

Recruitment of a Nicotinic Acetylcholine Receptor Mutant Lacking Cytoplasmic Tyrosine Residues in Its β Subunit into Agrin-Induced Aggregates

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During synaptogenesis at the vertebrate skeletal neuromuscular junction, acetylcholine receptors (AChRs) form high-density aggregates opposite the presynaptic terminal in response to nerve-derived agrin. Agrin has been shown to stimulate tyrosine phosphorylation of a muscle-specific receptor tyrosine kinase MuSK and of the AChR β subunit, and tyrosine kinase inhibitors and a tyrosine kinase-deficient mutant of MuSK prevent AChR aggregation. To evaluate the role of tyrosine phosphorylation of the AChR β subunit in receptor aggregation, we replaced all three putative cytoplasmic tyrosine residues of the AChR β subunit with phenylalanine residues and expressed the mutant receptors in cultured myotubes. Upon agrin treatment, transfected myotubes formed AChR aggregates that contained receptors with mutant β subunits. Thus, AChRs can be recruited into agrin-induced specializations by protein–protein interactions that do not depend on tyrosine phosphorylation of the AChR β subunit.

INTRODUCTION

Fast signal transmission between neurons and their targets depends on subcellular specializations at the points of synaptic contact. For example, neurotransmitter vesicles and the proteins mediating transmitter release accumulate in the presynaptic nerve terminal, while a prominent feature of the postsynaptic apparatus is the concentration of neurotransmitter receptors in the membrane of the postsynaptic cell.

At the developing vertebrate skeletal neuromuscular junction, the aggregation of nicotinic acetylcholine receptors (AChRs)² is induced by the nerve-derived signal

molecule agrin (McMahan, 1990). Agrin, a proteoglycan that is concentrated in the synaptic portion of the basal lamina that surrounds the muscle fiber, is synthesized by both neurons and muscle fibers. Agrin exists in several isoforms generated by alternative splicing (Ruegg *et al.*, 1992). Isoforms efficient in inducing AChR aggregation are expressed by motor neurons (Reist *et al.*, 1992); muscle cells express isoforms that are less potent in inducing AChR aggregation (Ruegg *et al.*, 1992). The importance of agrin in inducing postsynaptic specializations is demonstrated in agrin-deficient mice, which show a strong impairment of synaptic differentiation at the neuromuscular junction (Gautam *et al.*, 1996).

The phenotype of agrin-deficient mice is similar to that of mice deficient in a muscle-specific kinase, MuSK, which was identified by its homology to the family of receptor tyrosine kinases (Jennings *et al.*, 1993; Valenzuela *et al.*, 1995; DeChiara *et al.*, 1996). Several lines of evidence suggest that MuSK is a signal-transducing component of the agrin receptor. MuSK becomes tyrosine phosphorylated in response to agrin (Glass *et al.*, 1996), activation of MuSK by specific single-chain variable-region antibodies leads to the formation of AChR aggregates *in vitro* (Xie *et al.*, 1997), agrin can be chemically cross-linked to MuSK on the surface of myotubes (Glass *et al.*, 1996), and overexpression of a dominant-negative mutant of MuSK in myotubes inhibits AChR aggregation (Glass *et al.*, 1997). The physiological substrates of MuSK, however, are not yet known.

In addition to MuSK, the only protein known to be phosphorylated in response to agrin is the AChR (Wallace *et al.*, 1991; Meier *et al.*, 1995; Ferns *et al.*, 1996). The AChR is a pentameric complex comprising two α subunits and one β subunit, one γ or ϵ subunit, and one δ subunit. Agrin induces an increase in phosphorylation of the AChR β , γ , and δ subunits in chick and mouse

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² Abbreviations used: AChR, acetylcholine receptor; MuSK, muscle-specific receptor tyrosine kinase; GFP, green-fluorescent protein.

myotubes (Wallace *et al.*, 1991; Meier *et al.*, 1995). Of the changes in phosphorylation, only tyrosine phosphorylation of the AChR β subunit has been shown to correlate closely with AChR aggregation. For example, in chick myotubes the protein serine kinase inhibitor H-7 blocks agrin-induced phosphorylation of the γ and δ subunits, but does not prevent AChR aggregation. On the other hand, agrin-induced AChR aggregation and tyrosine phosphorylation of the β subunit are both abolished by treatment of myotubes with tyrosine kinase inhibitors (Wallace, 1994; Ferns *et al.*, 1996). Agrin-induced AChR aggregation and tyrosine phosphorylation of the β subunit share the same dose dependence for agrin (Wallace, 1992; Ferns *et al.*, 1996), β subunit tyrosine phosphorylation precedes aggregate formation and parallels aggregate dispersal (Wallace *et al.*, 1991; Wallace, 1992; Ferns *et al.*, 1996), and tyrosine phosphorylation of the β subunit is closely correlated with attachment of AChRs to the cytoskeleton (Meier *et al.*, 1995; Wallace, 1995). A conserved tyrosine phosphorylation site on the β subunit and one on the δ subunit together appear to account for all of the phosphotyrosine present in the AChR (Wagner *et al.*, 1991; Qu *et al.*, 1996). Results of studies in a heterologous expression system indicate that activation of MuSK causes phosphorylation of the conserved site (Y390) on the β subunit (Gillespie *et al.*, 1996). Agrin-induced tyrosine phosphorylation of the AChR δ subunit, which appears to occur in chick (Qu and Haganir, 1994) but not mouse myotubes (Meier *et al.*, 1995; Ferns *et al.*, 1996), has not been characterized further. Thus, there is considerable evidence consistent with the hypothesis that tyrosine phosphorylation of the AChR β subunit mediates receptor aggregation.

In the experiments described here we sought to test directly the hypothesis that tyrosine phosphorylation of the AChR β subunit is crucial for the recruitment of receptors into agrin-induced aggregates in myotubes, using a site-directed mutagenesis approach. We found that mutant AChRs that lack any tyrosine residues in the cytoplasmic portion of the β subunit accumulated in receptor aggregates. Thus, our results demonstrate that tyrosine phosphorylation of an AChR's β subunit is not required for incorporation of the AChR into agrin-induced specializations and emphasize the importance of protein-protein interactions that do not depend on AChR β subunit phosphorylation in the formation of receptor aggregates.

RESULTS

Our aim was to determine whether tyrosine phosphorylation of the AChR β subunit is required for agrin-induced AChR aggregation using site-directed mutagenesis to remove all possible tyrosine phosphorylation

sites. Although AChR aggregates have been shown to form in a variety of heterologous expression systems (Yu and Hall, 1994; Qu *et al.*, 1996; Gillespie *et al.*, 1996; Apel *et al.*, 1997), agrin-induced AChR aggregation is best characterized in muscle cells. Therefore, we sought to express recombinant AChRs in myotubes, to develop a way to discriminate between recombinant mutant and endogenous AChRs, and to determine whether mutant receptors accumulate in agrin-induced aggregates.

Species Specificity of a Monoclonal Anti-AChR β Subunit Antibody

We explored two ways of identifying recombinant AChRs in transfected myotubes. The first method was introduction of an epitope tag into the AChR β subunit, but this was found to interfere with the expression of recombinant AChRs. Second, we sought to use species-specific differences in the structure of the AChR β subunit to distinguish recombinant from endogenous AChRs by immunocytochemistry. We tested several monoclonal antibodies (mAbs) directed against internal epitopes of the AChR β subunit (Tzartos *et al.*, 1986) on myotubes in cultures derived from the mouse C2C12 muscle cell line and from chick myoblasts. Of the antibodies tested, mAb 148 (Tzartos *et al.*, 1986, Ratnam *et al.*, 1986) stained agrin-induced AChR aggregates in mouse myotubes (Figs. 1a and 1b), but not in chick myotubes (Figs. 1c and 1d). This result is consistent with previous observations that mAb 148 specifically recognizes the β subunit in mouse AChRs (Meier *et al.*, 1995), but does not recognize any AChR subunit in chick (Wallace *et al.*, 1991). Therefore, mAb 148 was used to detect recombinant AChRs of mouse origin expressed in chick myotubes.

Expression of Mouse AChRs in Chick Myotubes

Because the species-specific detection of recombinant AChR was dependent on the expression of recombinant mouse AChRs in chick myotubes, our next aim was to develop a protocol for transfecting chick muscle cultures. Of several protocols tested, the best results were obtained with liposome-mediated transfection of chick myoblasts 18–20 h after isolation and plating. In such cultures, $9.7 \pm 1.7\%$ (mean \pm SEM, $N = 7$) of the myoblasts expressed recombinant protein, determined by using the green fluorescent protein (GFP) as reporter. As a consequence of the subsequent fusion of transfected and nontransfected myoblasts to form multinucleated myotubes, $22.1 \pm 3.4\%$ (mean \pm SEM, $N = 7$) of myotubes expressed recombinant GFP after 6 days in culture.

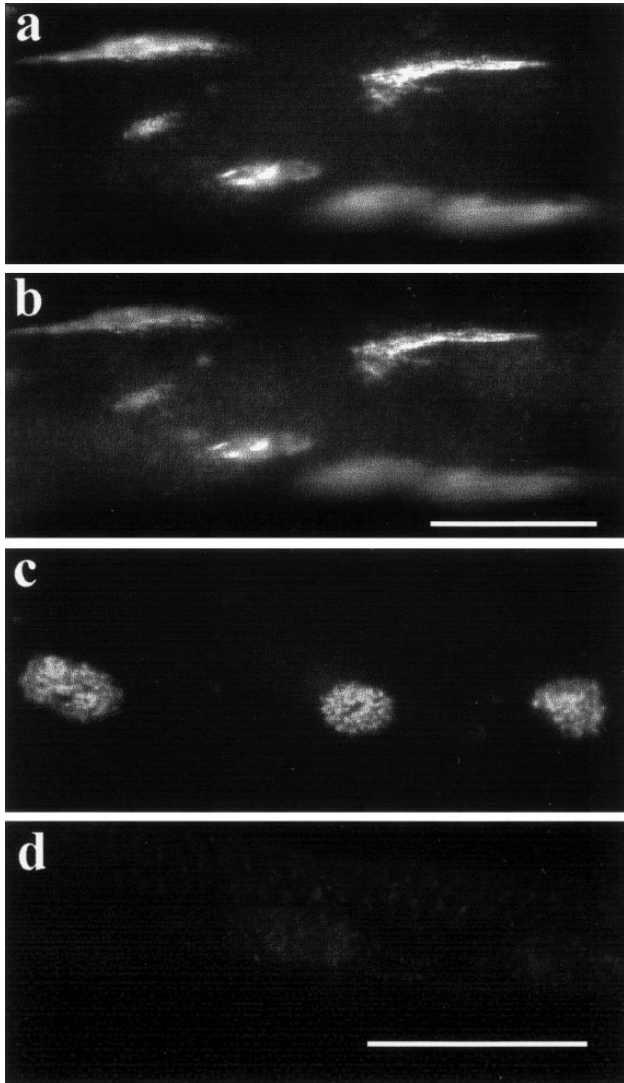


FIG. 1. Immunostaining of mouse, but not chick, AChRs with mAb 148. Myotubes of the mouse C2 cell line (a and b) or primary chick muscle cultures (c and d) were treated with agrin and labeled with rhodamine-conjugated α -bungarotoxin (a and c) and mAb 148 (b and d). AChR aggregates in mouse myotubes were labeled by mAb 148 (a and b), whereas those in chick myotubes were not (c and d). A faint background fluorescence of myotubes, especially of nuclei, is seen with mAb 148 staining. Bars, 20 μ m.

Transfection of a cDNA for the mouse AChR β subunit alone into chick myoblasts led to a large pool of recombinant subunits located intracellularly, suggesting that mouse β subunits did not coassemble efficiently with endogenous chick α , γ , and δ subunits. Therefore, cDNAs for mouse AChR α , β , γ , and δ subunits were cotransfected. Myotubes were stained with mAb 148 to label recombinant mouse AChRs and with α -bungarotoxin to label all AChRs. The density of recombinant

and endogenous AChRs that are diffusely distributed on the myotube surface was too low for reliable detection; therefore myotubes were treated with agrin to induce the formation of AChR aggregates, which were easily visualized. Aggregates containing recombinant AChRs were labeled by mAb 148 on $16.8 \pm 5.8\%$ (mean \pm SEM, $N = 5$) of myotubes, in agreement with the transfection efficiency determined by expression of GFP. Transfected myotubes showed regions which stained strongly for mAb 148, and in such regions every AChR aggregate was colabeled by rhodamine-conjugated α -bungarotoxin and mAb 148. However, the intensity of mAb 148 staining decreased with increasing distance from the region of highest intensity, suggesting that recombinant AChRs derived from a transfected nucleus did not diffuse along the whole length of the myotube.

We next sought to determine whether transfection and expression of mouse AChRs in chick myotubes altered the response to agrin. Agrin-treated cultures were colabeled with rhodamine-conjugated α -bungarotoxin and mAb 148. Transfected myotubes were identified by the presence of at least one AChR aggregate labeled by mAb 148. Because recombinant receptors were not present uniformly along the length of transfected myotubes, we could not assay AChR aggregation by counting aggregates in randomly selected segments of transfected myotubes. Accordingly, we measured agrin-induced AChR aggregation by taking advantage of the fact that spontaneous aggregates are widely scattered along myotubes while agrin-induced aggregates often lie in close proximity to one another. For each transfected myotube, the 200- μ m-long segment with the highest concentration of aggregates containing recombinant AChRs was selected and the number of aggregates within the segment was counted. In myotubes transfected with wildtype recombinant AChRs, regions containing the highest concentration of aggregates showed 4.0 ± 2.0 aggregates per myotube segment (mean \pm SD, $N = 262$ myotubes from nine experiments). In agrin-treated cultures transfected with empty vector, regions containing the highest concentration of aggregates (in this case labeled only with rhodamine-conjugated α -bungarotoxin) showed 4.2 ± 2.1 aggregates per myotube segment (mean \pm SD, $N = 161$ myotubes from six experiments), whereas without agrin treatment regions with the highest concentration of aggregates had 1.3 ± 0.6 aggregates per myotube segment (mean \pm SD, $N = 213$ myotubes from three experiments). Therefore, transfected myotubes could still respond to agrin. Moreover, the appearance of AChR aggregates in transfected myotubes was similar to that in nontransfected cultures (Figs. 1b and 2a–2d).

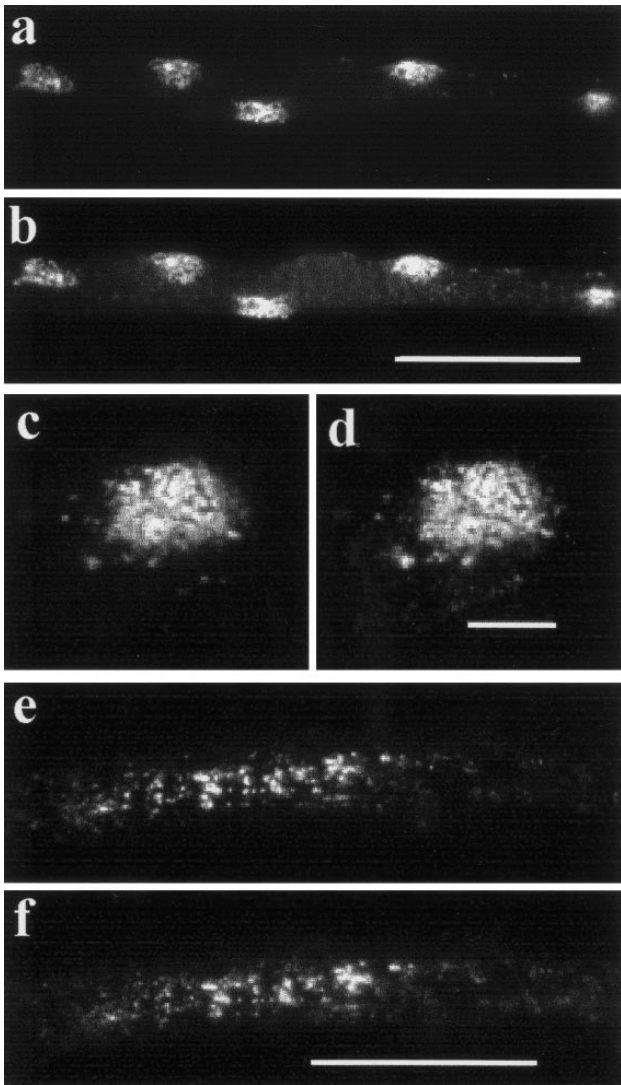


FIG. 2. Expression of mouse AChRs in chick myotubes. Chick myoblasts were transfected with cDNAs for the mouse AChR subunits and the formation of AChR aggregates was induced in myotubes by agrin. Myotubes were labeled with rhodamine-conjugated α -bungarotoxin (a, c, and e) and mouse AChR β subunits were immunostained with mAb 148 (b, d, and f). Aggregates had the size and shape typical for agrin-induced AChR aggregates (a and b; bar, 20 μ m). The aggregates showed subdomains of different staining intensity, which were precisely colabeled by α -bungarotoxin and mAb 148 (c and d; bar, 5 μ m). Some transfected myotubes showed, in addition to large agrin-induced AChR aggregates, much smaller microaggregates that were also labeled by both α -bungarotoxin and mAb 148 (e and f; bar, 20 μ m).

Thus, most of the aggregates we observed in agrin-treated transfected myotubes must have been induced to form by agrin and the response to agrin did not appear to have been changed by expression of recombinant AChRs.

In addition to the typical large agrin-induced AChR aggregates, some transfected myotubes showed diffusely distributed microaggregates (Figs. 2e and 2f). Scattered AChR microaggregates were also observed in myotubes overexpressing rapsyn, a peripheral membrane protein associated with the intracellular face of AChR aggregates at the neuromuscular junction (Yoshihara and Hall, 1993). Such microaggregates were not included in our analysis.

Expression and Aggregation of an AChR Mutant That Lacks All Cytoplasmic Tyrosine Residues in Its β Subunit

The primary structure of the AChR β subunit predicts a topology containing four transmembrane-spanning regions (Buonanno *et al.*, 1986). The predicted intracellular portion of the subunit consists of a short and a long cytoplasmic loop. The short loop, between transmembrane domains I and II, has no tyrosine residues; the long cytoplasmic loop, between transmembrane domains III and IV, contains three tyrosine residues. Previous results from other laboratories indicated that tyrosine 390, which lies within the long III-IV loop and is conserved in all β subunits thus far examined, is the site for tyrosine phosphorylation (Wagner *et al.*, 1991; Gillespie *et al.*, 1996; Qu *et al.*, 1996). Phosphopeptide analysis of cyanogen bromide fragments of the AChR β subunit from C2 myotubes confirmed that agrin-induced increases in phosphorylation occurred in the long III-IV cytoplasmic loop (data not shown). However, preliminary analysis of V8 phosphopeptides suggested that tyrosine residues within this loop in addition to tyrosine 390 may be phosphorylated in response to agrin (data not shown). Therefore, to eliminate any possibility of tyrosine phosphorylation of the AChR β subunit, we chose to mutate all three tyrosines within the long III-IV cytoplasmic loop.

We removed all three possible tyrosine phosphorylation acceptor sites by replacing each tyrosine with a phenylalanine residue (Y357F, Y390F, and Y442F). To test whether the mutations interfered with the folding of the β subunit or the assembly of the AChR complex, we coexpressed the mutant β subunit (Y357F, Y390F, and Y442F) with wildtype α , γ and δ subunits in human embryonic kidney 293 cells. Rhodamine-conjugated α -bungarotoxin labeled surface AChRs on cells only upon cotransfection with all four subunits (data not shown), indicating that AChR complexes bearing mutant β subunits reached the cell surface.

Cotransfection of the mutant β subunit cDNA along with the α , γ , and δ subunit cDNAs in chick myotubes

led to recombinant receptor expression in $18.6 \pm 5.6\%$ of the myotubes (mean \pm SEM, $N = 4$). Agrin treatment of transfected myotubes resulted in AChR aggregates that contained the mutant β subunit (Fig. 3). The low yield of myotubes in transfected cultures prohibited us from confirming directly the lack of agrin-induced tyrosine phosphorylation of mutant AChRs. The appearance of agrin-induced aggregates containing AChRs with mutant β subunits was indistinguishable from aggregates containing AChRs with recombinant wildtype β subunits or aggregates of chick AChRs in nontransfected cultures (Figs. 1–3). Similar results were obtained for cultures expressing mutant β subunits containing only single (Y357A or Y390F) or double (Y357F and Y390F) tyrosine replacements (data not shown).

In agrin-treated myotubes transfected with the mutant β subunit (Y357F, Y390F, and Y442F) along with

wildtype α , γ , and δ subunits, identified by the presence of AChR aggregates that were colabeled with rhodamine-conjugated α -bungarotoxin and mAb 148, regions containing the highest concentration of aggregates showed 4.4 ± 3.5 aggregates per myotube segment (mean \pm SD, $N = 222$ myotubes from nine experiments). This result is similar to that found in myotubes containing recombinant wildtype AChRs and endogenous AChRs (see above). Thus, the presence of AChRs containing mutant β subunits did not appear to alter agrin-induced AChR aggregation.

To estimate the fraction of mouse receptors in agrin-induced aggregates, we compared the intensity of the fluorescein fluorescence, derived from mouse receptors, to the rhodamine fluorescence, which measures total AChRs. The ratio of fluorescein to rhodamine fluorescence within aggregates in myotubes transfected with mutant mouse AChRs was similar to that seen either in chick myotubes transfected with wildtype mouse AChRs or in normal mouse C2 myotubes (Figs. 1–3). This indicates that mutant receptors contribute significantly to the total population of receptors in agrin-induced aggregates. In summary, AChRs containing β subunits that lack all intracellular acceptor sites for tyrosine phosphorylation were still recruited into agrin-induced receptor aggregates.

DISCUSSION

To examine the role of tyrosine phosphorylation of the AChR β subunit in receptor aggregation, we constructed cDNA for a mutant β subunit in which all cytoplasmic tyrosine residues were replaced by phenylalanine, thereby removing all acceptor sites for tyrosine phosphorylation. AChRs bearing such mutant subunits were expressed in myotubes. When the myotubes were treated with agrin, mutant receptors became incorporated into aggregates that had a normal size, shape, and appearance. Thus, tyrosine phosphorylation of an AChR's β subunit is not required for recruitment of the receptor into an agrin-induced aggregate.

AChR Aggregation and Tyrosine Phosphorylation of the AChR β Subunit

Several lines of evidence are consistent with the hypothesis that agrin triggers the formation of domains of increased protein tyrosine phosphorylation in the vicinity of active agrin receptors within which AChRs are phosphorylated and interact more strongly with the underlying cytoskeleton (Wallace, 1995; Meier *et al.*,

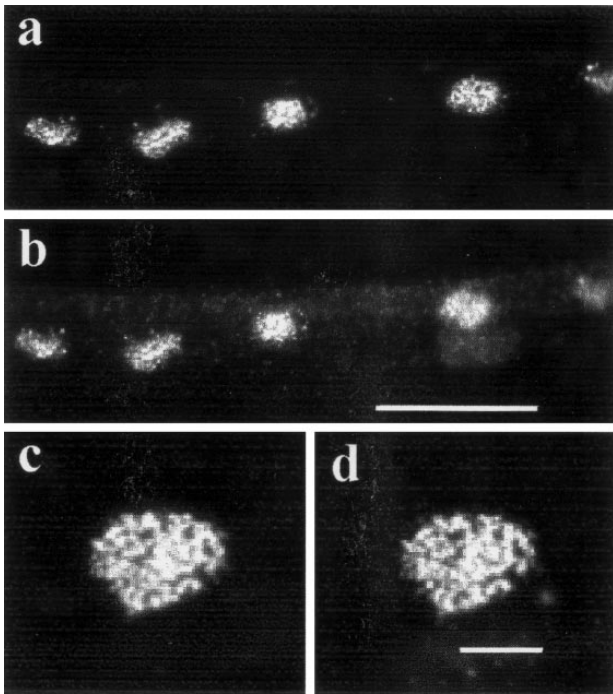


FIG. 3. Expression of mouse AChRs containing mutant β subunits (Y357F, Y390F, and Y442F) in chick myotubes. Chick myoblasts were transfected with cDNAs for a mouse AChR β subunit (Y357F, Y390F, and Y442F) mutant along with mouse AChR α , γ , and δ subunits and the formation of AChR aggregates was induced in myotubes by agrin. Myotubes were labeled with rhodamine-conjugated α -bungarotoxin (a and c) and mouse AChR mutant β subunits were immunostained with mAb 148 (b and d). As with wildtype recombinant AChRs, AChRs with mutant β subunits appeared in aggregates that had the size and shape characteristics of agrin-induced AChR aggregates in chick myotubes (a and b; bar, 20 μ m), including subdomains that contained precisely for α -bungarotoxin and mAb 148 (c and d; bar, 5 μ m).

1995). For example, sites of agrin-induced AChR aggregation label with anti-phosphotyrosine antibodies, agrin induces tyrosine phosphorylation of the AChR β subunit, and clustered AChRs are less mobile and more resistant to detergent extraction than diffusely distributed receptors. AChR phosphorylation and reduced detergent extractability are closely correlated, suggesting that phosphorylation of the AChR itself might be the cause of receptors binding more tightly to the cytoskeleton. If this were so, then AChR phosphorylation would be an obligate step in AChR aggregation (Fig. 4a). The result presented here, that mutant AChRs lacking acceptor sites for tyrosine phosphorylation on the β subunit still become incorporated into agrin-induced aggregates, argues against such a simple model as the only mechanism for receptor aggregation. Unfortunately, the very low yield of myotubes in transfected cultures made it impossible to determine whether agrin caused a change in the detergent extractability of mutant AChRs.

Agrin has also been shown to induce tyrosine phosphorylation of the AChR δ subunit in chick myotubes (Qu and Haganir, 1994). Tyrosine phosphorylation of the δ subunit does not appear to be required for AChR aggregation, however, as it is not seen in agrin-treated mouse C2 myotubes (Meier *et al.*, 1995; Ferns *et al.*, 1996). In addition, activation of MuSK in a heterologous expression system was shown to cause tyrosine phosphorylation of the AChR β subunit, but not the δ subunit (Gillespie *et al.*, 1996). A possible role for AChR δ subunit phosphorylation is not addressed in the experiments described here, but could be tested by identification and mutation of sites of agrin-induced tyrosine phosphorylation in the δ subunit.

Contribution of Mutant and Wildtype Receptors

In our experiments myotubes contain not only mutant receptors, but endogenous wildtype receptors as well. Thus, one explanation for the results reported here is that agrin first induces aggregation of endogenous wildtype receptors by a mechanism that requires tyrosine phosphorylation of the AChR β subunit and that this creates a scaffold into which mutant AChRs become incorporated by interactions that do not depend on phosphorylation (Fig. 4b). Consistent with such a scheme is the finding that in chick myotubes AChR tyrosine phosphorylation reaches a steady state after 3 h of agrin treatment, while the total number of aggregated AChRs continues to increase until 16 h after agrin addition (Wallace *et al.*, 1991).

The contribution that such a phosphorylation-independent pathway can make to the overall density of aggregated AChRs is significant. In our experiments, the fraction of mouse receptors within agrin-induced aggregates, estimated by comparing the fluorescence from anti-mouse β subunit antibodies and that from rhodamine α -bungarotoxin, was similar in chick myotubes transfected with mutant mouse receptors, chick myotubes transfected with wildtype mouse receptors, and normal C2 myotubes. This indicates that AChRs containing β subunits that cannot be tyrosine phosphorylated can constitute a substantial fraction of the receptors in agrin-induced aggregates.

Recruitment of Nonphosphorylated AChRs into Aggregates by Rapsyn

One candidate for mediating the recruitment of non-phosphorylated AChRs into agrin-induced aggregates is rapsyn, a peripheral membrane protein of 43 kDa that closely associates with AChRs (Hill, 1992; Froehner,

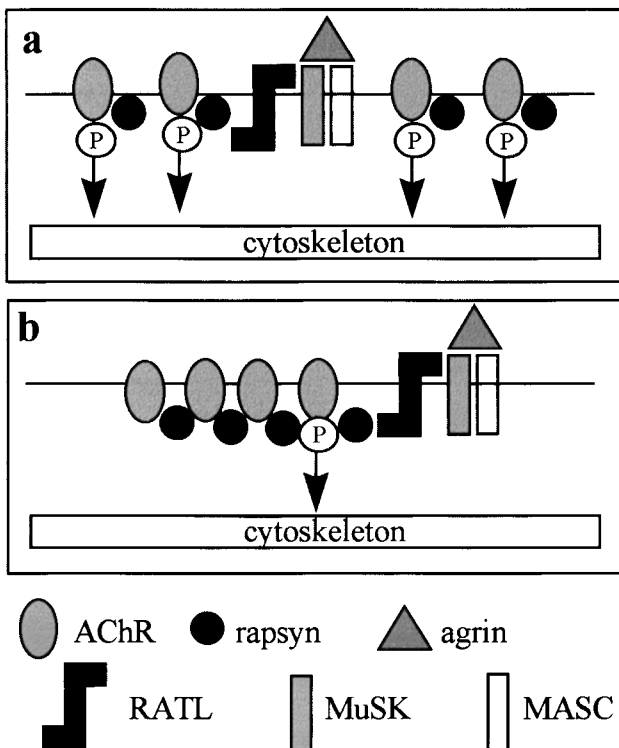


FIG. 4. Models for recruitment of AChRs into agrin-induced specializations. (a) Each AChR within an agrin-induced aggregate is bound to the cytoskeleton by interactions that require tyrosine phosphorylation of the AChR β subunit. (b) Some aggregated AChRs are bound to the cytoskeleton by interactions dependent on tyrosine phosphorylation, while other AChRs are recruited to aggregates by protein-protein interactions that do not require β subunit tyrosine phosphorylation. MuSK, MASC (Glass *et al.*, 1996), and RATL (Apel *et al.*, 1997) are depicted as components of the agrin receptor.

1993). Rapsyn plays a crucial role in postsynaptic differentiation, as demonstrated by the lack of AChR aggregates and other postsynaptic specializations at developing neuromuscular junctions in rapsyn-deficient mice (Gautam *et al.*, 1995). Rapsyn has been shown to colocalize with AChRs in heterologous expression systems by a mechanism that is not dependent on tyrosine phosphorylation of either the AChR α or the AChR β subunit (Yu and Hall, 1994; Qu *et al.*, 1996). Similarly, we found that AChRs containing mutant β subunits (Y357F, Y390F, and Y442F) colocalize with aggregates of rapsyn/GFP fusion protein on coexpression in human embryonic kidney 293 cells (G. Meyer and B. G. Wallace, unpublished observations). Thus, agrin-induced interactions between phosphorylated AChRs and the cytoskeleton could create a rapsyn-containing scaffold into which nonphosphorylated receptors are recruited.

The mechanisms of rapsyn aggregation and interaction with AChRs in heterologous expression systems are not yet known. It was recently reported that GABA_A receptors also colocalize with rapsyn aggregates in such coexpression systems (Yang *et al.*, 1997). The cytoplasmic domains of GABA_A receptors have little sequence homology to AChRs, suggesting that mechanisms other than direct protein-protein interactions between rapsyn and the cytoplasmic domains of the receptor can mediate rapsyn-receptor coaggregation in such heterologous expression systems. The relationship between the coaggregation of rapsyn and AChRs in heterologous expression systems and the formation of agrin-induced receptor aggregates in muscle cells also remain to be determined. However, it is clear that the regulation of rapsyn distribution and of the interaction between rapsyn and AChRs is more complex in muscle cells than in nonmuscle cells. For example, rapsyn does not form large aggregates, such as those seen in nonmuscle cells, in variants of the C2 muscle cell line that are deficient in AChRs or when rapsyn is overexpressed in C2 myotubes (LaRochelle *et al.*, 1989; Yoshihara and Hall, 1993). Nevertheless, the rapsyn-AChR interactions that occur in nonmuscle cells could play a role in recruiting receptors to a rapsyn-containing scaffold in agrin-treated myotubes.

Role of MuSK in Agrin-Induced AChR Aggregation

Agrin-induced AChR aggregation requires the presence of MuSK, a muscle-specific kinase that belongs to the family of receptor tyrosine kinases (Glass *et al.*, 1996). Antibodies to its extracellular domain activate MuSK and lead to formation of AChR aggregates (Xie *et al.*, 1997). MuSK becomes tyrosine phosphorylated in response to agrin, presumably by an autocatalytic mech-

anism (Glass *et al.*, 1996). Overexpression of a dominant-negative mutant of MuSK in myotubes inhibits AChR aggregation, suggesting that agrin-induced MuSK-catalyzed tyrosine phosphorylation is required in receptor aggregation (Glass *et al.*, 1996, 1997). When C2 myotubes are pretreated with the kinase inhibitor staurosporine, agrin causes phosphorylation of MuSK but not of AChRs, and aggregation is blocked (Fuhrer *et al.*, 1997). This suggests that a phosphorylation event in addition to tyrosine phosphorylation of MuSK is required for receptor aggregation. The physiological substrates of MuSK have not been identified; the only other protein shown to be tyrosine phosphorylated in response to agrin treatment is the AChR (Wallace *et al.*, 1991; Meier *et al.*, 1995; Ferns *et al.*, 1996). However, phosphorylation of MuSK together with AChRs is still not sufficient to induce AChR aggregation, as shown by results of experiments with a chimeric protein constituting the extracellular domain of the trkC receptor tyrosine kinase and the intracellular domain of MuSK (Glass *et al.*, 1997). When activated by NT-3, such chimeric receptors cause phosphorylation of the MuSK intracellular domain and of the AChR β subunit, but do not induce AChR aggregation. Thus, the extracellular domain of MuSK appears to play a role in agrin-induced AChR aggregation.

MuSK accumulates at sites of nerve-muscle contact in rapsyn-deficient mice, suggesting that MuSK forms a primary scaffold with which other components of the postsynaptic apparatus become associated (Apel *et al.*, 1997). Rapsyn has been shown to colocalize with MuSK through an indirect interaction involving the extracellular domain of MuSK and an as yet unidentified rapsyn-associated transmembrane linker RATL (Apel *et al.*, 1997). In addition, AChRs have been reported to associate with agrin-activated MuSK in a manner that does not depend on AChR phosphorylation (Fuhrer *et al.*, 1997). Such interactions between MuSK, rapsyn, and AChRs could be responsible for the recruitment of nonphosphorylated receptors into agrin-induced aggregates reported here. Thus, agrin-induced AChR aggregation may involve several activities of MuSK: tyrosine phosphorylation catalyzed by the intracellular domain, activation of additional intracellular kinases, formation of a primary postsynaptic lattice, association with AChRs, and interactions mediated by rapsyn, RATL, and the extracellular domain of MuSK.

Laminin-Induced AChR Aggregation

Another candidate for recruiting nonphosphorylated AChRs to agrin-induced aggregates is laminin. Lami-

nin-1 can induce AChR aggregation on primary rat myotubes, G8-1 clonal rat muscle cells, and mouse C2 myotubes (Vogel *et al.*, 1983; Sugiyama *et al.*, 1997; Montanaro *et al.*, 1998). Laminin-induced AChR aggregation occurs without tyrosine phosphorylation of either MuSK or AChRs (Sugiyama *et al.*, 1997; Montanaro *et al.*, 1998). Moreover, when agrin is added to myotubes in culture, laminin accumulates at sites of AChR aggregation, although with a slower time course than that of AChRs (Nitkin and Rothschild, 1990; Montanaro *et al.*, 1998). Thus, agrin may initially induce specializations containing AChRs and, with a delay, laminin, by a mechanism requiring AChR tyrosine phosphorylation; as laminin accumulates it could then recruit additional, nonphosphorylated receptors to the same sites, perhaps by a mechanism that involves interaction with α -dystroglycan (Montanaro *et al.*, 1998).

Alternative Roles for AChR Tyrosine Phosphorylation

Even if tyrosine phosphorylation of the AChR β subunit played no direct role in AChR aggregation, it might recruit to developing receptor aggregates proteins that are involved in other aspects of postsynaptic differentiation. Recently, three proteins well known for their role in signal transduction processes were reported to interact with AChRs. In the electric organ of Torpedo, for example, the adapter protein Grb2 binds to the AChR δ subunit in a phosphorylation-dependent manner (Colledge and Froehner, 1997). The binding site for Grb2, however, is not conserved in AChRs of chick or mouse. Also in Torpedo electric organ, the nonreceptor tyrosine kinases fyn and fynk associate with AChRs (Swope and Haganir, 1993). This association occurs via the SH2 domains of fyn and fynk, is dependent on tyrosine phosphorylation of the AChR, and is mediated by the AChR δ subunit (Swope and Haganir, 1994). In mouse myotubes, fyn and the related kinase src have been shown to bind to the AChR β subunit (Fuhrer and Hall, 1996). However, such interactions do not appear to be regulated by agrin-induced AChR phosphorylation (Fuhrer *et al.*, 1997), and the role they play in agrin-induced postsynaptic differentiation is not clear.

CONCLUSION

Our results demonstrate that mutant AChRs containing β subunits that lack sites for tyrosine phosphorylation can be recruited into agrin-induced aggregates at densities comparable to those of wildtype receptors.

Thus, protein-protein interactions that do not depend on tyrosine phosphorylation of the AChR β subunit may play a role in agrin-induced receptor aggregation. In order to determine whether agrin-induced aggregates can form *de novo* from nonphosphorylated receptors, it will be necessary to express mutant AChRs in myotubes that lack wildtype receptors.

EXPERIMENTAL METHODS

Site-Directed Mutagenesis

The mutant AChR β subunit (Y357F, Y390F, and Y442F) was constructed by oligonucleotide-directed mutagenesis (Transformer kit, Clontech, Palo Alto, CA) of the mouse AChR β subunit cDNA (Buonanno *et al.*, 1986). Three mutagenic primers (5'-GCTCCCTCCATTCCTGGGTCTG-3', 5'-GGAAGTATGAATTTTTTCATCCGGAAG-3', 5'-CCTCAATCAGCTTCATGGCCCGAC-3') allowing the replacement of tyrosine-357, tyrosine-390, and tyrosine-442 by phenylalanine residues, respectively, and a selection primer for removal of a unique *Xba*I restriction site of pBK-CMV (5'-GAGAGTACTTCGAGAGCGGCC-3') were annealed simultaneously. The mutations were confirmed by automated dideoxy sequencing (Applied Biosystems, Foster City, CA). All AChR subunit cDNAs were expressed from the vector pBK-CMV (Stratagene, La Jolla, CA), where the prokaryotic promoter was removed using the enzymes *Spe*I and *Nhe*I.

Myotube Cultures and Transfection

Myotube cultures were prepared from hindlimb muscles of 12-day-old White Leghorn chick embryos by the method of Fischbach (1972) with minor modifications (Wallace, 1989). Myoblasts were plated at a density of 4×10^5 cells per 35-mm-dish in 1 ml culture medium and transfected 18–20 h after being plated with 1.5 mg plasmid per milliliter of medium. Transfection was mediated by the liposomal reagent DOSPER (Boehringer Mannheim, Indianapolis, IN) in a DNA/liposomal reagent ratio of 1:4. AChR subunit cDNAs were mixed in an α : β : γ : δ ratio of 2:2:1:1. The culture medium containing the lipid was diluted 1:2 with medium 6 h after transfection and replaced by fresh culture medium 24 h after transfection and by culture medium containing 10^{-5} M cytosine β -D-arabinofuranoside (Sigma Chemical Co., St. Louis, MO) to select for differentiating cells 32 h after transfection. Transfected cultures produced fewer myotubes than did nontransfected control cultures. Experiments were made on 6- to 7-day-old myotube cultures.

Transfection efficiencies were determined using the green fluorescent protein (pEGFP-C1, 1.5 mg cDNA/dish, Clontech) as reporter. Fluorescent cells were counted 2 days (myoblasts) or 5 days (myotubes) after transfection. Experiments were performed in triplicate.

The mouse muscle cell line C2C12 was maintained and differentiated to myotubes as described (Meier *et al.*, 1995).

AChR Aggregation Assay and Immunocytochemistry

Cultures containing differentiated myotubes were incubated with 3 units of the recombinant agrin fragment c95A4B8 (Meier *et al.*, 1996) for 16 h. AChRs were labeled with 4×10^{-8} M rhodamine-conjugated α -bungarotoxin (Molecular Probes, Eugene, OR) for 1 h and rinsed in phosphate-buffered saline (PBS). For immunostaining, cultures were fixed in 1% (w/v) paraformaldehyde in PBS for 10 min, permeabilized by 1% (v/v) Triton X-100 in PBS for 10 min, and blocked by 3% (w/v) bovine serum albumin (BSA) in PBS. The cultures were incubated with mAb 148 at a concentration of 128 nM in PBS containing 3% (w/v) BSA, rinsed, incubated with a biotinylated goat anti-rat antibody (Jackson ImmunoResearch, West Grove, PA) at a concentration of 1.2 mg/ml in PBS containing 2% (v/v) normal goat serum, rinsed, incubated with fluorescein-conjugated streptavidin (Molecular Probes) at a concentration of 1 mg/ml in PBS containing 2% (v/v) normal goat serum, dehydrated, and mounted in Citifluor (Citifluor, Ltd., London, UK). Myotube segments (200 μ m) were viewed with a Nikon Optiphot microscope equipped with phase and fluorescence optics and the number of AChR aggregates was counted by eye at 400 \times magnification or imaged with a Star I chilled CCD camera (Photometrics, Ltd., Tucson, AZ) using a 100 \times oil-immersion objective. The number of aggregates per myotube segment was expressed as mean \pm standard deviation; *N* equals the number of myotube segments counted.

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