Calcium Plays a Critical Role in Determining the Acetylcholine Receptor-clustering Activities of Alternatively Spliced Isoforms of Agrin*

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Neural agrin, an extracellular matrix protein secreted by motor neurons, plays a key role in clustering of nicotinic acetylcholine receptors (AChR) on postsynaptic membranes of the neuromuscular junction. The action of agrin is critically dependent on an eight-amino acid insert (z8 insert) in the third of three consecutive laminin-like globular (G3) domains near the C terminus of neural agrin. Alternatively spliced agrin isoforms in non-neural tissue including muscle lack the z8 insert and are biologically inactive. Extracellular calcium has been shown to be imperative for the AChR-clustering activity of neural agrin. It is unclear, however, whether calcium preferentially interacts with the neural isoform or whether it acts solely as an intracellular messenger that mediates agrin signaling. Here, we report the G3 domain of rat neural agrin (AgG3z8) expressed in Pichia pastoris promoted AChR clustering on surface of C2C12 myotubes in a calcium-dependent manner. Direct binding of calcium to AgG3z8 was demonstrated by trypsin digestion and thermal denaturation experiments. Moreover, calcium induced a significant change in the conformation of AgG3z8, and the effect was correlated with an enhanced binding affinity of the protein to muscle receptor. Mutation of calcium-binding residues in the G3 domain diminished the conformational change of neural agrin, reduced its binding affinity to muscle membrane, and inhibited AChR-clustering activity. Conversely, the G3 domain of muscle agrin (AgG3z0) displayed little structural change in the presence of calcium, bound poorly to muscle surface, and was inactive in AChR-clustering assays. We conclude that distinct interactions of the G3 domain with calcium determine the biological activities of alternatively spliced agrin isoforms during synapse formation.

Agrin, a heparin sulfate proteoglycan (1, 2), is the major signaling molecule that triggers the formation and development of the neuromuscular junction (3–5). Motor neurons in the spinal cord synthesize agrin and secrete it from axonal terminals into the basal lamina of synaptic cleft (6). Agrin acts on muscle cell surface and is responsible for the induction of several major aspects of postsynaptic specialization at the developing neuromuscular junction. The most studied action of agrin is the clustering of nicotinic acetylcholine receptors (AChRs) on the postsynaptic muscle cell membrane (7–10). In addition, agrin is also required for the organization of other synaptic proteins, recruitment of cytoskeletal components, and the formation of junctional folds at the neuromuscular synapse (11–14). The action of neural agrin is mediated by a high affinity agrin receptor on muscle cell membrane. The identity of the receptor remains unknown, but MuSK, a muscle-specific receptor tyrosine kinase, is believed to be an important component of the receptor complex (15–17). Agrin induces rapid tyrosine phosphorylation of MuSK in cultured myotubes. The downstream signaling cascade that leads to AChR clustering is poorly understood. It involves tyrosine phosphorylation of the β-subunit of AChR and the recruitment of rapsyn, a 43-kDa protein, to the postsynaptic membranes of the neuromuscular junction (7, 8).

In mammals, a single agrin gene with three alternative splicing sites (x, y, and z sites) encodes multiple isoforms of agrin in a variety of tissues (18, 19). The AChR-clustering activity of agrin protein is critically influenced by amino acid sequences contained within the z site. This site is located in the third of three consecutive laminin-like globular (G3) domains near the C-terminal end of agrin. An exon, which encodes a short insert of eight amino acid residues (ELTNEIP; z8), is found only in neural agrin and is required for the AChR-clustering activity (see Fig. 1A) (20, 21). Splicing variants of agrin expressed by non-neural tissues including muscle all lack the z8 insert. They displayed little biological activity in AChR-clustering assays in vitro (22–25). How alternative splicing at the z site results in a striking difference in the functional activity of agrin isoforms is unclear.

Although agrin is a large (>200 kDa) protein with multiple domains, the AChR-clustering activity resides entirely in the C-terminal portion of the protein (~75 kDa) (22, 26). This region contains four copies of epidermal growth factor-like repeats and three copies of globular domains (G1, G2, and G3) initially described in the C terminus of laminin α2 chains (see Fig. 1A). Deletion analysis has revealed that the C-terminal 21-kDa fragment of agrin, which consists of only the G3 domain and z8 insert (AgG3z8), is necessary and sufficient for binding to agrin receptor on muscle cells and for inducing AChR clustering (22). The other laminin-like domains (G1 and G2), as

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† The abbreviations used are: AChR, acetylcholine receptor; Ni-NTA, nickel-nitritotriacetic acid; BuTx, bungarotoxin; DMEM, Dulbecco’s modified Eagle’s medium.
well as the epidermal growth factor-like repeats, are involved in agrin binding to α-dystroglycan and heparin (18). They are completely dispensable without significantly altering the receptor-clustering activity of neural agrin (27).

An important factor that influences the biochemical activity of neural agrin is calcium. Several studies have shown that calcium concentration is critical for the induction of ACR clustering by motoneuron and agrin in cultured myotubes and for the maintenance of cluster stability (28–30). Removal of extracellular calcium completely blocked agrin-induced activation of MuSK. Clamping of intracellular calcium with BAPTA had little effect on MuSK activation but inhibited tyrosine phosphorylation of AChR β-subunit (30, 31). These findings provide circumstantial evidence that extracellular calcium may promote agrin binding to the membrane receptor, whereas intracellular calcium appears to regulate the downstream signaling events subsequent to the formation of agrin-receptor complex. Nonetheless, the mechanism and precise site of action by calcium remain unknown.

The G3 domain sequence of agrin is homologous to the globular domain of α2 laminin (see Fig. 1B). Crystallographic studies reveal that this domain of laminin binds calcium through the side chains of two aspartic acids and two main chain carbonyls (32, 33). Because the aspartates are conserved in agrin G3 domain, and the z8 insert provides two additional negatively charged residues (Glu), it has been speculated that calcium might bind neural agrin, which contains the z8 sequence (32, 33). The present study has been undertaken to address the following: 1) whether calcium directly binds to the G3 domain of agrin, 2) whether the z8 insert affects the protein interaction with calcium, and 3) whether the interaction plays a role in regulating the AChR-clustering activity of alternatively spliced isoforms of agrin protein.

We report that the G3 domain of rat neural agrin (AgG3z8) expressed in Pichia pastoris promoted AChR clustering on the surface of C2C12 myotubes in a calcium-dependent manner. Direct binding of calcium to AgG3z8 was demonstrated by trypsin digestion and thermal denaturation experiments. Calcium induced a significant change in the conformation of AgG3z8, and the effect was correlated with an enhanced binding affinity of the protein to muscle receptor. Mutation of the calcium-binding site in G3 domain diminished the conformational change of neural agrin, reduced its binding affinity to muscle membrane, and inhibited AChR-clustering activity. Conversely, the G3 domain of muscle agrin (AgG30) displayed little structural change in the presence of calcium, bound poorly to muscle surface, and was inactive in AChR-clustering assays. We conclude that calcium specifically alters the conformation of G3 domain of neural agrin. The distinct effects of calcium on agrins with or without the z8 sequence determine the biological activities of alternatively spliced isoforms of the protein.

**Experimental Procedures**

cDNAs, Expression Vectors, and Strains—cDNAs encoding the C-terminal portion of the rat neural (CaG4,8) and muscle agrin (CaG0,0) were kindly provided by Dr. Michael Ferns (McGill University, Montreal, Quebec, Canada). The yeast expression vector pPICZαA and the KM71 mutS strain of P. pastoris were purchased from Invitrogen.

Chemicals and Reagents—Restiction and modification enzymes for DNA cloning were purchased from either New England Biolabs (Beverly, MA) or Invitrogen. Synthetic oligonucleotide primers were synthesized by Integrated DNA Technology (Coralville, IA). Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity resin was the product of Qiagen, Inc. (Valencia, CA). Rhodamine-α-BuTx was purchased from Molecular Probes (Eugene, OR). 125I-Na was obtained from Amersham Biosciences. Cell culture media and supplements were the products of Invitrogen. General chemicals including protein sequencing grade tryptic sin (EC 3.4.21.4) were purchased from Sigma.

**Construction of Expression Plasmids—** Sequences encoding the G3 domain of neural (amino acids 1756–1948) or muscle agrin (amino acids 1756–1940) were each amplified by PCR using CagG4,8 or CagG0,0 as the template (see Fig. 1). The forward and reverse primers used in the PCR are 5’-AGG AAT TCC CAG TGG GGG ACC TAG AAA CAC TG-3’ and 5’-CCC TCT AGA TTA GTG GTG ATG ATG GTG GGG AGT GGT GCA GGG TCT-3’, respectively. The PCR products were double-digested with EcoRI and XhoI and ligated into identically digested pPICZαA. Ligation mixtures were used to transform electrocompetent Top 10 Plasmid. DNA clones were prepared from colonies grown on low salt LB plates with Zeocin (25 μg/ml). Mutation of the putative calcium-binding residue (Asp1885) to alanine was carried out using sequence-specific oligonucleotides primed by following the QuickChange™ PCR mutagenesis protocol (Stratagene, La Jolla, CA). All of the constructs were verified by restriction digestion and dideoxynucleotide DNA sequencing (kit from Epicentre Technologies, Madison, WI).

**Protein Expression—** The pPICZαA-AgG3z8 or -AgG30 construct was linearized by PmeI digestion and transformed into P. pastoris KM71 strain by electroporation. Positive yeast clones were selected on YPD plates with Zeocin (0.1 mg/ml). A single colony was used to inoculate a 50-ml overnight culture in buffered glycerol complex medium (1% yeast extract, 2% peptone, 3.4 g/liter yeast nitrogen base, 0.1 m potassium phosphate, pH 6.0, 0.4 mg/liter biotin, and 1% glycerol). The initial culture was then expanded to 1 liter in buffered methanol complex medium (BMMY, 1% yeast extract, 2% peptone, 3.4 g/liter yeast nitrogen base, 0.1 m potassium phosphate, pH 6.0, 0.4 mg/liter biotin). Methanol was added daily to the BMMY medium at a final concentration of 0.75% to induce and maintain protein expression. On the fourth day of induction, the culture supernatant was collected, and proteins were salted out in 70% ammonium sulfate by centrifugation at 6000 × g for 30 min at 4 °C. The precipitates were dissolved in 40 ml binding buffer (250 mM NaCl, 50 mM imidazole, 50 mM sodium phosphate, pH 7.4), loaded onto a 10-ml Ni-NTA column, washed consecutively with 40 ml of binding buffer and 40 ml of washing buffer (250 mM NaCl, 25 mM imidazole, 50 mM sodium phosphate, pH 7.4), and finally eluted in 40 ml of phosphate-buffered saline containing 250 mM imidazole. After the imidazoles were removed by dialysis against two liters of 50 mM phosphate buffer, pH 7.4, the eluent was concentrated by gentle dehydration using Aquacide II (Calbiochem-Novabiochem). The agrin protein was then further purified using the Rotorof isoelectric focusing system (Bio-Rad). The recovered fractions were passed through a HiPrep 26/10 desalting column to remove the ampholytes (Amersham Biosciences).

**Computer Modeling of Agrin Structure—** The structural models were built by using the homology modeling function of Swiss-PdbViewer V3.7 (34). The crystal structure of αL2LG5 (Protein Data Bank code 1DYKA) was selected as the template, and the sequence alignment of AgG3 and αL2LG5 was adjusted according to their secondary structures (32). The modeling request was submitted to Swiss-Model server, and the modeling results were presented by Pdb viewer.

**Cell Culture and AChR-clustering Assay—** C2C12 myoblast cultures were maintained in the growth medium (DMEM containing 20% fetal calf serum, 2 mM glutamine, 0.5% chick embryo extract (Invitrogen), and penicillin/streptomycin). Fusion was induced by switching myoblasts at ~70% confluence to the fusion medium (DMEM containing 5% horse serum, 2 mM glutamine). AChR clusters were induced by adding AgG3 proteins to fully differentiated myotube cultures. 5 h later, the cells were fixed in 2% paraformaldehyde (5 min), stained with rhodamine-conjugated α-bungarotoxin, rinsed in phosphate-buffered saline, and viewed under a ×400 lens of an Olympus IX-70 fluorescent microscope. The results were photographed with a SPOT-2e camera, and the density of ACR clustering was analyzed with NIH Image software.

To remove extracellular calcium, we replaced the standard DMEM media that contains ~2 mM calcium with DMEM that lacked both calcium and magnesium. To increase extracellular calcium, we added to the standard DMEM an additional 3, 8, and 98 mM CaCl2, giving a final concentration of 5, 10, and 100 mM, respectively.

**Dissection and Dichroism Spectroscopy—** Circular dichroism spectra were collected at 25 °C on an Aviv 202 spectrometer. Protein was diluted in either 6 mM Tris, pH 7.4, 15 mM NaCl or to ~0.2 mg/ml to reduce UV absorbance in wavelength scanning experiments or in 1 × Tris-buffered saline for titration and temperature denaturation experiments. At least six scans per sample were recorded at 1-nm intervals from 280 to 165 nm. The protein concentration was determined using a BCA protein assay kit (Pierce). Mean residual ellipticity values were calculated based on the known protein sequences. The spectra were analyzed by the CDSSTR program using an IBasis 4 reference set (35–37). The results presented in this study were averaged values from
four separate experiments. Calcium titration curves of 0 to 100 mM CaCl₂ with 5 mM concentration steps were measured at wavelength 215 nm with 2 min of equilibration time and 1 min of average time. Thermal denaturation curves were also measured at 215 nm, with a 1 °C temperature step, a 0.2-min equilibration time, and a 1-s averaging time.

Limited Trypsin Digestion—AgG₃z8 or AgG₃z0 protein was first dialyzed against Tris-buffered saline with either 5 mM CaCl₂ or 5 mM EDTA and 5 mM EGTA. Trypsin digestion was carried out at enzyme/substrate ratios (w/w ratio) of 1:100, 1:50, 1:25, and 1:12.5 for 30 min at 37 °C. Digestion was stopped by boiling the proteins in SDS sample buffer for 10 min, and the digestion pattern was analyzed on a 15% SDS-polyacrylamide gel.

Radioligand Binding Assays—Recombinant agrins were iodinated using the chloramin T method described by Hunter and Greenwood.
The labeling was carried out by adding 1 mCi of $^{125}$I-Na (Amersham Biosciences) to 0.5 mg of protein in potassium-phosphate buffer, pH 7.0. The reaction was started with 20 µl of a chloramin T solution (2 mg/ml). After 45 s, 20 µl of 2 mg/ml Na$_2$S$_2$O$_5$ was added to terminate the labeling reaction. Free $^{125}$I was separated from labeled proteins by gel filtration on Sepharose-G25 columns. Approximately 85% of the proteins were recovered after gel filtration. The biological activity of neural agrin was retained after iodination as assayed by their ability to induce AChR clustering in cultured C2C12 myotubes.

Iodinated agrin proteins were added at indicated concentrations to cultured myotubes on six-well plates in the fusion medium. After incubation for 60 min at room temperature, cells were rinsed four times with 50 mM phosphate buffer, pH 7.4, and lysed with 0.2 M NaOH. Bound radioactivity was counted using a $^{32}$P-counter.

RESULTS

Characterization of Agrin G3 Domain—Using truncated fragments of agrin expressed by mammalian cell lines, several previous studies have shown that the C-terminal 21-kDa sequence of agrin, which contains only the G3 domain with the z8 insert, is the minimal domain sufficient for inducing AChR clustering in cultured myotubes (22, 39, 40). To obtain protein materials sufficient for structural and functional analysis, we expressed the G3 domains of neural (AgG3z8) and muscle agrin (AgG3z0), respectively, in P. pastoris (Fig. 1). PCR-amplified rat cDNA encoding these domains were subcloned into the yeast expression vector, pPICZA. The signal sequence from yeast mating factor and a hexahistidine tag were added in-frame to the 5' and 3' ends of agrin cDNA, respectively. AgG3z8 and AgG3z0 were each secreted as a soluble protein into the culture medium (30 mg/liter). They were purified to 95% by using the Ni-NTA column chromatography. On a reducing SDS-polyacrylamide gel, the proteins migrated as a single band of 26 and 24 kDa, respectively (Fig. 2A). The sizes were not changed upon digestion with endoglycosidase H or PNGase F (in 10% SDS at 95°C for 2 h), suggesting they were not modified by N-linked glycosylation (data not shown).
parative isoelectric focusing electrophoresis on the Rotofor apparatus separated the samples into 20 fractions, pH 3–10. Both proteins were concentrated in fraction 9 and 10, pH 6.0–6.5 (see Fig. 2, B and C), in agreement with a calculated pI of 6.31 for AgG3z0 and 6.04 for AgG3z8. The yield after the purification was \( \frac{1}{20} \text{mg/liter culture} \) for each of the proteins.

The following two experiments were carried out to determine whether the yeast-expressed agrin G3 domains were properly folded. First, we measured their spectrum with a CD spectrophotometer. The composition of protein secondary structures was calculated using the CDSSTR program (35, 36). The spectra of neural and muscle agrins were slightly different, but both had deep minima at 215 nm (Fig. 2 C). AgG3z8 had 30% \( \beta \)-sheet, 25% \( \beta \)-turn, and 2% \( \alpha \)-helix. These values are consistent with a model predicted based on the crystal structure of the homologous domain of \( \alpha 2 \) laminin (see Fig. 1B and Fig. 3). Similarly, AgG3z0 had a structural composition of 27% \( \beta \)-sheet and 26% \( \beta \)-turn with no \( \alpha \)-helix. The results of CD studies thus suggest that the recombinant G3 domains of neural and muscle agrins both assumed a folded conformation rich in \( \beta \)-structures.

Next, we assessed the function of the protein domains using an AChR-clustering assay with C2C12 myotubes grown in standard culture medium. In the absence of agrin, staining of myotubes with rhodamine-conjugated \( \alpha \)-BtTx revealed few, if any, spontaneous receptor clusters on the cell surface. Treating the cells for 3 h with AgG3z8 at concentrations as low as 0.01 \( \mu \text{g/ml} \) (\( \sim 0.4 \text{nM} \)) induced the formation of numerous AChR clusters. The number of clusters approached maximal when 1 \( \mu \text{g/ml} \) (\( \sim 100 \text{nM} \)) AgG3z8 was added to the culture (Fig. 4). The efficacy of yeast AgG3z8 protein was thus comparable.
with that of a chick agrin G3 fragment expressed in COS and HeLa cells (EC$_{50}$ ~13 nM) (40, 41). In contrast, muscle agrin (AgG3z0) at similar concentrations failed to induce AChR cluster formation. Only when the myotubes were treated with extremely high concentrations (>50 μg/ml, ~2 μM) of AgG3z0, a few receptor aggregates became visible (data not shown). Thus, the G3 domain of neural agrin expressed in *Pichia* acted potently to induce AChR aggregation, whereas the muscle isoform displayed little, if any, biological activity.

**Extracellular Calcium Alters the Binding and Biological Activity of G3 Domain**—Previous studies have demonstrated that calcium enhances the binding of G1 and G2 domains of agrin to α-dystroglycan and heparin on muscle membrane. The role of these interactions has not been defined, but they appear to be not required for agrin-induced AChR cluster formation. Only when the myotubes were treated with extremely high concentrations (>50 μg/ml, ~2 μM) of AgG3z0, a few receptor aggregates became visible (data not shown). Thus, the G3 domain of neural agrin expressed in *Pichia* acted potently to induce AChR aggregation, whereas the muscle isoform displayed little, if any, biological activity.

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C2C12 myotubes. In the absence of extracellular calcium, $^{125}$I-AgG3z8 bound poorly to muscle cell surface ($K_D \sim 375$ nM). In standard DMEM medium (~2 mM calcium), however, the binding affinity of agrin was increased by nearly two orders of magnitude ($K_D \sim 9.3$ nM). At higher calcium concentrations (5 and 100 mM), a further but less dramatic increase in AgG3z8 binding was detected (see Fig. 5C and Table I). In contrast, iodinated G3 domain of muscle agrin ($^{125}$I-AgG3z0) bound muscle cells with significantly lower affinity than the neural isoform regardless of extracellular calcium concentrations. These results therefore suggest that extracellular calcium promotes direct binding of the G3 domain to agrin receptor.

**Calcium Induces a Change in the Conformation of Neural Agrin**—The enhanced binding affinity of AgG3z8 to muscle cells implicated that the metal ion may alter the structure of the G3 domain of agrin or its putative receptor on the postsynaptic membrane. Alternatively, calcium may play a role in promoting the assembly of an agrin receptor complex, which involves MASC (MuSK-associated synaptic component), a putative muscle membrane protein, and MuSK (15, 30). To test these hypotheses, we measured CD spectra of the recombinant proteins at various calcium concentrations. In the absence of calcium, the G3 domains of neural and muscle agrin both displayed a similar composition of secondary structures (Fig. 2C). Interestingly, calcium induced a prominent change in the CD spectrum of AgG3z8 (Fig. 6, A and C). A change of the ellipticity at wavelength 215 nm was apparent at 5 mM calcium ($p < 0.01$, $n = 4$). Raising the concentration of calcium to 100 mM resulted in a further reduction in the ellipticity at 215 nm. Plotting the change of $[\theta]_{215}$ of AgG3z8 against calcium concentration indicated that the $K_D$ for the interaction was ~10 mM (Fig. 6C). Net secondary structure analysis using CDSSTR revealed that at 100 mM calcium, AgG3z8 protein had a significant 11% increase in $\beta$-sheet content with a concomitant reduction in unordered structure. In contrast, the CD spectrum of AgG3z0 exhibited little change in the presence of calcium (Fig. 6, B and C). It was unlikely that AgG3z0 was already saturated by background calcium, as addition of 5 mM EDTA/EGTA did not alter the protein spectrum (data not shown). Based on these results, we conclude that calcium binding specifically increases the content of $\beta$-structures in the G3 domain of neural but not muscle agrin.

**Muscle Agrin Binds Calcium but Does Not Undergo Conformational Changes**—In the CD spectroscopic studies, we did not detect any major change in the conformation of AgG3z0 protein in response to calcium. The result suggested that this domain of muscle agrin might not interact with calcium. Alternatively, it may bind calcium but does not undergo a significant change in the net conformation. To test these assumptions, we first examined whether radioactive calcium ($^{45}$CaCl$_2$) would directly bind AgG3z0. Unfortunately, the rather low binding affinity of the interaction (in mM range, see below) made it difficult to obtain consistent and accurate results. To circumvent this problem, we performed limited trypsin digestion experiments on agrins incubated with or without calcium. The patterns of enzymatic digestion were relatively simple on the SDS gel.
when the ion concentration was increased to 100 mM (Fig. 7). Moreover, additional intermediates of neural and muscle proteins were partially protected from trypsinolysis. A larger portion of the full-length AgG3z8 and AgG3z0 remained intact at various trypsin concentrations tested (Fig. 7A, lanes 8–11, arrows). Calcium also enhanced the intensity of protein bands corresponding to the intact AgG3 proteins in each of the lanes shown in A. The pixel intensity of each band on the gel was measured using NIH ImageJ software and normalized against that of protein in the absence of trypsin.

because of the small size of the G3 domain. In the absence of calcium (with 5 mM each of EDTA/EGTA), incubation of AgG3z8 with 0.1, 0.2, or 0.4 μg of trypsin resulted in two digestion intermediates, one at 20 kDa and the other at 18 kDa (Fig. 7A, lanes 3, arrowheads). AgG3z0 treated in the same manner displayed one digestion product of 20 kDa (Fig. 7A, lower panel, lanes 3, arrowhead). Almost all (>90%) of the full-length AgG3z8 and AgG3z0 were degraded when trypsin concentration was raised to 0.8 μg (Fig. 7, A (lanes 4–6, arrows) and B). In the presence of calcium (5 mM), however, both the neural and muscle proteins were partially protected from trypsinolysis. A larger portion of the full-length AgG3z8 and AgG3z0 remained intact at various trypsin concentrations tested (Fig. 7A, lanes 8–11, arrows). Calcium also enhanced the intensity of protein bands corresponding to the 20- and 18-kDa digestion products (Fig. 7A, lanes 8–11, arrowheads). Moreover, additional intermediates of ~14 kDa also appeared on the gel in the presence of calcium (Fig. 7A, lanes 8–11, asterisks). The protective effect was more striking when the ion concentration was increased to 100 mM (Fig. 7B). The action of calcium on AgG3 proteins was specific, as other divalent ions including Mg2+ did not exhibit any pronounced protection (data not shown). These data thus suggested that the G3 domain of muscle agrin might also interact with calcium.

Further evidence for interaction of both forms of agrin with calcium was observed in thermal denaturation studies. Upon unfolding, the ellipticity at wavelength 215 nm decreased significantly. In the absence of calcium, the proteins were totally unfolded at about 60 °C (Fig. 8). Based on the change of ellipticity in the range of 25 to 72 °C, we estimated that the Tm for AgG3z8 and AgG3z0 were both ~55 °C. In the presence of 100 mM calcium, both proteins became more stable, and the two thermal denaturation curves were shifted to higher temperature. As a result, the Tm increased to 62 °C for neural agrin and 63 °C for muscle agrin. These data, together with those from the trypsin digestion experiments, suggest that the AgG3 protein might interact with and be stabilized by calcium. However, calcium binding induces a conformational change only in the G3 domain of neural agrin.

**Mutation of Calcium-binding Site Impairs the Function of Neural Agrin**—Our experiments described above established that calcium binds to the G3 domain and induces a striking change in the conformation of neural agrin. Sequence alignment and computer modeling based on crystal structure of a homologous domain in laminin indicated that two aspartate residues (Asp1820, Asp1889) in the G3 fragment of agrin may be part of the calcium-binding sites (see Fig. 1B and Fig. 3). We thus replaced residue Asp1889 with alanine by site-directed mutagenesis and examined whether the mutation impairs the function of AgG3z8. Limited trypsin digestion experiments revealed that calcium failed to protect the D1889A mutant from degradation, suggesting the protein was deficient in calcium binding (Fig. 9A). It was unlikely that the mutation disrupted the overall folding of the G3 domain, as the CD spectrum of mutant was indistinguishable from that of wild-type protein in a calcium-free solution. Unlike the wild-type AgG3z8, however, the mutant protein displayed no conformational change upon addition of calcium to the sam-
Moreover, calcium failed to increase the binding affinity of the mutant G3 domain to muscle cell membrane (see Fig. 9C and Table I). Consistent with results of the CD and binding studies, mutation of the calcium-binding residue significantly reduced the AChR-clustering activity of neural agrin measured in the presence of 2, 50, or 100 mM calcium (Fig. 9, D and E). We thus conclude that the calcium-induced conformational change in the G3 domain is closely related to receptor binding and AChR-clustering activity of neural agrin.

**DISCUSSION**

Using the *Pichia* expression system, we have achieved high yields of the G3 domains of neural and muscle agrin. The fragments were generated as secreted proteins, and they remained soluble even after being concentrated to 90 mg/ml (3.5 mM). The yeast AgG3z8 induced aggregation of AChRs in C2C12 myotubes at low nM concentrations with an activity comparable with that of the same protein expressed in mammalian cells (40, 41). CD spectroscopic studies indicate that the recombinant proteins have net secondary structures rich in β-sheet as expected because of the homology to αL2LG5 (Fig. 1B). The results of biochemical, functional, and structural studies thus converge to the conclusion that the recombinant G3 domains of agrin we expressed in *P. pastoris* are properly folded proteins, which are amenable to future structural determination at high resolution.

It is well documented that calcium is required for induction of postsynaptic specializations in cultured myotubes by motoneuron or full-length agrin (28–30). The fact that calcium chelator BAPTA inhibited tyrosine phosphorylation of AChR β-subunit has led to the idea that the ion might act as an intracellular messenger for agrin signaling (31). Borges et al. (30) reported recently that removal of extracellular calcium prevented activation of MuSK by agrin, whereas clamping intracellular calcium had little effect on this process. The data provide circumstantial evidence that extracellular calcium may promote agrin binding to the membrane receptor or the assembly of agrin receptor complex. Calcium has been shown to enhance agrin binding to α-dystroglycan and heparin on muscle cell surface. These interactions, however, are mediated by the G1 and G2 domain of agrin, which are not required for the AChR-clustering activity. In the present study, we uncover a novel role of calcium in mediating the action of agrin. The metal ion induces a striking change in the CD spectrum of neural agrin. Such change is closely related to enhanced binding affinity and AChR-clustering activity of the protein. Thus, we postulate that binding of the ion alters the conformation of neural agrin and converts the protein from a low affinity, inactive precursor to an active, high affinity ligand. By promoting interaction of agrin with its muscle receptor, extracellular calcium plays a critical role in the postsynaptic specialization induced by agrin.

The results of our study also provide insight into the mechanism responsible for the striking difference in biological activities of alternatively spliced isoforms of agrin. The AChR-clustering activity of neural and muscle agrin differs by at least 5,000-fold because of the presence of an extra eight amino acids within the G3 domain of neural agrin (see the Introduction and Fig. 1). A possible explanation for this difference is that the z8 insert might constitute all or part of the binding site for agrin receptor. However, we found that a synthetic peptide of z8 sequence by itself was neither active in receptor-clustering assay using C2C12 myotubes nor did it inhibit the activity of neural agrin. In addition, substituted-cysteine scanning mu-

**Fig. 9.** Mutation disrupting the calcium-binding site impairs the function of AgG3z8. A, calcium was unable to protect the AgG3z8 (D1889A) mutant from trypsin digestion. B, the mutant protein displayed identical CD spectra in the presence or absence of 100 mM calcium. C, mutation of the calcium-binding residue decreased the binding affinity of AgG3z8 to muscle cells in the presence of 2 mM extracellular calcium. D, compared with the wild-type AgG3z8, the D1889A mutant (1 µg/ml) induced fewer AChR clusters on myotube surface in the presence of 2 mM calcium. Scale bar, 50 µm. E, the AChR-clustering activity of D1889A mutant was consistently lower than that of the wild-type AgG3z8 in the presence of higher concentrations of calcium. ***, p < 0.01 compared with wild-type AgG3z8, n = 4.
...tagenesis experiments revealed that modification by methanethiosulphonate reagents on cysteine residues introduced in z8 sequence did not significantly change the activity of AgG3z8 protein. Thus, the z8 insert itself is unlikely to be a major element involved in direct binding to agrin receptor on the postsynaptic membrane. Instead, results of our present studies suggest that the interaction between the z8 sequence and calcium determines the biological activity of alternatively splice isoforms of agrin. The G3 domain of agrin is homologous to the globular (LG5) domain of σ2 laminin (Fig. 1B). This domain of laminin binds calcium through the side chains of two aspartic acids and two main chain carbonyls (32, 45). The two aspartates are conserved in agrin. Superimposing the sequence of agrin G3 domain on the crystal structure of laminin suggests the z8 insert is situated in close proximity to the presumed calcium-binding site. In addition, the z8 insert contains two negatively charged residues (Glu) that may promote z8 interaction with calcium (see Fig. 1B and Fig. 3). Consistent with these predictions, our CD studies show that calcium specifically alters the secondary structures of the G3 domain of neural agrin. The metal ion significantly reduces unordered structure and increases the content of β-sheets in AgG3z8. In contrast, muscle agrin displayed little change in the net secondary structure at the same concentration of calcium. The Kd of the response deduced from changes of ellipticity in CD spectra of neural agrin is ~10 μM (Fig. 6C). Because raising calcium concentration from 2 to 5 or 10 μM increases the number of agrin-induced AChR clusters in C2C12 myotubes (Fig. 5), our result indicates that binding of calcium to AgG3z8 is probably not saturated at physiological calcium concentration (2.2 μM). Because neural and muscle agrins differ only by the z8 insert, this short sequence must be involved, either directly or indirectly, in mediating the conformational changes of AgG3z8 by calcium. Modeling of agrin G3 domain based on the crystal structure of laminin suggests the z8 insert is situated in close proximity to the presumed calcium-binding site (Fig. 3). In preliminary experiments, moreover, we found that substitution of the 4th residue (asparagine) in z8 insert with alanine diminished both the calcium-induced conformational change of AgG3z8 and its AChR-clustering activity. Because the calcium-induced conformation change is quite significant (a net increase of β-sheet by 11%), there must be structural changes that occur outside the z8 insert, which only constitutes 4% of total protein. Taking these together, our data are compatible with a model in which the z8 insert is essential for agrin to undergo a conformational change upon calcium binding. The structural change consequently brings together the key residues required for agrin binding to its receptor.

Depicting the precise effect of calcium on the G3 domain will require NMR or x-ray crystallographic analyses of the three-dimensional structure of agrin protein. In a preliminary NMR study, Alexandrescu et al. (46) have reported the backbone assignments for a bacteria-expressed chick AgG3z0, but the detailed structure of neural and muscle agrins remains to be determined. By establishing a system that could generate large quantities of properly folded AgG3z8, our present experiments set a solid foundation for such experiments in the near future. In addition, our work shall also facilitate discovery of the putative agrin receptor(s) on postsynaptic membranes of the muscle cells. Agrin is known to signal through a complex involving MuSK and a co-receptor MASC (15, 47). The identity of MASC remains mysterious partly because of the difficulties in obtaining sufficient quantities of agrin protein for affinity chromatography. The approach we introduced here makes such experiments feasible. As agrin is the only molecule known to regulate many aspects of synaptic structure and function, elucidating the mode of its action should contribute significantly to our understanding of the molecular mechanisms underlying synaptogenesis in the nervous system.

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