Immunofluorescence localization at the mammalian neuromuscular junction of the M_r 43,000 protein of *Torpedo* postsynaptic membranes

(peripheral protein/synapses/nicotinic acetylcholine receptor)

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ABSTRACT Highly purified cholinergic postsynaptic membranes from *Torpedo* electric tissue contain, in addition to the acetylcholine receptor (AcChoR), major proteins of M_r 43,000 and $M_r \approx 90,000$ and minor proteins that can be removed from the membranes by alkaline treatment. We have prepared an antiserum to these alkaline-extractable proteins that reacts with the M_r 43,000 protein but not with any of the other major membrane proteins, including the AcChoR subunits. Immunofluorescent staining of sections of *Torpedo* electric tissue shows that this antiserum binds to the innervated but not the uninnervated surface of the electrocytes. In rat diaphragm muscle, the antigens recognized by this antiserum are highly concentrated at the synapse. Synaptic staining of muscle is eliminated by prior incubation of the antiserum with the M_r 43,000 protein but not by incubation with affinity-purified AcChoR. This antiserum stains end plates of muscles denervated for 7 days. Antiserum to AcChoR binds to the subsynaptic membranes of electrocytes and muscle but does not react with the M_r 43,000 protein. Purified AcChoR blocks staining of synapses by anti-AcChoR but the M_r 43,000 protein does not. These results indicate that the M_r 43,000 protein is located in the innervated membrane of *Torpedo* electrocytes and that an immunologically similar component is highly concentrated in the postsynaptic membrane of mammalian muscle.

In its detergent-solubilized affinity-purified form, the nicotinic acetylcholine (AcCho) receptor (AcChoR) from *Torpedo* electric tissue is a complex composed of polypeptides of M_r 40,000 (α), 50,000 (β), 60,000 (γ), and 65,000 (δ) (1–6). Isolated postsynaptic membranes from the same tissue contain, in addition to the receptor polypeptides, proteins of M_r 43,000 and $\approx$90,000 and other minor components (7–11). In contrast to the other nonreceptor peptides, the M_r 43,000 protein remains with the postsynaptic membranes throughout several purification steps. That protein has an amino acid composition distinct from that of skeletal muscle actin (12), and unlike actin, it does not interact with deoxyribonuclease I (13). Spectroscopic (12) and photolabeling experiments (14) suggested that the M_r 43,000 protein bound local anesthetics, raising the possibility that it might be part of the AcCho-activated cation channel. However, Neubig *et al.* (9) demonstrated, and others have confirmed (10, 15, 16), that the M_r 43,000 protein can be removed from the membranes by treatment at pH 11 without altering either the binding of cholinergic ligands or the control of ion permeability.

Although receptor function is unaltered by alkaline extraction, the structural properties of the membranes are affected. Alkaline-extracted membranes have a smaller internal volume

(9) and a lower isopycnic density (17). Furthermore, the AcChoR in treated membranes is more sensitive to proteolysis (11, 17) and heat inactivation (18). The binding of anti-AcChoR antibodies to the membrane-bound receptor increases 4-fold after alkaline extraction of the membranes (unpublished data). Finally, the rotational mobility of the membrane-bound receptor is greatly enhanced by alkaline extraction (19, 20). These results suggest that the M_r 43,000 protein may interact with the AcChoR in the membrane.

The M_r 43,000 protein is associated with the most highly purified preparations of nicotinic postsynaptic membranes, but its cellular location *in situ* is unknown. We have prepared an antiserum to the alkaline-extractable proteins and have shown that it reacts predominantly with the M_r 43,000 protein. Immunofluorescence studies of *Torpedo* electric tissue and mammalian muscle suggest that the antigens recognized by this antiserum are highly concentrated in the postsynaptic membrane of both tissues.

MATERIALS AND METHODS

Isolation of Membranes and AcChoR. Receptor-rich membranes were prepared from fresh *Torpedo* electric organ as described by Sobel *et al.* (7) and purified further by velocity sedimentation on an 8–20% sucrose gradient (unpublished results). Affinity purification of AcChoR from frozen *Torpedo* electric organ was carried out with N-ethylmaleimide (MalNEt) as described (5). The specific activities were 3 and $\approx$8 μmol of α-toxin per g of protein for the membranes and purified AcChoR, respectively. Protein concentrations were determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard.

Preparation of Alkaline-Extracted Membranes and Alkaline-Extractable Proteins. Treatment of membranes at pH 11 was carried out at 4°C as described (9). After removal of the membranes by centrifugation, the extract was adjusted to pH 7. Integration of peak areas on Coomassie blue-stained-NaDodSO$_4$/polyacrylamide gels indicated that the M_r 43,000 protein comprised at least 70% of the protein in the alkaline extract used for the absorption experiments and that no other single peptide comprised more than 8%.

Preparation of Antisera to AcChoR and Alkaline-Extractable Proteins. Antiserum to affinity-purified AcChoR was prepared as follows (unpublished results). Briefly, rabbits were injected with AcChoR ($\approx$100 μg) in complete Freund's adjuvant

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Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; MalNEt, N-ethylmaleimide; P$_i$/NaCl, phosphate-buffered saline; FITC-anti-IgG, fluorescein isothiocyanate-conjugated anti-rabbit IgG; rhod-αBuTx, rhodamine-conjugated α-bungarotoxin.

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and 21 days later in incomplete adjuvant. Antiserum obtained 5 days later precipitated 55 pmol of ^{125}I-labeled AcChoR per μl of serum and was used after heat inactivation without further purification. Antiserum to the alkaline-extractable proteins was prepared as follows. Rabbits were injected subcutaneously at several spots on the back with neutralized supernatant of the alkaline extract ($\approx$70 μg of protein) in complete Freund's adjuvant. Four injections were administered at 7-day intervals. Preimmune serum was collected prior to the first injection, and serum samples were obtained weekly after the third administration of antigen.

Reaction of Antisera with Polypeptides Separated by NaDodSO$_4$ Electrophoresis. The method of Adair *et al.* (22) was used with some modifications. Proteins were separated by NaDodSO$_4$ microslab gel electrophoresis (23). The gels were stained reversibly, cut into strips, and destained. After equilibration in 50 mM Tris·HCl, pH 7.4/50 mM NaCl/0.02% NaN$_3$, the gel strips were placed in 10 ml of buffer containing gelatin (1 mg/ml), and 50 μl of antiserum was added. After incubation with gentle shaking for 24 hr, the gel strips were washed for 3 days in equilibration buffer (changed twice daily). Gel strips were then incubated in 10 ml of gelatin solution containing ^{125}I-labeled protein A (7 μg; 4 × 10^7 cpm/μg) for 24 hr. After washing for 3 days, the gels were dried and subjected to autoradiography at $-$70°C with an intensifying screen.

Immunofluorescence Studies of *Torpedo* Electric Tissue and Rat Muscle. Sections (14 μm) of *Torpedo* electric organ (stored at $-$70°C) were cut in a cryostat and dried on slides at room temperature for 30 min. The sections were fixed for 1.5 min at room temperature in 4% formaldehyde and washed for 5 min with 10 mM Na phosphate, pH 7.4/0.15 M NaCl (P$_i$/NaCl). Strips of rat diaphragm containing end plates were rolled up like a scroll and frozen in isopentane in liquid nitrogen. Transverse sections (8 μm) were cut, air dried on slides, and used without fixation. Some sections were stained for cholinesterase activity (24) to verify that end plates were present. Unstained sections cut from the same region of the muscle were used for the antibody incubations. In some cases, the left hemidiaphragm was denervated by transection of the phrenic nerve 7 days before use.

Approximately 100 μl of antiserum (diluted 1:200 or 1:400 in P$_i$/NaCl containing bovine serum albumin at 1 mg/ml) was placed on a section and the slides were incubated for 30–60 min in a moist chamber at room temperature. The slides were then washed twice for 5 min in P$_i$/NaCl and P$_i$/NaCl/bovine serum albumin containing fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (FITC-anti-IgG) (50 μg/ml; Cappel Laboratories, Cochranville, PA) and 0.1 μM rhodamine-conjugated α-bungarotoxin (rhod-αBuTx) (25) was placed on the section. After 30–60 min, the sections were washed with P$_i$/NaCl and mounted in P$_i$/NaCl/glycerol (1:1). The sections were examined with a Zeiss universal fluorescence microscope equipped with filters that permitted viewing each fluorochrome without interference from the other. Specimens were photographed with Kodak Tri-X film using identical exposures and developing and printing procedures.

RESULTS

As described (9), receptor-rich membranes from *Torpedo* electric organ are composed of four receptor polypeptides (α, β, γ, δ) plus two other major components of M_r 43,000 and $\approx$90,000 (Fig. 1, lane B). In addition, other components are present in smaller amounts. Most of the α-subunit of affinity-purified AcChoR (Fig. 1, lane A) migrates more slowly than its counterpart in the membranes because MalNEt, present in the buffers in which the receptor was solubilized and purified, in-
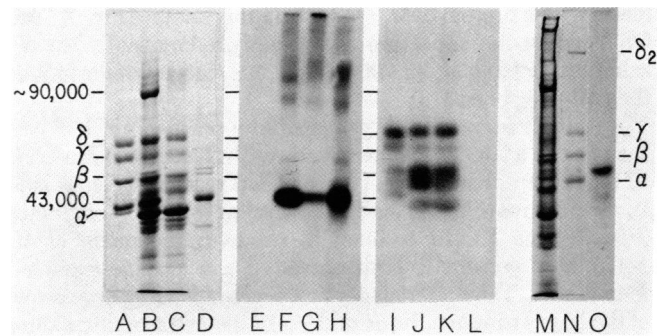

FIG. 1. Autoradiograms of gel strips from NaDodSO$_4$ gel electrophoresis of affinity-purified AcChoR (lanes A, E, I, and N), receptor-rich membranes (lanes B, F, and J), alkaline-extracted membranes (lanes C, G, and K), proteins removed by alkaline extraction (lanes D, H, and L), and total electric tissue proteins (lanes M and O). Lanes A–D, M, and N were stained for protein with Coomassie blue. The other lanes were incubated first with anti-p43 serum (E–H and O) or anti-AcChoR (I–L), both at a 1:200 dilution, and then with ^{125}I-labeled protein A. Note that the α subunit of affinity-purified AcChoR (lane I) migrates more slowly than its counterpart in membranes (lanes J and K). Samples for lanes A–L were denatured in 2.5% NaDodSO$_4$ sample buffer/5 mM dithiothreitol without boiling, while those for lanes M–O were boiled in 2.5% NaDodSO$_4$ sample buffer/10 mM MalNEt/1 mM EDTA/1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid/0.5 mM phenylmethylsulfonyl fluoride containing Trasylol at 10 units/ml and pepstatin at 5 μg/ml. In the latter case, the δ chain of AcChoR migrates as a dimer (δ_2).

creases the apparent molecular weight of this subunit in NaDodSO$_4$ gels (5). Alkaline extraction of the membranes removes almost all of the M_r 43,000 and $\approx$90,000 polypeptides, as well as minor components (Fig. 1, lane C; also see ref. 9). The M_r 43,000 polypeptide and some minor components are found in the alkaline supernatant (Fig. 1, lane D). The M_r $\approx$90,000 component is not recovered.

The alkaline-extractable material (Fig. 1, lane D) was used as antigen for the production of antisera. Two rabbits were injected and the production of antibody activity was monitored by double diffusion (26). Detectable antibodies were observed in serum obtained 4 weeks after the initial injection and the titer increased in the following weeks. One of these antisera (the third bleeding from one rabbit) was selected for further analysis.

Antiserum Labeling of Proteins on NaDodSO$_4$ Gels. The specificity of this antiserum was investigated by allowing it to react with electric organ proteins that had been separated by NaDodSO$_4$ gel electrophoresis. Gel strips containing the resolved polypeptides were treated first with antiserum and then with ^{125}I-labeled protein A. Reactive polypeptides were detected by radioautography. An autoradiogram of a gel treated with antiserum against alkaline-extractable material is shown in Fig. 1 (lanes E–H). This antiserum shows no reactivity with affinity-purified AcChoR (Fig. 1, lane E). It reacts predominantly with the M_r 43,000 protein in membranes and, to a much lesser extent, with material of apparent M_r 85,000–110,000 (Fig. 1, lane F). Neither the M_r $\approx$90,000 component nor the doublet of M_r 50,000–55,000 is reactive with this antiserum. The larger reactive material is not associated with peptides readily identified by staining with Coomassie blue and in part may be aggregated M_r 43,000 protein. Most of the reactive material is absent from membranes that had been subjected to alkaline extraction (Fig. 1, lane G) but is present in the alkaline-extractable proteins (Fig. 1, lane H). In another experiment, total electroplax protein was dissolved by boiling in NaDodSO$_4$ in the presence of MalNEt and protease inhibitors and then subjected to gel electrophoresis (Fig. 1, lane M). The antiserum

reacts with a protein of $M_r \approx 43,000$ in this sample (Fig. 1, lane O). Because the antiserum recognizes predominantly the alkaline-extractable M_r 43,000 polypeptide, we will refer to it as the anti-p43 serum.

An antiserum prepared against affinity-purified AcChoR was analyzed in a similar manner. Anti-AcChoR serum reacts with all four receptor subunits of the purified receptor (Fig. 1, lane I), membranes (Fig. 1, lane J), and alkaline-treated membranes (Fig. 1, lane K). In addition, a reactive component of M_r $\approx 47,000$ is present in both untreated and alkaline-extracted membranes. This material may be a degradation fragment of one of the larger subunits. None of the alkaline-extractable proteins is recognized by anti-AcChoR (Fig. 1, lane L).

Antiserum Labeling in *Torpedo* Electric Organ and Rat Skeletal Muscle. The *Torpedo* electric organ is composed of parallel stacks of large flat cells (electrocytes). Each electrocyte is ≈ 0.5 cm in diameter and 10 μm thick and is richly innervated only on its ventral surface. AcChoR are present in the postsynaptic membrane of the ventral face but are absent from the dorsal surface of the cell (27). We have examined the distribution of the binding sites for anti-p43 serum by immunofluorescence and compared it with the binding sites for anti-AcChoR and rhod-αBuTx. The use of fluorescein-conjugated antibodies and rhod-αBuTx permits unambiguous comparison of their binding sites in the same tissue section (28).

Sections of *Torpedo* electric tissue were cut perpendicular to the faces of the cells and treated with the antiserum to be tested and then with a mixture of FITC-anti-IgG and rhod-αBuTx. As expected, each electrocyte was stained on only one surface by rhod-αBuTx (Fig. 2). When stained with anti-AcChoR and viewed under fluorescein optics, the same face of

the cell is intensely fluorescent, although the rest of the cell shows a low amount of staining (Fig. 2 A and A'). Staining of the electrocyte by anti-p43 serum and rhod-αBuTx is virtually indistinguishable (Fig. 2 B and B'). Preimmune serum from the same rabbit does not react with the electrocytes (Fig. 2 C and C'). Thus, like the AcChoR, the antigens recognized by anti-p43 serum are preferentially located in the innervated membrane of the electrocyte.

Antibodies directed against the purified AcChoR of *Torpedo* also recognize the AcChoR of vertebrate skeletal muscle. We wished to determine whether the nonreceptor protein(s) recognized by the anti-p43 serum has a counterpart in mammalian muscle and, if so, whether it is localized at the motor end plate. The subsynaptic area of rat muscle, in which the AcChoR is highly concentrated, comprises <0.1% of the cell surface while, in *Torpedo*, $\approx 50\%$ of the innervated electrocyte surface is covered with nerve terminals. Labeling in rat muscle, therefore, permits a more precise correlation between the subcellular localization of AcChoR and the antigens recognized by anti-p43 serum. Sections of innervated rat diaphragm containing end plates were incubated with anti-p43 serum and then with a mixture of FITC-anti-IgG and rhod-αBuTx. As shown in Figs. 3 and 4 A and A', the synapses on the muscle fibers are intensely stained with the anti-p43 serum. The staining patterns of anti-p43 serum and rhod-αBuTx in the synaptic region are virtually identical. In addition, the anti-p43 serum stains the extrasynaptic membranes of the muscle fibers but only very faintly. Preimmune serum showed no staining (not shown). It is clear that the antigen(s) recognized by anti-p43 serum are highly concentrated at the neuromuscular junction.

We have characterized the muscle components recognized by anti-p43 serum by competition of the binding to tissue sections with purified *Torpedo* AcChoR (which has no detectable M_r 43,000 protein) and with an alkaline extract from *Torpedo*
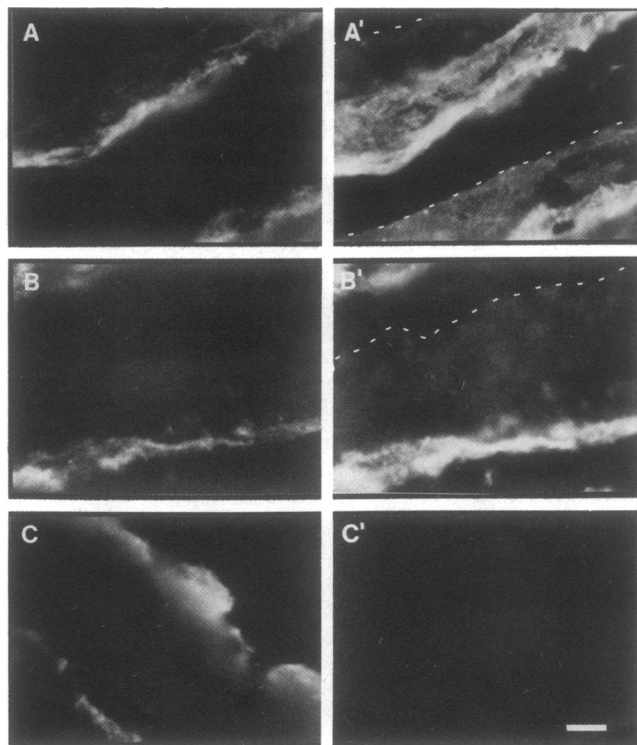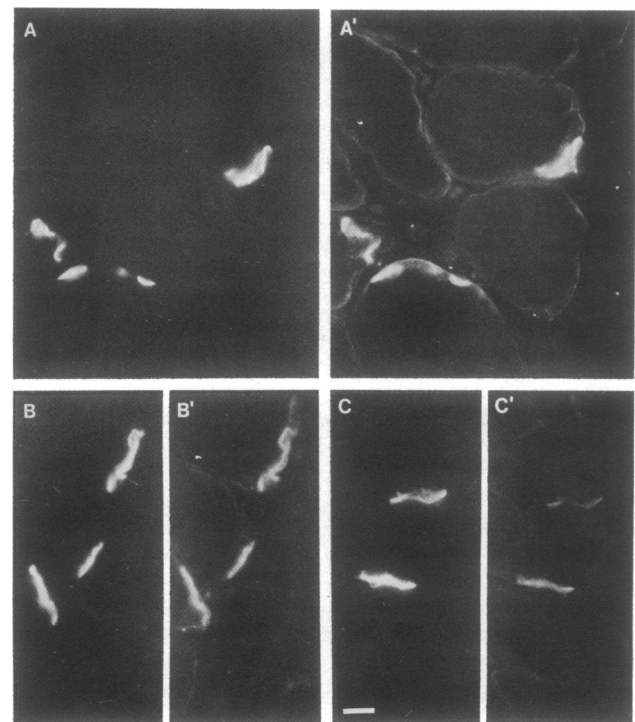

FIG. 2. Immunofluorescence images of *Torpedo* electric tissue. Sections were incubated with anti-AcChoR serum (*A* and *A'*), anti-p43 serum (*B* and *B'*), or preimmune serum (*C* and *C'*), all at a dilution of 1:400, and then with rhod-αBuTx and FITC-anti-IgG. The same field was photographed with either rhodamine (*A–C*) or fluorescein optics (*A'–C'*). Dashed lines (*A'* and *B'*) indicate the noninnervated electrocyte surface. (Bar = 5 μm.)

FIG. 3. Immunofluorescence images of normal rat diaphragm muscle. Sections were incubated with anti-p43 serum (1:200 dilution) and then with rhod-αBuTx and FITC-anti-IgG. The same field was photographed under rhodamine (*A–C*) or fluorescein (*A'–C'*) optics. (Bar = 10 μm.)
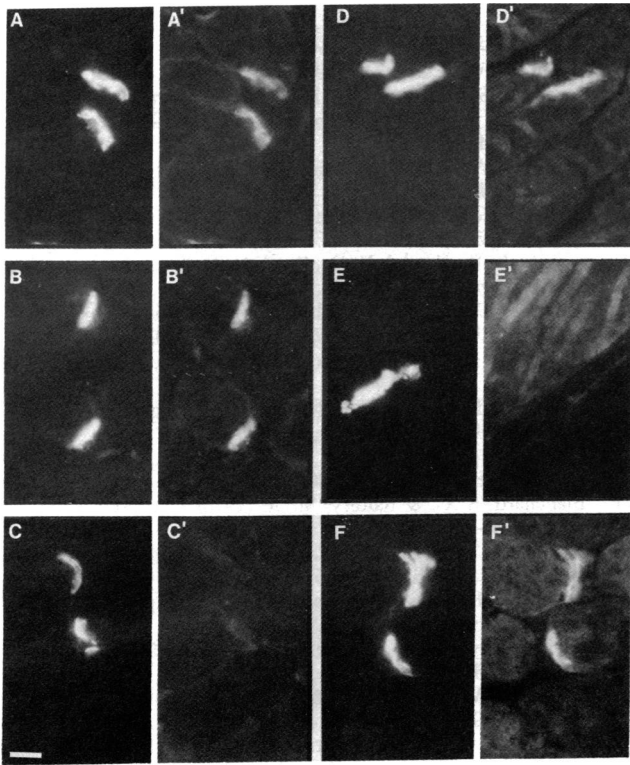
FIG. 4. Immunofluorescence studies of competition by AcChoR and alkaline-extractable proteins. Sections of muscle were incubated with anti-p43 serum (*A, A', B, B', C,* and *C'*) or anti-AcChoR (*D, D', E, E', F,* and *F'*) without (*A, A', D,* and *D'*) or with prior incubation with 50 μg purified AcChoR (*B, B', E,* and *E'*) or 25 μg of alkaline-extractable proteins (*C, C', F,* and *F'*). Sections were then incubated with rhod-αBuTx and FITC-anti-IgG and the same field was photographed under rhodamine (*A–F*) or fluorescein (*A'–F'*) optics. Antisera were incubated with competitors for 90 min at 37°C at a 1:200 dilution and then centrifuged at 12,000 × *g* for 15 min. (Bar = 10 μm.)
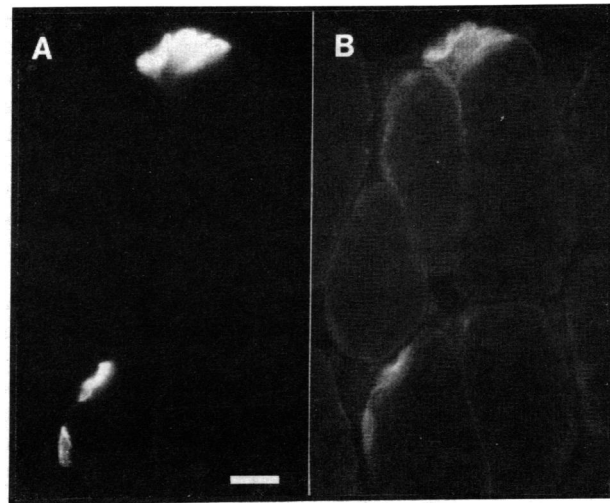

FIG. 5. Immunofluorescence images of denervated rat diaphragm muscle. A section was incubated with anti-p43 serum (1:200 dilution) and then with rhod-αBuTx and FITC-anti-IgG. The same field was photographed with rhodamine (*A*) or fluorescein (*B*) optics. (Bar = 10 μm.)

membranes. Anti-p43 serum staining of muscle fibers is not detectably affected by prior incubation of the antiserum with AcChoR (Fig. 4 *B* and *B'*). However, the synaptic membrane staining is almost completely eliminated (Fig. 4 *C* and *C'*) by prior incubation with proteins removed from membranes by alkaline extraction (>70% M_r 43,000 protein; see Fig. 1, lane D). It is our impression from several experiments that the faint extrasynaptic membrane labeling by anti-p43 serum is also reduced by previous incubation of the antiserum with alkaline-extractable proteins. More sensitive techniques will be required to answer this question conclusively. The anti-AcChoR serum labels synapses very intensely (Fig. 4 *D* and *D'*) but, in contrast to the anti-p43 serum, also stains other regions of the muscle cells. The latter staining is probably nonspecific, since it is not eliminated by previous incubation of the antiserum with purified AcChoR (Fig. 4 *E* and *E'*). Purified AcChoR blocks the synaptic staining by anti-AcChoR (Fig. 4 *E* and *E'*) but alkaline-extracted proteins do not (Fig. 4 *F* and *F'*). Thus, the muscle antigens recognized by anti-p43 serum appear to be immunologically similar to the alkaline-extractable proteins of *Torpedo* membranes but distinct from affinity-purified AcChoR.

The virtual identity of the synaptic staining patterns of rhod-αBuTx and anti-43K serum suggests that this antiserum reacts with the post synaptic membrane. To investigate this point further, we stained muscles that had been denervated 7 days earlier. This period of denervation is sufficient for nerve terminal degeneration (29). As shown in Fig. 5, anti-p43 serum stains end plates of denervated muscles with a pattern identical to that

of rhod-αBuTx. Thus, the major antigens recognized by anti-p43 serum are not contained within the nerve terminal. The amount of extrasynaptic AcChoR is substantially increased in denervated muscles, but we are unable to detect it with rhod-αBuTx (Fig. 5) or with anti-AcChoR (not shown) because the density of extrajunctional AcChoR (200–500 αBuTx sites per μm^2) is much less than that of receptors at the end plate (30,000 sites per μm^2). Thus, we are currently unable to comment on changes in the extrasynaptic concentration of the M_r 43,000 protein after denervation.

DISCUSSION

The antiserum used for these studies reacts predominantly with the M_r 43,000 protein of *Torpedo* postsynaptic membranes. It shows no reactivity with any of the four subunits of the receptor, nor does it react with the M_r 52,000–55,000 or M_r ≈90,000 proteins, peptides of membranes that commonly contaminate post-synaptic membrane preparations (9). Furthermore, its reactivity with purified AcChoR labeled with ^{125}I-labeled αBuTx in Triton is <0.3% of that of anti-AcChoR (S. Porter, unpublished results). The high molecular weight components recognized by anti-p43 serum under some conditions may be aggregated M_r 43,000 protein or other membrane proteins. They are present in very small amounts in the membranes and are barely visible in Coomassie blue-stained gels. Since the antiserum was used at a dilution of 1:400 on the electroplax and 1:200 on muscle, it seems unlikely that a minor population of antibodies are responsible for the fluorescent staining. Competition experiments showed that the muscle antigens cross-react with alkaline-extractable proteins (predominantly the M_r 43,000 protein) but not with AcChoR. Thus the anti-p43 serum recognizes a postsynaptic membrane protein(s) other than the receptor that is probably the M_r 43,000 protein.

Like anti-AcChoR and rhod-αBuTx, anti-p43 serum binds to the innervated but not the uninnervated membrane of *Torpedo* electrocytes. In muscle, the antigen recognized by anti-p43 serum is highly concentrated at the end plate but is also present in the extrajunctional membrane in much smaller amounts. It seems unlikely that the brighter staining of the end plate is due solely to the increased membrane surface area of the junctional folds (≈6-fold; ref. 30). We cannot eliminate the

possibility that the anti-p43 serum recognizes different antigens in the synaptic and extrasynaptic regions.

Several other membrane and cell surface components are concentrated in the synaptic region of the neuromuscular junction. A specialized form of AcCho esterase (31) is found only at the synapse attached to the basement membrane (32). Antigens recognized by antiserum prepared against anterior lens capsule are restricted to the synaptic basal lamina (28) and thus, like the esterase, are predominantly if not entirely extracellular components. Another synaptic component, junctional AcChoR, is an integral membrane protein that, by analogy to the *Torpedo* receptor (refs. 11, 33, and 34; unpublished results) spans the membrane.

In tissue sections, both the extracellular and cytoplasmic sides of the subsynaptic membrane are accessible to antibodies. Therefore, our results provide no information about the topological orientation of the M_r 43,000 protein. However, recent studies have provided evidence that it is located on the cytoplasmic face of the isolated *Torpedo* postsynaptic membranes. Wennogle and Changeux (11) showed that, under certain conditions, the receptor polypeptides in sealed right-side-out vesicles could be proteolyzed with trypsin while the M_r 43,000 polypeptide remained intact. Introduction of protease to the interior of the vesicles by sonication resulted in cleavage of the M_r 43,000 protein. Lactoperoxidase-catalyzed radioiodination has been used to assess the orientation of peptides in normal right-side-out *Torpedo* vesicles (35, 36). In vesicles that are impermeable to lactoperoxidase, the peptides of the AcChoR but not the M_r 43,000 protein incorporate iodine, but for vesicles made permeable to lactoperoxidase by the use of saponin, the M_r 43,000 protein is iodinated as well. If the muscle antigen recognized by anti-p43 serum is similar to the M_r 43,000 protein from *Torpedo* membranes, it may be the first example of a peripheral protein that is highly concentrated on the cytoplasmic side of the muscle postsynaptic membrane.

Anti-p43 serum raised against *Torpedo* protein stains both *Torpedo* and muscle synaptic membranes with similar intensities at dilutions of 1:200–1:400. The antigen, therefore, must be evolutionarily conserved and is likely to be an important component of the neuromuscular junction. The availability of a specific characterized antiserum will be useful in further studies of its role in synaptic structure and function.

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