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Non-neural agrin codistributes with acetylcholine receptors during early differentiation of *Torpedo* electrocytes

A. Cartaud[1,*], M. A. Ludosky[1], M. Haasemann[1], D. Jung[2], K. Campbell[2] and J. Cartaud[1]

[1]Biologie Cellulaire des Membranes, Département de Biologie Supramoléculaire et Cellulaire, Institut Jacques Monod, CNRS, Université Paris 7, 2 Place Jussieu, 75251 Paris Cédex 05, France
[2]Howard Hughes Medical Institute, Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242 USA

*Author for correspondence

SUMMARY

Agrin, an extracellular matrix protein synthesized by nerves and muscles is known to promote the clustering of acetylcholine receptors and other synaptic proteins in cultured myotubes. This observation suggests that agrin may provide at least part of the signal for synaptic specialization in vivo. The extracellular matrix components agrin, laminin and merosin bind to α-dystroglycan, a heavily glycosylated peripheral protein part of the dystrophin-glycoprotein complex, previously characterized in the sarcolemma of skeletal and cardiac muscles and at the neuromuscular junction.

In order to understand further the function of agrin and αDG in the genesis of the acetylcholine receptor-rich membrane domain, the settling of components of the dystrophin-glycoprotein complex and agrin was followed by immunofluorescence localization in developing *Torpedo marmorata* electrocytes. In 40-45 mm *Torpedo* embryos, a stage of development at which the electrocytes exhibit a definite structural polarity, dystrophin, α/β-dystroglycan and agrin accumulated concomitantly with acetylcholine receptors at the ventral pole of the cells. Among these components, agrin appeared as the most intensely concentrated and sharply localized. The scarcity of the nerve-electrocyte synaptic contacts at this stage of development, monitored by antibodies against synaptic vesicles, further indicates that before innervation, the machinery for acetylcholine receptor clustering is provided by electrocyte-derived agrin rather than by neural agrin.

These observations suggest a two-step process of acetylcholine receptor clustering involving: (i) an instructive role of electrocyte-derived agrin in the formation of a dystrophin-based membrane scaffold upon which acetylcholine receptor molecules would accumulate according to a diffusion trap model; and (ii) a maturation and/or stabilization step controlled by neural agrin. In the light of these data, the existence of more than one agrin receptor is postulated to account for the action of agrin variants at different stages of the differentiation of the postsynaptic membrane in *Torpedo* electrocytes.

Key words: Agrin, Dystrophin, Dystroglycan, Synaptogenesis, Electrocyte

INTRODUCTION

Differentiation of the neuromuscular junction (NMJ) depends on intricate interactions between sets of molecules synthesized by both partners: the motoneuron and the muscle fiber (reviewed by Hall and Sanes, 1993, Cartaud and Changeux, 1993). A crucial event in the genesis of the neuromuscular junction is the recruitment of nicotinic acetylcholine receptor (AChR) on the muscle cell surface beneath the nerve contact. Studies on molecules that direct the accumulation of AChRs at the neuromuscular junction led to the discovery of agrin, a protein of the synaptic basal lamina that was originally isolated from the *Torpedo marmorata* electrogenic organ on the basis of its ability to induce the clustering of acetylcholine receptors in vitro (reviewed by McMahan, 1990). The AChR clusters formed in this way are strongly reminiscent of those found at the developing vertebrate neuromuscular junctions in vivo and contain several other characteristic components of the nerve-muscle synapse suggesting that agrin plays a cardinal role in directing the postsynaptic differentiation (Nitkin et al., 1987; Smith et al., 1987; McMahan and Wallace, 1989; Fallon and Gelfman, 1989; Reist et al., 1992). Several agrin cDNA clones have been isolated (Rupp et al., 1991; Tsim et al., 1992) and it has been shown that agrin exists in various alternatively spliced forms in nerve and muscle that differ in their efficiency to induce AChR clusters (Ferns et al., 1992, 1993; Tsim et al., 1992). The most active isoform ($agrin_{4,8}$) with 4 and 8 amino acids inserts at the Y and Z splice sites in the rat (corresponding to A and B splice sites in the chick) is expressed by motoneurons and is quantitatively regulated during development (Magill-Solc and McMahan, 1988, 1990; Cohen and Godfrey, 1992; Reist et al., 1992; Hoch et al., 1993; Gesemann et al., 1995). These data confirm the so called 'agrin hypothesis', in which agrin

is believed to be one of the key instructive molecules for AChR recruitment (McMahan, 1990).

The molecular mechanism(s) by which agrin induces the AChR clustering was until now poorly understood. In the past decade the search for the agrin receptor (Nastuk et al., 1991; Ma et al., 1993) has received much attention. By convergent experimental approaches, several groups recently identified α-dystroglycan (αDG) as a high affinity binding site for agrin in the sarcolemma of mammalian muscle and in the postsynaptic membrane from *Torpedo* electrocytes (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; reviewed by Hoch et al., 1994). α-Dystroglycan is a peripheral membrane component of the dystrophin-glycoprotein complex (DGC) which also binds laminin and merosin in a Ca^{2+} dependent manner (Ervasti and Campbell, 1993) and which forms, via dystrophin, a network with the submembraneous actin-based cytoskeleton (Ervasti et al., 1991; Ibraghimov-Beskrovnaya et al., 1992). As such, α-dystroglycan represents an hypothetical key element in the signalling pathway between nerve and muscle. In agreement with this hypothesis, agrin and components of the DGC accumulate in the synaptic cleft and the postsynaptic membrane of the neuromuscular junction (Reist et al., 1987; McMahan, 1990; Matsumura et al., 1992). Thus, as in many other systems exhibiting membrane protein segregation (reviewed by Luna and Hitt, 1992; Nelson, 1992), the cytoskeletal proteins of the spectrin superfamily, to which dystrophin and utrophin belong, are likely to be engaged in the differentiation and the maintenance of the postsynaptic membrane domain.

Quite remarkably, in vitro binding experiments have shown that both nerve and muscle-derived agrin isoforms bind to α-dystroglycan from *Torpedo* electric organ or myotube membranes with a similar apparent affinity, raising the question of the respective physiological role(s) of the various agrin variants in synaptogenesis and of the existence of other functional agrin receptors (Sugiyama et al., 1994). In this line, one could hypothesize that α-dystoglycan would represent, by analogy with other systems (basic FGF receptor, TGF-β receptor), a sort of 'helper protein' which could serve to present agrin to a still unknown signalling receptor which requires the proteoglycan for high affinity binding (see Sugiyama et al., 1994, for discussion). Morever, the role of agrin isoforms in synaptogenesis is getting complicated by the observation that muscle-derived agrin also possesses AChR clustering activity and that agrin immunoreactivity is present in muscle prior to innervation and colocalizes with AChR clusters in developing muscle in the absence of motor innervation (Godfrey et al., 1988; Fallon and Gelfman, 1989). Taken together, these findings suggest that both nerve and muscle-derived agrin isoforms have their own function in the complex interplay leading to the establishment of the neuromuscular junction. Indeed, in rodents, the clustering of AChR begins within hours of neuronal contact at the neuromuscular junction, yet the stability of receptor clusters continues to increase for several weeks (Salpeter and Loring, 1985). This suggests that distinct mechanisms account for both the initiation and the stabilization of AChR clusters over the time course of synaptogenesis and that the agrin/DG partners may be involved at different stages of this process.

We previously reported that dystrophin appears early during *Torpedo* electrocyte differentiation, concomitant with clusters of AChRs (Jasmin et al., 1990). Therefore, as an initial step in understanding the role of agrin and dystroglycan in the differentiation of the postsynaptic membrane domain, we have taken advantage of the developing *Torpedo* electrocyte which presents a very slow and well characterized differentiation of its innervated membrane to determine immunohistochemically the settling of agrin and components of the DGC along with AChR. Our data emphasize a transient role of a non-neural agrin form in the earliest phase of AChR clustering and point to a two-step process of differentiation of the subsynaptic membrane possibly involving several agrin receptors.

MATERIALS AND METHODS

Antibodies

Polyclonal antisera against *Torpedo* dystrophin were raised in rabbits and characterized previously (Cartaud et al., 1992). Antiserum against α/β-dystroglycan (affinity purified sheep polyclonal antibody) was obtained from a fusion protein encoding α/β-dystroglycan from rabbit skeletal muscle (anti-FP-B). Monoclonal antibody (II H6) against α-dystroglycan (Ibraghimov-Breskrovnaya et al., 1992; Ervasti and Campbell, 1993) was also used. Monoclonal anti-agrin antibody (5 B1) previously characterized by Reist et al. (1987) was a gift from Dr McMahan. Monoclonal anti-synaptic vesicle antibody (SV2) was a gift from Dr Buckley (Buckley and Kelly, 1985).

Preparation of acetylcholine receptor-rich membranes and alkali extracts

Acetylcholine receptor-rich membranes were prepared from freshly dissected electric tissue obtained from *Torpedo marmorata* (Institut de Biologie Marine, Arcachon, France) as previously described (Saitoh and Changeux, 1980) with the addition of the protease inhibitors leupeptin (Sigma, 5 μg/ml) and pepstatin A (Sigma, 5 μg/ml), EDTA (5 mM) and EGTA (5 mM), phenylmethylsulfonyl fluoride (Sigma, 0.1 mM) and *N*-ethylmaleimide (Sigma, 10 mM). Typically 10-20 mg of membrane proteins were obtained from 100-200 g of fresh electrogenic tissue. The protein concentration was estimated by the method of Bradford (1976). Peripheral membrane proteins were removed by alkali treatment according to the method of Neubig et al. (1979). The alkaline extract (S11) was neutralized to pH 7.6 with 2 M Tris-HCl, pH 7.0, and the alkali-stripped membrane pellet (P11) was resuspended in 20 mM Tris-HCl, pH 7.6.

Purification of agrin

Agrin was partially purified (over 3,000-fold) from frozen *Torpedo* electric organ as described by Godfrey et al. (1984) and Nitkin et al. (1987). Briefly, the electric tissue (1,500 g) was homogenized in 10 mM Tris-HCl, pH 7.5, 450 mM NaCl, 1 mM EGTA, and 1 mM EDTA containing the protease inhibitors leupeptin (Sigma, 5 μg/ml) and pepstatin A (Sigma, 5 μg/ml), EDTA (5 mM) and EGTA (5 mM), and phenylmethylsulfonyl fluoride (Sigma, 0.1 mM) at 4°C and then centrifuged (17,500 ***g***, 30 minutes). The supernatant was discarded and the pellet was homogenized and centrifuged twice under the same conditions. The pellet was resuspended in 3% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA, stirred for 30 minutes, and centrifuged. The pellet was resuspended again in 3% Triton buffer solution and then centrifuged. The final pellet was resuspended and homogenized in 0.2 M sodium bicarbonate, pH 9, 5% glycerol, 0.02% sodium azide and stirred overnight at 4°C, rehomogenized, and centrifuged. The supernatant was loaded on a 15 ml Cibacron Blue 3GA-Agarose column (Affi-blue gel, 100-200 mesh; Bio-Rad laboratories, Richmond, CA) equilibrated in bicarbonate buffer and eluted with 1.5 M NaCl in bicarbonate buffer. Agrin containing fractions were pooled and frozen until used.

Detection of α/β-dystroglycan by immunoblotting

Purified AChR-rich membranes, alkali extracts (S11), and stripped membrane pellets (P11) were run on 1-D, 8% SDS-PAGE (Laemmli, 1970), using a Bio-Rad slab cell Mini Protean II. After transfer onto nitrocellulose, immunoblots were carried out according to the method of Towbin et al. (1979) with the peroxidase detection method.

Agrin overlay assay

Ligand blot overlay was achieved as described by Carr and Scott (1992). Briefly, proteins from AChR-rich membranes, alkali extracts (S11), and stripped membrane pellets (P11) were separated by 8% SDS-PAGE and electroblotted on nitrocellulose. Strips were blocked in 10% skimmed milk in PBS and incubated with 5×10^{-8} M agrin in the presence of 1 mM Ca^{2+} or 1 mM EGTA. The binding of agrin was revealed using anti-agrin antibodies 5B1 (1/1,000) and goat horseradish peroxidase-conjugated secondary anti-mouse antibodies (1/10,000) followed by enhanced chemiluminescent (ECL) detection (Amersham, UK; see Cartaud et al., 1993).

Immunofluorescence experiments

For immunofluorescence microscopy, pieces of adult and embryonic (40-45 mm body length) electrogenic tissue were fixed in 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, impregnated with 20% sucrose and rapidly frozen in liquid nitrogen-cooled Freon R22. Double fluorescence experiments were performed on cryostat sections (4 μm thick) using the anti-dystrophin (1/1,000), anti-agrin (1/1,000), anti-SV2 or anti-α/β-dystroglycan antibodies (FP-B:1/20) and tetramethylrhodamine (TMR) or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. TMR-conjugated α-bungarotoxin (1 μg/ml; Sigma) was used to label the AChRs in the membranes.

Immunogold labeling experiments

The immunogold labeling of the electric tissue was carried out on cryostat sections (see above) as previously described (Nakane, 1982; Kordeli et al., 1986) with the following modifications. To improve the probability of detection of well labelled and preserved areas, tissue sections were first treated as for immunofluorescence (see above), observed in the fluorescence microscope and the best areas marked out. Sections were then incubated with Protein A-gold (10 nm), fixed with 2.5% paraformaldehyde-1% tannic acid in 0.1 M phosphate buffer, postfixed with 1% OsO_4, dehydrated and finally embedded in Epon-Araldite resin. Thin sections were obtained after careful recovery of the preselected areas in the blocks and directly observed in the electron microscope (Philips CM12).

RESULTS

α-Dystroglycan: a high affinity agrin receptor in AChR-rich membranes

Proteins from purified AChR-rich membranes (Mb), alkali extract (S11) and residual membrane pellet (P11) were separated on 8% SDS-PAGE and immunoblotted with antibodies directed against α and β-dystroglycan. Monoclonal antibody II H6 against α-dystroglycan and affinity purified α/β-dystroglycan FP-B antibody cross reacted with two components of M_r 180,000 and M_r 43,000, respectively. These proteins were present in purified AChR-rich membranes and in alkali extracted membranes (Fig. 1). Immunodetection with anti-FP-B appears puzzling since it recognizes both α and βDG in rabbit skeletal muscle. As recently shown by Apel et al. (1995), one could suggest that the absence of immunoreactivity at the level of αDG in *Torpedo* tissue, likewise in quail, may be due to an incompletely or differently glycosylated form of the protein.

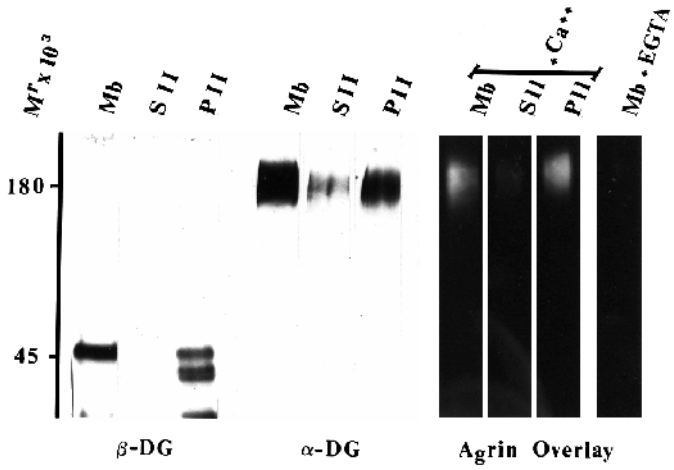

Fig. 1. α-Dystroglycan: a high affinity agrin binding component in AChR rich membranes from *Torpedo* electrocytes. SDS/8% PAGE-immunoblot analysis of α/β-dystroglycan in an AChR-rich membrane fraction (Mb), alkali extract supernatant (S11), and from alkali-stripped membranes (P11) from adult *Torpedo Marmorata* electrogenic tissue (sizes at left in kDa). From left to right: lanes 1-3, β-dystroglycan: immunodetection with anti-FP-B antibodies. Lanes 4-6, α-dystroglycan immunodetection with anti-α-dystroglycan (antibodies II H6). All fractions were recovered in the same volume of sample buffer, and an equal volume was deposited in each well to allow direct comparison. Immunodetection was achieved via horseradish peroxidase-conjugated IgG and the color reaction was developed with 4-chloro-1-naphtol/H_2O_2. Lanes 7-10, agrin overlay: an agrin binding assay was carried out in the presence of 1 mM Ca^{2+} or in the presence of 1 mM EGTA on AChR-rich membrane fraction (Mb), alkali extract supernatant (S11) and on alkali stripped membranes (P11). Agrin binds to the α-dystroglycan present in the AChR-rich membrane fraction and in the alkali stripped membranes in a Ca^{2+} dependent manner. Detection of bound agrin was achieved via anti-agrin 5B1 antibody and horseradish peroxidase-conjugated IgG followed by enhanced chemiluminescence (ECL) detection.

Using an in vitro agrin-binding assay we showed, in agreement with the data recently reported by Bowe et al. (1994) and Sugiyama et al. (1994), that the α-dystroglycan is the only detectable high affinity binding site for agrin in AChR-rich membranes in *Torpedo* electrocytes. The binding of agrin was effective at agrin concentrations as low as 5×10^{-8} M and is Ca^{2+} dependent (Fig. 1).

Immunofluorescence localization of α/β-dystroglycan and agrin in adult and developing *Torpedo* electrogenic tissue

In order to better understand the respective functions of the various elements of the DGC in the assembly of the AChR-rich membrane, we followed the distribution of agrin and of its high affinity receptor (α/βDG) by double immunofluorescence experiments in the adult *Torpedo* electrocyte and during its embryonic differentiation. AChR, agrin and dystroglycan were visualized by TMR-conjugated α-bungarotoxin, monoclonal anti-agrin antibody 5B1 and affinity purified anti-dystroglycan (FP-B), respectively.

Agrin distribution

In cryosections of adult electrogenic tissue, agrin immunoreactivity was detected at the level of the AChR-rich membranes (Fig. 2B). Agrin was detected in nerve trunks as well (Fig. 2B, arrows; see Reist et al., 1987). Double immunofluorescence experiments further disclosed that dystrophin colocalized with agrin at the AChR-rich membranes domain of the electrocytes (Fig. 2C,D). It is interesting to note that this specific location of dystrophin in the post synaptic membrane contrasted with that found in muscle, where dystrophin is distributed over the entire surface of the fiber, with the exception of NMJ where it is replaced by utrophin. Thus, the colocalization of agrin and dystrophin in the electrocyte corresponded to the synaptic location of agrin and utrophin in muscle (Reist et al., 1987). In 40-45 mm *Torpedo* embryos, a stage of development at which the electrocytes exhibit a definite structural polarity characterized in particular by the accumulation of AChRs at the ventral pole of the cells (Fig. 2E,G; see Witzemann et al., 1983; Kordeli et al., 1989), a strong agrin immunoreactivity can be detected at the ventral poles of the polarized electocytes (Fig. 2F,H). Compared to AChR labeling, agrin localization appeared much more focalized and was restricted to the ventral pole of the electrocytes (compare Fig. 2G and H). Particularly striking is the sharp extinction (arrows in Fig. 2H) of the fluorescence at the edges of the ventral pole membrane domain. This pattern of labeling contrasted with that of most of the other components of the AChR-rich membrane including those of the DGC (see below) which displayed an AChR-like distribution. Remarkably, agrin did appear (as estimated from immunofluorescence experiments) more concentrated in developing than in adult electrocytes. These data are in agreement with northern blot analysis showing that agrin transcripts are more abundantly expressed during embryonic development than in the adult (Campanelli et al., 1992).

A close examination of the 40-45 mm electrocyte sections reveals that agrin immunoreactivity was also present within the electrocytes (Fig. 3). As a secretion product, intracellular agrin

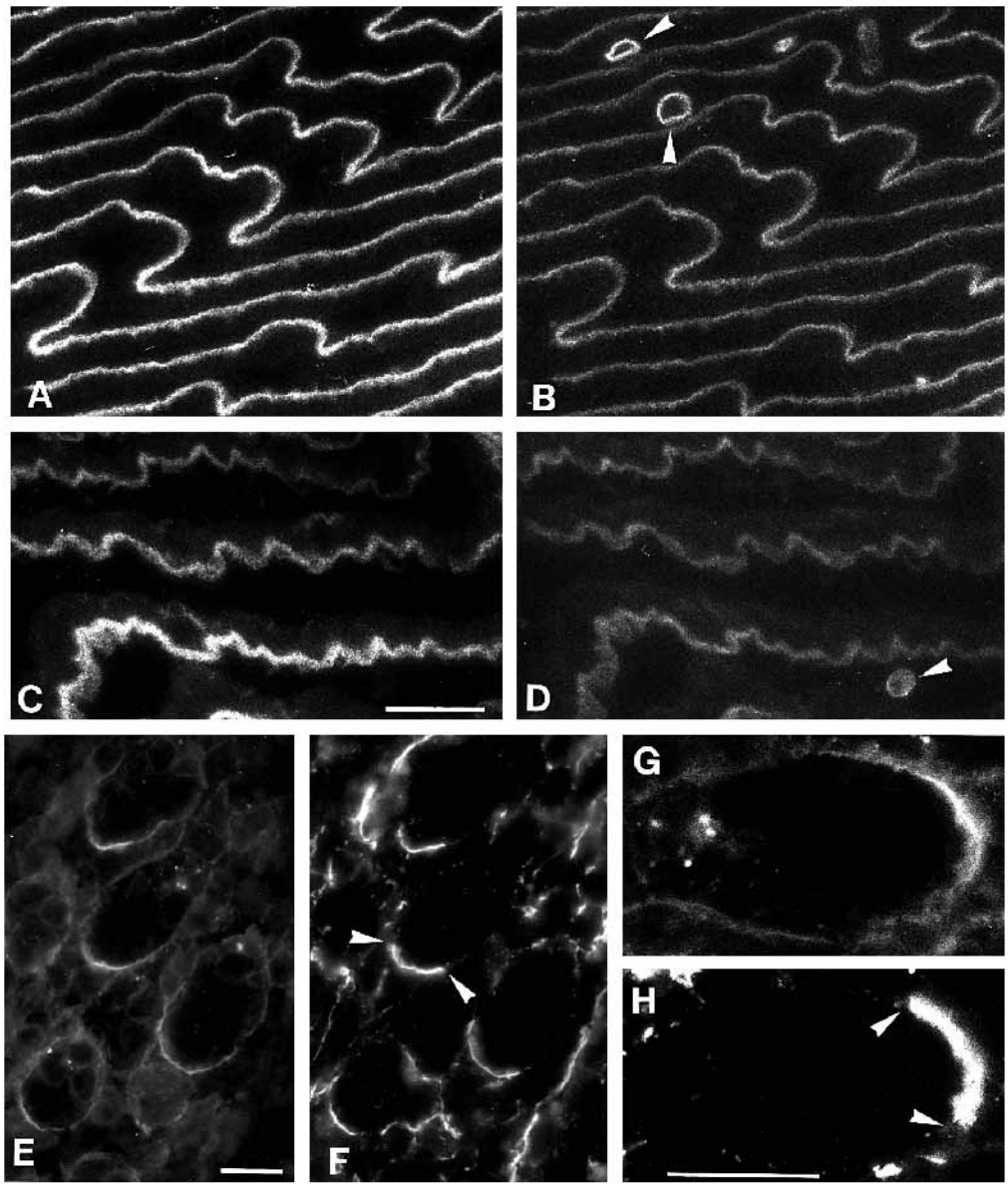

Fig. 2. Localization of AChR, agrin and dystrophin in adult and developing *Torpedo* electrocytes. In cryostat sections of adult electrogenic tissue, agrin immunoreactivity was detected at the innervated face of the electrocytes as visualized by double labelling with the AChR as well as around nerve trunks (arrowheads in B and D, α-bungarotoxin staining in A, anti-agrin antibody staining in B). Dystrophin and agrin also codistribute in the innervated membrane of adult electrocytes, (anti-dystrophin staining in C, anti-agrin staining in D). During embryogenesis, a strong agrin immunoreactivity (arrowheads in F and H) was detected at the ventral pole of 40-45 mm developing electrocytes (F), concomitant with the accumulation of AChRs (α-bungarotoxin labeling in E and G). Note the sharp extinction (arrowheads in H) of the agrin immunoreactivity compared to AChR (detail in G and H). Bars, 20 μm.

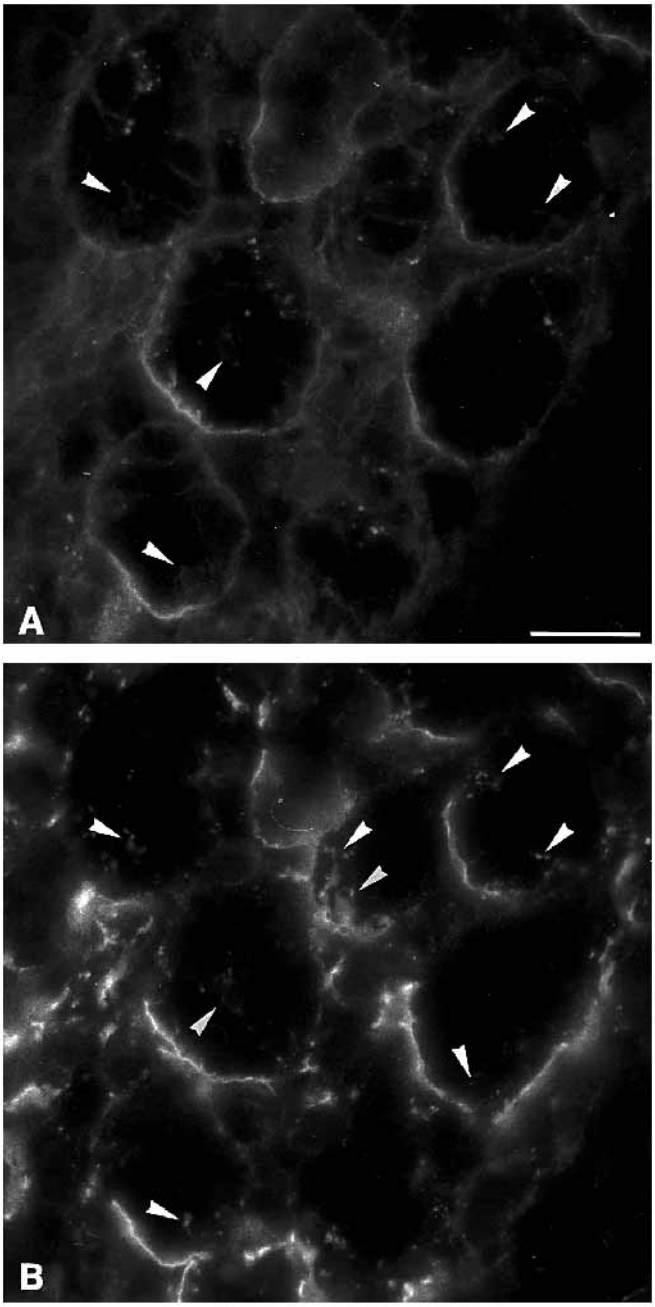

Fig. 3. Intracellular distribution of agrin in electrocytes from 45 mm embryos. In dorsoventral sections through the electrogenic tissue double-labeled with α-bungarotoxin and anti-agrin antibodies, agrin immunoreactivity was detected both at the ventral pole of the cells (see Fig. 2) and within intracellular compartments (arrowheads in B). Note that agrin staining often codistributes with intracellular, newly synthesized, AChRs (arrowheads in A) in the exocytic pathway. Bar, 20 μm.

was restricted to discrete compartments which comprise the Golgi apparatus (see Kordeli et al., 1989). Some of these structures were also associated with α-toxin fluorescence and therefore contained newly synthesized AChRs en route to the cell surface. These pictures indicate that the electrocytes are actively synthesizing agrin at this stage of development.

Dystroglycan distribution

Although both anti-dystroglycan antibodies (IIH6 and FP-B) did cross react with electrocyte α and β-dystroglycan, respectively, in immunoblot experiments, only FP-B antibody gave consistent immunofluorescence staining in *Torpedo* tissue. As α and βDG are derived from a single polypeptide precursor, and since αDG is tightly associated with the innervated *Torpedo* membrane (see Fig. 1), it is likely that they remained associated in the electrocyte membrane. We are thus confident that the labeling with anti-FP-B is indeed representative of the two proteins and, consequently we have used it throughout this work and will refer to it as 'DG-labeling'. In cryostat sections of adult electrogenic tissue, double immunofluorescence experiments show that most of the dystroglycan immunoreactivity (Fig. 4B) codistributed with AChRs in the innervated membrane (Fig. 4A). A prominent spotty pattern of distribution of dystroglycan was observed in the plane of the innervated membrane in tangential cryostat sections (Fig. 4C). In addition, a weak DG immunoreactivity was detected on the non-innervated membrane of the electrocyte (Fig. 4B, arrowheads). A close examination of this DG labeling reveals that it is limited to the edge of the cell, this suggesting that the dystroglycan is not distributed within the totality of the infoldings of the non-innervated membrane (see below for EM labeling). Until now, each protein identified in the plasma membrane or in the membrane-bound skeleton of *Torpedo* electrocyte, was localized exclusively either in the innervated (i.e. AChR, 43 kDa, dystrophin, syntrophin...) or in the non-innervated (Na^+/K^+-ATPase, ankyrin, spectrin...) membrane domain (see Kordeli et al., 1986). Thus, this observation represents, to our knowledge, the first evidence for a membrane-bound protein that distributes in both innervated and non-innervated domains of the electrocyte. It further emphasizes that the receptor for agrin may be present in membrane domains which do not contain dystrophin or utrophin (Jasmin et al., 1990; Sealock et al., 1991; Cartaud et al., 1992). Electron microscopy (EM) analysis of immunogold-labeled sections using FP-B antibodies reveals that dystroglycan immunoreactivity is confined to the cytoplasmic surface of the AChR-rich membrane domain underlying the nerve endings (Fig. 4D). This labeling is coherent with the immunofluorescence pattern of staining and with the notion that FP-B antibodies recognized almost exclusively a cytoplasmic epitope in β-dystroglycan. In agreement with the immunofluorescence experiments, EM pictures show that dystroglycan is also present, though at a lower level, at the cytoplasmic face of the non innervated membrane folds, precisely at the surface of the cell in contact with the basement membrane (Fig. 4E). In 40-45 mm developing embryos, immunofluorescence experiments show that dystroglycan was already present in the plasma membrane and start to accumulate in the AChR-rich domain (Fig. 5A,D). Thus, AChR, dystrophin and dystroglycan display a comparable subcellular localization while agrin is definitely more sharply localized and polarized.

SV2 distribution

Owing to the well established differential contribution of the various tissue specific variants of agrin in AChR clustering (see Hoch et al., 1994) it was of major concern to ascertain whether agrin detected at the early stages of electrocyte differentiation

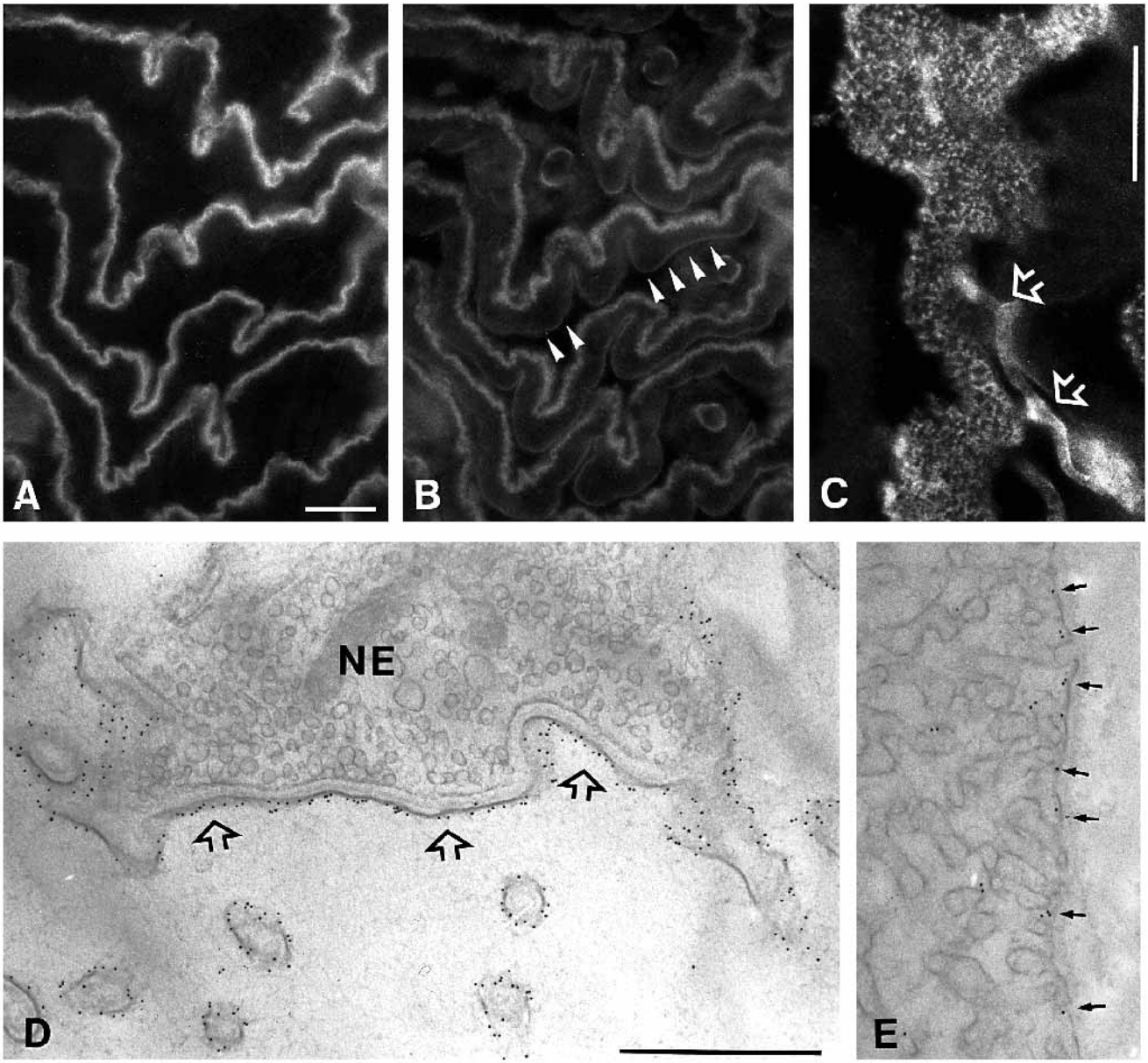

Fig. 4. Localization of AChR and dystroglycan in adult electrocytes. In adult electrogenic tissue, most dystroglycan (FP-B antibodies) immunoreactivity was detected in the postsynaptic membrane of the electrocytes. Close examination of a tangential view of the post synaptic membrane showed that dystroglycan immunostaining appears as spots distributed in the plane of the membrane (C). Note that nerves (arrows) are also labeled. (A) α-Bungarotoxin labeling, (B) anti-dystroglycan staining. The non-innervated membrane was also faintly labeled, in particular at the edge of the cells (arrowheads in B). At the EM level, immunogold labeling of dystroglycan was associated with the postsynaptic membrane (D) underlying the nerve ending (NE). Its association with the cytoplasmic surface of the membrane was coherent with immunoblotting experiments showing that the β-dystroglycan was recognized by FP-B antibodies. The localization of dystroglycan in infoldings of the non innervated membrane (arrows in E), matched the basement membrane deposited along the cell surface. Bars: (A and B), 20 μm; (C), 10 μm; (D and E), 1 μm.

derived from neural or electrocyte cells. The synaptogenic phase in developing electrogenic tissue begins at stage 50-55 mm. Neurofilament immunofluorescence experiments (Kordeli et al., 1989) and EM analysis (Mellinger et al., 1978), showed that at stage 40-45 mm, although nerve fibers began to invade electrogenic tissue, very few, if any, synaptic contacts were detected. The emergence of reliable markers of presynaptic differentiation (antibodies directed against the membrane-bound synaptic vesicle protein SV2) allowed us to reinvestigate this issue. The SV2-immunostaining pattern indeed confirmed that very few nerve endings were detected at developmental stage 40-45 mm (Fig. 6A,B). To verify that synaptic contacts would have been detected accurately with this marker, we provide a rare picture of such an innervation along the ventral pole of two electrocytes (Fig. 6C,D). From these data we conclude that most of agrin present in the developing electrogenic tissue at stage 40-45 mm was not secreted by nerve endings. More likely, agrin accumulation at the ventral pole of

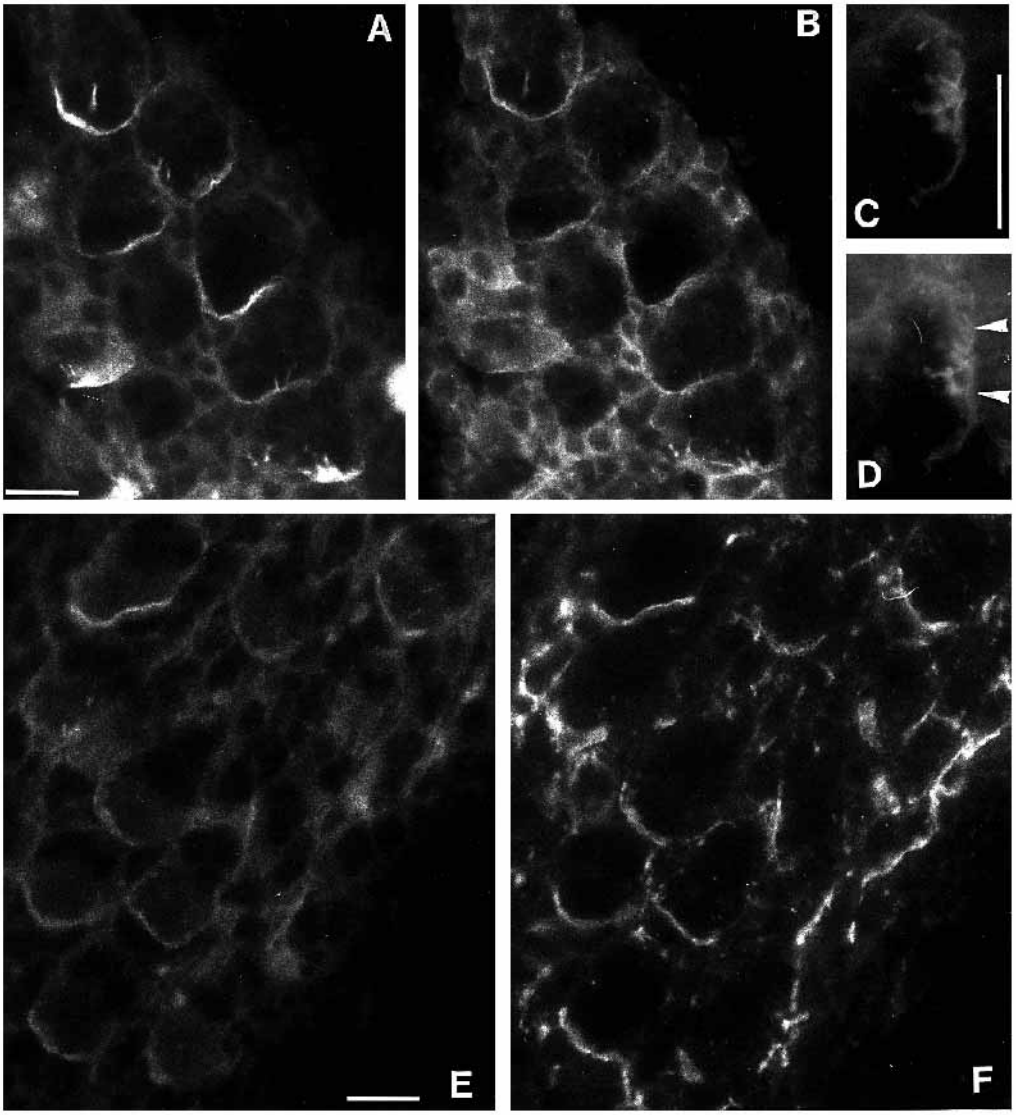

Fig. 5. Localization of agrin, DG and dystrophin in developing (45 mm) electrocytes. During embryogenesis, dystroglycan immunoreactivity (FP-B antibodies) was detected at the ventral pole of developing electrocytes (B) concomitant with the accumulation of AChR (α-bungarotoxin labeling in A). (C,D) Detail showing the codistribution of dystroglycan (arrowheads in D) and AChR (C) at one pole of a cell. (E,F) Immunofluorescence localization of dystrophin and agrin in developing electrocytes: anti-dystrophin staining in E, anti-agrin staining in F. Bars, 20 μm.

the electrocytes is contributed by the electrocytes themselves (see Fig. 3).

DISCUSSION

The data presented here show that agrin, dystroglycan and dystrophin, components which are specifically associated with the innervated membrane domain of adult *Torpedo* electrocyte, accumulate at an early stage of the differentiation of the developing electrocytes. In 40-45 mm embryos, electrocytes display a definite polarity characterized by the clustering of AChRs at the ventral pole of the syncytia before the onset of synaptogenesis. The main issue of this work, mainly derived from immunofluorescence experiments in situ, is that agrin accumulates very early during ontogenesis of the electrogenic tissue and appears as the most polarized and precisely localized component of the ventral surface of developing cells. Since innervation of the electrogenic tissue was not achieved at this developmental stage, we conclude that an electrocyte-derived agrin form rather than a neural agrin form is implicated in the initial step of differentiation of the AChR-rich membrane domain.

Several recent studies stressed the role of dystrophin-associated proteins in the process of agrin-mediated AChR clustering. In adult innervated muscle fibers, dystrophin and dystrophin-associated proteins are associated with the sarcolemma and accumulated at the folds of the NMJ (Watkins et al., 1988; Byers et al., 1991; Love et al., 1991; Yeadon et al., 1991). Utrophin, the autosomal homologue to dystrophin, is specifically localized at synaptic sites in normal muscles as well as dystrophin-associated proteins (Love et al., 1991; Khurana et al., 1990; Ohlendieck et al., 1991), from the earliest time in development (Phillips et al., 1993). Recently, α-dystroglycan, an extracellularly exposed peripheral membrane protein of the DGC involved in heparin inhibited and Ca^{2+}-dependent binding of extracellular matrix components laminin, merosin and agrin, was proposed as a potential functional agrin receptor (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Gee et al., 1993, 1994; Bowe et al., 1994; Campanelli et al., 1994; Sugiyama et al., 1994). This latter observation is likely to be of importance for synaptogenesis, since agrin is the

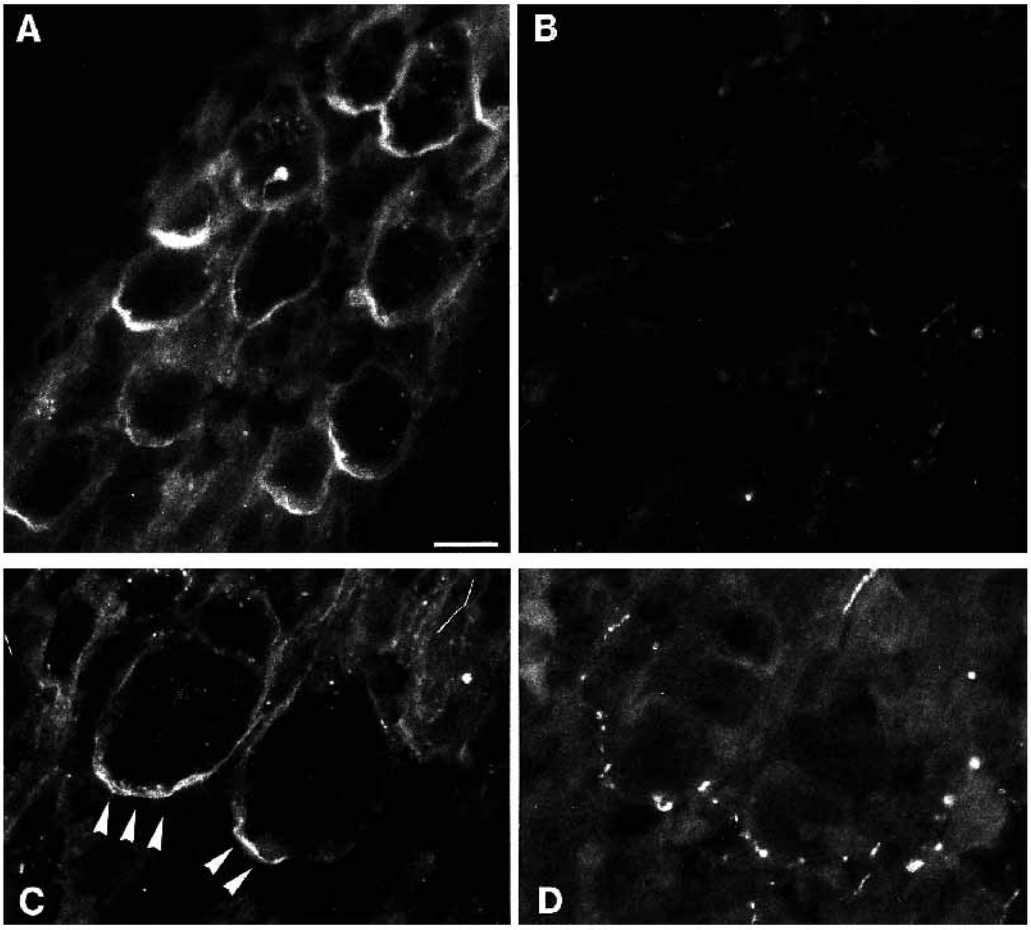

Fig. 6. Localization of AChR and synaptic vesicles in developing (45 mm) electrocytes. During embryogenesis, AChR was detected at the ventral pole of developing electrocytes forming dorso ventrally oriented columns (α-bungarotoxin labeling in A). At this stage of development, usually no nerve endings were detected with antibodies against synaptic vesicles (α-SV2) (B). Exceptionally, nerve endings running (α-bungarotoxin labeling, arrowheads in C) along the ventral pole of some electrocytes were detected with anti-SV2 antibody (D). Bar, 20 μm.

most potent AChR aggregating factor. Being synthesized and secreted by motoneurons in a developmentally regulated manner (Hoch et al., 1993; Ferns et al., 1992, 1993; Gesemann et al., 1995), agrin is believed to represent a signalling neural factor responsible for the clustering of AChRs during NMJ formation (reviewed by McMahan, 1990). Yet, until now, there is little information on the settling of dystroglycan during NMJ formation in vivo. Here, we show that during the early differentiation of the AChR-rich membrane domain of the developing electrocyte, DG accumulates concomitantly with dystrophin and AChR. These observations in vivo are in agreement with several recent observations (Campanelli et al., 1994; Cohen et al., 1995), showing that αDG codistributes with most agrin-induced or nerve-induced AChR clusters in vitro.

An interesting observation of the present study is that agrin accumulating at the vental pole of the differentiating electrocytes appears more sharply localized than DG, dystrophin or AChR. This observation, which has not been reported in other model animals, clearly illustrates the instructive role of agrin in organizing the recruitment of AChR via the DGC along a diffusion trap model. Further, agrin accumulates at an early stage of differentiation of the electrocytes prior to the establishment of innervation. Indeed, an intracellular pool of agrin was detected by immunofluorescence inside the differentiating electrocytes. One should thus consider that a muscle-derived agrin isoform is engaged, at least transiently, in the recruitment of AChR, via the conventional DG/dystrophin complex. The present data are in agreement with earlier observations in aneural muscles or premuscle masses that muscle-derived agrin codistributes with AChR clusters in vivo (Godfrey et al., 1988; Fallon and Gelfman, 1989). Yet, the physiological relevance of non-neural agrin in synaptogenesis remained puzzling. Our data suggest that a muscle-derived agrin variant is likely to be involved in the initial step of the differentiation of the postsynaptic membrane, prior to the establishment of innervation by presynaptic electromotor neurons. It will be of interest to determine by PCR analysis which of the few possible agrin transcripts (Gesemann et al., 1995) is actually expressed in developing electrocytes.

In the light of these findings, the existence of a unique functional receptor for various agrin variants could be questionned. Such an issue has already been emphasized given: (i) the similar affinity of muscle and nerve agrin variants for αDG determined by in vitro binding experiments; (ii) their dramatically different abilities to aggregate AChR; and (iii) the discrepancy between the concentration at which it is biologically active and the concentration at which it binds to DG in vitro (see Sugiyama et al., 1994; Fallon and Hall, 1994, for discussion). The present data point to a two-step model of AChR clustering. In this model, αDG would mediate, possibly in association with other signalling agrin receptor(s), both the initiation and the stabilization of AChR clusters. First, an electrocyte-derived agrin form would trigger the initiation of AChR clustering mediated by the DGC machinery. Upon innervation, a nerve-derived agrin would then induce the

stabilization of the clusters, via the same, additional or different receptor(s). In support of this two-step process is the present finding that the first AChR clusters are observed in these cells, concomitant with the DGC machinery, before innervation and independently of the presence of the peripheral 43 kDa protein (rapsyn), which in this system stabilizes the AChR only after its clustering in the ventral membrane domain (Kordeli et al., 1989; LaRochelle et al., 1990; Nghiêm et al., 1991).

Also, and most interestingly, tyrosine phosphorylation of the AChR-β subunit, which is required for agrin-induced aggregation (Wallace, 1990, 1994, 1995), is completed belatedly during biogenesis of the *Torpedo* postsynaptic membrane domain (Cartaud et al., 1995). This suggests that in contrast with neural agrin the primary effect of electrocyte-derived agrin in AChR clustering in vivo would not require tyrosine phosphorylation. Later in development i.e. after innervation, neural agrin would then induce tyrosine phosphorylation of the AChRs, a posttranslational modification likely to promote their association with the cytoskeleton (Wallace, 1995). Interestingly, the recent observation that βDG interacts with Grb2, an ubiquitous adapter protein containing SH2 and SH3 domains (Yang et al., 1995), is in agreement with the involvement of DG in signal transduction.

In the course of this work, we have also observed that DG is present, though at a lower concentration, in the non-innervated membrane, which does not contain dystrophin or dystrophin related components but does contain spectrin (Jasmin et al., 1990, and references therein). At this location, DG is likely to associate with laminin (Kordeli et al., 1989). Yet, the presence of DG in domains of the plasma membrane devoid of dystrophin raises the possibility that DG may serve different functions in various membrane domains; this in agreement with its known location in non-muscle tissues (Ibraghimov-Breskovnaya et al., 1993; Kadoya et al., 1995).

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