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AChR phosphorylation and aggregation induced by an agrin fragment that lacks the binding domain for α-dystroglycan

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Agrin induces both phosphorylation and aggregation of nicotinic acetylcholine receptors (AChRs) when added to myotubes in culture, apparently by binding to a specific receptor on the myotube surface. One such agrin receptor is α-dystroglycan, although binding to α-dystroglycan appears not to mediate AChR aggregation. To determine whether agrin-induced AChR phosphorylation is mediated by α-dystroglycan or by a different agrin receptor, fragments of recombinant agrin that differ in affinity for α-dystroglycan were examined for their ability to induce AChR phosphorylation and aggregation in mouse C2 myotubes. The carboxy-terminal 95 kDa agrin fragment agrin-c95$_{A0B0}$, which binds to α-dystroglycan with high affinity, failed to induce AChR phosphorylation and aggregation. In contrast, agrin-c95$_{A4B8}$, which binds less strongly to α-dystroglycan, induced both phosphorylation and aggregation, as did a small 21 kDa fragment of agrin, agrin-c21$_{B8}$, that completely lacks the binding domain for α-dystroglycan. We conclude that agrin-induced AChR phosphorylation and aggregation are triggered by an agrin receptor that is distinct from α-dystroglycan.

Keywords: acetylcholine receptor/agrin/dystroglycan/neuromuscular junction/protein tyrosine phosphorylation.

Introduction

An important step in the formation of the vertebrate skeletal neuromuscular junction is the nerve-induced accumulation of nicotinic acetylcholine receptors (AChRs), acetylcholinesterase (AChE) and other components, in the post-synaptic apparatus. Agrin, a protein originally isolated from *Torpedo* electric organ, appears to play a crucial role in this process (McMahan and Wallace, 1989; McMahan, 1990; Hall and Sanes, 1993). Agrin is synthesized by motor neurons, is transported anterogradely along their axons and is deposited in the synaptic basal lamina. When added to myotubes in culture, agrin induces the formation of specializations at which many components of the post-synaptic apparatus accumulate, including AChRs and AChE. Both nerve- and agrin-induced AChR aggregation are blocked by anti-agrin antibodies (Reist *et al.*, 1992). These and other findings suggest that agrin is the neural signal that triggers the formation of post-synaptic specializations at developing neuromuscular junctions.

Agrin is a 400–600 kDa heparan sulfate proteoglycan with a multi-domain structure (Denzer *et al.*, 1995; Tsen *et al.*, 1995). Agrin's AChR-aggregating activity is localized to the 95 kDa carboxy-terminal portion of the protein, which contains three domains homologous to the globular (G) domains of the laminin α chain (Nitkin *et al.*, 1987; Sasaki *et al.*, 1988; Tsim *et al.*, 1992; Ferns *et al.*, 1993; Gesemann *et al.*, 1995). Various isoforms of agrin result as a consequence of alternative splicing within this region. Inserts at two sites near the carboxy-terminal end, designated as A and B in chick and ray and y and z in rat, appear to regulate the ability of agrin to induce AChR aggregation (Rupp *et al.*, 1991, 1992; Ferns and Hall, 1992; Ferns *et al.*, 1992, 1993; McMahan *et al.*, 1992; Ruegg *et al.*, 1992; Smith *et al.*, 1992). Each of the splice sites lies within or adjacent to a G-domain. For the A site, the presence or absence of a four amino acid insert results in A4 and A0 variants, whereas for the B site there are 0, 8 and/or 11 amino acids inserted leading to B0, B8, B11 and B19 isoforms (see Figure 1). In chick and rat, agrin isoforms that contain amino acid inserts at the B site always have the four amino acid insert at the A site. Recombinant carboxy-terminal fragments of agrin isoforms with inserts at both splice sites, such as A4B8, A4B11 and A4B19 are capable of inducing AChR aggregation on cultured muscle cells. Motor neurons express high levels of such isoforms (Tsim *et al.*, 1992; Hoch *et al.*, 1993; Ma *et al.*, 1994, 1995; Smith and O'Dowd, 1994). Muscle cells, on the other hand, synthesize agrin isoforms that lack inserts at one or both sites [A0B0, A4B0 (Ruegg *et al.*, 1992; Hoch *et al.*, 1993; Ma *et al.*, 1994)]; such isoforms are only weakly active or inactive in AChR aggregation assays when added in a soluble form to cultured myotubes (Ruegg *et al.*, 1992; Gesemann *et al.*, 1995).

Previous work suggested that agrin- and nerve-induced AChR aggregation is mediated by an increase in protein tyrosine phosphorylation (Wallace *et al.*, 1991; Qu and Huganir, 1994; Meier *et al.*, 1995). Thus, treatment of cultured myotubes with agrin leads to the formation of domains within myotubes that stain intensely with antibodies to phosphotyrosine (Wallace *et al.*, 1991; Meier *et al.*, 1995). Agrin-induced AChR aggregates co-localize with such phosphotyrosine-enriched domains. Protein kinase antagonists that inhibit agrin-induced tyrosine phosphorylation also block agrin-induced AChR aggregation (Wallace, 1994). One component of the myotube membrane that becomes phosphorylated on tyrosine residues in agrin-treated myotubes is the AChR itself, most conspicuously the AChR β subunit (Wallace *et al.*, 1991; Qu and Huganir, 1994; Meier *et al.*, 1995). These and other findings suggest that binding of agrin to a receptor on

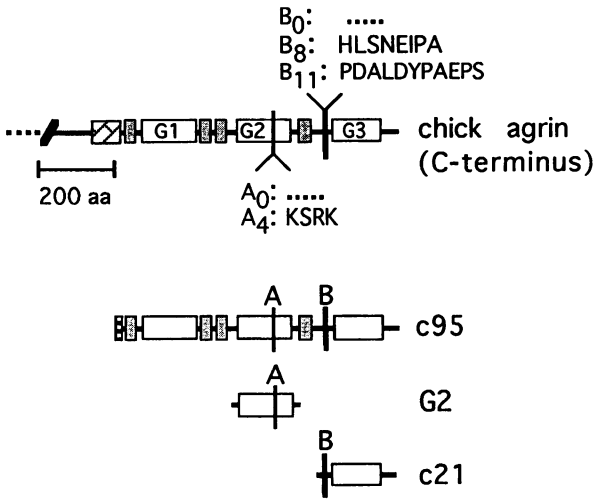

Fig. 1. Structural domains of chick agrin fragments. Simplified representation of the carboxy-terminal part of full-length chick agrin. Alternative splicing occurs at site A (0 or 4 amino acid insert) and site B (0, 8 and/or 11 amino acid insert). Shaded boxes represent epidermal growth factor (EGF)-like domains. The crosshatched box indicates a Ser/Thr-rich domain. Laminin α-like G-domains are indicated as G1, G2 and G3. In this study, recombinant fragments corresponding to the 95 kDa C-terminal portion of agrin (c95), the second laminin α-like domain (G2) and the third laminin α-like domain (c21) were used (for more details, see Gesemann *et al.*, 1995).

the myotube surface activates a protein tyrosine kinase, resulting in a localized increase in protein tyrosine phosphorylation of AChRs and perhaps other proteins as well. If one consequence of this increase in tyrosine phosphorylation were that AChRs became attached to the cytoskeleton, then AChRs would accumulate near the site of the activated kinase, thereby creating a receptor aggregate (Meier *et al.*, 1995; Wallace, 1995).

A crucial step in testing such a hypothesis is the identification of the agrin receptor. Motivated by the observation that binding of laminin to α-dystroglycan is mediated by G-domains for which homologs exist in agrin, several laboratories have shown that agrin binds to α-dystroglycan, a component of the dystrophin-associated glycoprotein complex (for reviews, see Matsumura and Campbell, 1994; Tinsley *et al.*, 1994). This observation led to the hypothesis that binding of agrin to α-dystroglycan triggers the formation of AChR aggregates (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). However some experimental results appear inconsistent with this hypothesis: (i) Agrin isoforms that are inactive in AChR aggregation assays bind to α-dystroglycan with an affinity that is similar to or higher than that of isoforms with AChR-aggregating activity (Sugiyama *et al.*, 1994; Gesemann *et al.*, 1996). (ii) Excess amounts of the inactive agrin isoform $c95_{A0B0}$ do not block agrin-induced AChR aggregation, but cause only a slight shift in the dose–response curve (Gesemann *et al.*, 1996). (iii) Experiments using mAb IIH6, which blocks binding of agrin to α-dystroglycan, give conflicting results. While Gee *et al.* (1994) reported that mAb IIH6 inhibited agrin-induced AChR aggregation, Sugiyama *et al.* (1994) and Cohen *et al.* (1995) saw little or no effect on agrin- or nerve-induced receptor aggregation. Campanelli *et al.* (1994) found that mAb IIH6 influenced the size but not the number of agrin-induced receptor patches. (iv) Recent studies using recombinant agrin show that a 21 kDa fragment, comprising the B splice site with the eight amino acid insert and the most carboxy-terminal G-domain ($c21_{B8}$, see Figure 1), does not bind to α-dystroglycan but is sufficient to induce AChR aggregation on cultured myotubes (Gesemann *et al.*, 1995, 1996). This led to the conclusion that binding to α-dystroglycan is not required for agrin-induced AChR aggregation (Gesemann *et al.*, 1995, 1996). Here we report that in mouse C2 myotubes the ability of different agrin fragments and isoforms to induce AChR phosphorylation varied in parallel with their ability to induce AChR aggregation rather than with their ability to bind to α-dystroglycan. Isoforms that lack inserts at the A and B splice sites and bind with high affinity to α-dystroglycan caused neither AChR phosphorylation nor aggregation, while the small $c21_{B8}$ fragment, which does not bind to α-dystroglycan, induced both AChR phosphorylation and aggregation. These findings support the hypothesis that the functional agrin receptor triggers both phosphorylation and aggregation and is distinct from α-dystroglycan.

Results

Agrin induces aggregation of AChRs on vertebrate skeletal myotubes and causes phosphorylation of the AChR β subunit. Agrin also binds to α-dystroglycan, although such binding does not appear to mediate AChR aggregation (Gesemann *et al.*, 1996). As a step towards identifying the agrin receptor mediating AChR phosphorylation, we have compared directly and quantitatively the ability of agrin fragments that differ in their affinity for α-dystroglycan to induce AChR aggregation and phosphorylation.

Binding to α-dystroglycan is not sufficient for induction of AChR phosphorylation

To study changes in the phosphorylation of AChR β subunits, cultured C2 myotubes were incubated overnight with medium containing [^{32}P]orthophosphate and treated for 4 h with various isoforms of agrin. AChRs were purified, the subunits separated by SDS–PAGE and the incorporation of radioactive phosphate measured by autoradiography. As illustrated in Figure 2, 85 pM of the recombinant agrin isoform $c95_{A4B8}$, which is known to induce receptor aggregation, increased phosphorylation of the AChR β subunit to the same extent as was observed with saturating amounts of *Torpedo* agrin (Meier *et al.*, 1995). In contrast, 85 pM $c95_{A0B0}$, an agrin isoform that is inactive in AChR aggregation assays but, like $c95_{A4B8}$, binds to α-dystroglycan (Sugiyama *et al.*, 1994; Gesemann *et al.*, 1996), did not increase AChR β subunit phosphorylation over control levels. Likewise, laminin-1, another ligand for α-dystroglycan (Gee *et al.*, 1994; Yamada *et al.*, 1994; Gesemann *et al.*, 1996) did not induce β subunit phosphorylation, at concentrations as high as 200 nM (data not shown). Thus, binding of agrin or laminin-1 to α-dystroglycan is not sufficient for the induction of phosphorylation of the AChR β subunit.

As demonstrated previously, *Torpedo* agrin not only induces AChR β subunit phosphorylation but also causes phosphorylation of AChR γ and δ subunits (Meier *et al.*, 1995). A comparable increase in AChR γ and δ subunit

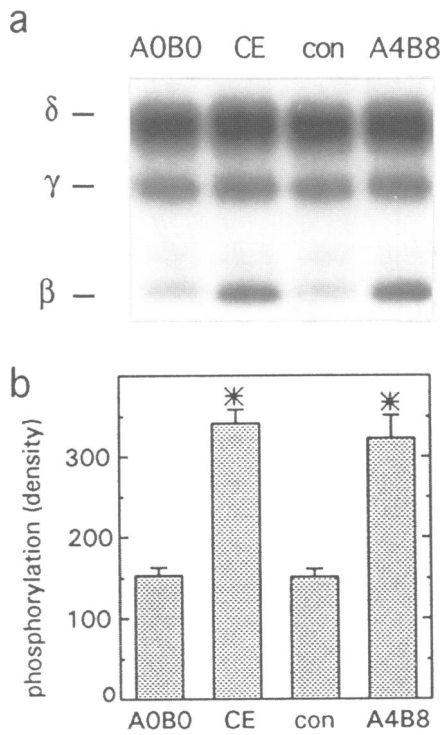

Fig. 2. Agrin isoforms differ in their ability to induce AChR phosphorylation. (**a**) C2 myotube cultures were labeled overnight with [^{32}P]orthophosphate, treated for 4 h with agrin and the AChRs isolated and analyzed by SDS–PAGE and autoradiography. AChR β, γ and δ subunits were identified according to their M_r as described (Meier *et al.*, 1995). Compared with control cultures (con), addition of 4 U of *Torpedo* agrin (CE) and 85 pM $c95_{A4B8}$ (A4B8) induced phosphorylation of the AChR β subunit. No such increased level of phosphorylation was induced with 85 pM $c95_{A0B0}$ (A0B0). (**b**) Quantitative analysis of agrin-induced changes in AChR β subunit phosphorylation. The level of incorporation of [^{32}P]orthophosphate into AChR β subunits was measured by densitometric analysis of autoradiograms. Data from different experiments were combined by normalizing the results of each experiment to the δ subunit of control myotubes in normal medium (=1000; see Meier *et al.*, 1995). Compared with controls (con), *Torpedo* agrin (CE) and recombinant $c95_{A4B8}$ agrin, but not $c95_{A0B0}$, significantly increased phosphorylation of the AChR β subunit. Data are mean ± SEM, N = 14–18. *Differs significantly from control cultures, $P<0.05$ (one-way ANOVA, Tukey–Kramer HSD test).

phosphorylation was observed with $c95_{A4B8}$, but not with $c95_{A0B0}$ (data not shown). Phosphorylation of AChR γ and δ subunits occurs predominantly on serine residues, with little change in phosphotyrosine content (Meier *et al.*, 1995). Agrin-induced protein serine phosphorylation of the γ and δ subunits is not required for AChR aggregation (Wallace *et al.*, 1991); therefore, we restricted our analyses to changes of AChR β subunit phosphorylation.

AChR-aggregating activity of agrin isoforms parallels their potency for inducing phosphorylation

As a step towards determining if both AChR aggregation and phosphorylation are triggered by the same agrin receptor, we measured the extent of AChR aggregation and phosphorylation as a function of concentration for the $c95_{A4B8}$, $c95_{A4B11}$ and $c95_{A0B0}$ agrin isoforms. Previous receptor aggregation assays on chick myotubes had demonstrated that $c95_{A4B8}$ was the most active isoform, $c95_{A4B11}$ was ~140-fold less active and $c95_{A0B0}$ was completely

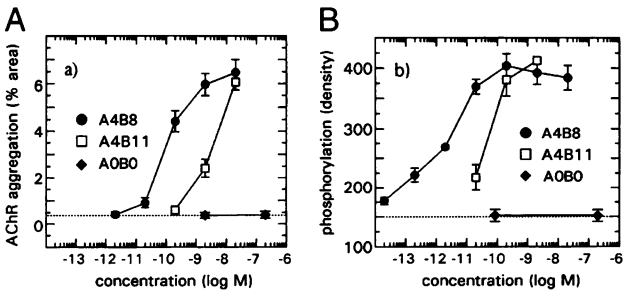

Fig. 3. The relative potency of agrin isoforms is similar in AChR aggregation and phosphorylation assays. (**A**) Dose–response curve for AChR aggregation. Activity is expressed as the percentage of the cell surface area occupied by AChR clusters following an overnight incubation with the indicated c95 agrin isoform. Each data point represents the mean ± SEM of 20 myotube segments analysed. The half-maximal response for $c95_{A4B8}$ (A4B8) was ~100 pM and for $c95_{A4B11}$ (A4B11) was ~4 nM. No AChR-aggregating activity was observed for $c95_{A0B0}$ (A0B0) at concentrations up to 200 nM. The dotted line represents the percentage area occupied by AChR aggregates in control cultures [mean: 0.38 ± 0.11 (SEM); N = 20]. (**B**) Dose–response curve for AChR β subunit phosphorylation. The level of β subunit phosphorylation was determined by densitometric analysis after separation of ^{32}P-labeled AChR subunits from cultures incubated with agrin isoforms for 4 h. Half-maximal responses were reached at ~4 pM for $c95_{A4B8}$ and ~50 pM for $c95_{A4B11}$. Each data point represents the mean ± SEM with N = 3–10 for $c95_{A4B8}$, N = 3–6 for $c95_{A4B11}$ and N = 7 and 18 for $c95_{A0B0}$. The dotted line represents the level of β subunit phosphorylation in control cultures [mean: 152.0 ± 5.4 (SEM); N = 47].

inactive (Gesemann *et al.*, 1995). These isoforms had the same relative potency on mouse C2 cells (Figure 3A). The concentration of $c95_{A4B8}$ that induced half-maximal receptor aggregation was ~100 pM, while $c95_{A4B11}$ had a half-maximal response at ~4 nM and $c95_{A0B0}$ failed to induce aggregation at concentrations as high as 200 nM. When assayed for their ability to induce phosphorylation of the AChR β subunit (Figure 3B), these isoforms again had the same order of potency; $c95_{A4B8}$ reached a half-maximal response at ~4 pM, $c95_{A4B11}$ at ~50 pM and $c95_{A0B0}$ had no effect at concentrations as high as 200 nM. The finding that the ability of different agrin isoforms to induce AChR phosphorylation varied in parallel with their AChR-aggregating activity is consistent with the hypothesis that the same agrin receptor mediates both effects.

A 21 kDa agrin fragment that lacks the binding domain for α-dystroglycan is sufficient to induce AChR aggregation and phosphorylation

As previously shown, a short ~21 kDa agrin fragment, comprising the third G-domain (G3) and the B splice site ($c21_{B8}$, see Figure 1), induces AChR aggregation on chick myotubes (Gesemann *et al.*, 1995). To determine whether the third G-domain is sufficient to induce AChR phosphorylation and whether this effect is splice site dependent, we compared the ability of $c21_{B0}$ and $c21_{B8}$ agrin fragments to induce AChR aggregation and β subunit phosphorylation. Additionally, since agrin-induced AChR aggregation is inhibited by heparin (Wallace, 1990) and the second G-domain (G2) contains the heparin binding site (Gesemann *et al.*, 1996), we also tested individual second G-domain (G2) agrin fragments, with or without amino acid inserts ($G2_{A0}$ and $G2_{A4}$), for their effect on AChR aggregation and phosphorylation.

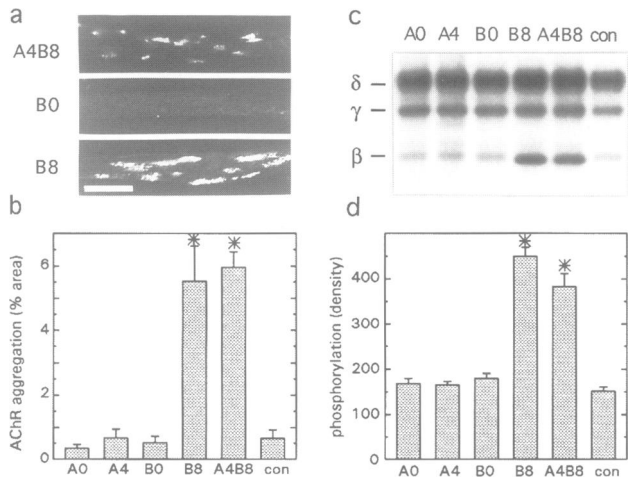

Fig. 4. AChR aggregation and phosphorylation by individual agrin G-domains. (**a**) AChR aggregates induced by agrin fragments. Fluorescence micrographs of segments of cultured C2 myotubes labeled with rhodamine–α-bungarotoxin. Overnight incubation with 20 nM $c95_{A4B8}$ (A4B8), 20 nM $c21_{B8}$ (B8) or 200 nM $c21_{B0}$ (B0). Bar: 40 μm. (**b**) Quantitative analysis of AChR-aggregating activity. For each fragment, the percentage of myotube surface area occupied by AChR aggregates was determined after overnight incubation with 400 nM $G2_{A0}$ (A0), $G2_{A4}$ (A4), $c21_{B0}$ (B0) or $c21_{B8}$ (B8) and compared with effects induced by 2 nM $c95_{A4B8}$ (A4B8) and with control cultures (con). Of the 21 kDa fragments, only $c21_{B8}$ caused a significant increase in AChR aggregation. (**c**) AChR β subunit phosphorylation induced by agrin fragments. Autoradiography of ^{32}P-labeled AChR subunits isolated after a 4 h incubation with 200 nM of the short agrin fragments ($G2_{A0}$, $G2_{A4}$, $c21_{B0}$, $c21_{B8}$) or 200 pM $c95_{A4B8}$. (**d**) Quantitative analysis of AChR β subunit phosphorylation. C2 myotubes were incubated for 4 h with 200 nM of each of the short agrin fragments and the extent of phosphorylation of the β subunit compared with that obtained with 200 pM $c95_{A4B8}$ and controls. The only 21 kDa fragment that significantly increased β subunit phosphorylation was $c21_{B8}$. There is no significant difference in the extent of β subunit phosphorylation induced by $c21_{B8}$ and $c95_{A4B8}$ (mean ± SEM; N = 6–12). *Differs significantly from control cultures, $P<0.05$ (one-way ANOVA, Tukey–Kramer HSD test).

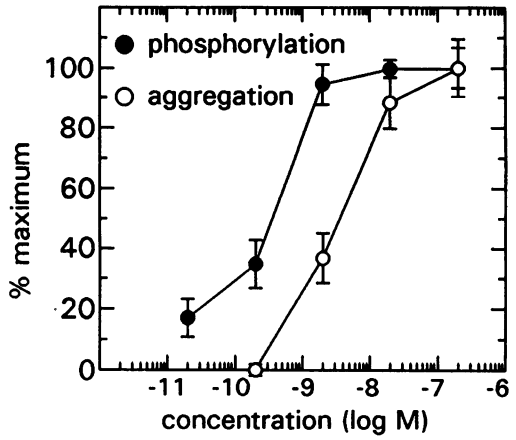

Fig. 5. Dose dependence of $c21_{B8}$-induced AChR aggregation and β subunit phosphorylation. Open symbols: AChR aggregation. Each data point represents the mean ± SEM of 20 myotube segments. The half-maximal response was reached at ~4 nM. Closed symbols: AChR β subunit phosphorylation. Data, expressed as the mean ± SEM (N = 5–7), represent the level of β subunit phosphorylation induced by $c21_{B8}$ as determined by densitometric analysis. The half-maximal response was reached at ~300 pM.

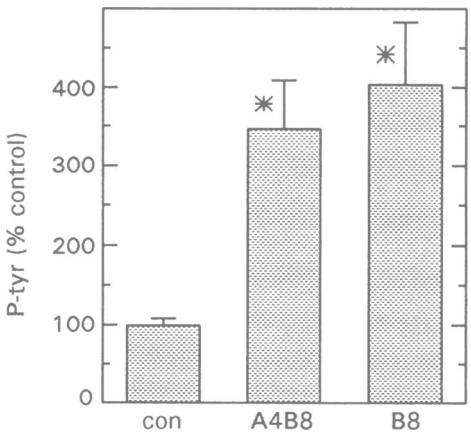

Fig. 6. The $c21_{B8}$ agrin fragment induces tyrosine phosphorylation of the AChR β subunit. Myotubes were labeled with [^{32}P]inorganic phosphate, treated with agrin isoforms, AChRs isolated and the phosphotyrosine content of the β subunit estimated. The results of each experiment were normalized to the phosphotyrosine content of β subunits from control cultures (=100%). Data are expressed as the mean ± SEM; N = 7 for control (con) and $c95_{A4B8}$ (A4B8), N = 6 for $c21_{B8}$ (B8). *Differs significantly from control, $P<0.05$ (one-way ANOVA, Tukey–Kramer HSD test).

As shown in Figure 4, the second G-domain was inactive in AChR aggregation and phosphorylation assays, regardless of the presence or absence of amino acid inserts at the A splice site. In contrast, the fragment containing the third G-domain induced both aggregation and phosphorylation, provided it contained an insert at the B splice site ($c21_{B8}$). The $c21_{B8}$ fragment does not bind to α-dystroglycan (Gesemann *et al.*, 1996). Thus, an agrin fragment that does not bind to α-dystroglycan induces both AChR phosphorylation and aggregation.

Next we compared the dose–response relationship for the formation of AChR aggregates and induction of β subunit phosphorylation for $c21_{B8}$ (Figure 5). Half-maximal receptor aggregation was reached at ~4 nM, whereas half-maximal β subunit phosphorylation occurred at ~300 pM. Thus, as was the case for c95 constructs, half-maximal phosphorylation occurred at a lower concentration of agrin than half-maximal receptor aggregation (see Discussion).

The $c21_{B8}$ agrin fragment induces tyrosine phosphorylation of the AChR β subunit

Results of previous experiments indicate that agrin causes conspicuous changes in the phosphotyrosine content of AChR β subunits (Wallace *et al.*, 1991; Qu and Huganir, 1994; Meier *et al.*, 1995) and that protein tyrosine phosphorylation is required for agrin-induced AChR aggregation (Wallace, 1994, 1995). To determine whether $c21_{B8}$ induces tyrosine phosphorylation of the AChR β subunit, mouse C2 myotubes were incubated in medium containing [^{32}P]inorganic phosphate and were treated with either $c21_{B8}$ or $c95_{A4B8}$. AChRs were isolated, the subunits separated by SDS–PAGE and the phosphotyrosine content of the β subunit examined by selective hydrolysis of phosphoserine residues with 1 M KOH (see Materials and methods; Cooper and Hunter, 1981). As illustrated in Figure 6, $c21_{B8}$ as well as $c95_{A4B8}$ agrin isoforms induced tyrosine phosphorylation of the AChR β subunit. Thus, as previously shown for *Torpedo* agrin, recombinant chick

agrin fragments, including $c21_{B8}$, trigger tyrosine phosphorylation of the AChR β subunit.

Discussion

The results reported here demonstrate that, when added to C2 myotubes in culture, the $c21_{B8}$ fragment of recombinant agrin, which does not bind to α-dystroglycan, causes AChR aggregation and tyrosine phosphorylation of the AChR β subunit. On the other hand, the $c95_{A0B0}$ fragment of recombinant agrin, which binds with high affinity to α-dystroglycan, does not induce either AChR aggregation or phosphorylation. We conclude that agrin-induced AChR phosphorylation and aggregation are triggered by a muscle surface agrin receptor that is distinct from α-dystroglycan.

Role of α-dystroglycan in mediating the effects of agrin

The changes in AChR distribution and phosphorylation induced by the agrin fragment $c21_{B8}$, which does not bind to α-dystroglycan, are qualitatively indistinguishable from those induced by longer agrin fragments, such as $c95_{A4B8}$, that do bind to α-dystrogylcan. This indicates that binding of agrin to α-dystroglycan is not required for AChR phosphorylation or aggregation. However, α-dystroglycan does accumulate at agrin- and nerve-induced specializations and may well play a role in their formation and/or stabilization (Phillips *et al.*, 1993; Campanelli *et al.*, 1994; Cohen *et al.*, 1995). For example, Sugiyama *et al.* (1994) suggested that binding of agrin to α-dystroglycan might act to increase the effective concentration of agrin in the vicinity of developing aggregates, thereby facilitating its interaction with the agrin receptor that mediates AChR phosphorylation and aggregation. Indeed, Gesemann *et al.* (1996) provide evidence that binding to α-dystroglycan reduces the concentration of agrin required for a half-maximal response by a factor of 2. Alternatively, α-dystroglycan, as well as other components of the dystrophin–utrophin complex, might be required for the interaction of AChRs with the cytoskeleton that is thought to mediate the immobilization of AChRs at sites of aggregate formation (Froehner, 1993; Apel and Merlie, 1995; Apel *et al.*, 1995; Cohen *et al.*, 1995). Thus, agrin-induced increases in protein tyrosine phosphorylation might alter the interactions between AChRs, the 43 kDa receptor-associated protein (rapsyn; Froehner, 1993) and proteins of the dystrophin–utrophin complex such that all of these components become tethered together to the underlying cytoskeleton. In fact, recent experiments using mutant mice with a targeted disruption of the *Rapsn* gene directly demonstrated the importance of rapsyn for the formation of AChR aggregates (Gautam *et al.*, 1995).

On the other hand, interaction of agrin with α-dystroglycan might play a role unrelated to AChR aggregation, both at synaptic sites and elsewhere. In muscle for example, myofibers synthesize agrin isoforms, lacking inserts at one or both A and B splice sites, that bind to α-dystroglycan but do not induce AChR aggregation. α-Dystroglycan, in turn, is found in extrasynaptic regions of muscle fibers (Cohen *et al.*, 1995). Whether or not agrin interacts with α-dystroglycan at such sites and, if so, what the functional consequences of such an interaction might be, remain to be determined.

Agrin-induced AChR aggregation and phosphorylation

Recombinant agrin fragments that induced AChR aggregation also caused AChR phosphorylation, while fragments that did not induce aggregation did not cause receptor phosphorylation. In particular, we found that the small recombinant chick agrin fragment $c21_{B8}$ induced both tyrosine phosphorylation of the AChR β subunit and AChR aggregation. Thus, based on the constructs described in this report, the domains within agrin that mediate AChR aggregation could not be separated from those that cause phosphorylation; the third G-domain containing an insert at the B splice site is both necessary and sufficient to induce AChR phosphorylation and aggregation. These findings provide support for the hypothesis that the same agrin receptor triggers both AChR phosphorylation and aggregation.

Dose dependence of agrin-induced AChR aggregation and phosphorylation

In previous experiments on chick myotubes, AChR aggregation and tyrosine phosphorylation were found to depend in the same manner on agrin concentration (Wallace, 1992), as would be expected if the combination of agrin with a receptor on the myotube surface triggered an increase in tyrosine phosphorylation that caused AChR aggregation. In the current study on C2 myotubes, however, agrin-induced AChR phosphorylation reached half-maximal levels at a concentration of agrin that was at least 10-fold lower than that necessary to induce half-maximal AChR aggregation. The apparent discrepancy may result from the manner in which AChR aggregation was measured. In the present study, the extent of receptor aggregation was determined by estimating the fraction of the C2 cell surface covered by receptor aggregates. For this analysis, small aggregates (<4 μm in their longest axis) were ignored, and the density of receptors within aggregates was not taken into account. Thus, relatively small, low density aggregates, such as might be produced by low concentrations of agrin, would not contribute to the measured extent of aggregation, artifactually shifting the dose–response curve for aggregation to higher agrin concentrations.

Of course, other explanations cannot be ruled out. If aggregation were a cooperative process, requiring the interaction of several phosphorylated receptors for example, this would also tend to create a difference in the dose–response curves for aggregation and phosphorylation. Another possibility might be that proteins other than AChRs must become phosphorylated to a certain level before aggregation can occur.

Regardless of the relationship between AChR phosphorylation and aggregate formation, the results reported here demonstrate that both agrin-induced AChR phosphorylation and aggregation are mediated by a receptor that is distinct from α-dystroglycan.

Materials and methods

Agrin constructs and expression

The recombinant chick agrin fragments used in this study were described by Gesemann *et al.* (1996). Fragments comprising the second G-domain with or without the four amino acid insert at the A splice site are

designated as $G2_{A4}$ and $G2_{A0}$ respectively, whereas fragments composed of the third G-domain and the associated B splice site are designated $c21_{B0}$ and $c21_{B8}$ (Gesemann *et al.*, 1995, 1996). Partially purified agrin from *Torpedo* electric organ (Cibacron pool) was prepared as described earlier (Nitkin *et al.*, 1987) and used at a saturating dose (4 U).

Cell cultures and aggregation assays

C2C12 cells (Yaffe and Saxel, 1977; Blau *et al.*, 1983) were grown in 35 mm tissue culture dishes in proliferation medium until they reached confluence. Cells subsequently were changed to differentiation medium as described earlier (Gesemann *et al.*, 1995; Meier *et al.*, 1995). After 4–6 days, cells had fused and were used for AChR aggregation and phosphorylation assays. For aggregation assays, cultures were incubated with agrin isoforms for 16 h. AChRs were labeled with 4×10^{-8} M rhodamine–α-bungarotoxin (Molecular Probes, Eugene, OR) and AChR aggregation was determined by estimating the fraction of the C2 cell surface covered by receptor aggregates. Only AChR aggregates with a longer axis of at least 4 μm were included in the counting (see Gesemann *et al.*, 1995 for details).

Measurement of AChR phosphorylation

AChR phosphorylation was assayed as previously described (Meier *et al.*, 1995) with minor modifications. Briefly, C2 cultures were rinsed with MEM without sodium phosphate (GIBCO BRL) supplemented with 1 mg/ml bovine serum albumin (RIA grade, Sigma Chemical Co.), 20 μg/ml conalbumin (type II, Sigma Chemical Co.), 100 U/ml penicillin and 100 μg/ml streptomycin, followed by a 16 h incubation in the same medium containing 0.25 mCi/ml [^{32}P]orthophosphate ([^{32}P]H_3PO_4 in H_2O, ICN Biomedicals Inc., Costa Mesa, CA). Agrin isoforms were added to this culture medium at the designated concentrations during the last 4 h of incubation. AChRs were labeled with biotinylated α-bungarotoxin (Wallace *et al.*, 1991; Meier *et al.*, 1995) and toxin–AChR complexes were solubilized by incubation for 10 min on ice in extraction buffer [20 mM sodium phosphate buffer, pH 7.4, supplemented with 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 40 mM sodium pyrophosphate, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) Triton X-100 and 0.25% (w/v) deoxycholate]. Extracts were cleared and toxin–AChR complexes purified on streptavidin-conjugated agarose beads. Bead complexes were washed thoroughly and eluted into SDS sample buffer at room temperature. AChR subunits were separated by SDS–PAGE on 7.5% gels. Gels were fixed, dried and exposed to pre-flashed autoradiography film (Hyperfilm-MP, Amersham, Arlington Heights, IL) and autoradiograms analyzed by densitometry as previously described (Wallace, 1994). AChR β, γ and δ subunits were identified by their position relative to pre-stained molecular weight markers and by criteria described previously (Meier *et al.*, 1995). For all densitometric analyses, data from different experiments were combined by normalizing the results of each experiment to the δ subunit of control myotubes in normal medium (=1000).

Assay for tyrosine phosphorylation

To estimate the level of tyrosine phosphorylation of AChR β subunits, isolated subunits were treated with base. Briefly, ^{32}P-labeled AChR β subunits were identified on SDS–polyacrylamide gels, gel pieces cut out and total ^{32}P incorporation determined by measuring Cerenkov radiation. Gel slices were treated with 1 M KOH at 60°C for 90 min to hydrolyze [^{32}P]PO_4 from serine residues, washed and counted again. Alkali hydrolysis reduced the amount of radioactive phosphate in AChR β subunits isolated from control cultures by 76% (SEM: ± 2.8; N = 7), for $c95_{A4B8}$-treated subunits by 55% (SEM: ± 3.0; N = 7) and for $c21_{B8}$-treated subunits by 52% (SEM: ± 8.8; N = 6). The AChR β subunit contains phosphotyrosine and phosphoserine, but no detectable phosphothreonine (Qu and Huganir, 1994; Meier *et al.*, 1995). Since base treatment hydrolyzes >90% of phosphoserine residues while ~40% of phosphotyrosine residues attached to proteins are recovered, we conclude that the radioactivity still associated with base-treated β subunits is due predominantly to phosphotyrosine (Cooper and Hunter, 1981). Similar results were obtained using anti-phosphotyrosine antibodies on Western blots (data not shown).

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