300-kD Subsynaptic Protein Copurifies with Acetylcholine Receptor-rich Membranes and Is Concentrated at Neuromuscular Synapses

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Abstract. Acetylcholine receptor–rich membranes from the electric organ of *Torpedo californica* are enriched in the four different subunits of the acetylcholine receptor and in two peripheral membrane proteins at 43 and 300 kD. We produced monoclonal antibodies against the 300-kD protein and have used these antibodies to determine the location of the protein, both in the electric organ and in skeletal muscle. Antibodies to the 300-kD protein were characterized by Western blots, binding assays to isolated membranes, and immunofluorescence on tissue. In *Torpedo* electric organ, antibodies to the 300-kD protein stain only the innervated face of the electrocytes. The 300-kD protein is on the intracellular surface of the postsynaptic membrane, since antibodies to the 300-kD protein bind more efficiently to saponin-permeabilized, right side out membranes than to intact membranes. Some antibodies against the *Torpedo* 300-kD protein cross-react with amphibian and mammalian neuromuscular synapses, and the cross-reacting protein is also highly concentrated on the intracellular surface of the postsynaptic membrane.

The neuromuscular synapse is a highly specialized structure whose formation and maintenance is dependent upon interaction between nerve and muscle. Presently we have only a very limited descriptive understanding of how nerve and muscle interact and how their interaction influences synaptic differentiation (Dennis, 1981).

One of the major specializations of the neuromuscular synapse is the high concentration of acetylcholine receptors (AChRs)[1] in the postsynaptic membrane. This high concentration of AChRs arises during embryonic development as a consequence of interaction between the developing nerve and developing muscle cell and results from a lateral movement of AChRs from extrasynaptic regions of the muscle cell to the newly formed synapse (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984). Thereafter, the synaptic site retains the ability to induce new synaptic AChR clusters, even in the absence of the nerve. The synaptic extracellular matrix has an important role in this process (Burden et al., 1979; Godfrey et al., 1984; Nitkin et al., 1983).

The muscle membrane is also morphologically specialized at the synaptic site: regular infoldings of the postsynaptic membrane increase the synaptic membrane surface area and thus contribute to the high concentration of synaptic AChRs (Birks et al., 1960; Couteaux, 1963; Fertuck and Salpeter, 1976). Moreover, the cytoplasm underlying the postsynaptic membrane is morphologically specialized (Couteaux and Pecot-Dechavassine, 1968; Couteaux, 1981; Sealock, 1982 *a*, *b*) and components of this subsynaptic sarcoplasm may have a role in establishing and/or maintaining the specializations of the postsynaptic membrane.

One approach to studying how synaptic differentiation is regulated and how AChRs become concentrated and subsequently maintained at synapses is to identify and characterize nonreceptor proteins that are associated with the postsynaptic membrane. Previously we had isolated postsynaptic membranes from the electric organ of *Torpedo californica* and identified two polypeptides that copurify with the membrane-bound AChR (Burden et al., 1983). One of these AChR-associated polypeptides is a 43-kD protein that is being actively studied by several laboratories (Sobel et al., 1978; Neubig et al., 1979; Gysin et al., 1981; Barrantes, 1982; Porter and Froehner, 1983; Burden et al., 1983; Sealock et al., 1984). The other polypeptide is 300 kD and remains largely uncharacterized. In this study we have produced monoclonal antibodies against the 300-kD protein and have used the antibodies to demonstrate that the 300-kD protein is a peripheral membrane protein located on the intracellular surface of the postsynaptic membrane, both in the electrocyte and at neuromuscular synapses.

Materials and Methods

Isolation of AChR-rich Membranes

AChR-rich membranes were isolated as previously described (Burden et al., 1983). Frozen *Torpedo* electric organ tissue was stored at −80°C and obtained either from Pacific Bio-Marine Laboratories, Inc., Venice, CA (*Torpedo californica*) or Biofish Associates, Gloucester, MA (*Torpedo*

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[1] *Abbreviations used in this paper*: AChRs, acetylcholine receptors; NGS, normal goat serum, TMR-alpha-bungarotoxin, tetramethylrhodamine-coupled alpha-bungarotoxin.

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nobiliana). The tissue was pulverized under liquid nitrogen and homogenized with a motor-driven teflon pestle in 1.5 vol of buffer A (400 mM sodium chloride, 50 mM Tris, 10 mM EDTA, 10 mM EGTA, 0.3 mM PMSF, 5.0 mM diisopropylfluorophosphate, 0.02% wt/vol sodium azide, pH 7.4) on ice. All subsequent procedures were performed at 4°C. The homogenate was centrifuged (5,000 g_{av} for 10 min), the supernatant was sonicated five times, 10 s on, 10 s off at setting 6 (model W185; Branson Sonic Power Co., Danbury, CT), and total membranes were collected by centrifugation (100,000 g_{av} for 1 h). We also prepared membranes without sonication and obtained identical fractionation and yield of AChR-rich membranes. The membrane pellet was resuspended in buffer B (10 mM sodium phosphate, 1 mM EDTA, 1 mM EGTA, 0.3 mM PMSF, 0.02% sodium azide, pH 7.4) with 10% sucrose, layered onto a 25-40% sucrose (in buffer B) gradient and centrifuged for 6.5-10 h at 25,000 rpm in an SW 28 rotor. Fractions (1 ml) were collected and analyzed by SDS PAGE (Laemmli and Favre, 1973). The membranes were stored at 4°C.

AChR-rich membranes were stripped of peripheral membrane proteins by treatment with either alkaline pH (pH 11) or 10 mM lithium diiodosalicylate as described previously (Neubig et al., 1979; Elliot et al., 1980; Burden et al., 1983). AChR-rich membranes were permeabilized with 0.03% wt/vol saponin (in buffer B, 30 min at 4°C), washed by centrifugation (in buffer B), and resuspended in buffer B (St. John et al., 1982). We cannot explain why other laboratories have not identified the 300-kD protein as a protein that copurifies with AChR-rich membrane; membranes, however, that were prepared in the absence of divalent ion chelators (Cohen et al., 1972; Sobel et al., 1977) contained similar quantities of the 43-kD protein and alpha subunit of the AChR, but had reduced amounts of beta, gamma, and delta subunits of the AChR and no 300-kD protein. It seems likely that the absence of the 300-kD protein and reduction in yield of AChR subunits in membranes prepared using this procedure is due to divalent ion-activated proteases.

Human erythrocyte spectrin was provided by Dr. J. Spiegel (Spiegel et al., 1984) and rabbit macrophage actin-binding protein was provided by Dr. J. Hartwig (Hartwig and Stossel, 1975). Affinity-purified rabbit antibodies to guinea pig brain spectrin were provided by Dr. V. Bennett (Davis and Bennett, 1983) and affinity-purified goat antibodies to rabbit macrophage actin-binding protein were provided by Dr. J. Hartwig (Hartwig and Stossel, 1983).

Isolation of the 300-kD Protein

The 300-kD protein was isolated by preparative SDS PAGE. Approximately 10 mg of AChR-rich membrane protein were layered on a 1.5-mm thick, 6% polyacrylamide SDS gel and electrophoresed at constant current (15 mA). The gel was rinsed in H_2O and the protein bands were visualized after staining for 10 min in ice-cold 0.25 M potassium chloride (Hager and Burgess, 1980). The region of the gel containing the 300-kD protein was excised and the protein was electroeluted in SDS gel running buffer into a 0.2-ml chamber. The protein was precipitated with 80% vol/vol acetone at −20°C and resuspended in 10 mM sodium phosphate, 150 mM sodium chloride (PBS). The concentration and purity of the protein were determined by SDS PAGE and staining with Coomassie Brilliant Blue; human erythrocyte spectrin was used as a standard. We estimated that we recovered 20% of the 300-kD protein that was present in the membranes. The protein was stored frozen at −80°C.

Production of Monoclonal Antibodies against the 300-kD Protein

Monoclonal antibodies were produced as described previously (Burden, 1982; Burden et al., 1983). 10-50 µg of 300-kD protein was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into either BALB/c or BALB/c/SJL mice. Mice were boosted intraperitoneally 3 wk later (10-50 µg of protein in incomplete Freund's adjuvant) and again after three more weeks with protein in saline. Fusion of spleen cells to SP2/0-Agl4 myeloma cells (Shulman et al., 1978) and selection, growth, and cloning of hybridomas were performed essentially as described previously (Burden, 1982).

Antibodies against the 300-kD protein were detected by a solid-phase binding assay in which AChR-rich membranes (∼250 ng of membrane protein), or denatured (1% SDS at room temperature) AChR-rich membrane proteins were adsorbed onto an ordered grid of nitrocellulose paper. The paper was incubated in 10% normal goat serum (NGS) in PBS (15 min), then incubated in hybridoma supernatant (1 h), washed with PBS (two times, 3 min per wash), further incubated in horseradish peroxidase-coupled goat anti-mouse IgG (diluted 1:1000 in 10% NGS in PBS, 1 h incubation) (Cappel Laboratories, Malvern, PA), and washed in PBS. Peroxidase activity was detected with 3,3'-diaminobenzidine as substrate (Burden, 1982). All procedures were performed at room temperature.

Monoclonal antibody to the alpha subunit of the *Torpedo* AChR (35.74) was provided by Dr. E. Hawrot. We characterized this antibody further: the antibody reacts with an extracellular determinant on the AChR, since it reacts with synaptic sites in intact, live amphibian muscle and AChR clusters in cultured *Xenopus* myocytes (data not presented).

Binding Assays

We used a solid-phase ELISA (Voller et al., 1976) to measure the amount of the 300-kD protein in gradient-fractionated membranes and a competition ELISA to determine whether the 300-kD protein was on the intra- or extracellular surface of AChR-rich membranes.

For direct binding assays, AChR-rich membranes (∼2 µg membrane protein in 50 µl of buffer B per well) were adsorbed to the wells of a 96-well polystyrene plate (Linbro Inc., Hamden, CT) overnight at 4°C and the wells were washed with PBS/0.05% Tween-20 (PBS-Tw) (three times, 3 min per wash). The wells were incubated subsequently in monoclonal antibody (hybridoma supernatant with 0.05% Tween-20) for 2 h at room temperature, washed, incubated in horseradish peroxidase-coupled goat anti-mouse IgG (diluted 1:1000 in PBS-Tw) for 2 h at room temperature, and washed. The enzyme activity was detected with 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) as substrate and the optical density of the reaction product was measured in a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA) with a 405-nm filter.

For competition assays, monoclonal antibody was incubated with AChR-rich membranes before addition of the antibody to the solid phase as described above. A fixed amount of monoclonal antibody (nonsaturating in a direct binding assay) was incubated with a variable amount of AChR-rich membranes (0.5-8.0 µg of protein in 100 µl of buffer B) for 30 min at room temperature. The membranes and adsorbed antibody were pelleted in an airfuge (14 psi, 3 min) and the amount of antibody remaining in the supernatant was determined in a direct binding assay as described above.

Western blotting was performed essentially as previously described (Towbin et al., 1979; Burden, 1982; Burden et al., 1983). Transfer of the 300-kD protein was inefficient, however, with the standard transfer buffer (80% 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3); the amount of transfer was increased by adding 0.01% SDS to the transfer buffer.

Immunofluorescence

Frozen sections from unfixed *Torpedo* electric organ, rat intercostal muscle, and frog (*Rana pipiens*) sartorius muscle were incubated with monoclonal antibody (hybridoma supernatant, 2 h at room temperature), washed 1 min in PBS, incubated in fluorescein-coupled goat anti-mouse IgG (1:200 dilution in PBS, for 2 h at room temperature; Cappel Laboratories), and washed. Sections were mounted in 80% glycerol, 50 mM sodium bicarbonate, pH 9, with 10 µg/ml *p*-phenylenediamine. Tetramethylrhodamine-coupled alpha-bungarotoxin (TMR-alpha-bungarotoxin) was included in the secondary antibody incubation to mark synaptic sites (Burden, 1982). Goat anti-mouse antibodies often cross-react with rat IgG, which is present in rat muscle and outlines the myofibers; reactivity against rat IgG was removed by adsorbing the goat anti-mouse IgG with rat serum.

Intact, or methanol-permeabilized (−20°C for 30 min) whole mounts of the rat diaphragm muscle (1-mo-old rat) were incubated with monoclonal antibody (hybridoma supernatant with 0.1% saponin), followed by biotin-coupled horse anti-mouse IgG (1:200 dilution in 10% NGS, 0.1% saponin in PBS) and fluorescein-coupled avidin (1:400 dilution in 10% NGS, 0.1% saponin in PBS) (Vector Laboratories, Inc., Burlingame, CA). All incubations were for 2 h at room temperature and the muscle was washed with PBS (three washes, 5 min per wash) between incubations. TMR-alpha-bungarotoxin was included with the fluorescein-coupled avidin to mark synaptic sites. To minimize interference from tissue autofluorescence, several layers of underlying muscle fibers were dissected away until a layer one to two muscle fibers thick remained. The pared-down muscle was mounted between two glass coverslips in 80% glycerol, 50 mM sodium bicarbonate, pH 9, with 10 µg/ml *p*-phenylenediamine.

The sections and whole mounts were viewed with filters selective for either rhodamine or fluorescein and with a Zeiss Planapo 63× objective (Burden, 1982).

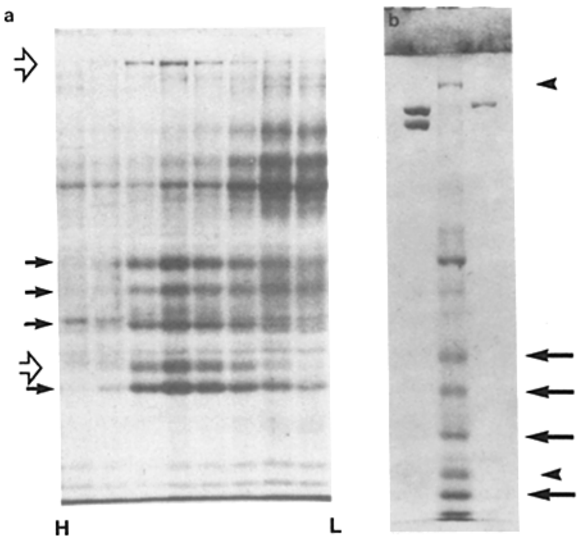

Figure 1. A 300-kD peripheral membrane protein copurifies with AChR-rich membranes. (*a*) Fractions from an equilibrium density sucrose gradient were analyzed by SDS PAGE. Proteins (6 μl) from membrane fractions 2–9 (14 fractions were collected) were resolved in a 9% polyacrylamide SDS gel and the gel was stained with Coomassie Brilliant Blue. The positions of the AChR subunits at 40, 49, 56, and 65 kD are indicated with arrows and the positions of the 43- and 300-kD proteins, which copurify with the AChR-rich membranes, are indicated with open arrows. The low (*L*) and high density (*H*) regions of the gradient are indicated. (*b*) The 300-kD protein in AChR-rich membranes (*center lane*) migrates more slowly in 6% polyacrylamide SDS gels than either subunit of human erythrocyte spectrin (*left lane*, 220, 240 kD) or rabbit macrophage actin-binding protein (*right lane*, 270 kD). The positions of the AChR subunits are indicated with arrows and the positions of the 43- and 300-kD proteins are indicated with arrowheads.

Results

A 300-kD Peripheral Membrane Protein Copurifies with Acetylcholine Receptor-rich Membranes

Membranes from the electric organ of electric fish can be fractionated by equilibrium density centrifugation into a membrane fraction that is enriched in postsynaptic membrane and a membrane fraction that is depleted of postsynaptic membrane (Fig. 1 *a*). The postsynaptic membrane-rich fraction is readily identified by SDS PAGE analysis of its protein composition: the postsynaptic membrane is enriched in the four different subunits of the AChR that migrate at 40, 49, 56, and 65 kD (Elliot et al., 1980). In addition to the four AChR subunits, the postsynaptic membrane-rich fraction is enriched in two nonreceptor polypeptides that migrate in SDS PAGE at 43 and 300 kD (Fig. 1 *a*) (Burden et al., 1983).

The 43-kD protein is a peripheral membrane protein located on the cytoplasmic surface of the postsynaptic membrane (Sobel et al., 1978; Hamilton et al., 1979; Neubig et al., 1979; Wennogle and Changeux, 1980; Gysin et al., 1981; Barrantes, 1982; St. John et al., 1982; Porter and Froehner, 1983; Sealock et al., 1984), where it is closely associated with the beta subunit of the AChR and present at 1 mol/mol of AChR complex (Burden et al., 1983). Moreover, the 43-kD protein is concentrated at neuromuscular synapses in both amphibians and mammals (Froehner, 1984; Burden, 1985).

The 300-kD protein is also a peripheral membrane protein: it is solubilized from the postsynaptic membrane by either alkaline pH or chaotropic salts (Burden et al., 1983). As described previously, there is 0.1-0.2 mol of 300-kD protein per mole of AChR complex in AChR-rich membranes (Burden et al., 1983). We had originally estimated the relative molecular mass of this high molecular mass protein to be 270 kD (Burden et al., 1983); more accurate determinations now estimate the relative molecular mass to be 300 kD. In 6% SDS PAGE the 300-kD protein migrates more slowly than either subunit of human erythrocyte spectrin or rabbit macrophage actin-binding protein (Fig. 1 *b*).

The 300-kD Protein Is on the Cytoplasmic Surface of the Postsynaptic Membrane

We produced monoclonal antibodies against the 300-kD protein to determine more precisely the location of the protein in both the electric organ and skeletal muscle. Several hundred micrograms of the protein were isolated by preparative SDS PAGE and mice were immunized with 10–50 μg of protein that we estimated to be >90% homogeneous. We produced approximately two dozen monoclonal antibodies against the 300-kD protein, which we characterized by Western blots and by immunocytochemistry on frozen sections of the electric organ of *Torpedo* and skeletal muscle of higher vertebrates. All of the monoclonal antibodies described in this study are of the IgG_1 subclass. Some of the antibodies

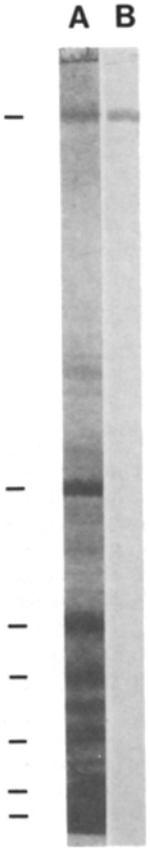

Figure 2. Monoclonal antibodies against the 300-kD protein react only with the 300-kD protein in Western blots of AChR-rich membrane proteins. AChR-rich membrane proteins were resolved by SDS PAGE (6% polyacrylamide gel), transferred to nitrocellulose, and the blot probed with a monoclonal antibody (hybridoma supernatant from cell line 601) to the 300-kD protein. AChR-rich membrane proteins stained by Coomassie Brilliant Blue are shown in *A* and the 300-kD protein band stained by the antibody is shown in *B*. The positions of AChR subunits (40, 49, 56, and 65 kD), the 43-kD protein, the heavy subunit of the Na^+, K^+ ATPase (92 kD) (Lindstrom et al., 1979), and the 300-kD protein are indicated.

could be distinguished from each other by their reactivity with other species and by their sensitivity to reaction with fixed tissue. The data for one monoclonal antibody (601) against the 300-kD protein is presented in Figs. 2 through 7; the data for two different monoclonal antibodies (602, 603) against the 300-kD protein is identical to that presented in Figs. 2 through 5.

Western blots of proteins from AChR-rich membranes demonstrate that the monoclonal antibodies are specific for the 300-kD protein (Fig. 2). Western blots of proteins from previous stages in isolation of AChR-rich membranes (low speed pellet, high speed supernatant; see Materials and Methods for details) demonstrate that the 300-kD protein is neither present in a substantial amount in other fractions nor proteolyzed during isolation of AChR-rich membranes. Thus, the stoichiometry of the 300-kD protein in AChR-rich membranes is likely to reflect the stoichiometry of the protein in the electric organ.

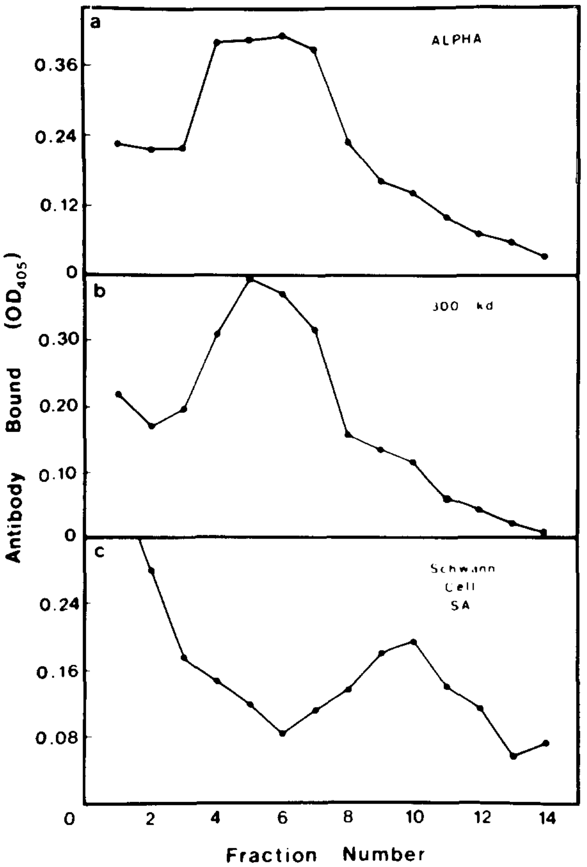

Figure 3. Monoclonal antibodies against the 300-kD protein recognize a protein that copurifies with AChR-rich membranes. Gradient-fractionated membranes were adsorbed to a microtiter plate and probed with a monoclonal antibody (hybridoma supernatant) to the (*a*) alpha subunit of the AChR (cell line 35.74; see Materials and Methods), (*b*) 300-kD protein (601), and (*c*) Schwann cell surface antigen (230, 250 kD; cell line IID2). The amount of bound antibody was detected as described in Materials and Methods. The distribution of the 300-kD protein in the gradient is identical to the AChR and different than the Schwann cell surface antigen. The protein concentration is approximately twofold greater in the peak AChR-poor fraction than in the peak AChR-rich fraction. In this experiment the 92-kD protein was most abundant in fraction number 10; this fraction number was designated the peak AChR-poor fraction. The heaviest membrane fraction is numbered 1 and the lightest membrane fraction is numbered 14. Each point is the mean of triplicate determinations.

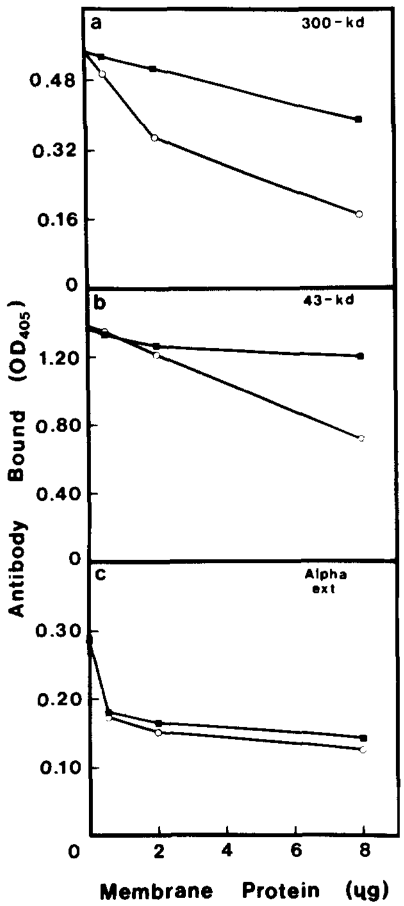

Figure 4. The 300-kD protein is on the intracellular surface of the postsynaptic membrane. Intact (*solid squares*), or saponin-permeabilized (*open circles*) AChR-rich membranes were incubated with monoclonal antibody to the (*a*) 300-kD protein (601), (*b*) 43-kD protein (ID2), or (*c*) an extracellular determinant on the alpha subunit of the AChR (35.74). The amount of free antibody was determined in an ELISA as described in Materials and Methods. Antibodies to either the 300- or 43-kD protein are more efficiently absorbed to permeabilized membranes, whereas antibodies to the extracellular domain of the alpha subunit are absorbed equally well to both intact and permeabilized membranes. Absorption of antibodies against either the 43- or 300-kD protein to intact membranes is likely to be due to membrane vesicles that are not sealed and/or to nonspecific absorption. Each point is the mean of triplicate determinations.

Since several different proteins may comigrate at 300 kD, it was important to establish that the monoclonal antibodies react with the 300-kD protein that copurifies with AChR-rich membranes. Therefore, we determined the distribution of the antigen in gradient-fractionated membranes. Fig. 3 demonstrates that the 300-kD protein recognized by the monoclonal antibody copurifies with AChR-rich membranes. In contrast, a monoclonal antibody directed against a Schwann cell surface protein recognizes an antigen (230, 250 kD) that is enriched in nonsynaptic membrane fractions (Fig. 3 *c*). Moreover, we did isolate two monoclonal antibodies that react with proteins of ~300 kD which do not copurify with AChR-rich membranes; one of these antibodies stains both the synaptic and nonsynaptic membranes of the electrocyte.

To determine whether the 300-kD, AChR-associated protein is located on the extracellular or intracellular surface of the postsynaptic membrane, we took advantage of the fact that AChR-rich membrane vesicles are largely right side out and sealed from macromolecules for several days after isolation (Hartig and Raftery, 1979). We determined whether monoclonal antibodies against the 300-kD protein could react with right side out AChR-rich membranes or whether detergent permeabilization of these membranes was required for antibody binding. As demonstrated in Fig. 4 *a*, antibodies against the 300-kD protein react poorly with intact, right side out AChR-rich membranes and more efficiently with saponin-permeabilized AChR-rich membranes. Similarly, a monoclonal antibody against the 43-kD protein (Burden et al., 1983; Burden, 1985) reacts more efficiently with saponin-permeabilized membranes (Fig. 4 *b*). In contrast, a monoclonal antibody against an extracellular determinant on the alpha subunit of the AChR reacts identically with intact and permeabilized AChR-rich membranes (Fig. 4 *c*). Thus, the determinants on the 300-kD protein that these antibodies recognize are inaccessible without permeabilization of the postsynaptic membrane. These results, along with protease studies of intact and permeabilized membranes (data not presented), strongly suggest that the 300-kD protein is located on the cytoplasmic surface of the postsynaptic membrane. Further evidence for an intracellular location of the 300-kD protein comes from the immunocytochemical studies on skeletal muscle presented below.

Antibodies against the 300-kD Protein Stain the Innervated Face of the Electric Organ

To determine whether the 300-kD protein is concentrated at the postsynaptic membrane in situ we probed frozen sections of *Torpedo* electric organ with antibodies against the 300-kD protein and detected the bound antibodies by indirect immunofluorescence. Postsynaptic membranes were located by probing the same sections with alpha-bungarotoxin, which was labeled with a distinguishable fluorochrome.

Fig. 5 demonstrates that the 300-kD protein is concentrated along the innervated face of the electrocyte. The staining pattern is identical with either antibodies against the

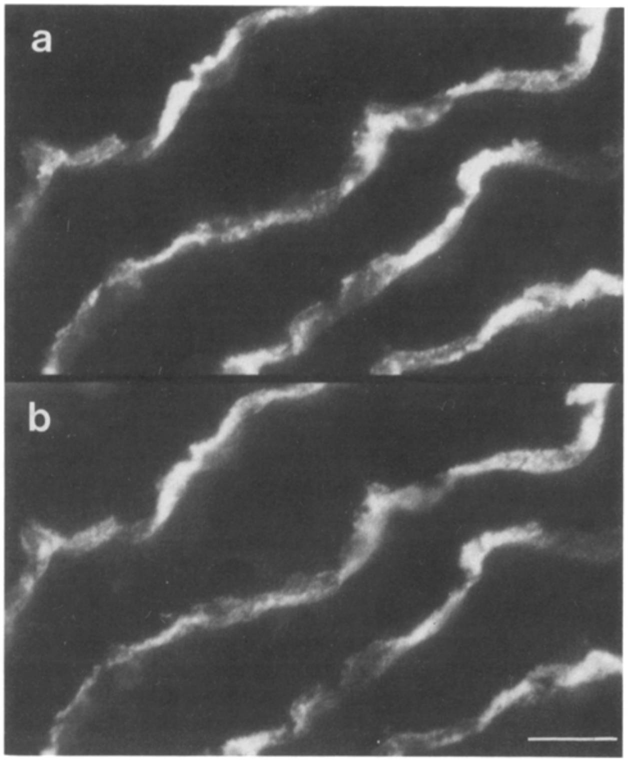

Figure 5. Monoclonal antibody against the 300-kD protein stains the innervated face of the *Torpedo* electric organ. Frozen sections of *Torpedo* electric organ were stained with (*a*) TMR-alpha-bungarotoxin and (*b*) antibody to the 300-kD protein (601). The sites stained with the antibody against the 300-kD protein are coincident with the sites stained with alpha-bungarotoxin. Bar, 15 μm.

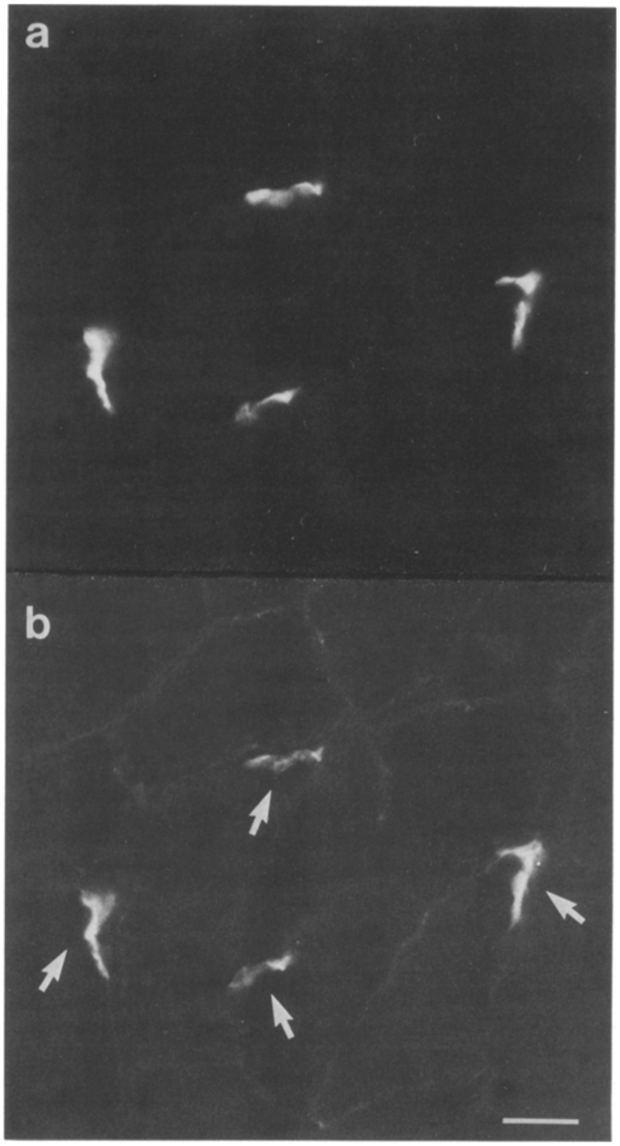

Figure 6. A monoclonal antibody against the *Torpedo* 300-kD protein cross-reacts with rat neuromuscular synapses. Frozen section of rat intercostal muscle was stained with (*a*) TMR-alpha-bungarotoxin and with (*b*) antibody to the 300-kD protein (601). The antibody stains synaptic sites (*arrows* in *b*) intensely and the extrasynaptic muscle fiber membrane less intensely. Bar, 15 μm.

300-kD protein or against the alpha subunit of the AChR. In contrast, a monoclonal antibody to intermediate filaments (Pruss et al., 1981) stains the entire electrocyte (Burden, 1982). Thus, the 300-kD protein is associated with the postsynaptic membrane in situ and does not artifactually associate with the postsynaptic membrane during its isolation.

Antibodies against the Torpedo 300-kD Protein Cross-react with Synaptic Sites in Mammalian Muscle

The postsynaptic membranes of the electric organ and skeletal muscle are similar in both their structure (Couteaux, 1963; Rosenbluth, 1975; Heuser and Salpeter, 1979; Sealock et al., 1982*a*, *b*) and in the composition of their constituent polypeptides. Moreover, antibodies against synaptic components from the electric organ often cross-react with synaptic components in higher vertebrates (Lindstrom et al., 1979; Sanes et al., 1979; Hooper et al., 1980; Burden, 1981; Froehner, 1984). Therefore, it seemed likely that a protein that is similar to the 300-kD protein would be present at neuromuscular synapses.

We probed frozen sections of rat skeletal muscle with monoclonal antibodies against the *Torpedo* 300-kD protein and found that one of these antibodies cross-reacts with mammalian muscle and that the cross-reacting antigen is concentrated at synaptic sites (Fig. 6). This antibody and several other antibodies against the 300-kD protein cross-react with amphibian neuromuscular synapses, and the pattern of staining is similar to that shown for the rat neuromuscular junction.

In addition to the staining at synaptic sites, there is less intense, but detectable, staining at the nonsynaptic muscle cell membrane. Although it is possible that the increase in membrane surface area at the synapse due to postjunctional folds produces the more intense synaptic staining seen in the light microscope, it is our impression that the intensity of synaptic staining exceeds that expected for a uniformly distributed membrane antigen. Immunoelectron microscopy will be required to resolve this issue. Nevertheless, no other staining is seen in frozen sections of muscle: fibroblasts, macrophages, Schwann cells, intramuscular nerves, perineural cells, and smooth muscle cells are not stained.

We were able to use whole mounts of skeletal muscle to determine whether the cross-reacting antigen was intracellular. We labeled intact whole mounts and permeabilized whole mounts of rat diaphragm muscle with an antibody against the *Torpedo* 300-kD protein and found that only in permeabilized whole mounts were synaptic sites labeled (Fig. 7). Thus, the cross-reacting antigen is on the intracellular surface of the postsynaptic membrane in skeletal muscle as well as in the electric organ.

Discussion

Our strategy to identify nonreceptor synaptic proteins requires that these proteins copurify with the postsynaptic membrane. This approach demands that these nonreceptor synaptic proteins be tightly associated with the postsynaptic membrane and that nonsynaptic proteins not associate artifactually with the postsynaptic membrane during its isolation.

The postsynaptic membrane from the electric organ of electric fish is enriched in two nonreceptor polypeptides which migrate in SDS PAGE at 43 and 300 kD (Burden et al., 1983). We have used monoclonal antibodies against the 300-kD polypeptide to demonstrate that it copurifies and is associated tightly with the intracellular surface of the postsynaptic membrane. Moreover, we have used antibodies in immunocytochemical experiments to demonstrate that the 300-kD protein is concentrated at the postsynaptic membrane in situ and does not artifactually associate with the postsynaptic membrane during isolation.

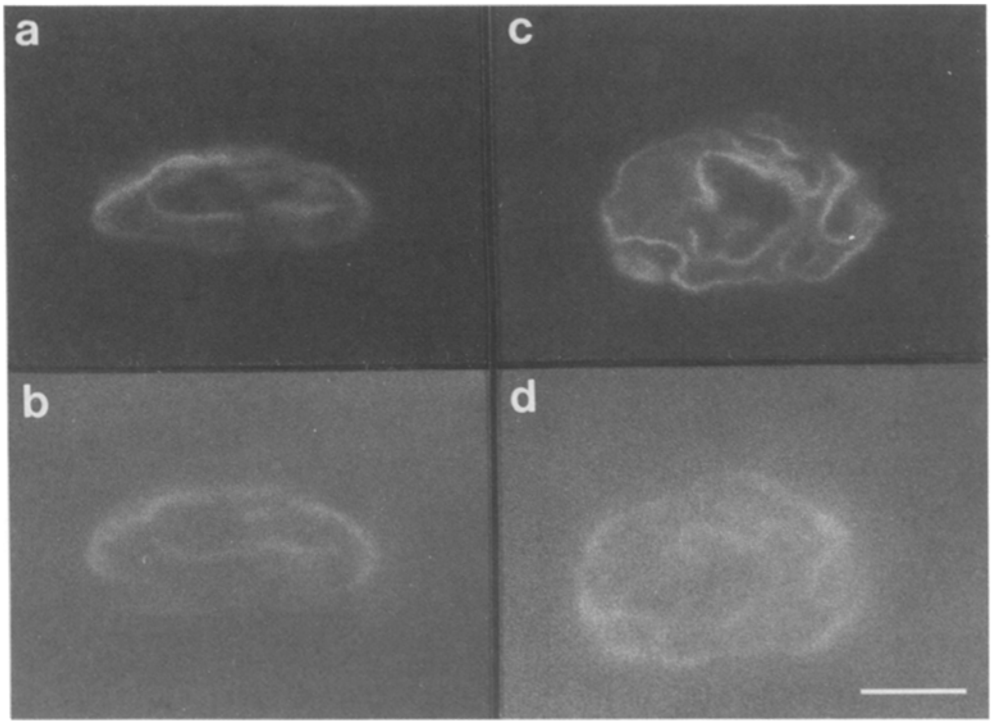

Figure 7. A monoclonal antibody against the *Torpedo* 300-kD protein stains the intracellular surface of the postsynaptic membrane in rat skeletal muscle. Rat diaphragm muscle was permeabilized with methanol and probed with (*a* and *c*) TMR–alpha-bungarotoxin and (*b* and *d*) antibody against the 300-kD protein (601) as described in Materials and Methods. Two muscle fibers are shown, one in *a* and *b* and the other in *c* and *d*, with optics selective for either rhodamine (*a* and *c*) or fluorescein (*b* and *d*). The pattern of the toxin staining and antibody staining at the synapse are indistinguishable. Antibody staining was also observed in muscle fibers that were permeabilized by cutting the muscle ~1 mm from the synaptic zone, but staining was not observed in intact muscle. Bar, 5 μm.

These results suggest that either both the 43- and 300-kD polypeptides are directly associated with the postsynaptic membrane or that one of these polypeptides is associated indirectly with the membrane through a linkage with the other polypeptide. The 1:1 stoichiometry of the 43-kD protein with AChR, and cross-linking experiments which demonstrate a close physical relationship between the 43-kD protein and the beta subunit of the AChR, suggest that the 43-kD protein is associated directly with the AChR (Burden et al., 1983). Direct evidence for such an association, however, is lacking. Because of the size of the 300-kD protein, its stoichiometry with AChR (0.1:1), and by analogy with the spectrin scaffold in erythrocytes (Branton et al., 1981; Marchesi, 1985) it is interesting to speculate that the 300-kD protein may serve as an underlying scaffold upon which the 43-kD protein is arranged. Further biochemical studies and immunoelectron microscopy may help to clarify this issue.

Both biochemical and immunocytochemical experiments demonstrate that the 300-kD protein is concentrated at the postsynaptic membrane in the electric organ (Figs. 3 and 5). Since our fractionation procedures result in a 10-fold increase in specific activity of the 300-kD protein in AChR-rich versus AChR-poor membrane fractions, we can conclude that the 300-kD protein is at least 10-fold more concentrated in the postsynaptic membrane than in the nonsynaptic membrane (Fig. 3). Autoradiographic experiments, however, have demonstrated that AChRs are concentrated several thousandfold in the postsynaptic membrane (Fertuck and Salpeter, 1976; Sealock and Kavookjian, 1980). Thus, although the data suggest that the 300-kD protein is concentrated in the postsynaptic membrane to the same extent as the alpha subunit of the AChR (Fig. 3), the present data is not sufficient to allow such a comparison.

In normal, adult rat and amphibian skeletal muscle the 300-kD protein is clearly detectable at extrasynaptic regions of the myofiber (Fig. 6). AChRs are not detectable at extrasynaptic regions of normal, adult myofibers by these techniques. Thus, in skeletal muscle the 300-kD protein is present at higher concentration at extrasynaptic regions than the AChR. These results suggest that the 300-kD protein is not associated with the AChR at extrasynaptic regions and has a role at the extrasynaptic membrane in addition to its role at the synapse.

In addition to the 43- and 300-kD proteins, a cytoplasmic isoform of actin (Hall et al., 1981), several actin-associated proteins (Bloch and Hall, 1983), and intermediate filaments (Couteaux and Pecot-Dechavassine, 1968; Burden, 1982) are concentrated at neuromuscular synapses. Neither actin nor intermediate filament proteins, however, copurify with AChR-rich membranes from the electric organ (Porter and Froehner, 1983; Burden et al., 1983). Intermediate filament proteins are not likely to be associated intimately with the postsynaptic membrane (Couteaux and Pecot-Dechavassine, 1968; Couteaux, 1981) and actin may be present at synapses in the electric organ but not concentrated there. Although actin-associated proteins have not been characterized in *Torpedo*, there is evidence that actin can associate with the 43-kD protein in vitro (Walker et al., 1984). In this regard, it will be interesting to determine whether the 300-kD protein can associate with the 43-kD protein and/or actin.

The 300-kD protein does not appear to be closely related to spectrin nor identical to actin-binding protein (data not presented). Unlike spectrin, the 300-kD protein is not solubilized from AChR-rich membranes by low ionic strength (0.1 mM sodium phosphate, 0.1 mM EDTA) (Tyler et al., 1980). Moreover, affinity-purified antibodies against pig brain spectrin (Davis and Bennett, 1983) react with a *Torpedo* protein(s) that is enriched in nonsynaptic membrane; in addition, these antibodies stain rat myofiber membrane uniformly, whereas antibodies against the 300-kD protein stain the synaptic site more intensely. A recent study of fodrin distribution in *Torpedo* electric organ demonstrates that fodrin is in fact concentrated on the noninnervated face of the electrocyte and in nerve endings (Kordeli et al., 1986). Affinity-purified antibodies against rabbit macrophage actin-binding protein (Hartwig and Stossel, 1983) do not react with membranes from *Torpedo* electric organ but stain rat neuromuscular synapses intensely, with less intense staining at extrasynaptic regions. These antibodies also stain macrophages present in skeletal muscle, whereas monoclonal antibodies to the 300-kD protein do not stain macrophages.

A first step in studying the structure and function of the subsynaptic sarcoplasm is to identify its components. We have fractionated postsynaptic membranes and used copurification as a criteria for identifying nonreceptor synaptic proteins. These procedures have allowed us to identify two polypeptides which copurify with AChR-rich membranes and are concentrated on the intracellular surface of the postsynaptic membrane. The precise physical relationship of these proteins to one another and to the AChR is not clear, but the simple structure of the postsynaptic membrane and the tight association of these proteins with the postsynaptic membrane suggest that these subsynaptic proteins could regulate properties of the AChR. Further study of these proteins may help to clarify how the specialized postsynaptic membrane is initially formed and subsequently maintained.

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