Asynchronous Assembly of the Acetylcholine Receptor and of the 43-kD \( \nu_1 \) Protein in the Postsynaptic Membrane of Developing Torpedo Marmorata Electrocyte

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Abstract. The assembly of the nicotinic acetylcholine receptor (AchR) and the 43-kD protein (\( \nu_1 \)), the two major components of the postsynaptic membrane of the electromotor synapse, was followed in \textit{Torpedo marmorata} electrocyte during embryonic development by immunocytochemical methods. At the first developmental stage investigated (45-mm embryos), accumulation of AchR at the ventral pole of the newly formed electrocyte was observed within columns before innervation could be detected. No concomitant accumulation of 43-kD immunoreactivity in AchR-rich membrane domains was observed at this stage, but a transient asymmetric distribution of the extracellular protein, laminin, which paralleled that of the AchR, was noticed. At the subsequent stage studied (80-mm embryos), codistribution of the two proteins was noticed on the ventral face of the cell.

Intracellular pools of AchR and 43-kD protein were followed at the EM level in 80-mm electrocytes. AchR immunoreactivity was detected within membrane compartments, which include the perinuclear cisternae of the endoplasmic reticulum and the plasma membrane. On the other hand, 43-kD immunoreactivity was not found associated with the AchR in the intracellular compartments of the cell, but codistributed with the AchR at the level of the plasma membrane. The data reported in this study suggest that AchR clustering in vivo is not initially determined by the association of the AchR with the 43-kD protein, but rather relies on AchR interaction with extracellular components, for instance from the basement membrane, laid down in the tissue before the entry of the electromotor nerve endings.

The postsynaptic membrane of the electromotor synapse from \textit{Torpedo} electric tissue is composed of two major proteins: a transmembrane protein, the acetylcholine nicotinic receptor (AchR)\(^1\) (reviewed in Popot and Changeux, 1984; Hucho, 1986; Changeux et al., 1987) and an extrinsic protein of apparent molecular mass 43,000 D, referred to as the 43-kD protein (reviewed in Kordeli et al., 1986; Changeux et al., 1984, 1987). This last protein was identified (Sobel et al., 1977) in purified postsynaptic membranes from \textit{T. marmorata} electric tissue to which it contributes \( \sim 40-60\% \) of the total extrinsic proteins extracted by mild alkaline or chaotropic agents (Neubig et al., 1979; Frohner et al., 1981). cDNAs coding for the \textit{T. californica} 43-kD protein have recently been cloned and sequenced (Frail et al., 1987).

Biochemical and immunocytochemical studies at the EM level showed that the 43-kD protein is localized on the cytoplasmic side of the membrane (Wennogle and Changeux, 1980; Elliott et al., 1980; St. John et al., 1982), takes part in the subsynaptic densities (Cartaud et al., 1981) and codistributes with the AchR (Nghiêm et al., 1983; Sealock, 1984; Kordeli et al., 1986, 1987; Bridgman et al., 1987). Extraction of the 43-kD protein by mild alkaline treatment (Neubig et al., 1979) from purified \textit{Torpedo} postsynaptic membrane fragments (Rousselet et al., 1979, 1982; Lo et al., 1980; Cartaud et al., 1981; Barrantes et al., 1980) and from myotube membranes (Bloch and Frohner, 1987) causes rotational and translational mobilities of the AchR molecules within the membrane plane. After such treatment, no significant change of the permeability response to Ach and of ligand binding to the AchR was noticed with \textit{T. californica} AchR-rich membranes (Neubig et al., 1979). From these data, it was suggested that the 43-kD protein and possibly

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1. Abbreviations used in this paper: AchR, the nicotinic acetylcholine receptor protein; \( \alpha \)-Bungtx, \( \alpha \)-bungarotoxin; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; 43-kD, the membrane-bound \( \nu_1 \) protein, NF, neurofilament.
other still unidentified peripheral components of the domain, has a structural role and, in particular, contributes to the immobilization of the AchR in the postsynaptic membrane (reviewed in Froehner, 1986). Moreover, structural (Cartaud et al., 1983; Kordeli et al., 1986, 1987b) and biochemical (Walker et al., 1984) evidences point to the possible association of the 43-kD protein with various elements of the cytoskeleton such as intermediate filaments or actin (Walker et al., 1984; see however Kordeli et al., 1987a).

Immunocytochemical studies showed that an immunologically related protein to the 43-kD protein is present at the mammalian motor end plate (Froehner et al., 1981; Froehner et al., 1983; Kordeli et al., 1986, 1987b) and biochemical mobilization of the AchR in the postsynaptic membrane (Walker et al., 1984) evidences point to the possible association of the protein and gave the best results in the early phase of embryonic development in which the expression level of the 43-kD protein is very low. Amplification of the fluorescence labeling of AchR with α-bungarotoxin (α-Bungtx) and EM immunolabeling was achieved with a rabbit anti-α-bungarotoxin antibody (provided by Dr. R. Sealock (University of North Carolina at Chapel Hill, Chapel Hill, NC).

Mouse anti-neurofilament (NF) antibody (monoclonal antibodies directed against the three NF proteins) was provided by D. Paulin (Institut Pasteur, Paris).

Double labeling experiments were carried out essentially as described in Kordeli et al. (1986). Enhancement of α-Bungtx staining of the AchR was achieved, when possible (in experiments in which the tested antibody was raised in a species different from rabbit), by an incubation with a rabbit anti-α-Bungtx antibody (1:1000) followed by the second fluorescent antibody.

Indirect Immunofluorescence

Double labeling experiments were carried out essentially as described in Kordeli et al. (1986). Enhancement of α-Bungtx staining of the AchR was achieved, when possible (in experiments in which the tested antibody was raised in a species different from rabbit), by an incubation with a rabbit anti-α-Bungtx antibody (1:100) followed by the second fluorescent antibody.

Immunoperoxidase and Immunogold EM Labelings

Immunoperoxidase or immunogold labelings were achieved on 3–4-μm-thick cryostat sections of paraformaldehyde-fixed tissue. Immunoperoxidase staining was achieved with immunoperoxidase (PO)-coupled goat anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands) revealed according to Sternberger (1979).

For immunogold labeling also carried out on cryostat sections, the secondary antibody was coupled to 5- or 10-nm gold particles (AuroProbe EM). In some experiments, immunogold labeling was checked by fluorescence microscopy after an additional labeling of the goat-IgG-gold complexes with rhodamine-coupled rabbit anti-goat IgG. After selection of the well-stained areas, the sections were fixed again with 1% glutaraldehyde 0.2% tannic acid, postfixed with 1% osmic acid, dehydrated in a graded series of alcohol, and embedded in epon-araldite. Thin white to yellow sections were observed without any staining in an electron microscope (Philips 400).

Results

I. Characterization of the Anti-43-kD Protein Antibodies

Immunocytochemical experiments and immunodetection of the 43-kD protein on gels were carried out with three different antibodies. First, a polyclonal antibody was raised in rab-

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bits against a purified preparation of the *T. marmorata* 43-kD protein and affinity purified on 43-kD protein blotted on one-dimensional gels. This antibody is specific for the 43-kD isoforms (Fig. 4, a–c), has a high affinity for the SDS-denatured form of the protein, and gave the best results on immunoblots with the early stages of embryonic development at which the levels of 43-kD protein are low.

A mAb developed in the mouse against the *T. californica* 43-kD protein was used in some double labeling experiments and was generously provided by S. Froehner (Dartmouth Medical School, Hanover, NH).

In most of the experiments, an antibody raised against a synthetic peptide corresponding to AA 340–354 of the known sequence of *T. californica* 43-kD protein (Frail et al., 1987; Carr et al., 1987) was used (see Materials and Methods). Its specificity was checked on two-dimensional gels of purified postsynaptic membranes as shown in Fig. 1, b–d. The antibody strongly reacted with the spots of the \( \alpha \) isoforms of the 43-kD protein. The other components (\( \nu_2 \) and \( \nu_3 \); Gysin et al., 1981) were not labeled. However, two additional small spots were also labeled. The antipeptide antibody was tested by immunofluorescence on cryostat sections of adult *T. marmorata* electric tissue. Double labeling experiments (Fig. 1, e and f) showed that \( \alpha \)-Bungtx and antipeptide fluorescence coincided. The antipeptide antibody almost exclusively recognized the innervated side of the electrocytes (see Nghiêm et al., 1983; Sealock, 1984). Moreover, preincubation of the serum with a 10-fold molar excess of the peptide resulted in an important reduction of the fluorescence associated with the innervated face of the electrocytes (Fig. 1, g and h).

II. Subcellular Distribution of AchR and 43-kD Protein in the Electrocytes of 45-mm Embryos

In an earlier work, Witzemann et al. (1983), using radioautography, showed that AchR accumulation at the ventral pole of the developing electrocyte was already detected at the stage of 40–45 mm. At that stage, the electrocyte precursors have a bag shape and are elongated dorsoventrally. They already disclose a remarkable structural asymmetry with the nuclei in the dorsal moiety of the syncytium and a clear cytoplasm in the ventral one (Fig. 2). The simultaneous visualization of the AchR and 43-kD protein (Figs. 2 and 3) was done by immunofluorescence using FITC \( \alpha \)-Bungtx with, or without, amplification with a rabbit-antitoxin antibody (see Materials and Methods). In all instances, this fluorescence was totally abolished when \( \alpha \)-Bungtx was omitted (not shown). Fig. 2 a shows the typical distribution of bound FITC \( \alpha \)-Bungtx within 45-mm embryos. In dorso-ventral sections of the electric lobe, only the columns containing electrocytes were stained, whereas skin and intercolumnar cells remained unstained. At higher magnification (Fig. 2 b), fluorescence was detected at the level of the plasma membrane of the electrocyte and found concentrated on the ventral pole of the cell. In addition, intracellular compartments displaying a significant amount of bound \( \alpha \)-Bungtx fluorescence were observed. EM pictures of electric tissue labeled with PO (Fig. 2 c) or immunogold (not shown) disclosed that this labeling was primarily associated with the perinuclear ER and dispersed cytoplasmic vesicles (ER and Golgi bodies). From these images, no evidence for an oriented intracellular targeting of the AchR-containing vesicles toward the ventral pole of the cells was obtained.

In double-fluorescence experiments (Fig. 3), the 43-kD protein immunoreactivity consisted of a faint and diffuse fluorescence, mostly with a cytoplasmic distribution (Fig. 3, a, c, and d). No particular association with the AchR-rich ventral membranes was observed (compare Fig. 3 a with Fig. 2 a and Fig. 3 b with Fig. 3 c). Inhibition experiments carried out with the antipeptide antibody preincubated with the peptide (see legend to Fig. 1) did result in only partial (30–40%), as measured with the photomultiplier of the microscope in position spot) extinction of the fluorescence (data not shown). Most likely, under these conditions, autofluorescence of the tissue and/or unspecific labeling becomes significant compared with the faint specific signal. EM pictures from immunogold experiments also did not reveal any significant accumulation of 43-kD protein immunoreactivity at the level of the plasma membrane, even at the ventral pole of the electrocytes (compare Fig. 3, e and f). To confirm further the absence of 43-kD protein immunoreactivity at the AchR-rich ventral pole of the developing electrocytes, another antibody was tested. Both recognized the 43-kD protein in the adult and 80-mm embryos and failed to label the AchR-rich domain in the electrocyte of the 45-mm embryos (Fig. 3, c and d).

Immunoblotsing experiments were carried out, in parallel, on homogenates from 45-mm embryos with a rabbit anti-43-kD antibody directed against the 43-kD protein (see Materials and Methods), which displays a high affinity for the denatured 43-kD protein on SDS gels (Fig. 4, a–c). At this stage, the 43-kD immunoreactivity was mainly detected in the cytosolic fraction (Fig. 4 d).

III. Subcellular Localization of AchR and 43-kD Protein in 80-mm Embryos

The general organization of the electric tissue in 80-mm embryos resembled that of the adult, except that the innervation was scarce. The electrocytes possess a flat discoid shape oriented in planes parallel to the plane of the body. The transition of the electrocyte from a bag to a disc along its main axis of symmetry was possibly achieved by lateral fusion of the smaller syncytial precursor (Fox and Richardson, 1978, 1979). The dorsoventral asymmetry of the electrocyte membrane, already evident in the earlier developmental stages, was preserved and even amplified resulting in fully differentiated dorsal and ventral membrane domains.

The fluorescence of bound \( \alpha \)-Bungtx in 80-mm electrocytes was detected essentially on the ventral face of the electrocytes (Fig. 5 a). Both the dorsal plasma membrane and the perinuclear ER membrane exhibited some labeling. The presence of AchR in the dorsal and perinuclear membranes was never observed in adult tissue. Fig. 5 b shows the same cryostat section stained with the anti–43-kD peptide antibody. The distribution of immunoreactivity corresponded almost exactly to that of the AchR, in particular at the level of the ventral plasma membrane domain. A faint cytoplasmic labeling also was detected that compared with the adult images, appeared significantly higher than background levels. Consistent with this observation, immunoblotting experi-

2. This antibody was not used in immunocytochemical experiments because of its low affinity for the native form of the 43-kD protein.
Figure 1. Characterization of the antipeptide antibody. (a) Sequence of the synthetic peptide (AA 340–354, Frail et al., 1987) used to immunize rabbits. (b) Ponceau staining of a blot of two-dimensional gel of purified T. marmorata AchR-rich membranes supplemented with cytoplasm in which \( \nu_1 \) protein is abundant (arrow). Two additional spots (different from \( \nu_2 \) and \( \nu_3 \)) were also faintly stained. (c) Alkaline phosphatase detection with the antipeptide antibody showing that the \( \nu_1 \) isoforms of the 43-kD protein were recognized (1:1,000 dilution). (d) Inhibition experiment: same conditions as in c, but with antipeptide antibody (1:1,000) preincubated with an excess of peptide. (e–h) Double fluorescence experiments on cryostat sections of adult T. marmorata electric tissue. Innervated faces were labeled with 1 \( \mu \)g/ml FITC-\( \alpha \)-Bungtx in e and g and f with antipeptide antibody (1:200 dilution) or antipeptide antibody (1:200 dilution) absorbed with (h) a 10-fold molar excess of peptide. Exposure times in the microscope and for printing are the same in f and h. The stained structure in h (arrow) probably represents blood vessels. Bar, 50 \( \mu \)m.
Figure 2. Subcellular distribution of AchR in electrocytes from 45-mm embryos. (a) Dorsoventral section through the electric tissue labeled with 1 μg/ml α-Bungtx, 1 μg/ml rabbit anti–α-Bungtx antibody, and 5 μg/ml fluorescein-coupled goat anti-rabbit IgG. AchR staining was associated with the plasma membrane of the electrocytes forming dorsoventrally oriented columns. Accumulation of AchR at the ventral pole was visible in most cells. Bar, 50 μm. (b) Detail showing the accumulation of the AchR at the ventral plasma membrane domain (arrows). Note that perinuclear membrane was also stained (open arrows). Control experiments in which α-Bungtx was omitted were totally negative (not shown). Bar, 50 μm. (c) Immunoperoxidase staining at the EM level: in addition to the ventral pole of the cell (open arrows), perinuclear ER membrane, Golgi body (G), and intracellular vesicles (arrows) were also stained. From these pictures, the AchR distribution in intracellular compartments does not suggest a targeted delivery of the newly synthetized AchR at the ventral membrane. Note the polar organization of the cell with the nuclei (N) at the dorsal pole and a clear cytoplasm containing mitochondria (M) at the ventral pole of the cells.
Figure 3. Comparative subcellular distribution of AchR and 43-kD protein in 45-mm embryos. (a) The fluorescence associated with the anti-43-kD immunostaining (mAb 1:600 dilution) was faint and diffuse. Comparison with the same section stained for the AchR (Fig. 2 a) illustrates that the 43-kD protein was not associated with the AchR rich ventral pole of the electrocytes. Bar, 50 μm. (b–d) Detailed views of electrocytes showing the diffuse fluorescence associated with the 43-kD protein staining. (b) α-Bungtx staining (arrows point to the Ach-R-rich ventral membranes); (c) anti-43-kD, mAb (1:600 dilution); (d) antipeptide antibody (1:200 dilution). Bars, 50 μm. (e–f) Immunogold localization at the EM level of the (e) AchR and (f) 43-kD protein (antipeptide antibody). Immunogold labeling experiment provided no indication for an accumulation of the 43-kD protein at the cytoplasmic side of the AchR-rich ventral membrane of the electrocytes.
Subcellular distribution of the 43-kD protein in electrocytes at different stages of development. (a–c) The affinity-purified anti-43-kD protein antibody (1:50 dilution) is directed against the 43-kD α1 protein of T. marmorata electrocyte (immunoperoxidase detections). (a) Immunostaining of a purified adult AchR-rich membrane (one-dimensional gel). (Arrowhead) Location of the 43-kD protein. (b) Ponceau red staining of a blot of purified AchR-rich membrane (two-dimensional gels). (c) Immunostaining demonstrating the specificity of the antibody. (d) The anti-43-kD antibody (1:200 dilution) recognizes a polypeptide with an apparent molecular mass of 43 kD in both cytosolic (c) and particulate (p) fractions at the three developmental stages tested (alkaline phosphatase immunodetection). Note the dramatic enrichment in 43-kD protein of the particulate fractions with age. Cytosolic and particulate fractions were obtained after homogenization and centrifugation (30,000 g, 30 min) of frozen electric tissue. Antiproteases were present during the whole purification steps. The same amount of protein (100 μg) from each fraction was layered in each well.

Figure 4. Subcellular distribution of the 43-kD protein in electrocytes at different stages of development. (a–c) The affinity-purified anti-43-kD protein antibody (1:50 dilution) is directed against the 43-kD α1 protein of T. marmorata electrocyte (immunoperoxidase detections). (a) Immunostaining of a purified adult AchR-rich membrane (one-dimensional gel). (Arrowhead) Location of the 43-kD protein. (b) Ponceau red staining of a blot of purified AchR-rich membrane (two-dimensional gels). (c) Immunostaining demonstrating the specificity of the antibody. (d) The anti-43-kD antibody (1:200 dilution) recognizes a polypeptide with an apparent molecular mass of 43 kD in both cytosolic (c) and particulate (p) fractions at the three developmental stages tested (alkaline phosphatase immunodetection). Note the dramatic enrichment in 43-kD protein of the particulate fractions with age. Cytosolic and particulate fractions were obtained after homogenization and centrifugation (30,000 g, 30 min) of frozen electric tissue. Antiproteases were present during the whole purification steps. The same amount of protein (100 μg) from each fraction was layered in each well.

Immunogold localization experiments at the EM level uncover additional information on the subcellular distribution of the AchR and 43-kD protein at this 80-mm stage. Taking advantage of the high biosynthetic activity expected to occur at this stage of embryonic development, we were able to follow both the terminal phase of AchR and 43-kD protein accumulation in the ventral membrane domain, and their main intracellular pathways. Besides its accumulation at the ventral domain, AchR immunoreactivity was detected in the dorsal domain and in the perinuclear ER membrane (Fig. 5 d). On the other hand, 43-kD protein immunoreactivity was observed exclusively at the level of the plasma membrane (Fig. 5 e and f). In particular, it was not found associated with the perinuclear ER membrane containing newly synthesized AchR. This observation suggests that: (a) the AchR, a typical integral membrane glycoprotein, and the 43-kD, a peripheral protein, follow different intracellular pathways, and (b) the 43-kD protein associates with the AchR only after receptor clustering has occurred in the plasma membrane.

IV. Immunocytochemical Detection of Laminin in 45-mm Embryos

Laminin is a component of the basement membrane generally assumed to play a role at the early stages of cell differentiation (reviewed in Martin and Timpl, 1987). We have looked for the presence of laminin in 45-mm embryos using an antilaminin antibody. As shown in Fig. 6, laminin immunoreactivity was detected in 45-mm electric tissue. Although most of the fluorescence was observed at the periphery of the growing columns, a significant labeling of the ventral faces of the electrocytes was consistently observed (Fig. 6 b). This latter distribution paralleled that of the AchR. (Compare Fig. 6, a and b.) However, the accumulation of laminin at the AchR-rich pole of the cell was a transient event. After electrocyte flattening, similar laminin staining was observed on both the ventral and dorsal surfaces of the cell (Fig. 6 c).

V. Immunocytochemical Detection of the Electromotor Innervation in Developing T. marmorata Electric Organ

Determining the precise timing of electric tissue innervation is of crucial importance to understand the mechanisms involved in the differentiation of the postsynaptic membrane domain. Controversial data were reported on the development of the innervation at the earliest stages of electrocyte differentiation. According to the most recent study (Fox and Richardson, 1979), at the 55-mm stage the intercolumnar nerves begin to send neurites that invade the electrocytes. However, Mellinger et al. (1978) claimed from EM pictures that traces of innervation were already detected at the 46-mm stage. This latter point is important because accumulation of AchR on the ventral face of the electrocyte is already evident at the 44–45-mm stage. In this study, we have tried to detect the onset of innervation in the electrocytes in developing embryos by following the immunofluorescence of NF labeled with anti–NF antibodies. Although NF are not a marker of the nerve endings, NF immunofluorescence is a very sensitive tool for the detection of axonal projections. In double fluorescence experiments carried out with permeabilized electric tissue from 45-mm embryos, anti–NF antibodies stained nerves running in the intercolumnar tissue in a direction perpendicular to the main dorsoventral axis of the columns. Yet, we never observed either axonal projections entering the columns at this stage (Fig. 7, a and b) or synaptic contacts in EM pictures. Whereas these observations did not exclude the occurrence of a few nerve terminals, they rule out the presence of a massive innervation at this developmental stage. At a later stage (70–80 mm) evidence for axonal projections going from the main nerve axis to the columns of electrocytes was obtained (Fig. 7, c and d). At this stage, NF staining within the columns was still scarce, a finding consistent with the low density of synaptic contacts revealed by EM in the same tissue. Finally, in the adult organ (Fig.
Figure 5. Comparative subcellular distribution of AchR and 43-kD protein in 80-mm embryos. (a-b) Double fluorescence experiment showing the localization of (a) AchR and (b) 43-kD protein (antipeptide antibody, 1:200 dilution), mainly associated with the ventral plasma membrane of the flattened electrocytes. AchR and 43-kD proteins were also present in the dorsal noninnervated membrane. Note that AchR was visualized at the periphery of nuclei (open arrows) and that some 43-kD immunoreactivity was visible in the cytoplasm. Bars, 50 μm. (c) Inhibition experiment achieved by preincubation of the antipeptide antibody with a 10-fold excess of peptide. An almost complete extinction of the fluorescence of the electrocytes was observed. (d) Immunogold staining revealed the accumulation of the AchR at the ventral plasma membrane domain (arrows). Intracellular compartments, i.e., the perinuclear ER membrane (open arrows), were also stained. N, nucleus. (e and f) Immunogold staining of the 43-kD protein (antipeptide antibody) showed the typical adult distribution (see Nghiêm et al., 1983; Sealock et al., 1984; Kordeli et al., 1986) at the cytoplasmic surface of the AchR-rich ventral membrane (arrows in e). NE, nerve ending. Some gold particles were also observed at the noninnervated membrane (arrow in f) and within the cytoplasm. Note the absence of labeling in perinuclear ER membrane.
Figure 6. Distribution of laminin in developing *T. marmorata* electric tissue. (a and b) Double fluorescence experiment on 45-mm electric tissue sections stained with (a) FITC-α-Bungtx and with (b) antilaminin antibody (1:200 dilution). The laminin immunoreactivity was observed at the periphery of the columns and was also associated with the ventral pole of the cells (compare with a, stained with FITC α-Bungtx alone). Bars, 50 μm. (c) Adult *T. marmorata* electric tissue. Laminin was associated with both the ventral and the dorsal surfaces of the electrocytes. (d and e) Inhibition experiment: in e, the antilaminin antibody (1:100 dilution) was preincubated with a 10-fold molar excess of pure laminin. Exposure times were the same in d and e. (f) Immunoblot detection of laminin on one-dimensional 6% PAGE of total proteins of 45-mm electric tissue. Lane 1, Ponceau red staining. Lane 2, a major component with an apparent molecular mass of ~200 kD was observed (antibody dilution 1:100). Lane 3, almost complete inhibition of the staining of the 200-kD band was achieved after preincubation with a 10-fold molar excess of pure laminin (antibody dilution 1:100). Alkaline phosphatase detection.
7, e and f), numerous axons running between the stacked electrocytes were consistently observed in all sections.

We thus conclude that the onset of AchR clustering and membrane differentiation on the ventral pole of the developing electrocytes occurs before massive innervation, and moreover, is not limited to areas of nerve contacts.

**Discussion**

We have followed during embryonic development the subcellular distribution and the assembly of two major components of the postsynaptic domain in *T. marmorata* electrocyte: the nicotinic AchR and the 43-kD protein. Using immunocytochemical techniques and a variety of antibodies, we show that the clustering of AchR that occurs on the ventral pole of electrocytes from 45-mm embryos is not accompanied by a parallel accumulation of the 43-kD protein. At best, the 43-kD protein was detected as a faint and diffuse cytoplasmic staining. However, the eventuality of a partial masking of the 43-kD protein or of the transient expression of a precursor slightly different from the mature form cannot be ruled out at this stage. More difficult to ascertain was the eventual presence of a soluble pool of 43-kD during embryogenesis. From the immunocytochemical data, we can only conclude for a faint diffuse labeling of the electrocyte cytoplasm that could only be partially inhibited, probably because of interference with autofluorescence of the tissue and nonspecific binding. Using a polyclonal antibody affinity purified on 43-kD from one-dimensional gels, which exhibits a high affinity for the SDS-denatured form of the protein, we observe that at all developmental stages studied so far, both cytosolic and particulate fractions of the electric tissue contain an immunoreactive form with an apparent molecular mass of 43 kD on a one-dimensional gel. The amount of protein detected in the cytosolic fraction decreases in favor of the particulate fraction in the course of development (Fig. 4 d) and might represent a soluble biosynthetic precursor of the 43-kD protein. However, it cannot be inferred from these experiments, with which the subcellular compartment the 43-kD protein is associated at this early development stage of the electrocyte. A detailed analysis of the subcellular distribution of the 43-kD protein during electrocyte development will be discussed elsewhere (Nghiêm et al., manuscript in preparation).

Additional evidence for a delayed association between AchR and 43-kD protein during development is suggested by their differential subcellular distribution in 80-mm embryos. As expected for a typical membrane-spanning protein (Devillers-Thiéry et al., 1983; Noda et al., 1982, 1983; Claudio et al., 1983) the synthesis and maturation of the AchR would occur in intracellular membrane compartments (see also Merlie and Smith, 1986) represented in the electrocyte primarily by the perinuclear ER membrane. Conversely, the 43-kD protein is an extrinsic protein lacking the signal peptide (Frail et al., 1987) and is thus synthesized on free polysomes. In the developing electrocyte, in which a high biosynthetic activity is expected to occur, pools of AchR and the 43-kD protein can be directly observed by conventional immunocytochemical methods within the cells. AchR was detected at the level of the plasma membrane but also within the perinuclear ER membrane and in ER or Golgi vesicles scattered within the cytoplasm. The 43-kD protein was never found associated with these intracellular membrane compartments. Since a codistribution of the two proteins was systematically found in 80-mm embryos at the plasma membrane level, in particular on the ventral face of the cell, it is legitimate to conclude that the 43-kD protein, which is probably synthesized in the cytoplasm becomes associated with the AchR, only after the clusters of AchR have formed in the plasma membrane. This suggests that the 43-kD protein contributes to properties of the AchR that develop after its recruitment such as its physical stabilization.

In the past few years, the association of the 43-kD protein with AchR clusters was investigated in several different systems. Mostly on the basis of immunofluorescence experiments, a codistribution between AchR clusters and the 43-kD protein was reported in mature and developing neuro-muscular junctions (Froehner et al., 1981; Froehner, 1984; Burden, 1985) as in spontaneous or experimentally elicited AchR clusters in aneural cultures of myotubes (Peng and Froehner, 1985; Bloch and Froehner, 1987). In *Torpedo* electric tissue and mammalian muscle, AchR and 43-kD proteins are found with an equivalent stoichiometry (La Rochelle and Froehner, 1986). In addition, morphological evidence from freeze fracture and gold immunolabeling (Bridgman et al., 1987; Kordeli et al., 1987b) and from chemical cross-linking (Burden et al., 1983) suggests a direct association between the two proteins. In contrast, the asynchrony that we observe between AchR and 43-kD clustering at the early stage of *Torpedo* electrocyte development demonstrate that the 43-kD is not primarily involved in the clustering of AchR.

The recruitment of AchR at the ventral pole of the developing electrocyte is a manifestation of the polarization of a cell that rapidly differentiate from a pool of nonpolarized myoblasts. Together with the differentiation of the membrane domains, cytoplasmic polarity develops, and, in particular, is characterized by the segregation of nuclei at the dorsal pole and the presence of a clear cytoplasm, in the ventral moiety of the cell. Accordingly, the differentiation of the ventral plasma membrane domain might be viewed as one of the manifestations of a global evolution of the electrocyte structure. In this process, extracellular factors, in particular, the nerve or extracellular matrix are expected to play a role in the differentiation of the excitable membrane domain.

Contradictory results based on morphological studies at
the EM level (Mellinger et al., 1978; Fox and Richardson, 1979) have been reported from two laboratories concerning the presence of synaptic contacts in 44–45-mm electrocytes. In a recent study, Richardson and Witzemann (1986) published evidence that AchE maturation and AchR accumulation in aneural primary cultures of Torpedo electrocytes is not under direct neural control. To clarify this point, we have carried out an immunocytochemical approach of the innervation of the electric tissue using anti-NF antibodies. This technique offers a sensitive test to monitor innervation within tissues although NF are not present in the growth cone and nerve endings.

The conclusion of this study is that at the 45-mm stage, a critical moment in our study, nerve fibers invading the electric lobes are only visible in the extracolumnar tissue. No evidence for axonal penetration within the columns of electrocytes was found at this stage, whereas later (70–80 mm) axonal invasion of stacked electrocytes was easily observed. However, even at this stage, at which elecrotocyte polarity is fully expressed and at which AchR and 43-Kd protein codistribution is established, the density of contacts between the nerve endings at the ventral surface of the electrocytes is low. AchR accumulation and association with the 43-Kd protein may thus occur in vivo in areas of the plasma membrane even not in contact with nerves. This observation is not surprising, however, since several experiments demonstrate that nerve per se is not required for AchR clustering (reviewed in Salpeter and Loring, 1986; Peng, 1987). In muscle, AchR clusters occur spontaneously or can be experimentally elicited by various extracellular factors such as agrin (Godfrey et al., 1984), or positively charged latex beads (Peng and Cheng, 1982). In all these instances AchR clusters were, most often, found associated with the 43-Kd protein (Peng and Froehner, 1985; Bloch and Froehner, 1987) and cytoskeletal components (Bloch, 1986; Bloch and Geiger, 1981; Connolly, 1984; Peng and Phehan, 1984, reviewed in Peng and Poo, 1986).

An eventual relationship between extracellular matrix proteins (Burden et al., 1979; Sanes and Chiu, 1983) and AchR clustering in vivo was also investigated. Accordingly, we looked for the presence of a component of the basement membrane, laminin, known to be involved in cell differentiation (Kleinman et al., 1985), neuronal survival and neurite development (Edgar et al., 1984). Laminin is also able to elicit AchR clustering in vitro (Vogel et al., 1983; Daniels et al., 1984) and was detected by immunocytochemical methods in Electrophorus electricus electric tissue (Labat-Robert et al., 1980). Accumulation of laminin and AchR at the ventral pole of the electrocyte was observed by the 45-mm stage. The polar distribution of laminin is, however, a transient event that occurs only at the onset of electrocyte differentiation since, later, laminin immunoreactivity was observed with the same intensity on both ventral and dorsal basement membranes. As suggested by various authors (reviewed in Martin and Timpl, 1987), the basement membranes may play a critical role in the differentiation of the membrane domains, and could also be involved in the guidance of axons which, by the 55-mm stage, establish stable contacts only at the level of the ventral surface of the electrocytes. Along these lines, one may recall that with the neuromuscular junctions the synaptic basement membrane is known to direct both pre- and postsynaptic specializations in the course of in vivo regeneration experiments (Burden et al., 1979; Sanes et al., 1978).

In summary, we observe in developing T. marmorata electric organs in situ that the recruitment of the AchR molecules at the presumptive innervated pole of the cell does not require the 43-Kd protein. The differentiation of the electrocyte, including that of the AchR-rich membrane domain occurs before detection of electromotor innervation. The transient accumulation of basement membrane components such as laminin at the site of AchR accumulation may, however, be relevant to the differentiation of the electrocyte polarity.

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