et al., 1985), an AChR clustering factor originally isolated from *Torpedo* ECM (Nitkin et al., 1987). We found that the active component from both ECM and conditioned media was >10 kD, heat labile, and mobilized AChR without clustering total cell surface glycoproteins. 1–3 μm² AChR clusters induced by ECM and conditioned media in All-11 cells were equivalent in size to agrin-induced AChR clusters reported on chick myotubes after a 2–4-h incubation in the presence of partially purified agrin (Wallace, 1988). Thus, it may be that the agent causing *Torpedo* AChRs to cluster, is agrin. This possibility is further supported by the observation that an ~1,000-fold purified preparation of *Torpedo* ECM also induced

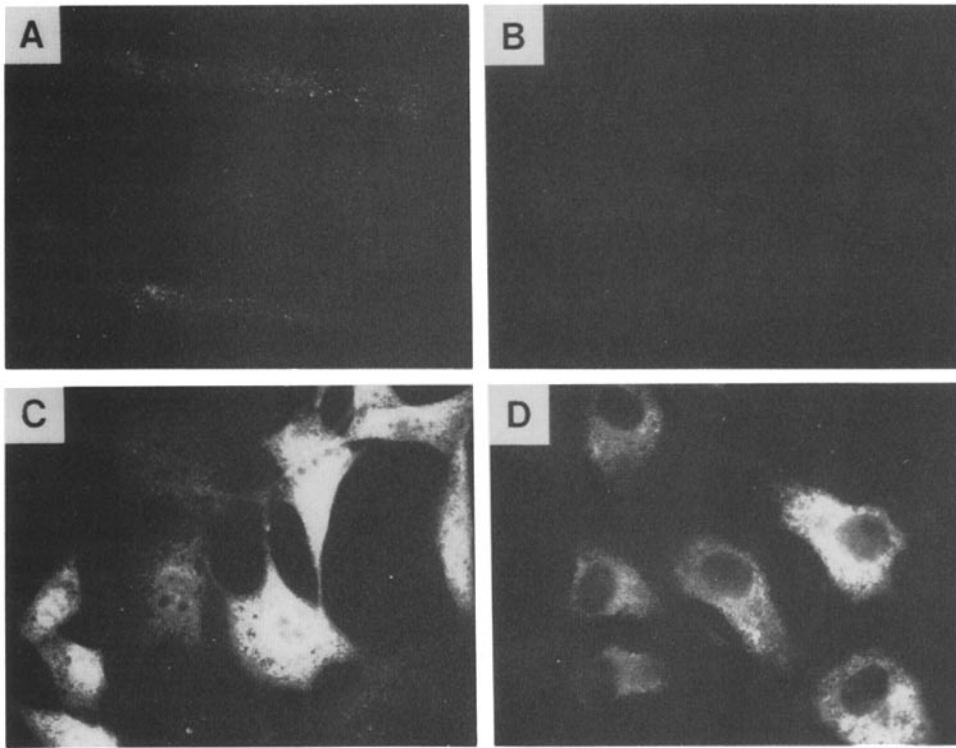


Figure 9. 43K protein is not detectable in all All-11 cells by indirect immunofluorescence. Cells were fixed and permeabilized, labeled with either a mAb directed against the 43K protein (mAb 1234A) or a mAb directed against assembled AChR (mAb 35), then fluorescently labeled (see Materials and Methods). C2 myotubes (A) show diffuse labeling for 43K protein, but none is detectable in All-11 cells shown in B. The same All-11 cells shown in B were double-labeled for internal AChR (D) and significant levels of AChR are clearly visible. In C, 3T3-43K cells (expressing *Torpedo* 43K) show strong, diffuse mAb 1234A labeling. A: $\times 160$; B-D: $\times 400$.

clusters in our system (Fig. 3). Although many factors have been identified that induce AChR clustering, it is not known whether these factors interact directly with the AChR molecule, or whether they induce AChR clustering through signalling mechanisms present in the muscle cell. The observations that AChR fibroblasts respond to factors isolated from both neuronal and extracellular matrix sources, that the surface distribution of glycoproteins is not affected, and that this clustering can occur in a non-muscle cell, all indicate that the AChR interacts directly with the clustering factor.

In cultured chick myotubes, small ($<4 \mu\text{m}^2$) AChR clusters appeared within 2 h of treatment with ECM and increased in number until 4 h (Wallace, 1988). During the next 12–20 h, the number of clusters per myotube decreased as the size of each cluster increased to $\sim 15 \mu\text{m}^2$. Other studies (using cultured rat muscle) have also indicated that AChR cluster formation in muscle cells is complex, occurring in multiple stages which begins with microaggregates ($<1 \mu\text{m}$ diameter) that coalesce to form aggregates $>10 \mu\text{m}^2$ (Olek et al., 1983). Our results show that the 1–3- μm^2 clusters are produced in both C2 myotubes and AChR-fibroblasts after incubation in ECM for 4 h (Fig. 3). As shown in Fig. 3 B, >30 of the 1–3- μm^2 clusters in C2 myotubes had aggregated to form a large cluster area $\sim 10 \mu\text{m}$ in diameter with a 4-h treatment in ECM, whereas in AChR-fibroblasts during this same time period, no aggregation of small clusters was observed. These results suggest that clustering events immediately subsequent to the initial aggregation of AChRs may require other muscle-specific proteins. Studies are currently underway to investigate the time course of cluster formation in AChR-fibroblasts, including incubation periods of 12–20 h.

Both lateral migration of preexisting AChRs (Anderson and Cohen, 1977; Styra and Axelrod, 1983; Ziskind-

Conhaim et al., 1984; Kuromi et al., 1985) and the directed insertion of new AChRs (Role et al., 1985; Dubinsky et al., 1989) have been suggested as mechanisms of producing AChR clusters. With All-11 cells, these two mechanisms can be separated since *Torpedo* AChR subunits cannot assemble at 37°C (Claudio et al., 1987). For all of our clustering experiments, we assembled and expressed AChRs on the surface of the fibroblasts by incubating at 20°C , but before incubating with clustering factors, the cells were shifted to 37°C . Once *Torpedo* AChRs are assembled at a permissive temperature and then shifted to 37°C , they remain fully functional and turn over on the surface of the cell with a half-life identical to that of mammalian AChR in muscle (Paulson and Claudio, 1990). As shown in Figs. 3–6, clusters were formed in All-11 cells after 2–4-h incubation at 37°C with clustering agents. We conclude that these clusters were all formed by the mobilization of preexisting AChRs on the cell surface, not by insertion of newly synthesized AChRs.

One protein that has long been thought to play a role in AChR clustering is the cytoplasmic 43K protein. When All-11 cells were screened for the presence of the 43K protein, none could be detected by either immunofluorescence or protein blotting techniques although both techniques revealed the presence of the 43K in C2 myotubes. Low levels of 43K mRNA could be detected in All-11 cells however, but the levels were significantly lower than those found in C2 cells. Although the ratio of 43K protein to AChR protein in C2 cells is approximately equivalent (LaRoche and Froehner, 1987), the ratio of 43K mRNA to AChR mRNA was ~ 150 -fold less in All-11 cells than it was in C2 cells. We conclude that if there are endogenous 43K proteins expressed in All-11 cells, they are in concentrations too low to have significant interactions with AChRs. The results presented in this study are entirely consistent with those

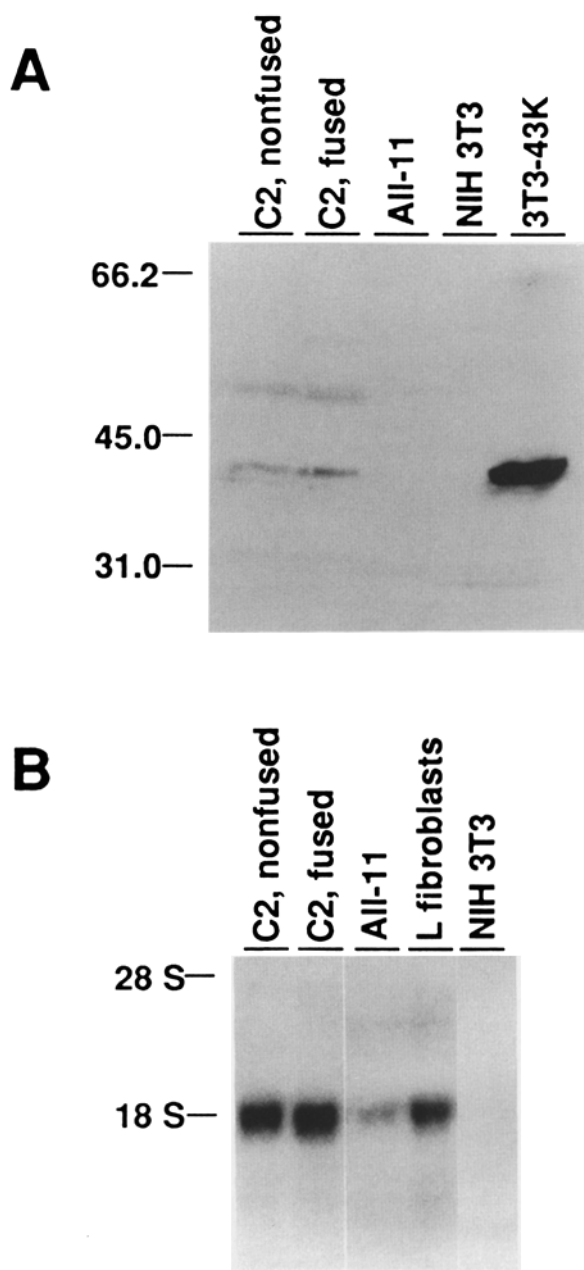


Figure 10. Immunoblot and RNA analysis of 43K in different cell lines. (A) Different cell lines were analyzed by immunoblot for the presence of 43K protein: C2 myocytes (C2, *nonfused*), C2 myotubes (C2, *fused*), temperature-shifted All-11 cells, NIH3T3 cells, and 3T3-43K cells. 43K protein was not detectable in either All-11 cells or 3T3 cells but it was detectable in C2 myocytes, myotubes, and 3T3-43K cells. Protein was separated on a 7.5% SDS polyacrylamide gel. (B) Northern analysis was performed on different cell lines to test for the presence of 43K mRNA: C2 myotubes, C2 myocytes, All-11 cells, L fibroblasts (the parental cell line of All-11 cells), and NIH3T3 cells. 43K mRNA is detectable in all cell lines except NIH3T3. Although detectable levels are seen in temperature-shifted All-11 cells, the amount is $\sim 10\%$ of that seen in a C2 muscle cell. 10 μg of total RNA was separated in each lane of a 1% agarose formaldehyde gel and hybridized to a 1,500-bp fragment of the mouse 43K cDNA.

presented earlier where we induced clustering of *Torpedo* AChRs stably expressed in NIH3T3 cells (All-15 cells; Claudio et al., 1989a). As shown in Fig. 10, neither 43K protein nor 43K mRNA are expressed in NIH3T3 cells, yet *Torpedo* ECM is capable of inducing AChR clusters in these cells. Although All-15 cells have been extensively characterized and shown to express a single population of proper ion channels (Sine et al., 1990), because All-15 cells did not adhere well to glass coverslips, it was difficult obtaining large numbers of cells for our clustering analyses. We preferred to present the results of hundreds of All-11 cells (in Fig. 7, the results from 400 cells are presented) with undetectable levels of 43K protein to just a few All-15 cells with undetectable levels of 43K mRNA. The central observation using either All-11 or All-15 cells, however, is that AChRs do not cluster unless external factors are added to the cells.

In a recent study by Froehner et al. (1990) when *Xenopus* oocytes were microinjected with cRNAs for mouse AChR and mouse 43K, the two proteins appeared to cocluster. Interestingly, if the 43K was expressed alone, it formed clusters by itself. The authors concluded that the 43K protein caused clustering of AChRs. However, in *Torpedo*, it has been shown that AChR aggregates appear at the ventral pole of newly formed electrocytes before innervation and before any 43K protein can be detected (Kordeli et al., 1989; LaRoche et al., 1990). These results suggest that in *Torpedo*, at least the initial clustering of AChR does not require association with the 43K protein. Further, in the mouse muscle BC3H-1 cell line, although equivalent amounts of AChR and 43K protein are present, AChRs fail to cluster (LaRoche and Froehner, 1987). Finally, we have shown here that the 43K is not required for clustering in fibroblasts, and that AChRs do not cluster unless an external agent is added.

A possible explanation for the apparent differences in cluster formation in the presence or absence of 43K, is that there are multiple mechanisms for inducing AChR clusters. It may be that during development, when the nerve first makes contact with a muscle cell, a factor secreted from the nerve terminal interacts with existing surface AChRs, triggering the clustering of these AChRs (analogous to the study we described here). Slightly later in development, when AChR synthesis is activated, the coexpression of AChR and 43K could result in associations between the two proteins and directed insertion at the site of the developing neuromuscular junction. It would be interesting to challenge mouse AChRs expressed in the absence of 43K with extracellular synaptic factors to see if clusters can be produced as they are in our system.

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