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Agrin-induced Phosphorylation of the Acetylcholine Receptor Regulates Cytoskeletal Anchoring and Clustering

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Abstract. At the developing neuromuscular junction, a motoneuron-derived factor called agrin signals through the muscle-specific kinase receptor to induce postsynaptic aggregation of the acetylcholine receptor (AChR). The agrin signaling pathway involves tyrosine phosphorylation of the AChR β subunit, and we have tested its role in receptor localization by expressing tagged, tyrosine-minus forms of the β subunit in mouse Sol8 myotubes. We find that agrin-induced phosphorylation of the β subunit occurs only on cell surface AChR, and that AChR-containing tyrosine-minus β subunit is targeted normally to the plasma membrane. Surface AChR that is tyrosine phosphorylated is less detergent extractable than nonphosphorylated AChR, indicating that it is preferentially linked to the cytoskeleton. Consistent with this, we find that agrin treatment reduces the detergent extractability of AChR that contains tagged wild-type β subunit but not tyrosine-minus β subunit. In addition, agrin-induced clustering of AChR containing tyrosine-minus β subunit is reduced in comparison to wild-type receptor. Thus, we find that agrin-induced phosphorylation of AChR β subunit regulates cytoskeletal anchoring and contributes to the clustering of the AChR, and this is likely to play an important role in the postsynaptic localization of the receptor at the developing synapse.

Key words: neuromuscular junction • synaptogenesis • agrin • tyrosine phosphorylation • cytoskeleton

Introduction

The development of the neuromuscular junction is triggered by agrin, a signaling factor that is deposited by the nerve terminal at the site of contact with the muscle cell. Motoneuron-derived agrin induces many aspects of synaptic differentiation and is required for the postsynaptic localization of many synapse-specific basal lamina, transmembrane, and cytoplasmic proteins (Burden, 1998; Sanes and Lichtman, 1999). In addition, agrin is sufficient to induce aggregation of these synaptic proteins in muscle cells in vitro (Wallace, 1989; Campanelli et al., 1991; Tsim et al., 1992), or at ectopic sites on muscle fibers in vivo (Cohen et al., 1997; Meier et al., 1997). Most notably, agrin induces a rapid aggregation of the acetylcholine receptor (AChR),[1] initially through a redistribution of preexisting receptors in the muscle membrane (Godfrey et al., 1984; Wallace, 1988). This agrin-induced clustering of the neurotransmitter receptor allows functional, synaptic transmission from early stages of neuromuscular junction formation.

The process by which the AChR becomes localized to the postsynaptic membrane is unclear, but it is known to require a cytoplasmic, peripheral-membrane protein called rapsyn. Rapsyn is present in ~1:1 stoichiometry with the AChR (LaRochelle and Froehner, 1987), and appears to be in close proximity with the AChR β subunit (Burden et al., 1983). Rapsyn can induce clustering of the AChR when they are coexpressed in heterologous cells (Froehner et al., 1990; Phillips et al., 1991) and, conversely, the AChR fails to cluster at developing neuromuscular junctions in rapsyn knock-out mice (Gautam et al., 1995). Thus, rapsyn mediates the postsynaptic aggregation of the AChR.

Synaptic localization of the AChR also involves some form of anchorage of the receptor to the cytoskeleton (Froehner, 1991). Upon aggregation in cultured myotubes, the AChR becomes immobilized in the plane of the membrane and is no longer free to diffuse like dispersed receptor (Stya and Axelrod, 1983; Meier et al., 1997). Clustered receptors in vitro are also less detergent extractable than dispersed receptor, again reflecting some cytoskeletal linkage (Prives et al., 1982; Stya and Axelrod, 1983; Podleski and Salpeter, 1988). Similarly, the AChRs at the developing neuromuscular junction in vivo are progressively stabilized and become resistant to dispersal after

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[1]*Abbreviations used in this paper:* AChR, acetylcholine receptor; CNS, central nervous system; HA, hemagglutinin; MuSK, muscle-specific kinase; wt, wild type.

various experimental treatments (Dennis, 1981; Slater, 1982). Consistent with this, the cytoskeleton beneath AChR clusters is known to be highly specialized, and contains several cytoskeletal proteins of the actin/spectrin family (Froehner, 1991). The specific components that mediate anchorage of the AChR are unknown, however.

Agrin induces postsynaptic localization of the AChR and other synaptic proteins by signaling through the receptor tyrosine kinase muscle-specific kinase (MuSK). Agrin and MuSK-deficient mice have a similar phenotype and both fail to form postsynaptic specializations at neuromuscular contacts (DeChiara et al., 1996; Gautam et al., 1996). Moreover, agrin rapidly activates MuSK in cultured myotubes (Glass et al., 1997), and MuSK becomes clustered together with the AChR (Bowen et al., 1998). Two signaling events have been identified downstream of MuSK that may regulate the aggregation of the AChR. Recently, agrin signaling has been shown to activate the guanosine triphosphatases, Rac and Cdc42, which could regulate cytoskeletal interactions involved in clustering (Weston et al., 2000). In addition, agrin/MuSK signaling induces a rapid tyrosine phosphorylation of the AChR β subunit, and this phosphorylation correlates in several ways with AChR clustering. First, agrin-induced phosphorylation of the β subunit precedes cluster formation and displays the same agrin dose dependence as AChR clustering (Wallace et al., 1991; Ferns et al., 1996). Second, tyrosine kinase inhibitors that inhibit β subunit phosphorylation also block AChR clustering, and cause the dispersal of preexisting clusters (Wallace, 1994; Ferns et al., 1996). Third, β subunit phosphorylation correlates with the linkage of the AChR to the cytoskeleton that is associated with receptor aggregation (Wallace, 1994; Meier et al., 1995).

These observations have led us to the working hypothesis that agrin-induced phosphorylation of the AChR β subunit plays some role in localizing the AChR in the postsynaptic membrane. For example, it could regulate the targeted insertion of AChR, the clustering of the AChR in the membrane, and/or the anchoring of the AChR to the cytoskeleton. To directly test the role of AChR phosphorylation in receptor localization, we expressed mutated forms of the β subunit, which lack the relevant tyrosine phosphorylation site(s), in cultured myotubes. We find that AChR β subunit tyrosine phosphorylation is required for linkage of the AChR to the cytoskeleton and that it also contributes to AChR clustering. We therefore propose that agrin-induced phosphorylation of the AChR plays a central role in regulating its postsynaptic localization at the developing neuromuscular junction.

Materials and Methods

Epitope Tagging and Mutagenesis of β Subunit

For expression of the AChR β subunit, we used the full-length cDNA coding for the mouse β subunit in a pSM expression vector (Yu and Hall, 1994). To epitope tag the β subunit, a KpnI site was introduced at the COOH-terminal extracellular tail, and double stranded oligonucleotides that coded for the hemagglutinin (HA) and 142 (Das and Lindstrom, 1991) epitopes were ligated into this site. Tyrosine 357, 390, and 442 were replaced by phenylalanines using PCR mutagenesis, and the accuracy of the mutagenesis was confirmed by sequencing of the amplified region.

Culture and Transfection of Muscle Cells

Myoblasts of the Sol8 mouse cell line were maintained in growth medium consisting of DME supplemented with 20% FBS, 2 mM L-glutamine, and penicillin/streptomycin. Differentiation into myotubes was induced in confluent cultures by replacing the growth medium with fusion medium consisting of DME supplemented with 5% horse serum and 2 mM L-glutamine. For transfection experiments involving immunoblot analysis, myoblasts were grown in 10-cm dishes and transfected at 90% confluence with 25 μg of DNA using the $CaPO_4$ method. After another 18 h in growth medium, the cultures were switched to fusion medium and allowed to differentiate for 5 d. For immunostaining analyses, myoblasts were grown in 6-cm dishes and transfected as above with 8 μg of DNA.

AChR Isolation and Immunoblotting

To assay for AChR phosphorylation, Sol8 myotube cultures were treated with 500 pM of neural agrin (C-Ag4,8; Ferns et al., 1993) for different time intervals. Control and agrin-treated cultures were then rinsed, scraped off in PBS containing 1 mM orthovanadate, and pelleted. The cell pellets were resuspended in extraction buffer containing 1% Triton X-100, 25 mM Tris, 25 mM glycine, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, and the protease inhibitors PMSF, benzamidine, *N*-ethylmaleimide, and $Na_2S_4O_6$. After incubation for 10 min on ice, the insoluble material was removed by centrifugation and the supernatant containing the solubilized AChR was retained.

To isolate total AChR (surface and intracellular), the cell extracts were incubated for 1 h with α-bungarotoxin conjugated to agarose beads. After washing the beads, the bound AChR was eluted in SDS-loading buffer. To selectively isolate surface AChR, live myotubes were incubated for 60–90 min with α-bungarotoxin conjugated to biotin (Molecular Probes). The AChR labeled with biotin-α-bungarotoxin was then precipitated from cell extracts with streptavidin-conjugated agarose beads.

For immunoblotting, the isolated AChR was separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Tyrosine phosphorylation was detected by immunoblotting with a mixture of monoclonal antibodies 4G10 (UBI) and PY20 (Transduction Laboratories), followed by an HRP-conjugated secondary antibody, and visualized using enhanced chemiluminescence. The total amount of AChR precipitated in the various experimental conditions was assayed by stripping the blots with low pH buffer (20 mM glycine, 0.1% Tween 20, pH 2.5, for 20 min), and reprobing with an antibody against the α subunit (mAb 210, provided by J. Lindstrom, University of Pennsylvania, Philadelphia, PA). To specifically assay AChR containing the tagged β subunit, we immunoblotted with an antibody to the HA epitope. Quantification of the results by densitometric analysis is described below.

Extractability Assay

To assay linkage of the AChR to the cytoskeleton, myotube cultures were sequentially extracted with low, and then high detergent buffer. First, myotubes were treated with extraction buffer (see above) containing 0.5% Triton X-100 for 10 min on ice, and nonsolubilized material was pelleted by centrifugation at 13,000 *g* for 4 min. The pellet was then resuspended in extraction buffer containing 1% Triton X-100 and incubated on ice for an additional 10 min, and insoluble material was precipitated as above. Surface AChR labeled with biotinylated-α-bungarotoxin was then isolated from each of the two soluble fractions using avidin beads. In control experiments, we found that a third extraction of the insoluble pellet with a more stringent buffer, containing 0.5% Na deoxycholate, 0.1% SDS, and 1% Triton X-100 for 10 min, did not solubilize any additional AChR. Thus, all of the detergent-extractable AChR is solubilized in the combination of the first two extractions (data not shown).

To assay the levels of tagged and endogenous AChR in each fraction, we immunoblotted for the HA-tagged β subunit and for the α subunit as described above. The distribution of the tyrosine phosphorylated AChR was then assayed by stripping the blot and reprobing with a mix of the antiphosphotyrosine antibodies PY20 and 4G10. To average the results of multiple experiments, we quantified the relative levels of α subunit, tyrosine-phosphorylated β subunit, and HA-tagged β subunit in the two fractions. To do this, we carried out densitometric analysis of the relevant immunoreactive bands using Sci-Scan 5000 Bioanalysis software (USB). For each condition, we determined the level of signal in the first and second extractions, and then expressed the distribution of the receptor in each as a percentage of the combined total. We previously confirmed the linearity of the densitometric analysis by running a dilution series of ty-

rosine-phosphorylated receptor in which we found that the measured densities closely matched the expected values (Jacobson et al., 1998). In addition, we always performed the analysis on films with subsaturating exposure levels.

Immunostaining and Clustering Assay

To assay agrin-induced AChR clustering, transfected Sol8 myotubes were treated with agrin for 5 and 24 h. For cluster dispersal assays, cultures were treated with agrin for 18 h, washed, and incubated in the absence of agrin for another 6 h. Total surface AChR and tagged AChR were then visualized by live staining with rhodamine-conjugated α-bungarotoxin and anti–tag antibody mAb142 (Das and Lindstrom, 1991), respectively. Cells were then rinsed in PBS, fixed with 2% paraformaldehyde for 20 min, blocked with 10% horse serum/PBS, and incubated with an FITC-conjugated anti–rat secondary antibody (Bio/Can). The immunostained cultures were viewed with a 630× fluorescence microscope (Carl Zeiss, Inc.), and the number of tag-positive clusters was counted for 20 random fields per experiment. All counting of myotubes transfected with the different β subunit constructs was done in a blinded fashion.

To assay the respective contribution of tagged and endogenous AChR to clusters, we quantified the relative intensity of staining for rhodamine-conjugated α-bungarotoxin and mAb142. Photographs were taken with equivalent exposure times of tag-positive clusters in random fields of transfected Sol8 cultures, and of COS cell cultures that were transfected with only tagged β subunit–containing AChR. Negatives were scanned with an AgfaArcusII using Adobe Photolook, and the relative pixel density was determined using the NIH Image program.

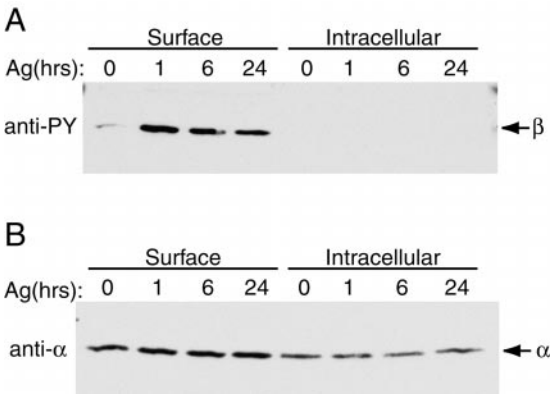

Figure 1. Agrin-induced phosphorylation of the β subunit occurs only on surface AChR. (A) Sol8 myotubes were treated with 500 nM neural agrin for 1, 6, and 24 h. Surface and intracellular AChR were then isolated separately from cell extracts and immunoblotted with antiphosphotyrosine antibodies 4G10 and PY20. Agrin induced a robust tyrosine phosphorylation of the β subunit on surface but not intracellular AChR. The level of phosphorylation was maximal after 1 h of agrin treatment, and then decreased to slightly lower levels by 6 and 24 h. (B) Reprobing the same immunoblot with an anti–α subunit antibody (mAb210) showed that there is a significant pool of intracellular AChR.

Results

Cellular Site and Time Course of AChR β-Subunit Phosphorylation

Agrin-induced phosphorylation of the AChR β subunit could contribute to receptor localization at the synapse by regulating one or more processes such as the insertion, clustering, anchoring, and/or stability of the AChR. To begin to define the role(s) of this signaling event, we first tested whether agrin-induced phosphorylation occurs on AChR localized only on the myotube surface or also in intracellular receptor pools. Secondly, we examined the time course of phosphorylation in relation to the formation of AChR clusters. To address these questions, we treated Sol8 mouse myotube cultures with soluble neural agrin for 1, 6, and 24 h and isolated surface and intracellular AChR pools. Surface receptor was selectively isolated by incubating the live myotubes with saturating concentrations of biotinylated α-bungarotoxin and purifying the bound receptor from cell extracts with avidin beads. Intracellular, fully assembled receptor was then isolated from the same extracts using α-bungarotoxin conjugated directly to beads. To assay agrin-induced β subunit phosphorylation, we immunoblotted the isolated AChR with a mix of the antiphosphotyrosine antibodies and, to assay levels of AChR, we reprobed the immunoblot with an α subunit antibody.

When we compared the levels of surface and intracellular AChR in Sol8 myotubes (Fig. 1 B), we found that a sizeable fraction (34 ± 5%, mean ± SD, $n = 4$) of the total receptor was intracellular, as observed previously (Devreotes et al., 1977; Gu et al., 1989). This intracellular receptor pool could potentially play a role in AChR localization if it were targeted specifically to agrin-induced clusters. We found that only surface AChR were tyrosine phosphorylated on the β subunit in response to agrin, however (Fig. 1 A). This was the case at all time points, ranging from initial agrin signaling (1 h) to well after the formation of AChR clusters (24 h). Thus, AChR phosphorylation is unlikely to directly regulate the insertion of receptors from the intracellular pool.

We also found that the time course of agrin-induced AChR β subunit phosphorylation in Sol8 myotubes was similar to that previously reported for C2 myotubes (Ferns et al., 1996). Neural agrin (500 pM) induced a rapid tyrosine phosphorylation of the β subunit that reached a maximum after 1 h, and then the level of phosphorylation declined with more prolonged agrin treatment (Fig. 1 A). In the present study, however, we used a combination of antiphosphotyrosine antibodies (4G10 and PY20) and still observed some tyrosine phosphorylation at 24 h (39 ± 20% of maximum, mean ± SD, $n = 4$). Reprobing of the immunoblots with an antibody to the AChR alpha subunit confirmed that levels of receptor were similar for each time point (Fig. 1 B). The time course of agrin-induced phosphorylation is slightly offset from that of AChR clustering, which is first observed after 2–4 h, peaks at 6 h, and then declines slightly (Ferns et al., 1996). Thus, phosphorylation of the β subunit occurs before clustering, and then persists at a somewhat lower level after clusters have formed. We therefore find that tyrosine phosphorylation occurs only on surface AChR, and that it correlates with both the formation and maintenance of AChR clusters. Together, these findings suggest that β subunit phosphorylation is most likely to regulate the surface aggregation and/or cytoskeletal anchoring of the AChR.

Assembly and Expression of AChR Containing Tyrosine-minus β Subunit

To test the role of AChR β subunit phosphorylation directly, we used a strategy of expressing mutant forms of the β subunit that lacked the relevant tyrosines in the major intracellular loop. To do this, we tagged the β subunit

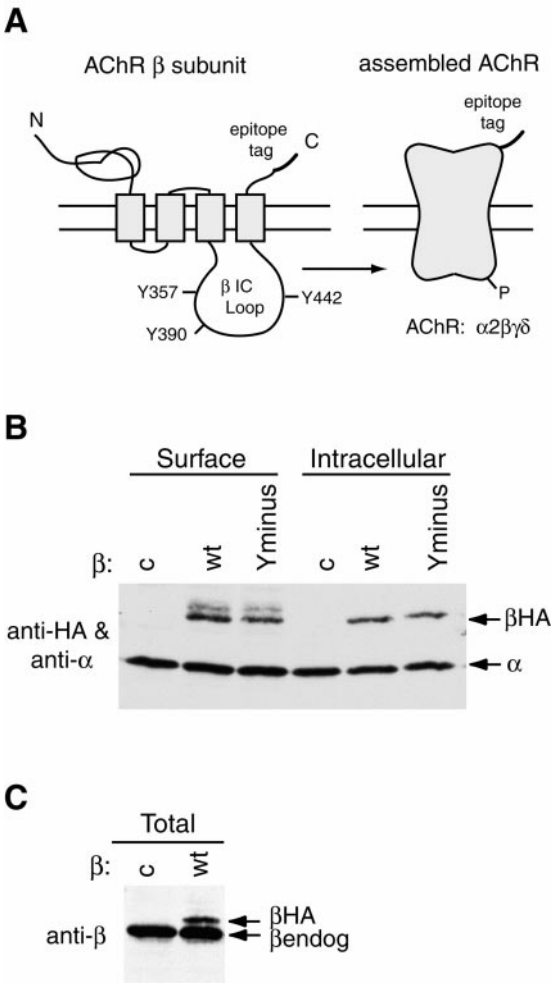

Figure 2. Assembly and expression of AChR containing tyrosine-minus β subunit. (A) The AChR β subunit was tagged at the COOH terminus with either an HA or 142 epitope. The tagged β subunit assembles together with the α, γ, and δ subunits to form AChR that can be distinguished from the endogenous AChR in muscle cells by its epitope tag. To test the role of tyrosine phosphorylation the three tyrosines in the β-subunit major intracellular loop were replaced by phenylalanines, either singly or in combination (Yminus). (B) Surface and intracellular pools of AChR were isolated from Sol8 myotubes that were transfected with either HA-tagged wt- or Yminus-β subunit, or from untransfected control myotubes. Immunoblotting with an anti–HA antibody shows that both HA-tagged wt- and Yminus-β subunits assemble into AChR, and that they are expressed at similar levels both in the surface and intracellular receptor pools. Simultaneous immunoblotting for the α subunit confirms that similar levels of AChR were present in each culture. (C) Total AChR was isolated from either control myotubes or from myotubes transfected with HA-tagged wt-β subunit. Immunoblotting with an anti–β subunit antibody (mAb124) shows that the HA-tagged β subunit is distinguishable as a band slightly above the endogenous β subunit.

at the COOH-terminal extracellular tail with a hemagglutinin epitope, and then substituted the three tyrosine sites in the major intracellular loop with phenylalanines (βYminus) (Fig. 2 A). Tagged β subunit constructs in wild-type (wt) or Yminus form were expressed in Sol8 mouse myotubes by transient transfection, and their expression was

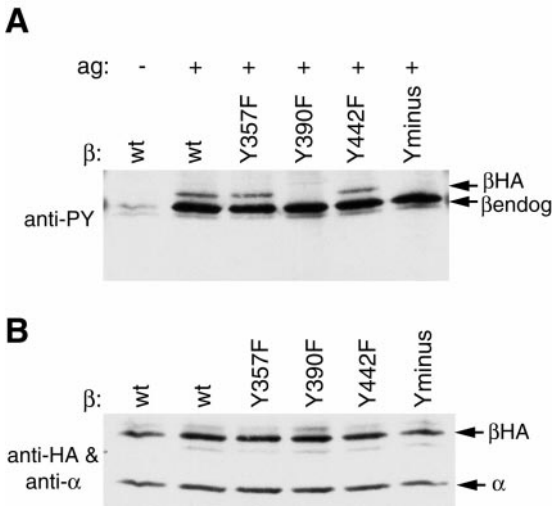

Figure 3. Agrin induces phosphorylation of the β subunit at tyrosine 390. (A) Sol8 cultures transfected with HA-tagged wt, Y357F, Y390F, and Y442F β subunit constructs were treated with agrin for 1 h, and then total AChR was isolated from cell extracts. Immunoblotting with antiphosphotyrosine antibodies (4G10 and PY20) shows that agrin induces phosphorylation of both endogenous and HA-tagged wt-β subunit. The Y390F mutation totally abolished this phosphorylation, whereas the Y357F and Y442F substitutions had no effect. (B) The immunoblot was reprobed with anti–HA and anti–α subunit antibodies to confirm that all of the β subunit variants were expressed at similar levels, and that equal amounts of AChR were isolated from all of the cultures.

assessed by immunoblotting of α-bungarotoxin–mediated precipitation of surface and intracellular AChR. Immunoblotting for the HA epitope showed that in transfected cultures both surface and intracellular AChR contained tagged β subunit (Fig. 2 B), indicating that the introduced β subunit assembles into AChR. In addition, we found that the tagged wt- and Yminus-β subunit were expressed at comparable levels in both the intracellular and surface pools of AChR. On average, 62 ± 8% of the βwt- and 60 ± 10% of the βYminus-containing AChR were expressed on the myotube surface (mean ± SD; $n = 10$). To determine the proportion of the total AChR in the transfected cultures that contained tagged β subunit, we immunoblotted for the β subunit (Fig. 2 C). The HA-tagged β subunit was distinguishable as a band slightly increased in size as compared with the endogenous β subunit, and typically made up 10–20% of the total receptor population. Together, these findings demonstrate that β subunit tyrosine phosphorylation is not required for assembly of the AChR, or for expression of the AChR on the myotube surface. Agrin-induced phosphorylation of the β subunit, therefore, does not regulate the insertion of intracellular pools of receptor into the surface membrane.

Agrin Induces Phosphorylation of the β Subunit at Tyrosine 390

The AChR β subunit has three putative tyrosine phosphorylation sites in the major intracellular loop between transmembrane domains 3 and 4 (Fig. 2 A). To identify the site(s) of agrin-induced tyrosine phosphorylation, we made single mutations of each of the tyrosines, expressed

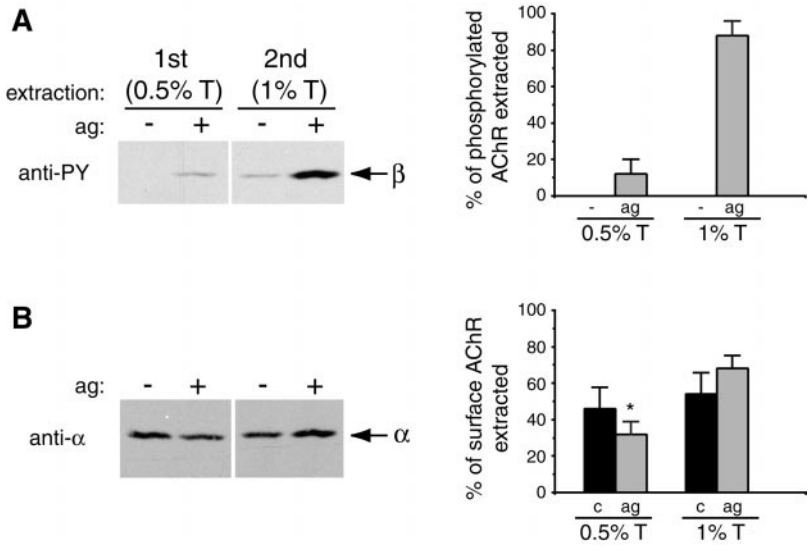

Figure 4. Phosphorylated AChR are less detergent extractable. (A) Control and agrin-treated Sol8 cultures were incubated with biotinylated α-bungarotoxin and sequentially extracted, first with low detergent buffer (0.5% Triton X-100), and then the insoluble pellet was reextracted with high detergent buffer (1% Triton X-100). Solubilized, surface AChR was isolated from each fraction and immunoblotted with antiphosphotyrosine antibodies. Quantification of the proportion of phosphorylated AChR that was recovered in the first and second extractions is shown in the associated histogram ($n = 8$). After agrin treatment, AChR containing tyrosine phosphorylated β subunit was predominantly solubilized in the second, less-extractable fraction (88 ± 8%, mean ± SD, $n = 8$). (B) The immunoblot was reprobed with an anti–α subunit antibody to determine the levels of total AChR, and the proportion of receptor that was recovered in each extraction is shown in the associated histogram ($n = 8$). Significantly less AChR was solubilized in the first extraction (0.5% Triton X-100) in agrin-treated cultures (32 ± 7%) than in control (46 ± 11%, mean ± SD, $*P = 0.0006$, paired Student's t test, $n = 8$). A correspondingly increased amount of receptor from agrin-treated cultures was then recovered in the second, less extractable fraction (1% Triton X-100).

the constructs in Sol8 myotubes, and treated with agrin. The AChR was then isolated from cell extracts and immunoblotted with antiphosphotyrosine antibodies. We found that agrin induces a pronounced tyrosine phosphorylation of the endogenous β subunit and the tagged wt β subunit (Fig. 3 A). Mutation of all three tyrosines (Yminus) completely abolished this agrin-induced phosphorylation of the β subunit, as did an individual mutation of tyrosine 390. In contrast, substitution of the two other tyrosines (357 and 442) had no effect on the level of phosphorylation (Fig. 3 A). All of the HA-tagged mutant β subunit constructs were expressed at similar levels (Fig. 3 B), and immunoblotting for the α subunit confirmed that equal amounts of AChR were present in all of the cultures (Fig. 3 B). Thus, agrin normally induces phosphorylation at tyrosine 390 in the β subunit intracellular loop.

β-Subunit Phosphorylation Correlates with Linkage of the AChR to the Cytoskeleton

It has been shown previously that clustered AChR are less mobile in the plane of the membrane and less detergent extractable than dispersed AChRs, suggesting that clustering involves the linkage of the AChR to the cytoskeleton (Prives et al., 1982; Stya and Axelrod, 1983; Podleski and Salpeter, 1988). Agrin-induced phosphorylation of the β subunit correlates with this decrease in the mobility and detergent extractability of the AChR (Meier et al., 1995), and we therefore tested whether it might regulate linkage to the cytoskeleton using the tyrosine-minus forms of the β subunit. As a starting point, we confirmed that agrin induces changes in the extractability of the endogenous AChR in the mouse Sol8 muscle cells. To do this, myotube cultures were treated with agrin and biotinylated α-bungarotoxin to label the surface AChR. The cultures were then sequentially extracted, first with a low detergent buffer (0.5% Triton X-100), and then the insoluble pellet fraction was reextracted in a high detergent buffer (1% Triton X-100). The solubilized, surface AChR was isolated independently from the two extractions and immunoblotted with antiphosphotyrosine antibodies to assay the distribution of phosphorylated AChR in each (Fig. 4 A). The proportion of phosphorylated AChR in each fraction was quantified in several such experiments and is shown in the associated histogram. After agrin treatment, which induces AChR β subunit phosphorylation, we found that little of the tyrosine-phosphorylated AChR is solubilized in the low-detergent extraction, but rather that 88% is recovered in the second, less-extractable fraction (Fig. 4 A). Similarly, the trace levels of tyrosine-phosphorylated AChR in control cultures is also recovered in the less-extractable fraction (not shown in histogram).

To assay the total levels of AChR recovered in each of the extractions, we reprobed the immunoblot with an anti–α subunit antibody (Fig. 4 B). Although we see some variability between experiments, in control cultures we found that roughly half of the AChR is recovered in the first extraction (46 ± 11%, mean ± SD, $n = 8$). In agrin-treated cultures, however, we found that a significantly lower proportion of the AChR was solubilized in the first, low-detergent extraction (32 ± 7%, mean ± SD; $P = 0.0006$, paired Student's t test, $n = 8$). We also assessed the agrin-induced decrease in extractability within each experiment. On average, agrin reduced the amount of surface AChR that is solubilized in the first extraction by 30 ± 9% as compared with control (mean ± SD; $P < 0.0001$, one sample Student's t test, $n = 8$).

Thus, agrin treatment results in tyrosine-phosphorylated AChR that is partitioned to the less-extractable fraction, and this correlates with a reduction in the amount of AChR that is solubilized in the first extraction. The shift in total AChR towards the less-extractable fraction is less pronounced than the partitioning observed for phosphorylated AChR, and suggests that only a proportion of the receptor is phosphorylated in response to agrin. Together, these findings confirm that agrin reduces the extractability of the AChR, and demonstrate a strong correlation be-

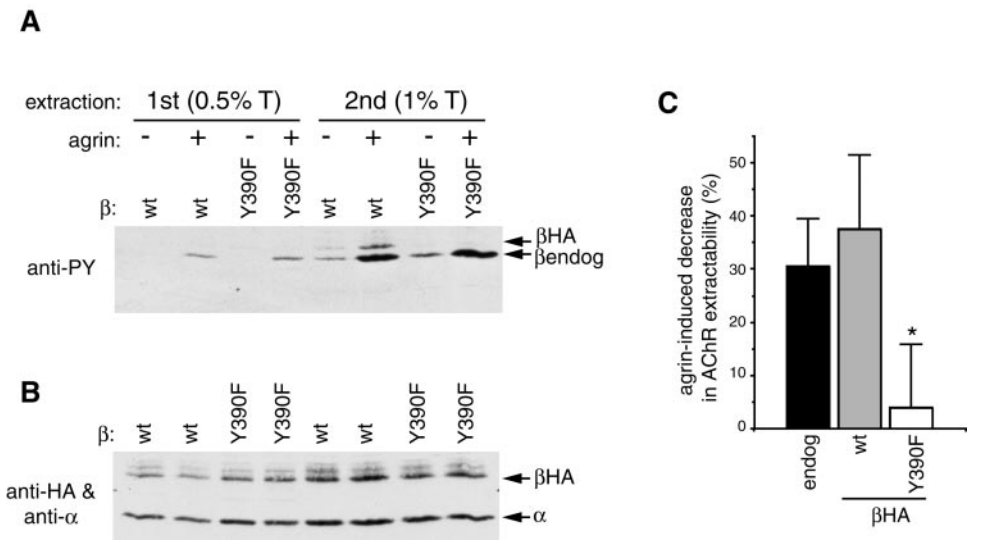

Figure 5. Phosphorylation of the AChR β subunit regulates linkage to the cytoskeleton. (A) Control and agrin-treated Sol8 cultures expressing HA-tagged wt- or Y390F-β subunit were sequentially extracted, and the solubilized AChR was isolated from each fraction. Phosphotyrosine immunoblotting of the surface AChR shows that both endogenous and tagged-wt AChR were phosphorylated in response to agrin treatment, and that the phosphorylated receptor was only recovered in the second extraction (1% Triton X-100). (B) The same immunoblot was probed with a mix of anti–HA and anti–α subunit antibodies to detect HAβ-AChR and total AChR, respectively. After agrin treatment, less βwt-AChR and total AChR were solubilized in the first extraction compared with control, reflecting a decrease in extractability. In contrast, agrin treatment did not affect the levels of βY390F-AChR that were solubilized in the first extraction. (C) Quantification of the agrin-induced decrease in the amount of AChR extracted by low detergent buffer. Agrin treatment caused a significant decrease in the extractability of endogenous AChR (30 ± 9%, mean ± SD, $P < 0.0001$, one sample Student's *t* test, $n = 8$) and HA-tagged βwt-AChR (37 ± 14, mean ± SD, $P = 0.0005$, one sample Student's *t* test; $n = 7$). No change in extractability was observed for βY390F-AChR after agrin treatment (4 ± 12%, mean ± SD, $n = 8$), indicating that phosphorylation at this site regulates the interaction with the cytoskeleton.

tween tyrosine-phosphorylation of AChR β subunit and linkage to the cytoskeleton.

AChR β Subunit Phosphorylation Regulates Anchoring to the Cytoskeleton

The agrin-induced decrease in AChR extractability correlates strongly with tyrosine phosphorylation of the AChR and can be interpreted as evidence that β subunit phosphorylation regulates the interaction of the AChR with the cytoskeleton. Alternatively, AChR that are already attached to the cytoskeleton could be more likely to be phosphorylated by kinases that are activated by agrin and present in this cellular compartment. In this case, phosphorylation would not induce the interaction with the cytoskeleton, but would merely be a consequence of a preexisting interaction. To directly test the role of the agrin-induced β subunit phosphorylation in regulating the interaction of the AChR with the cytoskeleton, we monitored the change in extractability of AChRs containing either tagged wt, or Y390F -β subunit. To do this, we performed the sequential extraction on transfected cultures and assayed levels of wild-type or mutant AChR in each fraction by immunoblotting for the HA-tagged β subunit (Fig. 5 B). The behavior of the total population of surface AChR was monitored by immunoblotting for the α subunit as shown before (Fig. 5 B), and the distribution of the phosphorylated receptor was followed by immunoblotting with antiphosphotyrosine antibodies (Fig. 5 A). We found that AChR containing wild-type tagged β subunit behaved in an identical manner to AChR containing the endogenous β subunit. Both the endogenous and tagged β subunit were phosphorylated in response to agrin, and the phosphorylated receptor was predominantly recovered in the less-extractable fraction (Fig. 5 A). Agrin treatment also decreased the amount of endogenous and wild-type tagged β subunit AChR that was solubilized in the low detergent extraction (Fig. 5 B). In contrast, AChR containing Y390F-β subunit was not phosphorylated by agrin, as was endogenous receptor (Fig. 5 A), and the amount of βY390F-AChR that was solubilized in the low-detergent extraction did not decrease with agrin treatment (Fig. 5 B). The combined results of several such experiments show that agrin significantly decreased the extractability of the tagged wt-AChR in low detergent buffer (37 ± 14%, mean ± SD, $P < 0.005$, one sample Student's *t* test, $n = 7$), but no significant change in extractability was observed with the tagged mutant-AChR (4 ± 12%, mean ± SD, $n = 7$; Fig. 5 C). Thus, AChR containing Y390F-β subunit behaves essentially like total AChR in control cultures, which is predominantly nonphosphorylated, and the mutant receptor clearly does not become linked to the cytoskeleton after agrin treatment. These results show that agrin-induced tyrosine phosphorylation of the β subunit regulates the interaction of the AChR with the cytoskeleton, and this could play an important role in synaptic localization of the receptor.

AChRs Containing Tyrosine-minus β Subunit Have Reduced Clustering Ability

To test whether β subunit phosphorylation is required for AChR clustering, we assayed the ability of AChR containing tagged wt, Y390F, and Yminus-β subunits to form agrin-induced AChR clusters. For these experiments, we used β subunit constructs that were tagged at the COOH terminus with the 142 epitope (Das and Lindstrom, 1991) rather than with HA. This epitope proved to be more accessible for immunostaining, but otherwise the 142-tagged receptor behaved exactly like HA-tagged forms of recep-

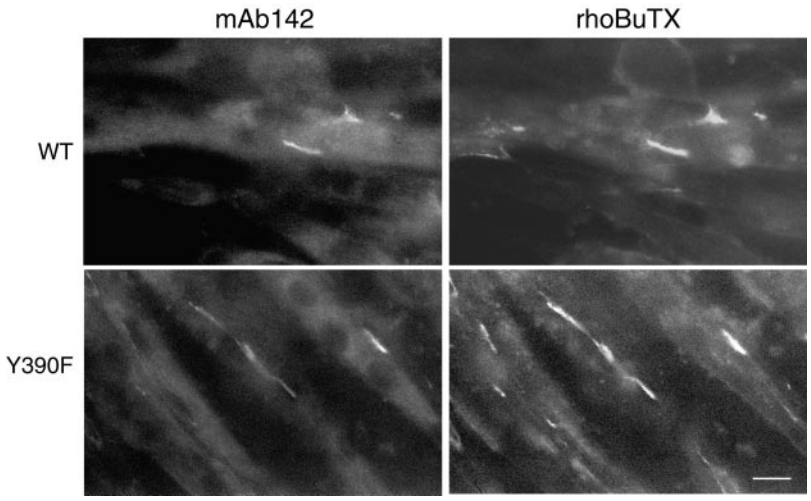

Figure 6. AChR containing Y390F-β subunit contributes to agrin-induced clusters. Sol8 cultures expressing tagged wt- and Y390F-β subunit were treated with agrin for 24 h to induce clustering, and then stained with mAb142 to detect tagged AChR, and with rhodamine bungarotoxin (rhoBuTX) to detect total surface AChR. A proportion of the AChR clusters that labeled with rhoBuTX also costained for the tagged wt- and Y390F-AChR. Thus, AChR containing Y390F-β subunit can contribute to clusters along with the endogenous AChR. Scale bar, 15 μm.

tor (see below). To examine clustering, cultures transfected with the different β subunit constructs were treated with agrin for 24 h, and then stained for the AChR with rhodamine-conjugated α-bungarotoxin and for the tagged receptor with mAb142. In control cultures that were transfected with tagged wt-β subunit, we found that a proportion of the α-bungarotoxin–labeled AChR clusters costained for tagged receptor (Fig. 6). Thus, tagged wt-AChR clusters normally in response to agrin. Surprisingly, tagged Y390F (Fig. 6) and Yminus-AChR (data not shown) also contributed to a proportion of the AChR clusters, and are therefore capable of forming AChR clusters together with the endogenous receptor. Thus, tyrosine phosphorylation of the β subunit is not an absolute requirement for recruitment of an individual AChR to a cluster.

One complication in these experiments is the presence of endogenous AChR in the transfected myotubes that will be normally phosphorylated in response to agrin. We therefore assessed the relative contribution of tagged and endogenous receptor in clusters by comparing the intensity of 142 tag and α-bungarotoxin (total AChR) staining. This was compared with parallel staining of COS cells transfected with tagged AChR where there is a constant 1:1 ratio of tag and α-bungarotoxin staining. We found that the ratio was considerably lower in transfected myotubes, and that tagged receptor always made up <50% of the total receptor in a cluster, with the mean level being 14% (n = 36 clusters).

We then examined whether βY390F-AChR formed agrin-induced AChR clusters as efficiently as wild-type receptor. Myotubes transfected with either tagged wt or Y390F-β subunit were treated with agrin, immunostained as above, and the number of tag positive clusters was determined for each. Agrin treatment for 5 h induced significantly more clusters containing βwt-AChR than βY390F-AChR (Fig. 7 A). On average, we find that the number of AChR clusters containing βY390F-AChR is reduced 49% compared with βwt-AChR (P = 0.0005; one sample Student's t test, n = 5). Similarly, after agrin treatment for 24 h, the number of βY390F-AChR clusters was 35% less than βwt levels (P = 0.005, one sample Student's t test, n = 6), indicating that there is a continuing rather than transient defect in clustering of the mutant receptor (Fig. 7 B). To ensure that the reduced level of agrin-induced clustering of βY390F-AChR was not due to lower levels of expression, we performed parallel transfections, and isolated and immunoblotted the tagged receptor. These immunoblots showed that βY390F-AChR is expressed at identical levels to βwt-AChR (104 ± 14% of βwt-AChR levels, mean ± SD, n = 5). Thus, AChR containing Y390F β subunit has a reduced ability to form AChR clusters, even in the presence of endogenous (wild-type) receptor. This finding demonstrates that agrin-induced tyrosine phosphorylation of the AChR β subunit has a direct, regulatory effect on AChR clustering.

Finally, we compared the stability of clusters containing βwt- and βY390F-AChR. Transfected myotubes were treated with agrin for 18 h to induce AChR clustering, and then agrin was removed for a further 6 h to initiate cluster dispersal. We found that the number of βwt-AChR clusters decreases ~40% during this period. Although fewer Y390F-AChR clusters are induced by the agrin treatment, they exhibit a similar decrease upon agrin removal. Clusters containing βwt- and βY390F-AChR therefore disperse with a similar time course.

Discussion

In this study, we tested the role of agrin-induced tyrosine phosphorylation of the AChR in the insertion, cytoskeletal anchoring, and clustering of the AChR. We find that AChR β subunit phosphorylation regulates linkage to the cytoskeleton and contributes to receptor clustering, and propose that this plays an important role in the synaptic localization of the AChR.

AChR Insertion

Our mutagenesis experiments on the mouse AChR β subunit show that agrin induces phosphorylation of tyrosine residue 390 in the major intracellular loop. This tyrosine phosphorylation consensus site is conserved in rat, mouse, chick, and torpedo β subunits, and was previously shown to be phosphorylated in adult *Torpedo* electric organ (Wagner et al., 1991) and in mouse AChR expressed in heterologous cells together with rapsyn and MuSK (Gillespie et al., 1996; Qu et al., 1996). We detected agrin-

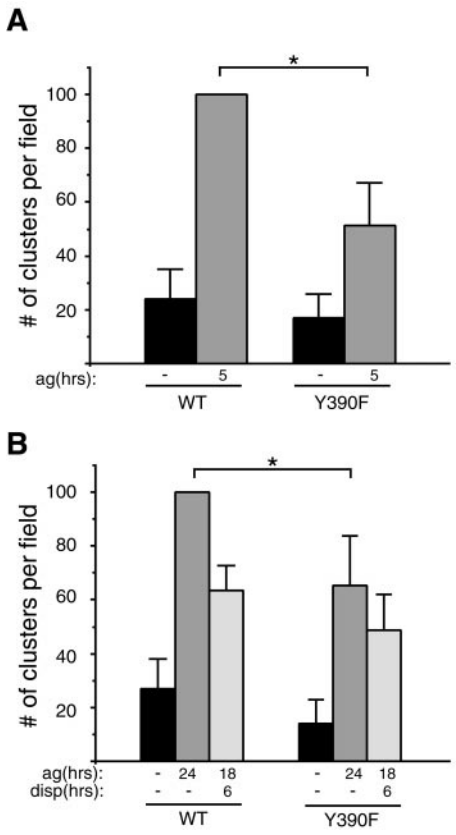

Figure 7. AChR containing Y390F-β subunit cluster less efficiently. (A) Quantification of agrin-induced clustering of βwt- and Y390F-AChR. Myotubes expressing wt- and Y390F-β subunit were treated with agrin for 5 h, and the number of clusters that contained tagged AChR was determined in random fields, and expressed as a percentage of maximum. Agrin-induced clustering was significantly lower for βY390F-AChR than βwt-AChR (51 ± 16% of wt levels, mean ± SD, $P = 0.0024$, one sample Student's *t* test, $n = 5$). (B) Myotubes expressing wt- and Y390F-β subunit were treated with agrin for the indicated periods to induce maximal AChR clustering, and then agrin was removed for 6 h to allow cluster dispersal. After a 24-h agrin treatment, there were significantly less βY390F-AChR clusters than βwt control (65 ± 18% of wt levels, mean ± SD, $P = 0.005$, one sample Student's *t* test, $n = 6$). After agrin removal, AChR clusters containing wt- and Y390F-β subunit dispersed at similar rates.

induced phosphorylation of the β subunit in cell-surface, but not intracellular pools of AChR. Moreover, AChR containing wild-type and tyrosine-minus forms of tagged β subunit were expressed at similar levels, and had identical proportions of surface and intracellular receptors. Together, these findings indicate that β subunit phosphorylation is not involved in insertion of the AChR into the plasma membrane, and therefore this signaling event is unlikely to target the AChR to developing receptor clusters.

AChR Linkage to Cytoskeleton

Agrin-induced phosphorylation of surface AChR could also mediate synaptic localization by anchoring the receptor to the cytoskeleton. Consistent with this idea, we found that agrin treatment reduced the detergent extractability of the AChR in Sol8 mouse myotubes, and this has been shown previously to correlate with an immobilization of the AChR in the membrane (Meier et al., 1995; Wallace, 1995). In addition, we found that AChR containing tyrosine-phosphorylated β subunit was significantly less extractable than nonphosphorylated receptor, consistent with β subunit phosphorylation regulating anchorage to the cytoskeleton. Alternatively, this correlation could stem from agrin-induced phosphorylation of other proteins such as other AChR subunits (Qu et al., 1990; Qu and Huganir, 1994; Meier et al., 1995), or to a preferential phosphorylation of AChR that is already linked to the cytoskeleton. We therefore used mutant forms of the β subunit to test these possibilities directly, and found that agrin reduces the extractability of AChR containing wild-type but not tyrosine390-minus β subunit. This finding demonstrates that agrin-induced phosphorylation of β subunit tyrosine 390 is required for linkage of the AChR to the postsynaptic cytoskeleton.

Phosphorylation could regulate anchoring of the AChR through a direct interaction with a cytoskeletal protein, or indirectly via a linker protein. Direct interactions of neurotransmitter receptors and cytoskeletal proteins have been demonstrated in the central nervous system (CNS), where, for example, the *N*-methyl-D-aspartate NR1 subunit binds to neurofilament subunit NF-L, yotiao, α-actinin, and spectrin (reviewed in Sheng and Pak, 1999, 2000). Interestingly, phosphorylation of the NR1 C1 region affects NR1 distribution in heterologous cells, and may do so by regulating the binding of α-actinin to this tail region. Generally, however, it appears that receptors are anchored via a wide variety of linker proteins (Sheng and Pak, 1999, 2000; Garner et al., 2000). For example, synaptic localization of the glycine receptor is dependent on gephryin (Feng et al., 1998), a protein that binds the receptor intracellular loop region (Meyer et al., 1995) and anchors it to the microtubule cytoskeleton (Kirsch et al., 1991).

At the neuromuscular synapse, anchorage of the AChR could well be mediated by rapsyn, which is known to be closely associated with the AChR β subunit (Burden et al., 1983). Rapsyn is required for the localization of the AChR at the synapse (Gautam et al., 1995), and extraction of rapsyn increases the mobility of the AChR in the membrane (Rousselet et al., 1982). Moreover, rapsyn has been reported to bind actin (Walker et al., 1984) and to interact with the dystrophin-glycoprotein complex that is anchored to the cytoskeleton by utrophin (Apel et al., 1995; Cartaud et al., 1998). We are currently investigating, therefore, whether β subunit phosphorylation modulates the interaction of rapsyn and the AChR, or perhaps the interaction of a preexisting rapsyn/AChR complex with the cytoskeleton. Other mechanisms are also consistent with our data, and we cannot discount the possibility that AChR phosphorylation might indirectly modify protein interactions that anchor the AChR. For example, tyrosine phosphorylation of the β subunit could recruit kinases through SH2 or PTB-domain interactions, and these kinases may then phosphorylate linker or cytoskeletal proteins involved in anchoring of the AChR.

The cytoskeleton is known to be highly specialized in the postsynaptic region, and several cytoskeletal proteins colocalize with the AChR and rapsyn at the tops of the junctional folds (Froehner, 1991). Of particular interest is

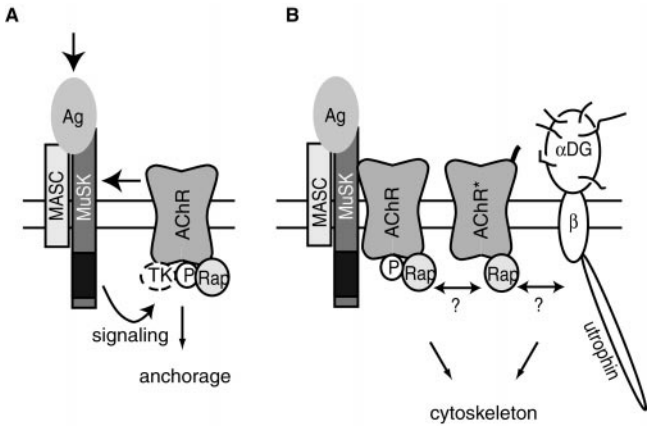

Figure 8. Model for regulatory role of β subunit phosphorylation in AChR localization. Motoneuron-derived agrin activates MuSK and triggers a localized signaling cascade that induces tyrosine phosphorylation of the β subunit of nearby AChR (A). This phosphorylation results in linkage of the AChR to the cytoskeleton and aids in progressively clustering the AChR at the nascent synapse (B). Cytoskeletal anchoring could occur via rapsyn-mediated linkage to the cytoskeleton or indirectly by association with membrane proteins such as α and β dystroglycan. Other interactions may also contribute to AChR aggregation, and these may be responsible for the recruitment of AChR containing tyrosine-minus β subunit to clusters (AChR*). MASC, myotube-associated specificity component; rap, rapsyn; TK, tyrosine kinase; P, β phosphorylation; α and β DG, α and β dystroglycan.

the actin cytoskeleton since the blockage of actin polymerization with Latrunculin B has been shown to inhibit agrin-induced AChR clustering (Dai et al., 2000; Weston et al., 2000). In addition, various components of the dystrophin-glycoprotein complex were recently implicated in several aspects of synaptic maturation, including maintenance of the postsynaptic density of AChR (Cote et al., 1999; Adams et al., 2000; Grady et al., 2000).

Although these studies support our contention that the AChR is anchored to the cytoskeleton, the change in detergent solubility of the AChR could potentially reflect other mechanisms, such as recruitment to lipid rafts (Simons and Ikonen, 1997). Lipid rafts are membrane microdomains rich in sphingolipids and cholesterol, which are typically insoluble in Triton X-100 at 4°C, but are solubilized with SDS (Brown and Rose, 1992; Simons and Ikonen, 1997). In our experiments, however, we found that the AChR is solubilized efficiently with Triton X-100, and that no additional receptor is extracted with SDS-containing buffer. We therefore believe that an agrin-induced association of the AChR with lipid rafts is unlikely.

AChR Aggregation

AChR containing tyrosine-minus β subunit not only failed to be anchored to the cytoskeleton but also showed a partial defect in receptor clustering. We observed significantly less agrin-induced clusters containing βY390F-AChR than βwt-AChR at early stages of cluster formation (~50% decrease). Moreover, this reduced contribution to AChR clusters was maintained even after clustering had reached maximal levels at 24 h. The difference in receptor clustering was not due to differences in the assembly of the β subunit constructs into AChR, and identical levels of βwt- and βY390F-AChR were expressed on the myotube surface. Thus, our findings demonstrate that agrin-induced tyrosine phosphorylation of the β subunit also regulates clustering of the AChR.

Although AChR containing mutant β subunit showed a decreased clustering ability, we found that it contributed along with the endogenous receptor to many clusters. This is in agreement with an earlier study that showed that mouse AChR containing tyrosine-minus β subunit coclustered with the endogenous AChR in transfected chick myotubes (Meyer and Wallace, 1998). Together, these findings indicate that phosphorylation of the β subunit of an individual AChR is not an absolute requirement for aggregation of that AChR.

Our finding of a partial defect in clustering of tyrosine-minus AChR is consistent with at least two different mechanisms for the clustering process. One possibility is that more than one agrin-induced process contributes to clustering of the receptor and, by mutating β tyrosine 390, we are only disrupting one of these. Clustering would therefore still occur in the absence of β subunit tyrosine phosphorylation, but with decreased efficiency. A second possibility is that agrin-induced β subunit phosphorylation is required for clustering, but that this was partially obscured by the presence of endogenous receptor in our experimental system. We found that tagged AChR containing tyrosine-minus β subunit made up less than half of the total receptor in a given AChR cluster, and phosphorylation of the endogenous receptor may be sufficient to initiate clustering of both wild type and mutant. In either case, coclustering of AChR containing tyrosine-minus β subunit presumably reflects either a passive trapping of the mutant receptor or additional interactions with other postsynaptic proteins (Fig. 8 B). Consistent with the second idea, the AChR has been found to be constitutively associated with both MuSK and dystroglycan in muscle cells (Fuhrer et al., 1999). Moreover, rapsyn coclusters the AChR with MuSK or dystroglycan in heterologous cells, and it can cluster AChR in the absence of agrin/MuSK signaling in nonmuscle cells (Apel et al., 1995, 1997; Gillespie et al., 1996). Thus, interactions of the AChR in addition to those involving the β loop might help localize the AChR in clusters. A definitive test of the requirement for β subunit phosphorylation in AChR clustering will require expression of tyrosine-minus forms of the β subunit in a null background.

Regulatory Role of β Subunit Phosphorylation in AChR Localization

We have found that agrin-induced tyrosine phosphorylation of the β subunit regulates AChR anchoring and clustering, and this is likely to play a critical role in the postsynaptic localization of the AChR at the developing neuromuscular junction. Motorneuron-derived agrin is thought to initiate this process by activating and clustering the MuSK receptor in the muscle membrane, forming a primary scaffold to which synaptic proteins are recruited (Apel et al., 1997). A straightforward possibility is that this localized MuSK signaling would lead to tyrosine phos-

phorylation of nearby AChR, and this in turn would anchor the AChR to the underlying cytoskeleton (Fig. 8 A). Thus, the AChR would be progressively clustered at the nascent synapse (Fig. 8 B), reflecting a direct mechanistic link between anchoring and localization. Consistent with such an idea, we found that agrin/MuSK signaling induces a rapid tyrosine phosphorylation of the AChR β subunit that results in immediate anchorage to the cytoskeleton, and that this occurs before the onset of detectable AChR clustering. Agrin-induced AChR β subunit phosphorylation may not be sufficient in itself to elicit clustering, however (Glass et al., 1997). Potentially, several mechanisms might combine to induce AChR clustering, with β subunit phosphorylation presumably contributing to the process by anchoring and immobilizing the AChR in the postsynaptic region (Fig. 8 B).

In addition to regulating the initial aggregation of the AChR, β subunit phosphorylation might also play a role in stabilizing developing AChR clusters. Consistent with this, we found that some level of β subunit phosphorylation was maintained well after AChR clusters had formed. A functional role for this prolonged phosphorylation is suggested by the finding that agrin-induced AChR clusters in cultured myotubes can be readily dispersed by staurosporine, which is known to inhibit β subunit phosphorylation (Wallace, 1994; Ferns et al., 1996). Thus, cytoskeletal anchoring or clustering interactions mediated by β subunit phosphorylation may be required to maintain AChR clusters, at least at early stages of development. The postsynaptic aggregate of AChR is known to become progressively stabilized during development (Dennis, 1981; Slater, 1982), and presumably additional mechanisms contribute at later stages.

Interestingly, cytoskeletal anchoring of the AChR at the mature neuromuscular junction has been suggested both to localize the receptor and to regulate its turnover time (reviewed in Salpeter, 1999). Potentially, β subunit phosphorylation might play some role in these processes as previous studies have shown that AChR from adult *Torpedo* electric organ and rat neuromuscular junctions are tyrosine phosphorylated on the β subunit (Qu et al., 1990; Wagner et al., 1991). If this is the case, it will be important to determine whether agrin/MuSK signaling is maintained at some level at the mature synapse, or if AChR β subunit phosphorylation is maintained by some other signaling event.

Finally, our findings suggest several parallels with the localization of neurotransmitter receptors at CNS synapses. In the CNS, different classes of ligand-gated ion channels appear to be anchored to the postsynaptic cytoskeleton by specific interactions, either with linker proteins or with cytoskeletal proteins themselves (reviewed in Garner et al., 2000; Sheng and Pak, 2000). The interactions of receptors and their intracellular binding partners is thought to stably anchor the receptors at synapses, and in some cases may regulate their insertion, clustering, and stability. Moreover, an emerging hypothesis is that regulation of these interactions plays an important role in localizing receptors at developing synapses and in modifying their distribution during synaptic plasticity. In this regard, many of the receptor interactions with linker or cytoskeletal proteins have been proposed to be regulated by intracellular signaling events, including modification of binding by phosphorylation or competitive binding of calcium/calmodulin (Sheng and Pak, 2000). Presumably, this form of regulation could be triggered by inductive signals during initial synaptogenesis or by neural activity at mature synapses. Our findings provide direct support for this hypothesis by showing that agrin signaling and the resulting phosphorylation of the AChR regulates postsynaptic localization, and that this occurs, at least in part, by regulating linkage of the receptor to the cytoskeleton.

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