Microinjection of a Monoclonal Antibody against a 37-kD Protein (Tropomyosin 2) Prevents the Formation of New Acetylcholine Receptor Clusters

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Abstract. We have shown previously that chick muscle cells transformed with Rous sarcoma virus are unable to form clusters of acetylcholine receptors (AChRs) (Anthony, D. T., S. M. Schuetze, and L. L. Rubin. 1984. Proc. Natl. Acad. Sci. USA. 81:2265-2269) and are missing a 37-KD tropomyosin-like protein (TM-2) (Anthony, D. T., R. J. Jacobs-Cohen, G. Marazzi, and L. L. Rubin. 1988. J. Cell Biol. 106:1713-1721). In an attempt to clarify the role of TM-2 in the formation and/or maintenance of AChR clusters, we have microinjected a monoclonal antibody specific for TM-2 (D3-16) into normal chick muscle cells in culture. D3-16 injection blocks the formation of new clusters but does not affect the preexisting ones. In addition, TM-2 is concentrated at rat neuromuscular junctions. These data suggest that TM-2 may play an important role in promoting the formation of AChR clusters.

Acetylcholine receptors (AChRs) are highly concentrated at the adult vertebrate neuromuscular junction. The preferential localization of AChRs at the postsynaptic membrane occurs during the early stages of nerve-muscle synapse formation (for reviews see 11, 13, 27). This region is also characterized by an intricate submembranous cytoskeletal network. In the last few years, much work has focused on the possible functional relationship between the components of this cytoskeletal network and the organization of the postsynaptic region. The search for cytoplasmic events involved in AChR clustering has been predominantly immunocytochemical to this point. A variety of cytoskeletal molecules have been found to be concentrated at the vertebrate neuromuscular junction or in association with AChR aggregates on cultured mammalian or avian muscle fibers (for review see 15). These include nonmuscle actin, various actin-binding proteins, and a 43-kD AChR-associated protein thought to have the ability to bind to actin and to the β subunit of AChR (4-8, 10, 16, 19, 22, 23, 26, 28, 30). It is likely that these proteins are involved in the organization of the subcluster region, but no direct evidence has yet been provided. Since the junctional region has many cellular specializations, including postsynaptic mem-

TM-2 in the formation and/or maintenance of AChR clusters, we have microinjected a monoclonal antibody specific for TM-2 (D3-16) into normal chick muscle cells in culture. D3-16 injection blocks the formation of new clusters but does not affect the preexisting ones. In addition, TM-2 is concentrated at rat neuromuscular junctions. These data suggest that TM-2 may play an important role in promoting the formation of AChR clusters.

Materials and Methods

Monoclonal Antibodies

A monoclonal antibody specific for the 37-kD tropomyosin-like protein (D3-16) was produced as described previously (2). IgG was purified from hybridoma supernatant by absorption to a protein A-Sepharose 4B affinity column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with 50 mM citrate buffer, pH 4, according to the manufacturer's conditions. Peak fractions were pooled, dialyzed in 10 mM Tris, pH 7.4, and concentrated on a Centricon 30 filter (Amicon Corp., Danvers, MA). The concentration
of the antibody solution was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) (29) and was adjusted to 7 mg/ml. The antibodies were then sterilized through a 0.22-μm filter and kept at 4°C.

**Muscle Cell Culture**

Muscle cells were obtained by enzymatic dissociation of 11-d chick embryo leg muscle as described previously (14). Cells were grown on collagen-coated coverslips in a growth medium consisting of MEM (Gibco Laboratories, Grand Island, NY) with 10% horse serum (Gibco Laboratories) and 2% chick embryo extract in an atmosphere containing 5% CO₂. For some experiments, muscle cells were treated with an AChR cluster-inducing factor (CF) isolated from *Torpedo californica* electric tissue using techniques already described (25).

**Microinjection**

Glass pipettes used in microinjection were made from Omega Dot tubing (internal diameter of 0.6 mm; Glass Company of America, Bargaintown, NJ) by two sequential pulls (244 and 188 V, respectively) with a vertical puller (David Kopf Instrument, Tujunja, CA). Pipettes were acid washed, sterilized in absolute ethanol (18), and kept under sterile conditions. During microinjection, cells were kept in a 1:1 mixture of MEM/L-15 media (Gibco Laboratories) with 10% horse serum and 2% chick embryo extract. D3-16 was a goat antiserum against an IgG class antibody derived from a BALB/c mouse using similar procedures to that of the untreated cells. After injection, cells were treated for 6 h with CF in growth medium and incubated for 1 h with rhodamine-conjugated α-bungarotoxin (Rh-α-Bgtx) prepared by the method of Radin and Axelrod (24) to localize AChRs. Cells were then fixed in methanol at -20°C and labeled with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA) to identify the injected cells. Cells were examined with a microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with phase and fluorescence optics.

In some cases, cells were pretreated for 5-6 h with 5 mM sodium azide to disperse the existing AChR clusters (4). Some cells were labeled and fixed after sodium azide treatment. Others were microinjected with D3-16, M1g, or with a monoclonal antibody. This latter antibody is an IgG class antibody derived from a BALB/c mouse using similar procedures to those used to derive D3-16. Hybridoma cells were grown, and antibody was purified from the supernatant and concentrated just as described for D3-16. As previously shown, the antibulin antibody binds to tubulin but does not interfere with its function (9a, 10). The cells were then allowed to recover for 5-6 h in the presence of CF in growth medium and then labeled as described above.

**Morphometry**

To analyze in detail the number and size of AChR aggregates, myotubes were selected at random and filmed under fluorescence optics with a silicon-intensified instrument camera (Dage-MTI Inc., Walbash, MI) interfaced with a video cassette recorder (VC-9507; NEC Home Electronics [U.S.A.] Inc., Elk Grove Village, IL). Myotube segments and bright Rh-α-Bgtx patches were traced from the video screen onto acetate sheets. Areas of patches and of myotube segments were measured by tracing their outlines on a digitizing tablet interfaced with a personal computer (IBM Instruments, Inc., Danbury, CT) using the MicroComp Planar Morphometry software (SMI, Inc., Atlanta, GA). Approximately 150 segments were analyzed for each treatment. Bright fluorescent patches of Rh-α-Bgtx were defined as clusters when their area was >25 μm² and as AChR aggregates when their area was >25 μm². We then calculated the ratio between the number of clusters (or aggregates) counted per segment and the area of the corresponding segment; this gave the number of clusters (or aggregates) per unit area.

**Immunochemistry**

Adult rat diaphragm or intercostal muscles were frozen in liquid nitrogen-cooled isopentane and mounted in OCT compound (Miles Scientific Div., Naperville, IL). 4-μm tissue sections were cut and mounted on gelatin-subbed slides. Slides were preincubated for 30 min in MEM containing 10% horse serum or 1% BSA (Sigma Chemical Co.) and then incubated for 1 h at room temperature in either D3-16 hybridoma supernatant or purified D3-16 IgG diluted in MEM plus horse serum. Sections were rinsed in PBS and incubated in Rh-α-Bgtx and fluorescein-conjugated goat anti-mouse IgG for 1 h at room temperature. Sections were rinsed again in PBS and mounted in UV-inert mounting compound (Atomicmer Chemetsals Corp., Plainview, NY) and photographed as described previously (2).

**Results**

**Microinjection of Normal Cells**

To determine the role of TM-2 in the clustering process, we injected normal cultured chick muscle cells with D3-16. We assumed that antibody binding to TM-2 would subsequently affect the ability of TM-2 to interact with other cytoskeletal components, thereby blocking its function. We used chick myotubes for these experiments because of our prior study on RSV-transformed chick cells (1). Cells were injected between days 5 and 7 after plating when the probability of spontaneous cluster formation is highest. It has been shown previously that chick or rat muscle cells in culture exhibit an increase in the number of AChR clusters when treated with a factor (or factors) derived from the extracellular matrix of *Torpedo* electric tissue (25, 31). After microinjection, cells were treated with *Torpedo* CF to test whether the antibody inhibited the formation of new AChR clusters.

Results were analyzed initially by immunofluorescence (Fig. 1), and subsequently these data were subjected to quantitative morphometric analysis. Using a planar morphometry system, we measured the areas of rhodamine-positive bright patches and of myotube segments (see Materials and Methods). We analyzed cells exposed to four different experimental conditions: (a) noninjected, untreated cells; (b) noninjected, CF-treated cells; (c) D3-16-injected, CF-treated cells; and (d) M1g-injected, CF-treated cells. The distributions of the frequencies of clusters and aggregates per unit area are presented in Figs. 2 and 3. Most noninjected, untreated myotubes did not have any AChR clusters (Fig. 2). Nevertheless, clusters and smaller fluorescent Rh-α-Bgtx patches were observed in some cells.

After 6 h of treatment with CF, the number of clusters per unit area increased markedly in the noninjected cells. Only a few segments had no clusters. To evaluate the effect of microinjection per se, we injected nonimmune M1g under the same conditions as for D3-16 (Fig. 1, e and f). As shown in Fig. 2, we observed that the ability of M1g-injected cells to respond to CF was only slightly diminished. In contrast, in D3-16-injected cells, treatment with CF did not increase the number of AChR clusters (Fig. 1, a–d, and Fig. 2). The distribution of cluster density was very similar to that of untreated cells, suggesting that the binding of D3-16 to TM-2 does not disrupt preexisting clusters but instead prevents the formation of new clusters induced by CF. There were no other obvious morphological effects on the injected cells.

We also analyzed the density of aggregates (Fig. 3). D3-16–microinjected cells had a density of aggregates similar to that of the untreated cells. After a 6-h treatment with CF, the number of segments without aggregates decreased in both noninjected and M1g-injected cells. In contrast, the number of segments without aggregates remained very high in untreated cells and D3-16–injected cells.

We were also interested in determining whether the size of AChR clusters was modified by any of the four different
Figure 1. Effect of D3-16 microinjection on AChR clusters formation. After injection, cells were treated for 6 h with CF, incubated for 60 min with Rh-αBgt to localize AChRs (a, c, and e), fixed in methanol at -20°C, and labeled with fluorescein-conjugated goat anti-mouse IgG to identify injected cells (b, d, and f). After a 6-h treatment with CF, no AChR clusters were observed in the bright D3-16-injected cells, whereas several clusters and aggregates were present on the nearby noninjected cell (a–d). The Mlg-injected cell was still able to cluster AChRs after treatment with CF (e and f). The Rh-αBgt staining pattern of this cell is comparable with that of the noninjected cells. Bar, 10 μm.

Experimental conditions. We therefore determined the distribution of cluster size on 150 muscle segments for each experimental condition. Under all four conditions, the majority (80–90%) of AChR clusters were between 25 and 75 μm². 5% of the clusters (total number of 692) in noninjected and Mlg-injected cells (516 clusters) treated with CF were very large (>125 μm²). We never observed such large clusters in D3-16-injected cells (192 clusters) and only saw 1 large cluster out of 216 in untreated cells.

Microinjection of Myotubes Treated with Sodium Azide

From these observations, we assumed that blocking TM-2 did not affect the preexisting clusters but prevented the induction of new clusters caused by the treatment with CF. To distinguish more easily between the effect of microinjection on preexisting clusters and on new cluster formation, we wanted to examine cells that had few or no clusters at the start of the experiment. A previous study (4) demonstrated that AChR clusters are dispersed by treating cells with a metabolic inhibitor such as sodium azide. This process is reversible, and cells are able to reform clusters rapidly after replacement of medium. We treated cells for 5 h with 5 mM sodium azide before microinjection. After sodium azide treatment, most of the cells did not have any clusters, although occasional small aggregates were present (Fig. 4a and Table I). After changing the medium, the cells were allowed to recover for 6 h in the presence of CF. After recovery, noninjected cells had clusters and large aggregates (Fig. 4b). Control antitubulin- and Mlg-injected cells were also able to reform clusters during the recovery from sodium azide treatment (Fig. 4, e–h). Cells injected with D3-16 after sodium azide treatment did not have any clusters, although very small aggregates were sometimes detected that were similar to those observed in cells labeled and fixed immediately after dispersal of AChR clusters (Fig. 4, c and d). Taken together, these data suggest that D3-16 microinjection does block the formation of new clusters.
Figure 2. Distribution of numbers of AChR clusters per unit area. Four different experimental conditions were analyzed: noninjected, untreated cells (CF\(^-\)); noninjected, CF-treated cells (CF\(^+\)); nonimmune MIg, CF-treated cells (MIg\(^+\)/CF\(^+\)); and D3-16-injected, CF-treated cells (D3-16\(^+\)/CF\(^+\)). Bright fluorescent patches of Rh-α-Bgtx were considered as clusters when their area was >25 \(\mu\)m\(^2\). We calculated the ratio between the number of clusters observed in each segment and the area of the corresponding segment. We then multiplied the values obtained by 10\(^4\). Each dot represents a myotube segment with an assigned density of AChR clusters. Dots at and beneath the zero line represent segments with no clusters at all. Most of the segments analyzed in noninjected, untreated myotubes (CF\(^-\)) do not have any clusters. The segments that did possess clusters had <10 clusters per unit area. Treatment with CF led to a marked increase in the number of clusters per unit area in noninjected cells (CF\(^+\)) and, although to a lesser extent, in MIg-injected cells (MIg\(^+\)/CF\(^+\)). Segments with no clusters at all were less frequent. In contrast, D3-16-injected cells did not show an increase in AChR cluster numbers after treatment with CF (D3-16\(^+\)/CF\(^+\)). The distribution of clusters is very similar to the one observed in noninjected, untreated cells, suggesting that the preexisting clusters were not disassembled.

Figure 3. Distribution of the number of AChR aggregates per unit area (see legend to Fig. 2). Aggregates were defined as bright Rh-α-Bgtx patches with an area between 12.5 and 25 \(\mu\)m\(^2\). Note that the number of segments with no aggregates is very high in both noninjected, untreated cells (CF\(^-\)) and D3-16-injected cells after treatment with CF (D3-16\(^+\)/CF\(^+\)). In contrast, in noninjected cells (CF\(^+\)) and MIg-injected cells (MIg\(^+\)/CF\(^+\)), treatment with CF for 6 h led to a decrease in the number of segments with no aggregates.
Immunocytochemistry of Adult Muscle

Results of our microinjection experiments suggested that TM-2 plays a role in the process of AChR clustering; therefore, we examined its distribution in adult muscle using immunocytochemistry on frozen sections of adult rat diaphragm and intercostal muscles. We found that TM-2 was distributed all along the lengths of the muscle fibers. However, it was most concentrated at the junctional region (Fig. 5, a and b). We did not observe any labeling along motor nerve processes. Our current efforts with frozen sections made from adult chicken muscle have so far not revealed any obvious staining anywhere in the fiber despite the ability of D3-16 to label TM-2 in cultured chick myotubes. We know that TM-2 is present in chicken muscle based upon results with Western blots (2); thus, either the accessibility of TM-2 to D3-16 is different in chicken muscle or TM-2's antigenicity is lost during the preparation of the frozen sections. However, we note that its distribution in rat muscle concurs strongly with a proposed role of TM-2 in clustering.

Discussion

Our previous work established that chick muscle cells transformed with a temperature-sensitive mutant of RSV cannot cluster AChRs even when maintained at the nonpermissive temperature for transformation (1). We examined possible differences between normal and RSV-transformed cells that might account for the inability of transformed cells to cluster AChRs. We found that transformed cells showed greatly decreased amounts of a nonmyofibrillar tropomyosin-like molecule, TM-2 (2). In the experiments described in this report, we present a functional assay that suggests that TM-2 participates in AChR clustering. Microinjecting a monoclonal antibody specific for this molecule (D3-16) interferes with new cluster formation. We calculated that the concentration of antibody introduced into the cells was ~0.5–1 μM. Based on our recent unpublished results with microsequencing of TM-2 and on the estimates of Wojcieszyn et al. (32), we estimate the concentration of TM-2 to be ~2 μM. We do not know how much of the TM-2 would have to be blocked by the antibody to block clustering since we do not know, for instance, the proportion of TM-2 that participates in this process. It seems quite possible that the amount of injected D3-16 was sufficient to bind to a large fraction of the TM-2. The antibody concentration is similar to that used on Western blots where D3-16 labels primarily TM-2, but also a 43-kD tropomyosin-like protein (TM-1). Of course, we can't truly predict what the specificity of D3-16 would be after its introduction into the cell, but previous experience has suggested that other antibodies injected into cells under similar circumstances retain their specificity (20, 21). Also, we favor the explanation that it is TM-2, rather than TM-1, that plays a role in clustering since the levels of TM-1 were not altered in virally transformed cells, but further experimentation will be needed to resolve this issue.

The first set of experiments we described focused on cells that already had some clusters and that were subsequently treated with CF derived from Torpedo electric tissue. Noninjected cells had a large increase in the number of clusters within a few hours of treatment with this factor. Cells microinjected with a control nonimmune Ig had only a slightly decreased capacity to form new AChR clusters, probably due to minor cell damage resulting from microinjection. Cells injected with D3-16, however, showed a dramatic reduction in their ability to cluster AChRs. In addition, very large clusters were never observed in either D3-16-injected or untreated cells. Changes were most marked 6–8 h after microinjection and decreased by 24 h, corresponding with the point at which we can no longer detect the presence of the injected antibody. There were no pronounced effects of the microinjection on general cell morphology, and cells survived for at least 24 h after injection. Also, the fact that microinjection seemed not to break up preexisting clusters argues against a generally deleterious effect of injection of any of the antibodies.

We also examined the effects of injection on AChR aggregates. Previously, we have seen that these small patches of AChRs are not as stable as clusters and are not necessarily associated with immobile nuclei (12). Presumably, they represent an intermediate stage in cluster formation, and we quantified them separately. Again, we found that D3-16-injected cells had more regions free of aggregates than did MIg-injected ones. This further supports the notion that D3-16 injection interferes with the process by which AChRs accumulate.

D3-16-injected cells had some clusters after treatment with CF. We interpret these to be clusters that were already present before injection. Evidence consistent with this includes our observation that cells both injected with D3-16 and treated with CF had approximately the same number of clusters and aggregates as untreated cells. The fact that the size distribution of clusters in the two types of cells was similar (i.e., very large clusters were absent from both) is consistent with this interpretation. In addition, clusters labeled before microinjection were still present 4 h after microinjection (data not shown).

A strong indication that D3-16 microinjection blocks the formation of new clusters derives from observations of cells incubated with sodium azide. This treatment had been shown previously by Bloch (4) to disrupt clusters reversibly. Cells treated with sodium azide and then injected with either of two control Igs (nonimmune IgG or an antitubulin monoclonal made and concentrated in the same way as D3-16) formed new clusters after sodium azide washout; however, cells microinjected with D3-16 before sodium azide removal were unable to form new clusters. The ability of D3-16 microinjection to block new cluster formation without affecting preexisting clusters might suggest that (a) once clusters are formed, TM-2 does not play any role in their maintenance; (b) once clusters are formed, the epitope is no longer accessible to the antibody; or (c) TM-2 is assembled rapidly during cluster formation but its disassembly is slow so that we cannot observe disruption of preexisting clusters 6 h after a single microinjection.

Evidence consistent with the putative role of TM-2 in AChR clustering is the observation that it is concentrated (but not exclusively localized) at mammalian neuromuscular junctions. Preliminary ultrastructural experiments confirm a postsynaptic localization of this molecule and also suggest that this preferential localization is not due simply to an increased surface area in the junctional region (Fumagalli, G., G. Marazzi, and L. L. Rubin, unpublished observations). Despite the fact that Western blot analysis using D3-16 has
Table 1. Effect of Microinjection on Azide-treated Cells

| Condition                              | Number of clusters per segment |
|----------------------------------------|-------------------------------|
| 1. Azide treated and fixed             | 0.28 ± 0.03 (68)              |
| 2. Azide removed, noninjected          | 2.33 ± 0.21 (45)              |
| 3. Azide removed, Mlg injected         | 2.32 ± 0.27 (49)              |
| 4. Azide removed, antitubulin injected | 2.15 ± 0.29 (38)              |
| 5. Azide removed, D3-16 injected       | 0.35 ± 0.13 (45)              |

Cells were treated as described in the legend to Fig. 4. After azide treatment, cells were either fixed immediately (condition 1), treated with CF (condition 2), or microinjected and treated with CF (conditions 3-5). The number of clusters and large aggregates per segment was then determined. Entries in the table represent the mean number of clusters per segment ± SEM. The numbers in parentheses are the number of segments for each condition. D3-16 injection blocks the recovery normally seen after azide washout, whereas injection of two control antibodies has no significant effect.

shown that TM-2 is present in adult chicken muscle (2), we do not know its distribution in that tissue because D3-16 does not label frozen sections of chicken muscle. In a previous report, we showed that TM-2 is present along the entire length of rat and chick cultured muscle fibers and is not concentrated near AChR clusters (2). The difference between cultured embryonic myotubes and adult innervated muscle fibers may reflect the greater degree of maturity of the adult subcluster region. Alternatively, the relatively large amounts of TM-2 seen away from clusters in cultured muscle fibers may reflect its participation in processes that are more prevalent in embryonic than in adult muscle. For example, as we suggested previously (2), assembly of myofibrils may be such a process.

How might TM-2 participate in AChR clustering? Several investigators have demonstrated a high concentration of actin beneath AChR clusters in cell culture or at the neuromuscular junction (5, 10, 19, 22). Since cluster formation appears to be blocked by cytochalasin D (12), it seems probable that this actin network contributes significantly to the process by which AChRs form stable aggregates. In other systems, it has been shown that tropomyosin can stabilize actin filaments by direct binding (3). Therefore, since TM-2 shares some properties with tropomyosins, it might be essential for stabilizing actin filaments that are actively polymerizing under newly forming clusters and thus eventually serve as a scaffold for the formation of AChR aggregates. As we have also mentioned, this subcluster scaffold is likely to be extensive, spanning the sarcolemma and associated organelles, such as nuclei and Golgi apparatus (12). If the tropomyosin-like behavior of TM-2 is confirmed (i.e., binding to actin), this will guide the search for other structural proteins that are involved in the establishment of the neuromuscular junction region.

In summary, we feel that we have provided three results consistent with a role for TM-2 in clustering: (a) virally transformed cells with decreased amounts of this polypeptide cannot cluster AChRs; (b) TM-2 is localized at neuromuscular junctions, at least in adult rat muscle; and (c) microinjection of an antibody directed against TM-2 blocks the ability of cultured muscle cells to form new AChR clusters. Our next set of experiments will be directed at introducing TM-2 into virally transformed cells to see if this enables them to cluster AChRs. This will strengthen the argument for TM-2's participation in the clustering process.

Figure 5. Immunofluorescence of adult rat neuromuscular junctions stained with D3-16. Sections of rat diaphragm were labeled with Rh-α-Bgtx (b) and D3-16 followed by fluorescein-conjugated goat anti-mouse IgG (a). Antibody labeling is concentrated at the junctional region but extends throughout the muscle fibers as well. Bar, 10 μm.

Figure 4. Effect of microinjection on cells recovering from azide treatment. (a and b) Rh-α-Bgtx labeling after sodium azide treatment. Cells were treated with 5 mM sodium azide for 5 h. Cells were then either immediately fixed (a) or allowed to recover from treatment in growth medium, in the presence of CF, for 6 h (b). Sodium azide treatment completely dispersed the AChR clusters, although punctate labeling was still present on some cells (a). After replacement of medium, cells were able to reform clusters (b). (c–h) Cells were treated as described above, but before recovery they were injected with either D3-16 (c and d), Mlg (e and f), or antitubulin (g and h). D3-16-injected cells did not form clusters after recovery from sodium azide treatment. The ability to reform clusters was not lost by Mlg- and antitubulin-injected cells. Bar, 10 μm.

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