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The Agrin Receptor

LOCALIZATION IN THE POSTSYNAPTIC MEMBRANE, INTERACTION WITH AGRIN, AND RELATIONSHIP TO THE ACETYLCHOLINE RECEPTOR*

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Agrin is a component of the synaptic basal lamina that induces the aggregation of acetylcholine receptors (AChRs) and other elements of the postsynaptic membrane. We have determined the localization, binding characteristics, and biochemical profile of the agrin receptor in *Torpedo* electric organ membranes and defined domains of agrin that bind this receptor. Postsynaptic membranes from *Torpedo* electric organ bind agrin as judged by depletion of AChR clustering activity from solution. A ligand-based radioimmunoassay shows that agrin binding to postsynaptic membranes is saturable and calcium-dependent. Half-maximal binding is observed at agrin concentrations $\leq 10^{-10}$ M. Identification of the bound agrin polypeptides shows that at least one membrane binding domain of agrin is located in a 70-kDa proteolytic fragment. Immunofluorescent visualization and radioimmunoassay of agrin binding demonstrates that the agrin receptor is selectively concentrated in postsynaptic membranes, with little binding detected on nonsynaptic or liver membranes. Agrin binding is greatly reduced if the membranes are pretreated with trypsin, but is unaffected by phosphatidylinositol-specific phospholipase C. Membranes stripped of peripheral proteins by alkaline treatment retain full ligand binding capacity. α-Bungarotoxin affinity columns bind AChRs but not agrin receptors. The ratio of agrin receptors to AChRs in postsynaptic membranes is ~1:200. We conclude that the agrin receptor is an integral membrane glycoprotein that is selectively concentrated in postsynaptic membranes, but that is not tightly complexed with the AChR. The results also indicate that the biological activity of agrin is mediated through intracellular signal transduction events triggered by ligand binding to the agrin receptor.

Synaptogenesis demands the precise ordering and stabilization of diverse molecular components to restricted domains on the postsynaptic cell surface. At the neuromuscular junction, innervation triggers the aggregation and subsequent maintenance of high concentrations of acetylcholine receptors (AChRs)[1] such that at maturity, junctional receptor density is greater than 1,000-fold higher than extrajunctional receptor density (1). The clustering of the AChRs is accompanied by the accumulation of several other postsynaptic components, including extracellular matrix, plasma membrane, and cytoskeletal elements (reviewed in Ref. 2).

The synaptic basal lamina contains molecular cues that direct postsynaptic specializations to form at regenerating neuromuscular junctions (3). Agrin, an extracellular matrix protein originally isolated from the electric organ of *Torpedo californica*, is likely to function as such a synapse organizing molecule (reviewed in Ref. 4). When applied to cultured muscle cells, agrin induces the formation of AChR clusters strongly reminiscent of the AChR aggregates found at vertebrate neuromuscular junctions *in vivo* (5). Agrin is concentrated in the basal lamina at developing and mature nerve-muscle synapses (6–10). Antibodies against agrin block the nerve-induced clustering of AChRs (11). Together, these data indicate that agrin plays a central role in the development and regeneration of the neuromuscular junction.

To unravel the mechanism of agrin's action, one must define its properties as a ligand and characterize its cell surface receptor(s). In recent studies we used cell biological approaches to study a putative agrin receptor on cultured myotubes. This receptor binds agrin in a calcium-dependent manner and redistributes with the AChR upon agrin stimulation (18). These experiments indicate that there is a distinct plasma membrane agrin receptor on myotubes, and serve as a springboard for further biochemical and subcellular localization studies. Furthermore, given agrin's large size and the existence of functionally distinct forms arising by alternative mRNA splicing, there is a particular need to identify the domains of agrin that interact with its receptor.

To address these questions, we have turned to the electric organ of the marine ray *T. californica*. Since agrin was originally purified from the extracellular matrix of this tissue, we reasoned that membrane fractions from the same tissue would be an excellent resource for the biochemical characterization of its receptor. In addition, since postsynaptic membranes are readily isolated from electric organ, the subcellular localization of this receptor could be determined. We show here that agrin binds to postsynaptic membranes from *Torpedo* electric organ with high affinity in a saturable and calcium-dependent

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[1] The abbreviations used are: AChR, acetylcholine receptor; MEM, minimal essential medium; MEM-H, MEM with HEPES; PI-PLC, phosphatylinositol phospholipase C; RIA, radioimmunoassay; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

manner. At least one of agrin's membrane binding domains is located in a region encompassed by a 70-kDa proteolytic fragment. Agrin binds to an integral membrane glycoprotein that is selectively concentrated in postsynaptic membranes, but that is not tightly associated with the AChR. Given the close correlation of agrin's binding properties to its biological activity, we will refer to the molecule characterized here as the agrin receptor. This receptor is likely to mediate agrin's AChR clustering activity through the initiation of intracellular signal transduction cascades.

EXPERIMENTAL PROCEDURES

Isolation of Torpedo Electric Organ Membranes—Membrane fractions from *Torpedo* electric organ were prepared as described (19) with some modifications. Electric organs were dissected from *T. californica* (Pacific Biomarine) that had been anesthetized with 3-aminobenzoic acid ethyl ester (MS 222) and pithed. The tissue was immediately frozen in liquid nitrogen and stored at −80 °C. The tissue (17 g) was ground to a fine powder under liquid nitrogen, homogenized in 25 ml of buffer A (400 mM NaCl, 50 mM Tris, 10 mM EDTA, 10 mM EGTA, 100 μg/ml phenylmethylsulfonyl fluoride, 1.06 mg/ml diisopropyl fluorophosphate, 0.02% sodium azide, pH 7.4). The homogenate was centrifuged twice for 10 min at 6,000 × *g* and the resulting supernatant then centrifuged for 1 h at 100,000 × *g*. The pellet was resuspended in buffer B (10 mM NaH_2PO_4, 1 mM EDTA, 1 mM EGTA, 100 μg/ml phenylmethylsulfonyl fluoride, 0.02% sodium azide, pH 7.4), homogenized, sonicated, layered onto a continuous 25%-40% sucrose gradient, and centrifuged for 6 h at 100,000 × *g*. AChR-rich fractions (determined by SDS-PAGE) were then pooled. The four AChR subunits were identified by comigration with purified AChR generously provided by A. Karlin. Protein concentrations, determined according to Bradford (20), ranged from 0.4 to 0.6 mg/ml for the AChR-rich pools. Membranes from *Torpedo* liver were prepared exactly as described above.

Alkaline pH Stripping—Alkaline stripping (pH 11.0, 1 h) of peripheral membrane proteins was performed as described (21). After treatment, the pellets were resuspended in the original volume of buffer B and used for further analysis. In some experiments, membranes were treated at pH 12.0 using the same protocol. Material extracted at pH 11 or 12 was analyzed for agrin binding and glycoprotein composition as described below. Membranes aggregated irreversibly after treatment at pH 12 and were not examined for agrin binding.

For identification of glycosylated peripheral membrane proteins extracted by alkaline treatment, supernatants were neutralized with 20 mM HEPES, pH 7.2, made 1 mM in $CaCl_2$, and incubated with wheat germ agglutinin (WGA)-coupled to agarose beads (Vector Laboratories, Burlingame, CA). The beads were washed and boiled in SDS sample buffer. Eluted glycoproteins were separated on 7.5% SDS-PAGE gels, blotted onto nitrocellulose, and incubated with biotinylated WGA (5 μg/ml, Vector) followed by avidin-biotin-horseradish peroxidase. WGA-binding glycoproteins were visualized by chemiluminescence (ECL, Amersham Corp.). For solubilization, membranes (untreated or alkaline-stripped) were incubated in 25 mM octyl-β-D-glucopyranoside in 50 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride in sucrose-free buffer B, pH 7.4 for 18 h at 4 °C. Detergent-treated membranes were centrifuged at 100,000 × *g* for 3 h and the supernatant collected for further analysis.

Agrin—Agrin was purified over 3,000-fold from *Torpedo* electric organ as described previously (18). Agrin bioactivity is expressed in units/ml, which refers to the amount of agrin required to induce half-maximal AChR clustering on myotubes cultured in 1 ml of media (5). One unit is estimated to correspond to 10^{-12} to 10^{-13} M agrin (7). The agrin polypeptides in this preparation migrate with an apparent molecular mass of 80 and 60 kDa under nonreducing conditions (Fig. 2; see also Ref. 22). These polypeptides are the same as those designated 95 and 70 kDa based on SDS-PAGE under reducing conditions (7). For consistency, we will use this original designation ("95 and 70 kDa"; the 150- and 135-kDa fragments are not present in our preparations, which have been size selected by gel filtration chromatography). The NH_2 terminus of these fragments is at amino acid residue 598 (23). The exact site of the 70-kDa fragment carboxyl terminus has not been determined. Cleavage between the second laminin A domain and the fourth EGF domain at residue 1083 seems most likely, based both on the predicted molecular mass of the fragments and that residues 1082–1083 are Arg and Lys, respectively. A protease with such a specificity is likely to be present in these preparations, since a dibasic pair at residues 597–598 is cleaved to form the NH_2 terminus of the 95- and 70-kDa fragments.

Antibodies—The monoclonal anti-agrin antibody used in these experiments, mAb 6D4, has been extensively characterized (6–8, 23). Binding of 6D4 to agrin is not calcium-dependent. This antibody immunoprecipitates agrin from extracts of *Torpedo* electric organ as judged by depletion of biological activity and by immunochemistry; mAb 6D4 also labels agrin *in situ*. Recent molecular cloning data have confirmed that the polypeptides recognized by this antibody correspond to agrin (23). mAb 6D4 was purified on protein A beads (MAPS II, Bio-Rad) and iodinated with ^{125}I using IODO-GEN (Pierce Chemical Co.) as per the manufacturer's instructions. Specific activities were 5–7 μCi/μg.

Binding of Bioactive Agrin to Membranes—Membranes were pelleted and resuspended in HEPES-buffered MEM (pH 7.2; MEM-H) with 10% horse serum, 1% bovine serum albumin and incubated for 30 min at 4 °C with 3 units of agrin in 20 μl of membranes with gentle agitation. Incubations (in duplicate) were carried out in the presence or absence of 2 mM EGTA. Maximal activity under these conditions was determined by incubating agrin without membranes. After incubation, membranes were then pelleted and 15 μl of each supernatant was used for overnight stimulation of separate chick myotube cultures. Myotubes were labeled with rhodamine-α-bungarotoxin and AChR clustering activity determined as described previously (18).

Identification of Agrin Polypeptides Bound to Membranes—Alkaline-stripped postsynaptic membranes were resuspended in 150 units of agrin for 1 h at 4 °C in 50 mM NaCl, 5% glycerol, 10 mM $NaPO_4$, pH 7.4, with 1 mM calcium or 1 mM EGTA. The membranes were collected, solubilized in SDS sample buffer with no reducing agents, separated on 7.5% SDS-PAGE gels, and blotted onto nitrocellulose. The blots were then incubated in anti-agrin monoclonal antibody 11D2 (7), biotinylated horse anti-mouse IgG, and avidin-biotin-horseradish peroxidase (Vector). Bound antibody was detected by chemiluminescence (ECL, Amersham). The binding of 11D2 to agrin and the stability of the agrin polypeptides were unaffected by the presence or absence of calcium.

Solid Phase Radioimmunoassay—Intact or solubilized membranes were plated onto polylysine-coated wells (Removawell 96-well strips, Dynatech). The plates were washed with two changes of MEM-H and blocked in MEM-H with 1% bovine serum albumin (MHB) with 10% horse serum. Wells were then incubated in agrin diluted in MHB (200 units/ml, unless otherwise noted) for 30 min, washed, and incubated a further 30 min in 1 μg/ml ^{125}I-anti-agrin antibody 6D4. Nonspecific antibody binding was determined by addition of 100 μg/ml unlabeled 6D4 during the incubation in iodinated antibody and was less than 10% of specific values. The wells were washed twice in MEM-H then immersed in two changes of Hanks' balanced salt solution with 0.1% bovine serum albumin for 5 min each, dried, separated, and counted. Three replicate wells per condition were analyzed in each experiment; data are expressed ± S.E. To quantitate the effects of calcium on agrin binding to AChR-rich membranes, the initial blocking step, the agrin incubation, and the subsequent wash in MEM-H were performed with or without 2 mM EGTA. In some experiments, the peptides GRGDSP (100 μM; Gly-Arg-Gly-Asp-Ser-Pro; Telios) and LRE (300 μM; Leu-Arg-Glu; a gift of J. Sanes) were added during the agrin binding step.

Enzyme Treatment—Membranes were treated for 12–16 h at 22 °C with PI-PLC (10 units/ml) or partially purified heparatinase (from *Flavobacterium heparinum*, used at concentrations known to degrade brain proteoglycans; (24). Membranes were washed twice in MEM-H and assayed for agrin binding by RIA. For protease treatment, aliquots of membranes were incubated for 30 min at room temperature with 0.25 mg/ml trypsin (Sigma) in phosphate-buffered saline. Soybean trypsin inhibitor (Type I-S, Sigma) was then added to inhibit further protease digestion.

Immunocytochemistry—Aliquots of membranes were spotted onto polylysine-coated glass slides. To inhibit nonspecific binding, slides were then blocked for 30 min in MEM-H with 10% horse serum and agrin (200 units/ml) MHB applied for 30 min. For experiments to assess the calcium dependence of agrin binding, this incubation and washing was carried out in the presence or absence of 2 mM EGTA. Subsequent incubations, each for 30 min, were in mAb 6D4 IgG (15 μg/ml), biotinylated horse anti-mouse Ig (15 μg/ml, Vector)/rhodamine-α-bungarotoxin (5×10^{-7} M, Molecular Probes), and streptavi-

din-fluorescein (10 μg/ml, Amersham), all in MHB. A 5-min fixation in 1% paraformaldehyde at room temperature preceded the last incubation step. Coverslips were air-dried, mounted in glycerol with Citifluor (Pella), observed with a Zeiss Axioplan fluorescence microscope, and photographed with Kodak TMax 400 film. When comparing agrin binding under different conditions (*e.g.* Fig. 5), antibody staining, photographic exposures, and printing conditions were identical.

Lectin Binding—Agarose-bound lectins were obtained from Vector. Beads were washed in MEM-H, then incubated with solubilized membranes for 18 h at 4 °C. After incubation the beads were centrifuged and the supernatants assayed for agrin binding by RIA. Identical conditions were used for heparin-agarose beads (Sigma).

α-Bungarotoxin Affinity Chromatography—Biotinylated α-bungarotoxin was obtained from Molecular Probes. As these preparations contained significant proportions of unconjugated ligand, biotinylated toxin was purified using monomeric avidin affinity chromatography according to the manufacturer's instructions (Pierce). To deplete AChRs, the solubilized membranes were incubated with 10^{-8} M biotinylated α-bungarotoxin for 1 h, followed by streptavidin-α-bungarotoxin-agarose beads for 2 h. The bound α-bungarotoxin-AChR complexes were removed by centrifugation. AChR depletion was >95% as assessed by scanning densitometry (LKB Ultrascan) of the AChR α-subunit polypeptide band following SDS-PAGE and Coomassie Blue staining.

RESULTS

Torpedo Electric Organ Membranes Bind Bioactive Agrin—Since agrin is highly enriched in the basal lamina of *Torpedo* electric organ, we reasoned that the adjacent plasma membranes would be a good source of agrin receptors. As a first step toward characterizing the agrin receptor in this preparation, we asked if electric organ membranes bind bioactive agrin polypeptides. Postsynaptic membranes (AChR-rich) were incubated with soluble *Torpedo* agrin, the membranes pelleted, and supernatants assayed for their ability to induce AChR clusters on cultured myotubes. Fig. 1 shows that postsynaptic membranes from *Torpedo* electroplax, but not liver, deplete AChR-aggregating activity from the agrin preparations. Furthermore, this binding is calcium-dependent. These results provide a direct correlation between agrin's bioactivity on cultured muscle cells observed previously and the binding of agrin to *Torpedo* electric organ membranes.

Identification of Agrin Polypeptides That Bind Torpedo Membranes—To determine which portions of the agrin molecule are required for interaction with its receptor, we used Western blotting to identify the agrin polypeptides that bind postsynaptic membranes. Native agrin has a molecular mass of ~200 kDa. Agrin purified from *Torpedo* electric organ exists in the form of stable proteolytic fragments from the its carboxyl-half of molecular mass 95 and 70 kDa (see "Experimental Procedures"). The 70-kDa fragment is a truncated form of the 95-kDa polypeptide (Fig. 2; Ref. 23). Fig. 2 shows that postsynaptic membranes bind both the 95 and the 70 kDa agrin polypeptides. All agrin binding to the membranes is calcium-dependent. Thus, at least one membrane binding domain of agrin is located in this 70-kDa proteolytic fragment.

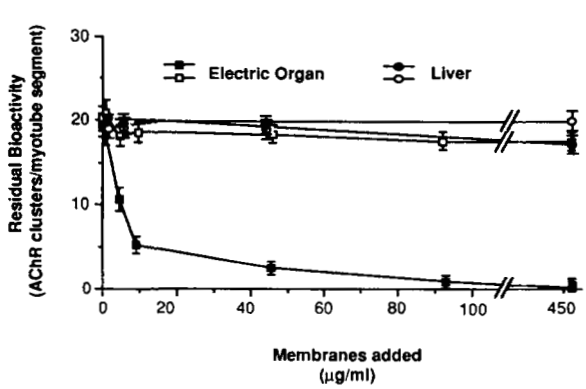

FIG. 1. ***Torpedo* electric organ membranes bind bioactive agrin.** The indicated concentrations of postsynaptic *Torpedo* electric organ (*squares*) or liver membranes (*circles*) were incubated in duplicate with agrin (3 units in 20 μl) in the presence (*filled symbols*) or absence (*open symbols*) of calcium. The membranes were then pelleted and 15 μl of the supernatants tested for residual AChR clustering activity. Agrin bioactivity is depleted only by electric organ membranes and only in the presence of calcium. Data are the average of three experiments and are expressed ± S.E.

Agrin Binding to Torpedo Electric Organ Membranes Is Calcium-dependent and Saturable—Extracellular calcium is required for nerve- and agrin-induced AChR clustering and for agrin binding to myotubes (25–27). We therefore further examined the calcium requirement for agrin binding to *Torpedo* membranes. Figs. 1 and 2 show that postsynaptic membranes bind agrin only in the presence of calcium. No other divalent cations (Mg^{2+}, Mn^{2+}, Zn^{2+}, Sr^{2+}, Ba^{2+}) tested supported agrin binding. The calcium dependence, which we observed in all of our assays for agrin binding (see below), provides a shared and unifying reference point for the study of agrin's interaction with the agrin receptor at the cellular, biochemical, and molecular levels.

To enable the quantitative assessment of agrin's interaction with its receptor under a range of conditions (*e.g.* in the presence of detergent), we developed a ligand-based agrin binding RIA to complement the bioactivity depletion method described above. We chose this method, since the yields of purified agrin are low; therefore, conventional binding techniques requiring large quantities of unlabeled ligand to determine nonspecific binding levels are impractical. In this approach, the binding of exogenous agrin to the membranes is measured as a function of bound radiolabeled anti-agrin antibody. As described below, we can consider the signal observed in the absence of calcium as a measure of nonspecific agrin binding. Fig. 3 shows that calcium-dependent agrin binding to *Torpedo* membranes is saturable. In the absence of calcium, binding is reduced by more than 5-fold and rises linearly with increasing ligand concentration. No agrin binding was observed on liver membranes, either in the presence or absence of calcium (not shown). Half-maximal agrin binding is observed at an agrin concentration of ~250 units/ml, corresponding to $\leq 10^{-10}$ M agrin. This low ligand concentration suggests that agrin binds to its receptor with high affinity; furthermore, the saturation curve presented here provides strong evidence for the specificity of calcium-dependent agrin binding.

We next determined the calcium and pH optima for agrin binding. Fig. 4 shows that agrin binding is half-maximal at ~0.5 mM calcium, saturates between 1 and 2 mM calcium, and declines gradually thereafter. Agrin binding also shows a striking pH dependence; little agrin binding is observed under even mildly acidic conditions, whereas robust binding is observed from neutral pH to as high as pH 10 (Fig. 4). These values are in agreement with those reported for agrin's AChR clustering activity on myotubes (26). The close parallels between the requirements for agrin binding to cell-free electroplax membranes and its biological activity on live cells indicate that the calcium- and pH-dependent interaction of agrin with its receptor is a critical aspect of agrin's function.

The deduced amino acid sequence for ray agrin shows that there is one RGD (Arg-Gly-Asp) and one LRE (Leu-Arg-Glu) tripeptide sequence in the COOH-terminal portion of the molecule (23). The RGD peptide is an important component

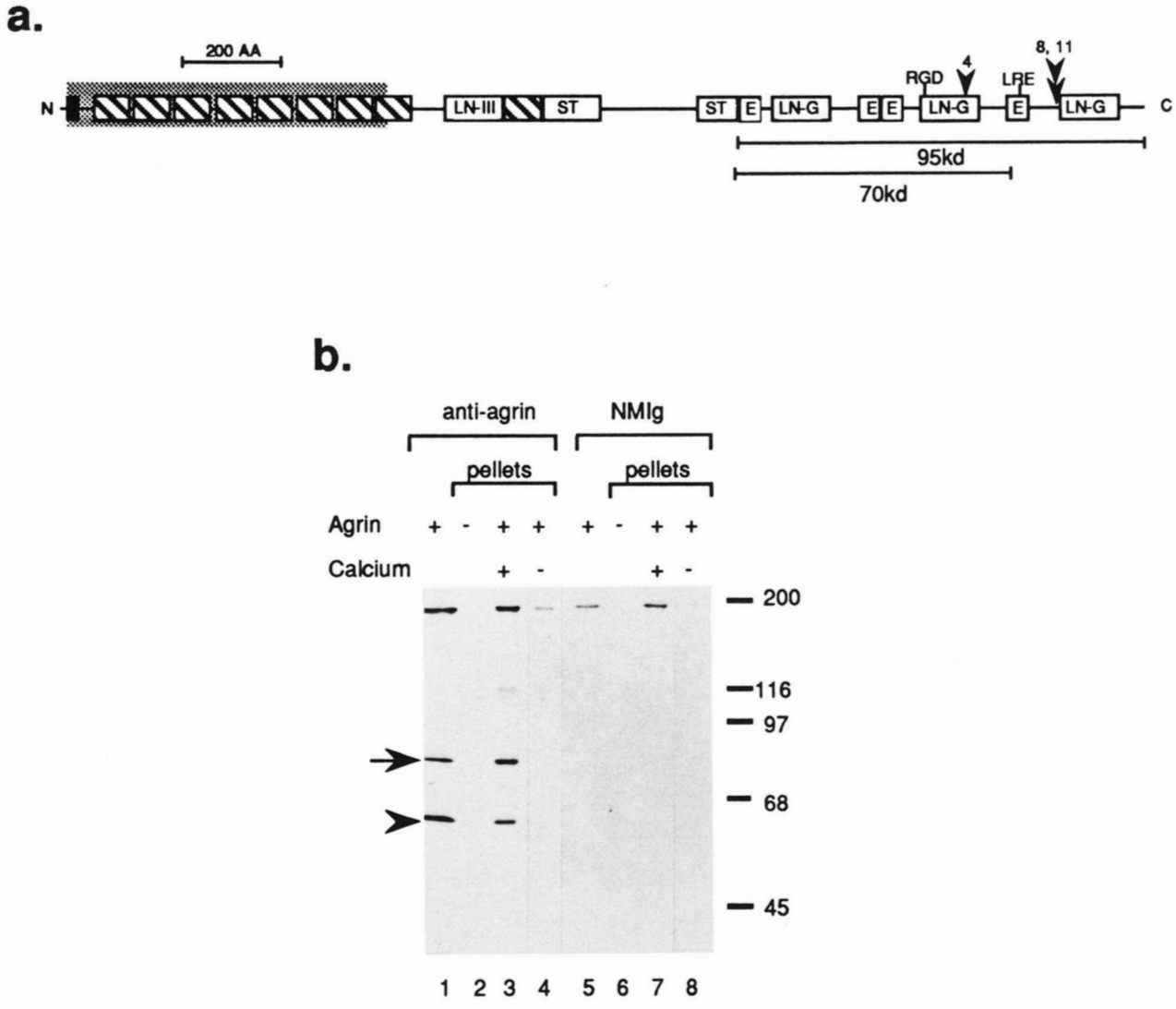

FIG. 2. **Mapping of the membrane binding domain of agrin.** *a*, schematic structure of agrin. The amino-terminal one-third of *Torpedo* agrin (*shaded portion*) has not been sequenced; a consensus domain organization for this region based on rat and chick sequence is shown for illustrative purposes. The locations of the 95- and 70-kDa agrin polypeptides derived from full-length agrin are indicated. Also shown are the known sites of alternative exon splicing (*arrowheads*; the number of amino acids encoded by each insert is indicated above them). Motifs predicted by sequence analysis: *filled box*, signal sequence; *hatched*, follistatin; *LNIII*, domain III of laminin; *ST*, serine-threonine-rich; *E*, EGF; *LN-A*, type A laminin. *b*, identification of agrin polypeptides that bind postsynaptic membranes. Shown is a Western blot probed with anti-agrin antibody 11D2 (*lane 1–4*) or an irrelevant IgG (*lanes 5–8*). *Lanes 1* and *5*, starting agrin preparation (150 units). *Lanes 2–8*, pellets of alkaline stripped postsynaptic membranes (25 μg) after incubation with: no agrin *(lane 2* and *6)* or agrin (150 units) in the presence (*lanes 3* and *7*) or absence *(lanes 4* and *8)* of calcium. Both the 95-kDa (*arrow*) and the 70-kDa (*arrowhead*) agrin polypeptide fragments bind postsynaptic membranes in a calcium-dependent manner. (The designations "95 kDa" and "70 kDa" are based on the mobility of these polypeptides in reducing gels and do not represent their mass under the conditions used here; see "Experimental Procedures"). Note also the lack of detectable agrin in the starting membrane preparations.

of the integrin binding site of many extracellular matrix molecules, and the LRE sequence is present in S-laminin and blocks its interaction with motor neurons (28, 29). However, neither peptide inhibited the binding of agrin to postsynaptic membranes (Table I).

Agrin Binds Selectively to Postsynaptic Membranes—Agrin is concentrated in the synaptic basal lamina at the neuromuscular junction and at the innervated face of *Torpedo* electrocytes (6, 8). We used two approaches to determine whether the agrin receptor is similarly concentrated in the adjacent postsynaptic membranes. First, we used double-label immunofluorescence to compare the localization of agrin binding and AChRs on isolated membrane vesicles. Fig. 5 shows that agrin binds to AChR-rich postsynaptic membrane vesicles in a calcium-dependent manner. Furthermore, agrin binds to the large majority of AChR-rich membrane vesicles. Little agrin binding is detectable on nonsynaptic membranes (not shown).

To confirm and to quantitate this selective localization of the agrin receptor in synaptic membranes, we turned to a biochemical approach. We separated crude membranes from *Torpedo* electric organ on sucrose gradients and measured both agrin and α-bungarotoxin binding across the resulting fractions. Fig. 6 shows that AChR-rich synaptic membrane fractions are also enriched in agrin receptors. In contrast, only low levels of agrin receptors are detected in membrane fractions from the noninnervated face of the electrocyte, which are poor in AChR and enriched in Na^+/K^+-ATPase (19). Similar results were obtained using activity depletion assays (not shown). The subcellular localization of the agrin

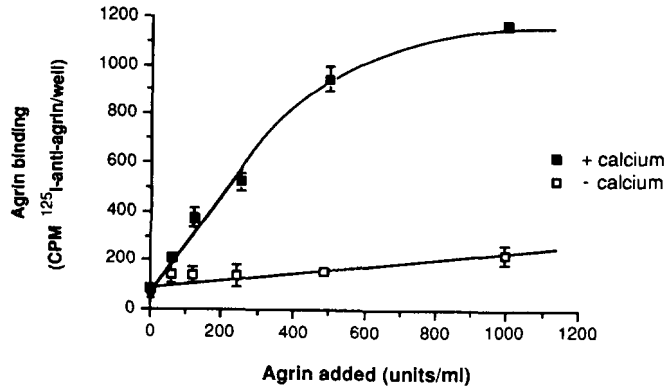

FIG. 3. **Agrin binding to *Torpedo* membranes is saturable and calcium-dependent.** Agrin binding was measured by RIA as described under "Experimental Procedures." In the presence of calcium half-maximal agrin binding is observed at approximately 250 units/ml, corresponding to an estimated agrin concentration of 10^{-10} to 10^{-11} M. Agrin binding in the absence of calcium is not saturable.

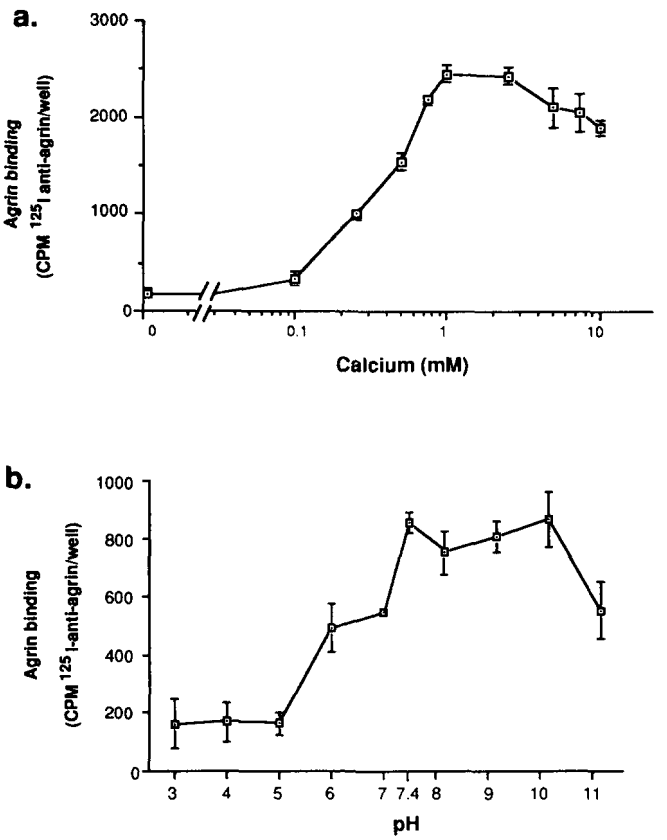

FIG. 4. **Calcium and pH dependence of agrin binding to postsynaptic membranes.** Agrin binding to postsynaptic membranes was measured under varying calcium concentrations (*a*) or pH (*b*), using the ligand-based RIA described under "Experimental Procedures." Optimal agrin binding is observed at 1–2 mM calcium, which agrees well the calcium optima for agrin's AChR clustering activity.

receptor in *Torpedo* electric organ therefore is consistent with its proposed role in mediating the differentiation of the postsynaptic membrane.

Biochemical Characterization of the Agrin Receptor—We have begun to construct a biochemical profile of the agrin receptor (Table I). Trypsin treatment of isolated electric organ membranes completely abolishes agrin binding, demonstrating that the agrin receptor is at least partly proteinaceous. However, treatment with heparatinase or PI-PLC does not affect subsequent agrin binding. Therefore, the receptor does not appear to be linked to the membrane by a glycosyl phosphatidylinositol anchor, nor do heparan sulfate GAGs appear to be necessary for its binding to agrin.

TABLE I

Enzyme and peptide sensitivity of agrin binding to postsynaptic membranes

Treatment	Agrin binding
	% control
Membrane pretreatment	
Trypsin	19 ± 2.2
PI-PLC	114 ± 7.1
Heparatinase	85 ± 3.2
Heparin[a] (immobilized)	97 ± 3.2
Competing peptides[b]	
GRGDSP (100 μM)	107 ± 1.1
LRE (300 μM)	101 ± 0.9

[a] Solubilized membranes, flow-through from heparin-agarose column.

[b] Present during incubation with agrin and membranes.

Agrin is synthesized by both motor neurons and their targets (13). We used several assays to determine whether endogenous agrin is present in the postsynaptic membrane preparations used here. Agrin could not be detected in alkaline stripped membranes by immunoblotting (Fig. 2), immunostaining (Fig. 5), or RIA (not shown). In addition, unlike agrin, the solubilized receptor does not bind to immobilized heparin (Table I). Thus, agrin is likely to bind to membranes via a heterophilic mechanism.

It is critical to determine whether the agrin binding observed here is to an integral or peripheral membrane protein. Alkaline treatment (pH 11) of *Torpedo* membranes is known to extract peripheral membrane proteins such as the 43-kDa, 58-kDa, and dystrophin-like polypeptides (21, 30–32). However, these stripped membranes retain integral membrane proteins with hydrophobic domains, such as the AChR. When we alkaline-stripped postsynaptic membranes, their agrin binding capacity was fully preserved (Fig. 7). Although pH 11 extraction is standard for *Torpedo* electric organ membranes, some rabbit sarcolemmal peripheral membrane glycoproteins, which could be related to those found at the synapse, are removed only at pH 12 (33, 34). We therefore compared the glycoproteins removed from postsynaptic membranes at both pH values. Fig. 7 shows that, for *Torpedo* electric organ, the same set of glycoproteins is extracted under both conditions. Based on these data, we conclude that the agrin receptor is an integral membrane protein.

To study the agrin receptor in more detail, and to allow its further biochemical purification, we isolated it from its native lipid environment and other membrane proteins. Fig. 8 shows that this receptor can be solubilized in the nonionic detergent octyl-β-D-glucopyranoside. Under these conditions, >90% of the agrin binding activity is recovered with complete retention of calcium dependence. We also determined the detergent and salt optima for the agrin receptor's solubilization. The optimal values for the agrin receptor are 25 mM octyl-β-D-glucopyranoside and 50 mM NaCl. These conditions differ significantly from the corresponding optima for the AChR (34 mM octyl-β-D-glucopyranoside, 0 mM NaCl, as determined both by SDS-PAGE and ^{125}I-α-bungarotoxin binding; not shown). The values for the AChR are in agreement with those reported previously (35). The solubilization experiments suggest that the agrin receptor is distinct from the AChR and provide

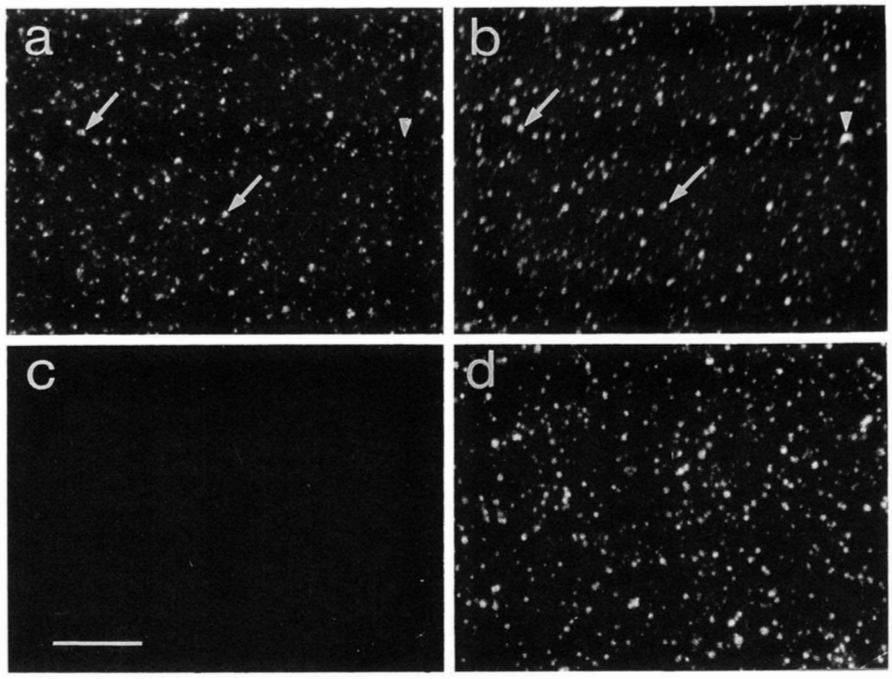

FIG. 5. **Immunofluorescent detection of agrin binding to isolated postsynaptic membranes.** Postsynaptic membranes were immobilized on coverslips and incubated with agrin in the presence (*a, b*) or absence (*c, d*) of calcium, followed by labeling with anti-agrin mAb, biotinylated anti-MIg, and streptavidin-fluorescein (*a, c*). AChRs in the same fields were detected by double labeling with rhodamine-α-bungarotoxin (*b, d*). Agrin binds to the large majority AChR-rich membrane vesicles (*arrows*); occasional AChR-rich vesicles do not exhibit detectable agrin binding (*arrowheads*). No agrin binding is observed in the absence of calcium (*c*). *Bar* = 10 μm

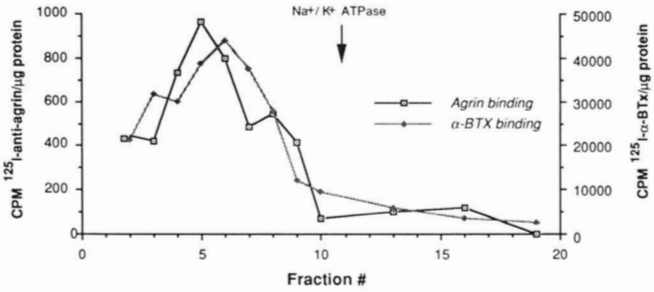

FIG. 6. **The agrin receptor is selectively concentrated in postsynaptic-rich membranes.** A crude membrane preparation from electric organ was separated on a 25–40% continuous sucrose density gradient. Fractions were collected and assayed for agrin binding by RIA, for ^{125}I-α-bungarotoxin (*BTx*) binding, and for total protein. The peak fraction enriched in nonsynaptic membranes, as judged by maximal levels of the Na^+/K^+-ATPase heavy subunit, is indicated (*arrow*).

further support that it is an integral membrane protein.

As a plasma membrane protein with an extracellular domain, the agrin receptor would be expected to have carbohydrate side chains. We tested the binding of the solubilized agrin receptor to immobilized lectins to determine its glycosylation pattern. Table II shows that the agrin receptor is retained on lectin columns with specificity for mannose (concanavalin A), *N*-acetylglucosamine (WGA), and galactosyl β(1,3) *N*-acetylgalactosamine (peanut agglutinin). On the other hand, the agrin receptor apparently does not have terminal fucose residues as judged by its failure to bind UEA1 and PSA. The binding to WGA and to PNA suggests that the agrin receptor has both *N*- and *O*-linked carbohydrate side chains.

Separation of the Agrin Receptor and AChR by α-Bungarotoxin Affinity Chromatography—The experiments presented above show that the agrin receptor is an integral membrane glycoprotein selectively concentrated in postsynaptic membranes. Since the AChR constitutes over 50% of the protein in these membranes, and the function of the agrin receptor is to direct the aggregation of AChRs, it is clearly important to assess directly the relationship between these molecules. Therefore, we solubilized postsynaptic membranes, applied them to α-bungarotoxin affinity columns, and measured the flow-through for levels of AChR and agrin receptors. These columns remove >95% of the AChR from the solubilized membranes without depleting the agrin receptor (Fig. 9). These experiments provide direct biochemical evidence that the agrin receptor and the AChR are different molecules. It is also noteworthy that the membranes used here were not alkaline-stripped and thus had a normal complement of peripheral membrane proteins, including cytoskeletal elements. Moreover, the AChR and the agrin receptor could be separated under mild detergent, salt, and pH conditions. These experiments show that the agrin receptor is readily separated from the AChR even under conditions that favor the preservation of macromolecular complexes. Together, these data suggest that AChRs and agrin receptors are not tightly associated with one another in the membrane.

DISCUSSION

We have characterized a plasma membrane receptor for agrin in postsynaptic membranes of *Torpedo* electric organ. This analysis has yielded insights into the domains of agrin that mediate receptor binding, the nature and localization of this receptor, and its relationship to the AChR. We will discuss these data with regard to the role of agrin receptors in synapse formation and the resultant implications for understanding the mechanisms of agrin's biological activity.

In previous studies we used cell biological methods to characterize agrin binding to the surface of cultured myotubes (18). We showed that agrin binds to these cells saturably in a calcium-dependent fashion. Moreover, the agrin binding sites redistribute during agrin-induced, but not antibody-induced, AChR clustering. Although we referred to the agrin binding sites as "putative" agrin receptors, the properties of these binding sites suggested that they served as authentic agrin receptors. These studies provided a strong basis for further work to elucidate the biochemical nature and *in vivo* localiza-

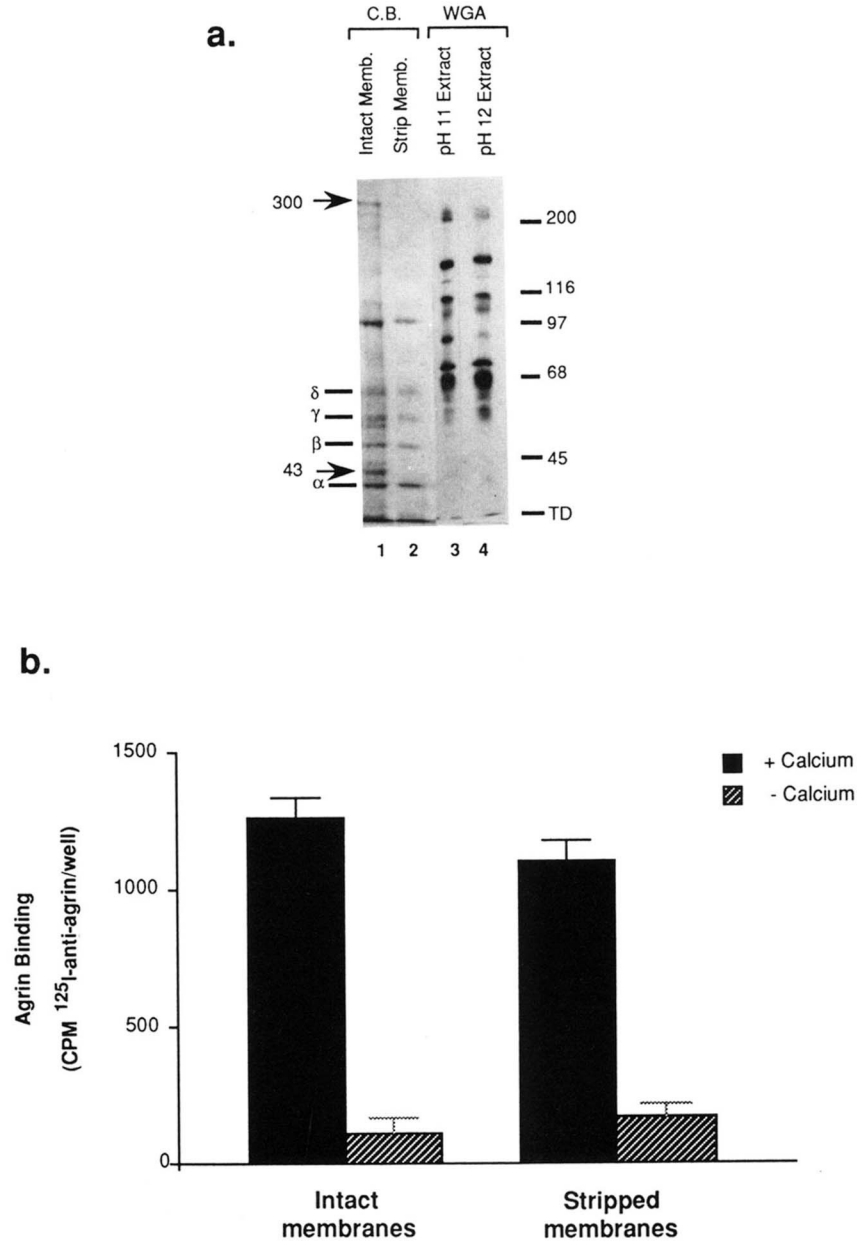

FIG. 7. **Agrin binds to alkaline-stripped membranes.** *a*, alkaline stripping extracts peripheral membrane proteins. Coomassie Blue (*C.B.*) staining of postsynaptic-rich membranes before (*lane 1*) and after (*lane 2*) pH 11 extraction. The well characterized 43- and ~300-kDa (dystrophin-like) cytosolic peripheral membrane proteins are removed by this treatment. The positions of the four AChR subunits are indicated. Western blot probed with WGA of proteins extracted at pH 11 (*lane 3*) and pH 12 (*lane 4*). The same set of peripheral membrane glycoproteins is extracted at both pH values. These glycoproteins are not readily detected by Coomassie Blue staining. *b*, agrin binding to intact and pH 11 stripped membranes. Equal volumes of intact or alkaline-stripped membranes were assayed for agrin binding by RIA. Agrin binding is quantitatively recovered following alkaline stripping, with complete retention of calcium sensitivity.

tion of such receptors. In particular, we wished to know whether the agrin receptor was an integral membrane protein, if it was localized to synapses *in vivo*, and its biochemical relationship to the AChR. We also sought to determine which domains of agrin mediate binding to its receptor.

Several lines of evidence indicate that the agrin receptor in *Torpedo* electric organ is comparable with the one we characterized previously on the myotube surface. First, agrin binds isolated electroplax membranes and intact myotubes saturably, in a calcium-dependent manner, and with similar affinities. The ligand-based RIA shows that half-maximal ligand binding in both cases is seen at concentrations of 10^{-10} to 10^{-11} M. Since our RIA is indirect, this figure is likely to be a low estimate of the actual affinity. Second, robust agrin binding to isolated postsynaptic membranes is observed at physiological ionic strength, pH, and calcium concentrations. Third, the calcium optimum reported for agrin's biological activity (26) is virtually identical to that observed here for agrin binding (Fig. 4). The sharp decrease in agrin binding we observe at acidic pH is also mirrored in agrin's biological activity. This concordance indicates that the calcium and pH optima for agrin's AChR clustering activity reflect the optima for agrin binding to its receptor. The calcium dependence of agrin binding provides common ground for the comparison to studies on nerve-induced AChR clustering, which also requires extracellular calcium and is mediated at least in part by agrin (11, 25). Together, these data show that agrin receptors on myotubes and *Torpedo* electric organ membranes are similar and indicate that the binding observed to the receptor characterized here represents an early and essential event in agrin's mechanism of action.

Agrin is selectively concentrated in the synaptic basal lamina of the electrocyte (6, 8). We find that agrin receptors have a similar synaptic localization, being richest in the innervated face of the electrocyte (Figs. 5 and 6). Receptors are sparse in membranes from the uninnvervated face as well as from tissues expressing low levels of agrin, *e.g.* liver (Fig. 1). This colocalization of ligand and receptor suggests that the agrin

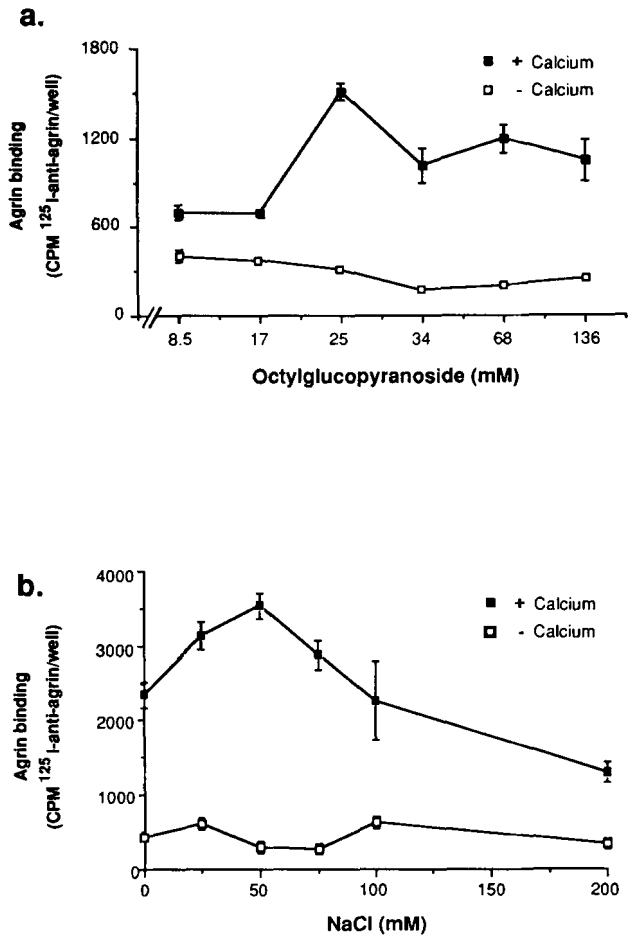

FIG. 8. **Solubilization of the agrin receptor in non-ionic detergent.** *a*, alkaline-stripped postsynaptic membranes were incubated in the indicated concentrations of octyl-β-D-glucopyranoside and centrifuged for 150,000 × *g* for 3 h. The supernatants were then assayed for agrin binding by RIA in the presence or absence of calcium. The agrin receptor is completely solubilized in 25 mM detergent. Maximal AChR solubilization was observed at 34 mM detergent as judged by SDS-PAGE analysis of the fractions (not shown). *b*, salt dependence of agrin receptor solubilization. Membranes were solubilized in 25 mM octyl-β-D-glucopyranoside in the indicated concentrations of NaCl, and agrin binding was measured by RIA under standard conditions (see "Experimental Procedures").

TABLE II

Lectin affinity chromatography depletes the agrin receptor

Alkaline-stripped detergent-solubilized postsynaptic membranes were incubated with the indicated lectin-substituted agarose beads. After incubation, the level of agrin binding activity in the supernatants was determined by RIA.

Lectin	Residual agrin binding activity
	%
Agarose beads only	100 ± 2.1
Peanut agglutinin	4.1 ± 3.4
Wheat germ agglutinin	5.2 ± 3.2
Ricinus communis 1	10.2 ± 4.1
Concanavalin A	14.5 ± 5.0
Dolichos biflorus	33.0 ± 4.8
Soybean agglutinin	78.6 ± 3.0
Ulex europeaeus	87.2 ± 4.5
Pisum sativum	96.3 ± 3.6

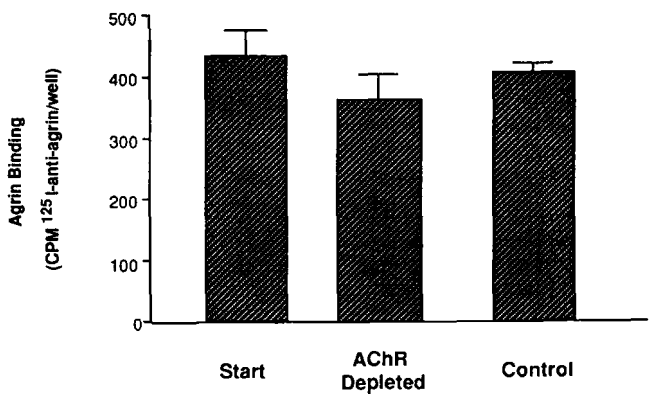

FIG. 9. **Affinity methods resolve the AChR and the agrin receptor.** Solubilized membranes were incubated with buffer only (*Start*) or with (*AChR-depleted*) or without (*control*) 10^{-8} M biotinylated α-bungarotoxin, followed by streptavidin-agarose beads. More than 95% of the AChR was removed by this treatment as judged by SDS-gel electrophoresis and scanning densitometry of the AChR α-subunit. The agrin receptor does not coprecipitate with AChRs. These results provide direct biochemical evidence that the agrin receptor is distinct from the AChR and that these receptors are not tightly associated with one another in the membrane.

receptor is monospecific in distinction, for example, to many integrins, which bind several ligands (29). The high concentration of agrin receptors in mature postsynaptic membranes seems likely to be important for agrin's function. It will be of interest to determine how this selective distribution is regulated during synapse development.

A key question in understanding agrin's AChR clustering activity is whether it binds directly to an integral membrane component or if it works indirectly through association with other extracellular matrix or peripheral membrane elements. Several lines of evidence indicate that the agrin binding observed here is to an integral membrane protein: 1) agrin binding is inhibited by trypsin pretreatment of the membranes (Table 1); 2) alkaline stripping of peripheral membrane proteins has no effect on subsequent agrin binding (Fig. 7), and no agrin binding is detected to the extracted material (not shown); and 3) the agrin receptor is readily solubilized in low concentrations of a mild nonionic detergent at neutral pH (Fig. 8). Thus, the agrin receptor has all the hallmarks of an integral membrane protein. Finally, since agrin binding is unaffected by PI-PLC treatment, it seems most likely that this receptor is a transmembrane protein.

One of the dystrophin-associated glycoproteins, dystroglycan, is localized at neuromuscular junctions and is reported to bind laminin (34). Agrin shares significant homology with laminin (36, 37), raising the possibility that dystroglycan may serve as its receptor. Although dystroglycan is a peripheral membrane protein, it is extracted from rabbit sarcolemmal membranes only at pH 12 (33). We compared the peripheral membrane glycoproteins extracted with standard methods and at pH 12 (Fig. 7). An identical set of polypeptides is observed in both extracts, including one with an apparent mobility of ~150 kDa, which may represent *Torpedo* dystroglycan. However, removal of these proteins had no effect on agrin binding to the membranes, and no agrin binding was detected to the extracted proteins. In addition, immunoprecipitation of solubilized (nonstripped) membranes with an antibody directed against the 58-kDa protein, a constituent of the dystrophin-dystrophin-associated glycoprotein complex, does not deplete the agrin receptor.[2] Thus, the agrin

[2] A. Vidal, M. Bowe, S. Froehner, and J. R. Fallon, unpublished observations.

receptor does not appear to be dystroglycan or a constituent of this complex.

Several molecules mediating cell-cell interaction, such as the cadherins and N-CAM, work through homophilic binding mechanisms (38, 39). Since agrin is synthesized both by neurons and muscle cells (12, 14), it is possible that agrin serves as its own receptor. However, three lines of evidence argue against such a mechanism. 1) The agrin polypeptides used here show no evidence of self-association as judged by gel filtration chromatography or sucrose density sedimentation (7); 2) we could detect no endogenous agrin in alkaline stripped membranes as assessed by western blotting (Fig. 2.), immunostaining (Fig. 5), or RIA (Fig. 7); and 3) unlike agrin, the solubilized agrin receptor does not bind to heparin (Table I; Ref. 40). We conclude that agrin binds to postsynaptic membranes via a heterophilic mechanism.

Agrin is a 200-kDa, multidomain protein possessing regions that are likely to mediate binding to basal lamina, heparin, and the cell surface. In addition, agrin mRNAs show alternative exon splicing at three sites. Alternatively expressed amino acid inserts at two of these sites have major effects on agrin's AChR clustering activity (15–17). To explore the relationship of agrin's domain structure and alternative splicing to its receptor binding properties, we took advantage of the stable polypeptide fragments that are generated during its purification. As illustrated in Fig. 2, the major polypeptide fragments are referred to as 95 and 70 kD (based on their molecular mass under reducing conditions; see "Experimental Procedures"). These fragments have identical amino termini, with the 70-kDa polypeptide being truncated at its carboxyl end. We find that both the 95- and 70-kD fragments bind to the membranes. Since all of our antibody-based assays detect both fragments, we conclude that the binding of both polypeptides is calcium-dependent, saturable, and specific to postsynaptic membranes. These results show that at least one of the receptor binding domains of agrin is located in the region delineated by the 70-kDa fragment.

The localization of a receptor binding domain in the 70-kDa fragment is highly informative in view of the known structure-function relationships of agrin. The 95-kDa fragment induces AChR clustering on myotubes, whereas the 70-kDa fragment does not (7, 22, 23). Consistent with this difference, one of the two alternative splice sites needed for optimal activity ("*8, 11*"; Fig. 2) is present in the 95-kDa fragment but likely to be missing in the 70-kDa fragment. However, the other essential site ("*4*") is present in the 70-kDa region. Together, these data suggest that a domain within the 70-kDa fragment is necessary for receptor binding, but not sufficient to induce AChR clustering. One possibility is that this domain could form part of a high affinity binding pocket along with the extreme carboxyl terminus unique to the 95-kDa fragment (37). It is also possible that agrin is multivalent and binds to more than one receptor. Perhaps the 95-kDa fragment can bind and dimerize two receptor monomers, whereas the 70-kDa polypeptide can only interact with one. This proposal is consistent with the requirement for many signaling receptors to oligomerize in order to be activated (41). Alternatively, the 70-kDa fragment may not bind with sufficient affinity to trigger the receptor. Indeed, membranes consistently bound higher levels of the 95 kDa as compared with the 70-kDa fragment (Fig. 2). The resolution of these questions must await purification and cloning of the agrin receptor and detailed binding studies with recombinant agrin fragments.

The best characterized function of agrin is to induce AChR clustering. In addition, in at least some cases agrin induces the phosphorylation of AChRs on tyrosine residues (42). It is important to understand the relationship between the agrin receptor and the AChR, two functionally intertwined integral membrane proteins concentrated in postsynaptic membranes. In previous work (18) we used cellular approaches and concluded that these molecules are distinct. In the current study we used biochemical methods to determine the possible relationship between these molecules. The salt and detergent optima for solubilization of the agrin receptor differ from those for the AChR (Fig. 8; see also Ref. 35). Most importantly, when the solubilized membranes are depleted of AChRs, agrin receptor levels are unchanged. It is noteworthy that the membranes used in these experiments had a full complement of peripheral proteins (*i.e.* the membranes were not alkaline-stripped) and were solubilized at neutral pH in mild nonionic detergent, conditions designed to preserve protein-protein interactions. Finally, we also compared the amount of bound anti-agrin antibody at saturating agrin concentrations (*cf.* Fig. 3) with α-bungarotoxin binding. The molar ratio of bound antibody to bound toxin is about 1:400 (~0.5 and 200 fmol/μg of protein, respectively). Thus, the approximate ratio of agrin receptor to AChR is 1:200. This calculation must be treated with caution, since bound anti-agrin antibody is an indirect indication of receptor levels, and the effect of immobilizing the membranes on plastic wells is not known. Interestingly, however, a similar estimate was obtained based on the amount of agrin needed to cluster AChRs on myotubes (7). Together, these data show that AChRs far outnumber agrin receptors in the postsynaptic membranes, and the two molecules are not tightly associated with one another. It seems most likely that intervening elements mediate the functional interaction of these proteins.

In sum, we have provided evidence that there is a unique agrin receptor in postsynaptic membranes of *Torpedo* electric organ. This integral membrane glycoprotein is ~200-fold less abundant than the AChR and is not tightly complexed with it. In addition, we have mapped at least one of agrin's receptor binding domains to a region encompassed by a 70-kDa fragment. We propose that agrin binding to a unique receptor is likely to initiate a signaling cascade, resulting in the redistribution of AChRs and other synaptic components to the neuromuscular junction.

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