Clustering and Immobilization of Acetylcholine Receptors by the 43-kD protein: A Possible Role for Dystrophin-related Protein

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Abstract. Recombinant acetylcholine receptors (AChRs) expressed on the surface of cultured fibroblasts become organized into discrete membrane domains when the 43-kD postsynaptic protein (43k) is co-expressed in the same cells (Froehner, S.C., C. W. Luetje, P. B. Scotland, and J. Patrick, 1990. Neuron. 5:403-410; Phillips, W. D., M. C. Kopta, P. Blount, P. D. Gardner, J. H. Steinbach, and J. P. Merlie. 1991. Science (Wash. DC). 251:568-570). Here we show that AChRs present on the fibroblast cell surface prior to transfection of 43k are recruited into 43k-rich membrane domains. Aggregated AChRs show increased resistance to extraction with Triton X-100, suggesting a 43k-dependent linkage to the cytoskeleton. Myotubes of the mouse cell line C2 spontaneously display occasional AChR/43k-rich membrane domains that ranged in diameter up to 15 μm, but expressed many more when 43k was overexpressed following transfection of 43k cDNA. However, the membrane domains induced by recombinant 43k were predominantly small (<2 μm). We were then interested in whether the cytoskeletal component, dystrophin related protein (DRP; Tinsley, J. M., D. J. Blake, A. Roche, U. Fairbrother, J. Riss, B. C. Byth, A. E. Knight, J. Kendrick-Jones, G. K. Suthers, D. R. Love, Y. H. Edwards, and K. E. Davis, 1992. Nature (Lond.). 360:591-593) contributed to the development of AChR clusters. Immunofluorescent anti-DRP staining was present at the earliest stages of AChR clustering at the neuromuscular synapse in mouse embryos and was also concentrated at the large AChR-rich domains on nontransfected C2 myotubes. Surprisingly, anti-DRP staining was concentrated mainly at the large, but not the small AChR clusters on C2 myotubes suggesting that DRP may be principally involved in permitting the growth of AChR clusters.

The specialized postsynaptic membrane of skeletal muscle cells, characterized at first by a cluster of acetylcholine receptors (AChRs), is thought to be induced to form by the presynaptic nerve terminal during embryogenesis, shortly after the arrival of the motor nerves (reviewed in Phillips and Merlie, 1992). Subsequently, the postsynaptic membrane develops into a large plaque of AChR-rich membrane at which the terminals of several motor axons make transient synapses (Steinbach, 1981; Slater, 1982). In the mouse, these transient polynervous synaptic inputs are withdrawn during the first three weeks after birth as the shape and extent of the AChR-rich membrane becomes precisely sculpted to match the overlying release sites of the one remaining motor nerve terminal. AChR clusters also occur spontaneously (in the absence of nerves) on myotubes in cell culture (Vogel et al., 1972), indicating that the mechanism of AChR clustering is intrinsic to the muscle cell. Despite evidence that the motor nerve is involved in controlling the incipient formation, spatial distribution and subsequent remodeling of the postsynaptic AChR cluster by secreting agents such as agrin and ARIA into the synaptic cleft (Nikitin et al., 1987; Wallace, 1991; Falls et al., 1993), the mechanisms by which these instructions are carried out within the muscle cell remain to be determined.

Studies on the AChR-rich membranes of electric fish and rays have identified several intracellular proteins likely to be involved in the mechanism of AChR cluster formation and/or stabilization (Froehner, 1991). One of these, a 43-kD peripheral membrane protein, was present in equimolar amounts with AChR in these membranes and was closely and consistently colocalized with aggregated AChR (Sealock et al., 1984). The cloned murine muscle homologue of this 43-kD postsynaptic protein (43k) has been shown to form specialized plasma membrane associated domains when expressed
in Xenopus oocytes (Froehner et al., 1990) or in quail fibroblasts (Phillips et al., 1991a). Co-expressed recombinant AChR became localized at these domains suggesting that 43k is the protein directly responsible for organizing AChRs into clusters. Studies of the developing mouse embryo showed that 43k mRNA is present before neuromuscular synapse clusters. Studies of the developing mouse embryo showed in blast cells (Phillips et al., 1991a). Co-expressed recombinant to exclude possible effects of chymotrypsin on 43k or the AChRs becoming more dispersed. While it was difficult bilizing AChR clusters. By isolating substratum-attached characterizes. There is indirect evidence that newly synthesized intracellular AChRs may be target preferentially to AChR-rich membrane domains (Dubinsky et al., 1989). On the other hand, labeling studies with the high-affinity ligand α-bungarotoxin (α-BGT) have shown that at least part of the growth of AChR clusters on myotubes is due to aggregation of AChRs that were originally dispersed across the muscle cell surface (Anderson and Cohen, 1977). Previous studies with myotubes in culture have suggested that the fraction of cell surface AChRs that become organized into AChR clusters also become relatively resistant to extraction with the nonionic detergent, Triton X-100 (Prives et al., 1982). In the first part of the present study we used the fibroblast transfection system to investigate the possible role of 43k in linkage of AChRs to the cytoskeleton. We demonstrate that membrane domains induced to form by recombinant 43k recruit AChRs that were already present on the cell surface and that the AChRs that become localized in these domains are resistant to Triton X-100 extraction, thus providing evidence that 43k itself constitutes the trap by binding AChRs on the cell surface and attaching them to some very large, inextractable complex that remains to be characterized.

Bloch and Morrow (1989) have argued that membrane associated spectrin-actin complexes may be involved in stabilizing AChR clusters. By isolating substratum-attached AChR clusters from cultured rat myotubes they showed that a β-spectrin-like protein was colocalized with 43k in AChR-rich domains. Treatment of the isolated membrane patches with chymotrypsin led to quantitative removal of the β-spectrin isoform but not AChRs or 43k. Nevertheless, the large AChR-rich domains characteristic of the substratum-associated AChR clusters were disrupted by this treatment, with AChRs becoming more dispersed. While it was difficult to exclude possible effects of chymotrypsin on 43k or the AChR, these results point to a possible contribution of β-spectrin or some other chymotrypsin-sensitive cytoskeletal protein in organizing AChRs in the AChR cluster. In mouse, several unique spectrin-like proteins are concentrated at the AChR clusters of mature neuromuscular synapses and therefore are in a position to immobilize membrane-associated 43k, and the AChRs with which 43k associates. These include dystrophin (Yedon et al., 1991; Byers et al., 1991) and the dystrophin-related protein (DRP; otherwise known as Utrophin; Tinsley et al., 1992), the product of a distinct gene that differs from dystrophin in its deduced amino acid sequence (Love et al., 1989; Ohlendieck et al., 1992; Tinsley et al., 1992). In the final part of this study we investigate the participation of DRP in the development of AChR clusters at embryonic synapses and in cultured muscle cells, and present evidence that DRP may be important for the growth of AChR clusters formed initially by 43k.

**Materials and Methods**

**Cell Culture and Transfection**

The QT-6 fibroblast cell line (Moscovici et al., 1977) was maintained in culture as previously described (Blount and Merlie, 1988). Cells were transfected by the calcium phosphate method of Chen and Okayama (1987) modified as previously described (Phillips et al., 1991a). Expression plasmd for the four subunits of the AChR (α, β, γ, and δ; 4-μg each; Phillips et al., 1991) plus or minus 4 μg of expression plasmid for wild-type or mutant 43k (M43ε, M43θ, or M43β; Phillips et al., 1991b) were combined with "filler" plasmid (pSKII+; Stratagene, La Jolla, CA) to give a total of 24 μg per 6-cm culture dish of subconfluent QT-6 cells. The experiments shown in Fig. 1 used the QT-6 cell clone Q-F18, that permanently expresses fetal type AChR (Phillips et al., 1991a). Q-F18 cells were plated at low density onto ethanol washed glass coverslips in a 6-cm culture dish and incubated overnight at 37°C. Cells were labeled with 5 nM c~125I-c~6-BGT for 1 h at 37°C and then washed four times with complete medium to remove free toxin; the final wash was carried out for 30 min at 37°C. A transfection solusion containing 4 μg of M43ε and 20 μg of pSKII+ was added, and the cells were incubated at 37°C under 3% CO2 for 15 h. The transfected cells were then rinsed with warm serum-free medium, fixed, and stained as described below. The C2 muscle cell line was maintained in culture as previously described (Frail et al., 1989) except that the growth medium consisted of DME supplemented with 10% calf serum plus 10% FBS. C2 cells were transfected in the same manner as the QT-6 cells except that the total plasmid DNA per 6-cm dish was reduced to 12 μg which gave optimal results with these cells. In general, lower transfection efficiencies were obtained in C2 cells (roughly 1% of cells expressed transfect M43ε) than in QT-6 cells (1~10%). Following overnight transfection with 4 μg of M43ε, cells were trypsinized and replated onto ethanol-washed, collagen-coated glass coverslips in a 24-well plate at a density of 5 x 10⁴ cells/well. After allowing >6 h for cells to adhere, growth medium was replaced with DME containing 5% horse serum, and cultures were incubated for 3~4 d until small myotubes first appeared. When C2 myotubes were co-transfected with AChR subunits, 2 μg of each subunit expression plasmid was used.

**Triton X-100 Extraction Experiments**

QT-6 fibroblasts were co-transfected overnight with expression plasmids for the four subunits of the AChR (α, β, γ, and δ) plus or minus M43ε, M43θ, or M43ε as described above. Cells were washed twice with complete medium and incubated with fresh medium for at least 2 h at 37°C. Transfected cells were trypsinized, replated onto ethanol-washed glass coverslips in a 24-well plate at a density of 1.5 x 10⁵ cells/well, and incubated for 1 d at 37°C. To label cell surface AChRs, 5 nM 125I-α-BGT was added to the medium, and the cells were incubated for 1 h at 37°C and then washed four times with PBS to remove unbound toxin. Cells were extracted with 0.5 ml of CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 10 mM Pipes, pH 6.8) containing 1% Triton X-100 (Hamaguchi et al., 1985) and washed with 0.5 ml of CSK buffer without Triton X-100. The extract and wash fractions were pooled and counted in a gamma counter. Extractions were performed on triplicate wells and were corrected for nonspecific binding by subtracting the average cpm from triplicate wells labeled in the presence of 500 nM non-iodinated α-BGT. Cells extracted with PBS in place of 1% Triton X-100-CSK retained most of their specific cpm (97 ± 1%; mean ± SD for cells transfected with α, β, γ, and δ subunits alone [n = 7 transfections]; 95 ± 2% for cells transfected with α, β, γ, and δ subunits plus M43ε [n = 10]). Experiments in which cells were grown on glass coverslips rather than directly on plastic yielded similar results.

**Immunoblot Analysis**

Rabbit crude surface membranes were prepared as previously described (Ohlendieck et al., 1991b). C2 cells were allowed to differentiate for 4 d
in the presence of 5% horse serum as described above. Whole cell pellets of QT-6 and C2 cell cultures were solubilized in 3 vol of 5% Laemmli sample buffer (Laemmli, 1970). Crude surface membranes or solubilized QT-6 or C2 cell pellets were fractionated on 3-12% SDS-polyacrylamide gels by the method of Laemmli (1970) and transferred to nitrocellulose according to Towbin et al. (1979). Immunoblot staining was performed as previously described (Ohlendieck et al., 1991b).

Immunofluorescent Staining

Cells were rinsed in warm serum-free medium and fixed for 20 min at room temperature in PBS containing 1% paraformaldehyde, 100 mM L-lysine, 10 mM sodium metaperiodate, and 0.1% saponin. After rinsing in PBS, cells were permeabilized for 10 min in PBS containing 1% Triton X-100 (LaRochelle et al., 1989), washed three times in PBS and stored overnight at 4°C in PBS containing 5-10% normal goat serum. Coverslips were stained by indirect immunofluorescence as previously described (Phillips et al., 1991a). Monoclonal rat anti-AChR antibody, mAb 35 (Tzartos and Lindstrom, 1980), was diluted 1:200 in PBS containing 5% normal goat serum. Affinity-purified rabbit antibodies anti-mouse 43k (Phillips et al., 1991b) and DRP (Ohlendieck et al., 1991a) were each used at a dilution of 1:50. After a 1-h incubation at room temperature, coverslips were washed in three changes of PBS over at least 30 min. Coverslips were then incubated for 1 h in a mixture of affinity-purified FITC-goat anti-rat IgG and TRITC-goat anti-rabbit IgG, each diluted 1:100, washed as above and mounted with an anti-fading mountant (Johnson and Nogueira Araujo, 1981). Photomicrographs were taken using either a 40 x objective (for myotubes) or a 100 x oil objective (for myoblasts). Measurements of the size of AChR clusters were made by projecting the negative images onto the enlarger baseboard (total magnification 2,000 ×) and tracing the outlines of all AChR clusters on a given cell that were within the focal plane of the photographic image. Lengths given in Fig. 5 were the largest dimension for each AChR cluster. For labeling cell surface AChRs, α-BGT (10 nM final) was added to the culture medium for 1 h. Cells were washed four times with complete medium with the final wash being continued for 30 min. Fixed and permeabilized cultures were then preblocked as above and were incubated for 1 h with affinity-purified anti-α-BGT (Merlie and Sebbane, 1981) together with 43k-specific mouse mAb 1234A (Pang and Froehner, 1985). The hybridoma conditioned medium was diluted 1:10. Secondary antibodies, FITC-goat anti-rabbit IgG and TRITC-goat anti-mouse IgG, were each diluted 1:100 as above. To control for fluorescent cross-bleed and secondary antibody specificity, parallel coverslips were stained in the absence of either one or the other of the primary antibodies.

For immunostaining of embryonic mouse muscles, embryos (staged as described in Noakes et al., 1993) were obtained from pregnant ICR female mice (Harlan Sprague Dawley). Two to three embryos for each stage described were prepared in the following manner. Embryos were placed in OCT mounting medium (Miles Elkhart, IN), were frozen and sectioned at 6-10 μm on a cryostat. Sections were stained overnight at 4°C with anti-DRP (Ohlendieck et al., 1991a) together with TRITC-conjugated α-BGT and affinity-purified secondary antibodies as previously described (Noakes et al., 1993). To test for the specificity of anti-DRP staining, the immunogen, a synthetic peptide representing the C-terminal 12 amino acids of DRP, was included with anti-DRP in the primary incubation step to block staining (Ohlendieck et al., 1991a). Specificity of TRITC-α-BGT staining was confirmed by preincubation with a 100-fold excess of unlabeled α-BGT.

Results

43k Clusters AChRs Already Present on the Cell Surface

Calcium phosphate transfection was used to introduce the expression vector for 43k into AChR-expressing Q-F18 fibroblasts. Using this transfection protocol between 1 and 10% of Q-F18 cells expressed 43k and of these about 79% displayed colocalized AChRs as judged by immunofluorescence (Phillips et al., 1991b). To investigate the cellular pathway by which AChRs are incorporated into these plasma membrane domains, preexisting cell surface AChRs on living QF-18 cells were labeled with the essentially irreversible ligand, α-BGT. After removing unbound α-BGT by extensive washing, cells were transfected with M43l. As expected, cells fixed immediately after addition of the transfection solution showed no anti-43k immunostaining (Fig. 1A). Co-staining with anti-α-BGT at this stage showed that AChRs were distributed diffusely across the cell surface.
(Fig. 1 B), as on nontransfected cells (Phillips et al., 1991a). Coverslips fixed 12–16 h after addition of the transfection solution showed cells with anti-43k immunostaining localized both in plasma membrane domains and intracellular aggregates (Fig. 1 C). The 43k-rich plasma membrane domains also stained strongly with anti-α-BGT antibody (Fig. 1 D). Control cultures that were not pre-incubated with α-BGT showed no staining in the anti-α-BGT fluorescence channel (Fig. 1, compare F to E). These results suggest that the 43k-induced AChR clusters in Q-F18 fibroblasts arise, wholly or in part, by recruitment of preexisting cell surface AChRs into 43k-rich membrane domains.

43k Reduces the Triton X-100 Extractability of Cell Surface AChRs

As a first step in exploring the nature of the AChR/43k-rich membrane domains, we tested the ability of Triton X-100 to extract AChRs from the fibroblast cell surface. To determine the effect of 43k on AChR extractability, it was found necessary to co-transfect M43wt together with expression vectors for all four subunits of the AChR (α, β, γ, and δ) into the parental fibroblast cell line, QT-6, to ensure that both AChR and 43k were co-expressed in the same cells. Double label immunofluorescence staining confirmed that a high proportion of co-transfected cells expressed both 43k and cell surface AChRs (Maimone and Merlie, 1993). Cell surface AChRs on living Q-F18 cells were labeled with [125I-α-BGT and treated for 6 min on ice with 1% Triton X-100 in CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM CaCl2, 10 mM Pipes, pH 6.8). When QT-6 cells were transfected with AChR subunits in the absence of M43wt, Triton X-100 treatment extracted all but 13.4 ± 2.5% of cell surface AChRs (mean ± SD for seven independent experiments; see Methods). Co-transfection of M43wt and AChRs into QT-6 cells increased the fraction of unextractable cell surface AChRs threefold (Fig. 2). We tested mutant forms of 43k that do not aggregate AChRs to determine their effects on the Triton X-100 extractability of AChRs. Co-transfection of AChRs with a mutant form of 43k that lacks the NH2-terminal signal for myristoylation and associates poorly with the plasma membrane (M43A2; Phillips et al., 1991b) had no effect on the Triton X-100 extractability of AChRs compared with AChRs alone (Fig. 2). Furthermore, a deletion mutant of 43k that forms 43k-rich membrane domains but does not efficiently cluster AChRs into these domains (M43ID1), caused only a slight increase in the percentage of non-extracted AChRs (Fig. 2). Thus, the resistance of cell surface AChRs to extraction by Triton X-100 correlated well with the ability of 43k to cluster AChRs into domains.

Triton X-100-extracted cells were examined by immunofluorescence to determine whether AChRs that were resistant to extraction had 43k co-localized with them. As expected, QT-6 cells co-transfected with the four subunits of the AChR and M43wt and permeabilized only after fixation showed close co-localization of anti-AChR (mAb 35) staining (Fig. 3 B) with anti-43k-stained membrane domains (Fig. 3 A). When cells were extracted with Triton X-100 prior to fixation, 43k-rich domains were still evident (Fig. 3 C) and stained intensely for co-localized AChRs (Fig. 3 D), consistent with the idea that the clustering of AChRs involves attachment to Triton X-100-insoluble structures. A non-myristoylated mutant of 43k (the M43A2 product) failed to associate with the plasma membrane and instead became concentrated in the nucleoplasm (n in Fig. 3 E; Phillips et al., 1991b). As previously reported, this mutant fails to cluster AChRs; anti-AChR staining revealed only diffuse cell surface staining and perinuclear intracellular (ER-like) staining in fixed-then-permeabilized cells (Fig. 3 F; Phillips et al., 1991b). Treatment with Triton X-100 before fixation extracted most of the diffuse nucleoplasmic anti-43k immunostaining (Fig. 3 G) as well as the dispersed anti-AChR immunostaining from the membranes (Fig. 3, compare H with F). The internal deletion mutant (M43ID1 product) formed anti-43k stained plasma membrane domains (Fig. 3 I) but only weak anti-AChR immunostaining was observed at these domains. The anti-43k–stained membrane domains formed by the M43ID1 mutant protein were resistant to Triton X-100 extraction (Fig. 3 K). Thus, the M43ID1 protein that was localized in discrete membrane domains like wild-type 43k protein was also resistant to Triton X-100 extraction, while the M43A2 protein, that lacks NH2-terminal myristoylation and is targeted instead to the nucleoplasm (Phillips et al., 1991b), could be substantially extracted. These results sug
gest that an important step in the formation of membrane domains may be the attachment of 43k to the cytoskeleton or to some other, unidentified, Triton X-100-insoluble structure localized at the cytoplasmic face of the plasma membrane.

**Overexpression of 43k in C2 Muscle Cells Leads to Formation of Small AChR Clusters**

In living muscles, membrane domains rich in AChRs and 43k are confined to a single site on the sarcolemma immediately underlying the presynaptic nerve terminal while in the cell culture experiments outlined above, these domains form at multiple sites on the fibroblast cell surface. Our working hypothesis to explain this difference was that overexpression of 43k can override whatever mechanism(s) normally restrict the number and spatial distribution of 43k-rich membrane domains. To test this we examined the effect of overexpressing 43k in the muscle cell line, C2. When maintained in high serum medium, C2 myoblasts express little or no AChR while after differentiation in low serum medium, expression of the AChR subunits is up-regulated at the transcriptional level (Frail et al., 1989). In contrast, 43k is expressed in both proliferating C2 myoblasts and differentiated C2 myotubes at similar levels (Frail et al., 1989). The four subunits of the AChR (α, β, γ, and δ) were first transfected into C2 myoblasts (which do not normally express AChR) to test whether the 43k expressed endogenously in these cells is capable, on its own, of clustering AChR. The transfection efficiency achievable with C2 cells was low (roughly 1% of cells expressing detectable anti-AChR staining). While diffuse anti-AChR staining was seen, no AChR clusters were observed (Fig. 4 B). Anti-43k immunostaining revealed only a dull, diffuse distribution of endogenous 43k in these cells (Fig. 4 A) suggesting either that 43k is insufficient in quantity or that these cells are lacking in some other requirement for AChR clustering. When M43α was co-transfected into myoblasts together with the AChR subunits a small proportion (~1%) displayed bright anti-43k staining, much of it localized in membrane associated domains (Fig. 4 C).

Most of the myoblasts that expressed transfected 43k (21/23; 100% transfection efficiency, Materials and Methods). Coverslips were then stained by double label immunofluorescence to reveal the distribution of 43k (left) or AChR (right). In cells co-transfected with AChRs and M43α, membrane domains rich in 43k and AChR were still present after Triton X-100 extraction of unfixed cells (C and D). In cells co-transfected with M43α, few anti-43k-stained membrane domains were formed (most 43k staining was localized in the nucleus, n in E). Anti-AChR staining revealed a combination of diffuse cell surface and intracellular membrane staining (F; Phillips et al., 1991b). Little staining for either 43k or AChR remained when M43α-transfected cells were Triton X-100 extracted before fixation (G and H). M43α formed anti-43k-stained membrane patches (J) as did M43α. Triton X-100 extraction of live cells failed to eliminate these anti-43k-stained patches (K). Only weak anti-AChR staining could be observed at the membrane domains formed by M43α, whether or not cells were fixed before extraction (J and L). In previous studies (Phillips et al., 1991b) with the AChR expressing cell line Q-F18, AChR immunostaining was not observed at membrane domains formed by M43D1 protein (Phillips et al., 1991). The higher level of AChR expression afforded in the present transient expression experiments may thus be revealing a weak residual affinity of the mutant for the AChR as in J. Bar, 10 μm.

**Figure 3.** Immunofluorescent staining of AChR clusters following Triton X-100 extraction of cells. QT-6 cells were co-transfected with the four subunits of the AChR (α, β, γ, and δ) together with M43α (A to D), M43α (E to H), or M43D1 (I to L). After 2 d, cultures were fixed either before (A and B, E and F, I and J), or after (C and D, G and H, K and L) Triton X-100 extraction (see Materials and Methods). Coverslips were then stained by double label immunofluorescence to reveal the distribution of 43k (left) or AChR (right). In cells co-transfected with AChRs and M43α, membrane domains rich in 43k and AChR were still present after Triton X-100 extraction of unfixed cells (C and D). In cells co-transfected with M43α, few anti-43k-stained membrane domains were formed (most 43k staining was localized in the nucleus, n in E). Anti-AChR staining revealed a combination of diffuse cell surface and intracellular membrane staining (F; Phillips et al., 1991b). Little staining for either 43k or AChR remained when M43α-transfected cells were Triton X-100 extracted before fixation (G and H). M43α formed anti-43k-stained membrane patches (J) as did M43α. Triton X-100 extraction of live cells failed to eliminate these anti-43k-stained patches (K). Only weak anti-AChR staining could be observed at the membrane domains formed by M43α, whether or not cells were fixed before extraction (J and L). In previous studies (Phillips et al., 1991b) with the AChR expressing cell line Q-F18, AChR immunostaining was not observed at membrane domains formed by M43D1 protein (Phillips et al., 1991). The higher level of AChR expression afforded in the present transient expression experiments may thus be revealing a weak residual affinity of the mutant for the AChR as in J. Bar, 10 μm.
Figure 4. Overexpression of 43k in mouse C2 muscle cells. Myoblasts, in which the AChR subunit genes are inactive, were co-transfected with expression vectors for the four subunits of the AChR (\(\alpha, \beta, \gamma, \) and \(\delta\)) + M43\(\text{wt}\). Myoblasts co-transfected with AChR subunits alone displayed a dispersed distribution of anti-AChR staining across the cell surface (B). Endogenous 43k was not seen to form membrane domains in these myoblasts (A). After co-transfection of AChRs and M43\(\text{wt}\) some cells displayed anti-43k-stained plasma membrane domains as well as some intracellular aggregates (C). Anti-AChR staining was concentrated at the membrane domains (D). (E-H) Lower magnification micrographs of C2 myotubes. AChR clusters formed spontaneously on nontransfected C2 myotubes (F), and endogenous 43k was co-localized at these AChR clusters (E). Myotubes overexpressing transfected M43\(\text{wt}\) displayed small anti-43k-stained membrane patches (G) and AChR became co-localized at these (H). Bar: (A-D) 10 \(\mu\)m, and (E-H) 25 \(\mu\)m.

91\%) displayed large numbers of membrane domains that stained intensely with both anti-43k and anti-AChR (Fig. 4, C and D), indicating that AChR clustering can be induced to occur in myoblasts when 43k is expressed at higher than normal levels.

Unlike C2 myoblasts, differentiated C2 myotubes express high levels of AChRs, some of which become organized into spontaneously forming 43k-rich membrane domains (Fig. 4, F and E, arrows; LaRochelle et al., 1989). The effect of overexpression of 43k on AChR cluster formation in myotubes was examined by transfecting myoblasts with M43\(\text{wt}\) then switching cells to low serum medium to induce differentiation. Myotubes overexpressing 43k could be readily identified in these cultures by intense and abundant anti-43k staining (Fig. 4 G), while non-transfected myotubes displayed only occasional anti-43k stained membrane domains (Fig. 4 E, arrows). The majority of the myotubes that overexpressed 43k also displayed AChR clusters (90 \(\pm\) 2\%, \(n = 4\) experiments) and these were co-localized with the membrane patches of 43k (Fig. 4 H). In contrast, only 38 \(\pm\) 4\% \(n = three experiments\) of mock-transfected myotubes displayed AChR clusters. Furthermore, the average number of AChR clusters on 43k-overexpressing myotubes was much higher (74 \(\pm\) 56 AChR clusters per myotube, \(n = 8\)) than on nontransfected or mock-transfected myotubes. For example, among those mock-transfected myotubes that displayed any AChR clusters, the average number of AChR clusters per myotube was only 7 \(\pm\) 5 (\(n = 39\)). Anti-43k staining was consistently found to be concentrated at these spontaneously forming AChR clusters (97\%; 38/39 clusters examined.

While there were many more AChR clusters on M43\(\text{wt}\) transfected myotubes, the AChR clusters were smaller than
spontaneously forming AChR clusters on mock-transfected or non-transfected cells (Fig. 4, H compared to F). AChR clusters on mock-transfected myotubes showed a skewed distribution of lengths ranging up to 15 μm (Fig. 5 A) but AChR clusters on myotubes overexpressing 43k were predominantly of the smaller size classes; 91% being <2-μm long (Fig. 5 B). Overexpression of 43k and AChRs in C2 myoblasts similarly gave rise to mainly the smaller size classes of AChR clusters (Fig. 5 C). These results suggest that while overexpression of 43k could induce the formation of additional small AChR clusters in C2 muscle cells, the enlargement of these AChR clusters was impared compared with spontaneously occurring AChR clusters.

**Figure 5.** C2 cells overexpressing M43α formed predominantly small AChR clusters. The frequency histogram in A shows the size range of AChR clusters (largest linear dimension) that occurred on mock-transfected myotubes. Myotubes overexpressing transfected M43α (B) and myoblasts cotransfected with the four subunits of the AChR (α, β, γ, and δ) together with M43α (C) showed mainly small type AChR clusters. Data pooled from two independent transfection experiments (A and B) or from a single experiment (C). Arrowheads above the frequency histogram in (A) indicate the mean sizes of spontaneously forming AChR clusters that either were not, or were accompanied by locally enhanced anti-DRP staining, respectively (see text).

**Distribution of Dystrophin-related Protein at AChR Clusters**

The formation of large membrane domains may involve the interaction of 43k with one or more of the cytoskeletal proteins that are concentrated beneath the postsynaptic membrane (see Introduction). One likely candidate is DRP, a protein with a predicted rodlike central domain and an NH2-terminal actin-binding region (Tinsley et al., 1992), that is reported to be closely co-localized with AChRs and 43k at adult neuromuscular synapses (Ohlendieck et al., 1991). To determine whether DRP might be involved in growth of AChR clusters, we examined its localization in developing mouse intercostal muscles at the stages when synaptic AChR clusters are beginning to form. At 15-d gestation, when large AChR clusters are already present in intercostal muscles, immunostaining with an antibody specific for the unique COOH-terminal sequence of DRP (Ohlendieck et al., 1991) was concentrated at these primitive synapses (Fig. 6, A and B). The first small AChR clusters with associated 43k immunostaining can be detected in the intercostal muscles at 13-d gestation (Noakes et al., 1993). Even at this earliest stage, anti-DRP staining was consistently co-localized with the newly formed AChR clusters (Fig. 6, E and F). In addition, anti-DRP immunostaining was often seen in punctate accumulations in parts of the membrane that showed no co-localized staining for AChR (Fig. 6, F, arrows). Immunostaining was specific for DRP, since addition of a peptide representing the COOH terminus of DRP to the antibody incubation abolished staining (Fig. 6, D and H). Since anti-DRP staining, like anti-43k staining, is present at the earliest stages of AChR cluster formation in vivo, DRP could conceivably be involved in the genesis and/or growth of AChR clusters.

The roles of DRP and dystrophin in the formation of AChR clusters in cultured C2 muscle cells was then examined. Immunoblot analysis with anti-DRP antibody showed that DRP is indeed expressed in C2 cells (Fig. 7). In contrast, two different anti-dystrophin antibodies failed to detect dystrophin in C2 cells (Fig. 7). The ability of large AChR clusters to form in C2 cells in the presence of DRP but absence of dystrophin confirms that dystrophin is not necessary for AChR clustering. It also raises the question of whether DRP is an essential component in the enlargement of AChR clusters or whether plays it instead only an accessory role in postsynaptic differentiation.

If DRP is part of a fundamental mechanism of AChR cluster formation it should be localized at AChR clusters on muscle cells in culture as it is in vivo. By immunofluorescence, DRP appeared to be distributed at low density throughout the plasma membrane of myotubes (Fig. 8, B and D). However, the prominent AChR clusters that formed spontaneously on nontransfected and mock-transfected myotubes stained more intensely than the surrounding myotube membrane, suggesting that DRP becomes concentrated beneath these large AChR-rich domains (Fig. 8, B compared with A) as it does in vivo. The presence of DRP (albeit at lower levels) throughout the C2 myotube membranes appears consistent with the observation of weak extrasynaptic staining for DRP in the muscles of Duchenne dystrophy patients and mdx mice and may be explained by the suggestion that in the absence of dystrophin, DRP can replace dystrophin in its interactions with the dystrophin-associated proteins throughout the membrane (Mann et al., 1991; Matsumura et al., 1992).
surfaces membranes (CSM), and SDS-solubilized whole-cell pellets of QT-6 or C2 cell cultures. For anti-DRP staining, 150 µg of crude surface membranes or 150 µg of solubilized QT-6 or C2 cell pellets were loaded per lane. For staining with DYS Abl or Ab2, 500 µg of solubilized QT-6 or C2 cell pellets were loaded per lane. After transfer, blots were stained with antibody to the COOH terminus of DRP (DRP; Ohlendieck et al., 1991a), or with antibodies directed against the central portion of rabbit dystrophin (DYS Abl; Matsumura et al., 1993) or the COOH-terminal 10 amino acids of human dystrophin (DYS Ab2; Ervasti et al., 1990). Anti-DRP confirms the presence of DRP in C2 cells. The presence of DRP in QT-6 cells is uncertain since this antibody fails to cross-react with quail DRP. Dystrophin could not be detected in C2 cells with either DYS Abl or 2. DYS Abl is reactive with both murine and avian (chicken) dystrophin, while DYS Ab2 does not cross-react with chicken dystrophin. Molecular weight markers (in kD) are indicated at left.

Figure 6. Double immunofluorescence staining of AChR and DRP in developing mouse muscle. Sections of intercostal muscles from embryos at 15- (A to D) or 13- (E to H) d gestation were doubly stained for AChRs with TRITC-α-BGT (A, C, E, and G) and for DRP with rabbit anti-DRP (B and F). AChRs and DRP co-cluster at 15- (A and B) and 13- (E and F) d gestation. In addition, punctate anti-DRP staining, not co-localized with AChRs, was observed (arrows, F). The specificity of immunostaining for DRP was confirmed by addition to the anti-DRP of a peptide representing the COOH-terminal sequence of DRP (D and H). Bar, 10 µm.

Figure 7. Immunoblot analysis of QT-6 (quail) and C2 (mouse) cells with antibodies specific for DRP and dystrophin. Lanes represent rabbit skeletal muscle crude surface membranes (CSM), and SDS-solubilized whole-cell pellets of QT-6 or C2 cell cultures. For anti-DRP staining, 150 µg of crude surface membranes or 150 µg of solubilized QT-6 or C2 cell pellets were loaded per lane. For staining with DYS Abl or Ab2, 500 µg of solubilized QT-6 or C2 cell pellets were loaded per lane. After transfer, blots were stained with antibody to the COOH terminus of DRP (DRP; Ohlendieck et al., 1991a), or with antibodies directed against the central portion of rabbit dystrophin (DYS Abl; Matsumura et al., 1993) or the COOH-terminal 10 amino acids of human dystrophin (DYS Ab2; Ervasti et al., 1990). Anti-DRP confirms the presence of DRP in C2 cells. The presence of DRP in QT-6 cells is uncertain since this antibody fails to cross-react with quail DRP. Dystrophin could not be detected in C2 cells with either DYS Abl or 2. DYS Abl is reactive with both murine and avian (chicken) dystrophin, while DYS Ab2 does not cross-react with chicken dystrophin. Molecular weight markers (in kD) are indicated at left.

Surprisingly, anti-DRP immunostaining was not found to be concentrated at the small AChR clusters induced to form on myotubes by 43k transfection (Fig. 8, C and D). Upon closer examination of the nontransfected myotubes, concentrations of anti-DRP staining were also found to be absent from many of the smaller spontaneously forming AChR clusters. Alignment of photomicrographs of five nontransfected myotubes allowed comparison of the distribution of anti-AChR and anti-DRP staining. Dull anti-DRP staining was dispersed throughout the membranes of all the myotubes but intense staining was locally concentrated beneath 74% (37/50) of the AChR clusters examined. AChR clusters with co-localized intense anti-DRP staining were, on average, larger (mean length 6.2 ± 5.4 µm) than those AChR clusters that did not show locally enhanced anti-DRP staining (1.4 ± 0.7 µm). (In Fig. 5 A, arrows indicate means.) Thus, DRP appears to become localized at large AChR clusters in culture but not necessarily at smaller (less mature) clusters, whether spontaneously forming or induced by 43k overexpression.

It remains unclear whether DRP is concentrated at the large AChR clusters formed after transfection of 43k into (quail) QT-6 fibroblasts. Immunoblotting analysis has shown that DRP is expressed in a variety of mammalian tissues and cell lines other than skeletal muscle (Khurana et al., 1990; Man et al., 1991). Unfortunately, the anti-DRP antibody used in this study, and which is the most definitively DRP specific, does not stain endplates in quail muscle. Thus we were unable to examine the distribution of DRP in QT-6 cells.

Discussion
When recombinant 43k was expressed together with recom-
Figure 8. Double immunofluorescence staining of DRP and AChRs in nontransfected (A and B) or M43" transfected (C and D) C2 myotubes. C2 myotube cultures were doubly stained for AChRs with mouse anti-AChR (A and C) and for DRP with rabbit anti-DRP (B and D). Myotubes showed membrane staining for DRP in an irregular pattern throughout their lengths (B and D). Anti-DRP staining was more intense at large, spontaneously forming AChR cluster domains (compare B with A). In contrast the small AChR clusters that were induced to form by overexpression of transfected M43" did not display levels of anti-DRP immunostaining brighter than the surrounding membrane (compare D with C). Bar, 25 μm.

In nonmuscle cells the AChRs, that would otherwise be dispersed across the cell surface, became organized with 43k into discrete membrane domains (Froehner et al., 1990; Phillips et al., 1991a). Here we have investigated the mechanism by which AChRs become aggregated by 43k and show that 43k forms AChR clusters at least in part, by aggregating AChRs that were already present on the cell surface before transfection of 43k into the cells. Aggregated AChRs became resistant to extraction with Triton X-100, suggesting that 43k-induced AChR clustering involved linkage of AChRs to the cytoskeleton or some other Triton X-100-resistant structure in the cell. To investigate the role of the cytoskeletal protein DRP in AChR cluster formation, 43k was transfected into the mouse muscle cell line C2 and the resulting AChR clusters were probed with antibody specific for DRP. In C2 myotubes, overexpressed transfected 43k induced the formation of additional small (but not large) AChR clusters. Anti-DRP staining was concentrated at the large AChR clusters that formed spontaneously on nontransfected myotubes but not at the small AChR clusters induced by 43k overexpression. Thus the results suggest that if DRP does play a role in AChR cluster formation, it may be in the enlargement of AChR clusters rather than in the initial stages of AChR cluster formation.

Several mechanisms have been proposed to explain how AChRs become clustered in muscle cells. Studies in cultured myotubes have provided indirect evidence that AChR may accumulate at growing AChR clusters due to targeting of newly synthesized intracellular AChRs (Bursztajn et al., 1985; Role et al., 1985; Dubinsky et al., 1989). Another
possible mechanism involves the “trapping” of molecules of AChR already present and diffusing laterally in the plasma membrane (Edwards and Frisch, 1976; Chao et al., 1981; Ngiem et al., 1991). Indeed, the reported diffusion coefficients for dispersed AChRs on cultured muscle cell membranes are sufficiently high to explain the rate of AChR clustering on the basis of a trapping mechanism (Stollberg and Fraser, 1990; Dubinsky et al., 1989). Since 43k-rich membrane-associated domains can form even in the absence of co-expressed AChRs (Froehner et al., 1990; Phillips et al., 1991a) it is conceivable that 43k-rich domains form first and then function to bind AChRs diffusing laterally in the overlying plasma membrane. In this context, it is interesting to note that the a deletion mutant lacking residues 16-254 (M43\textsuperscript{16-254}) will form membrane domains but cluster AChRs only poorly, suggesting that the central (deleted) portion may contain a binding domain for the AChR (Phillips et al., 1991b). Recent co-transfection experiments have shown that 43k can cluster even single co-transfected subunits (either \(\alpha\), \(\beta\), \(\gamma\), or \(\delta\) alone) in QT-6 cells, suggesting that each of the subunits contains at least one target for 43k binding (Maimone and Merlie, 1993). Thus the assembled pentameric AChR may have five or more sites for interaction with 43k, so maximizing the potential for trapping of AChRs during random encounters with 43k-rich domains in the membrane. Stya and Axelrod (1983) have presented evidence that immobilization of AChRs in a cluster and linkage of the AChRs to the cytoskeleton may involve separate molecular interactions. They observed that when cultured rat myotubes were exposed to sodium azide, a treatment that leads to the disaggregation of AChR clusters, the expected increase in Triton X-100 extractability actually preceded the increase in lateral mobility of the clustered AChR (as measured by fluorescence photobleaching recovery) suggesting that in addition to cytoskeletal linkage, other interactions may be involved in immobilization of AChRs. The observations, reported here, suggest that within the fibroblast expression system, at least, 43k-rich membrane domains serve both to immobilize AChRs and link them to the cytoskeleton. The possibility that membrane targeting may also contribute to the growth of 43k-induced AChR clusters requires further investigation.

The structure to which 43k may link AChRs remains to be determined. Studies with AChR clusters isolated from rat myotubes provide indirect support for the involvement of the actin-spectrin based membrane skeleton in stabilizing the AChR cluster (Bloch and Morrow, 1989; Bloch, 1986). Another possibility is that resistance to Triton X-100 extraction instead reflects the polymerization of 43k (and thereby AChRs) into very large and poorly extractable protein complexes as has been reported to occur with heterotrimetric G proteins (Coulter and Rodbell, 1992).

Transfection of 43k into C2 muscle cells provides evidence that 43k-induced AChR cluster formation may occur in two stages, with formation of micro clusters preceding the formation of larger membrane domains. Yoshihara and Hall (1993) have recently produced lines of C2C12 cells stably expressing recombinant 43k under the control of a human cyto-megalovirus promoter and enhancer. They report that individual myotubes within cultures of these lines express varying amounts of recombinant 43k. Formation of AChR clusters was found to be inhibited in myotubes expressing 43k at the highest levels, suggesting that an approximate stoichiometric relationship is required for the formation of AChR clusters. At intermediate levels of expression (comparable perhaps to those reported in the present study), additional small AChR clusters were reported to be formed. Thus our results confirm those of Yoshihara and Hall and highlight the distinction between the ability of 43k to interact with AChR to form small AChR clusters and the (apparently) more fastidious requirements for the growth of large membrane domains. The mature neuromuscular synapse is characterized by large AChR-rich membrane domains that extend 10 \(\mu\)m or more across the muscle fiber surface. However, embryonic muscle displays a skewed distribution of AChR cluster sizes, with the larger size classes, characteristic of mature synapses, becoming prominent only during the later stages of embryonic development (Smith and Slater, 1983; Phillips et al., 1985). Similarly in this study, nontransfected and mock-transfected C2 myotubes displayed AChR clusters ranging in length up to 15 \(\mu\)m. In addition, freeze fracture studies of AChR clusters on rat myotubes suggest that aggregation of AChR into very small AChR clusters (micro-aggregates) may precede their integration into large, well-organized AChR-rich membrane domains (Pumpkin and Bloch, 1987). It is not known why overexpression of 43k in C2 cells should lead to the formation of small, but not large AChR clusters. One possibility is that the cells contain only a limiting amount of some unidentified molecular component or enzymatic activity that is needed for the enlargement of newly formed AChR clusters. Alternatively, since 43k can induce the formation of large AChR clusters in QT-6 fibroblasts, its failure to do so in C2 cells may reflect some form of negative regulation of the growth of AChR clusters that is not active in QT-6 fibroblasts.

Whether or not 43k-induced AChR clustering involves interaction of 43k with the cytoskeleton, a number of cytoskeletal proteins do become concentrated beneath the postsynaptic membrane of muscle cells (reviewed by Froehner, 1991). While some of these proteins may be essential participants in the mechanism of 43k-mediated AChR clustering, others may serve downstream roles at the mature AChR cluster. Several spectrin related, actin-binding proteins have been implicated in AChR cluster formation. For example, a \(\beta\)-spectrin isofrom that is concentrated at AChR clusters on rat myotubes has been implicated in stabilizing AChR clusters (Bloch and Morrow, 1989). It is unclear whether this protein is also present at mouse AChR clusters. Two other spectrin-related proteins are known to be concentrated at AChR clusters in mouse muscle; dystrophin (Yeaden et al., 1991) and DR.P (Ohlendieck et al., 1991a). It seems unlikely that dystrophin is a crucial element in clustering AChRs since it does not co-localize precisely with clustered AChR (Sealock et al., 1991) and since AChR clusters can form in the muscles of \(m d x\) mice in the absence of functional dystrophin (Yeaden et al., 1991) and on cultured C2 myotubes, that express no detectable dystrophin by immunoblot (this study). On the other hand, DR.P, like 43k, is closely co-localized with clustered AChRs at the adult synapse (Ohlendieck et al., 1991a; Bewick et al., 1992) and at the very earliest stage of synapse formation (see Fig. 6). In nontransfected C2 myotubes the large, spontaneously occurring AChR-rich membrane domains also showed enhanced immunostaining for both DR.P and 43k compared with the

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surrounding membrane areas. While we cannot exclude the possibility that AChR clusters may correspond to regions of enhanced membrane infolding that might give the impression of enhanced staining for both anti-43k and anti-DRP at these sites, a simple explanation of this kind seems unlikely. The small AChR clusters on C2 myotubes showed enhanced immunostaining for 43k but not DRP, suggesting that within these cultured myotubes, DRP becomes localized at the clusters subsequent to accumulation of 43k in these domains.

While the results with C2 myotubes support the idea that 43k is directly involved in initial formation of small AChR clusters subsequent to accumulation of 43k in these domains, it is somewhat different. Even the smallest, newly formed, nerve-induced AChR clusters in vivo stained brightly both for 43k (Noakes et al., 1993) and for DRP (this study). While transfected 43k can induce the formation of small AChR clusters in culture, it is interesting to speculate that the accumulation of DRP at newly forming clusters (as occurs in vivo) may be important in permitting the progressive growth of small AChR clusters into the large, stable AChR-rich membrane domains that characterize the mature neuromuscular junction.

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