Peripheral Proteins of Postsynaptic Membranes
from *Torpedo* Electric Organ
Identified with Monoclonal Antibodies

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ABSTRACT   Highly purified postsynaptic membranes from *Torpedo* electric organ contain the acetylcholine receptor as well as other proteins. To identify synapse-specific components, we prepared monoclonal antibodies (mabs) to proteins extracted from the membranes with either lithium diiodosalicylate or alkaline treatment. 10 mabs specific for three different proteins were obtained. Seven mabs reacted with a major 43,000-mol-wt protein (43K protein). This protein is composed of isoelectric variants (pl = 7.2–7.8) and each of the mabs reacted with all of the variants. Analysis of these mabs by competition for binding to 43K protein and by reaction with proteolytic fragments of 43K protein in immunoblots showed that they recognize at least five different epitopes. Two mabs reacted with a protein of 90,000 mol wt (90K protein) and one with a protein of 58,000 mol wt composed of isoelectric variants (pl = 6.4–6.7) (58K protein). The 43K and 58K proteins appeared to co-purify with the receptor-containing membranes while the 90K protein did not. Immunofluorescence experiments indicated that the anti-43K mabs bind to the innervated face of *Torpedo* electrocytes and that a component related to the 43K protein is found at the rat neuromuscular junction. The anti-58K mab stained the innervated face, although rather weakly, while the anti-90K mabs reacted intensely with the non-innervated membrane. Thus, the 43K protein and possibly also the 58K protein are synaptic components while the 90K protein is predominantly nonsynaptic.

Membranes highly-enriched in the acetylcholine receptor (AChR)[1] can be isolated from the electric organ of *Torpedo*. In addition to the subunits of the AChR, these preparations contain a number of other proteins, including two prominent ones of apparent molecular weights of 43,000 (43K protein) and ~90,000 (90K protein) and numerous other components present in smaller amounts (8, 12, 14, 19, 21, 28, 29). It is important to identify those that are synapse specific since they may participate in the function of the postsynaptic membrane. The electrocytes of *Torpedo* are particularly useful in this regard since they provide sufficient material for biochemical studies as well as for production of monoclonal antibodies.

Some of the nonreceptor proteins can be separated from the membranes by treatment at alkaline pH (8, 19) or by extraction with low concentrations of lithium diiodosalicylate (LIS) (8, 21). Several observations indicate that these peripheral membrane proteins are important in synaptic function. First, some properties of the receptor change when the membranes are treated at alkaline pH. Increases in rotational (17, 22) and translational (2, 6) mobility, in sensitivity to proteolysis (15) and heat denaturation (24), and in the number of exposed antigenic sites (9, 10) have been described. These alterations occur without any detectable changes in ligand binding or ion influx properties of the receptor (8, 19). Second, morphological studies have demonstrated that cytoplasmic specializations co-extensive with the receptor can be removed by alkaline extraction (25), suggesting that the molecular constituents of this specialization occupy the same region of the postsynaptic membrane as the receptor and thus may interact with it. Finally, antiserum prepared against alkaline-extracted proteins reacts strongly with the synaptic membranes of electrocytes and rat muscle in immunofluorescence

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[1] *Abbreviations used in this paper:* 43K, 58K, and 90K proteins, 43,000-, 58,000-, and 90,000-mol-wt components, respectively, of receptor-rich *Torpedo* membranes; AChR, acetylcholine receptor; αBuTx, α-bungarotoxin; CK, creatine kinase; LIS, lithium diiodosalicylate; mab, monoclonal antibody.

experiments (11).

We have prepared monoclonal antibodies (mabs) to three proteins of alkaline and LIS extracts of receptor-rich membranes and have used them to show that the 43K protein and possibly a 58K protein are synaptic components. The 90K protein is located predominantly in the nonsynaptic membrane of *Torpedo* electrocytes.

MATERIALS AND METHODS

Preparation of Antigens for Injection: Receptor-rich membranes were isolated from frozen *Torpedo nobiliana* electric organ as previously described (8, 21). LIS extracts were prepared according to procedures described by Porter and Froehner (21) using these membranes at a concentration of 5 mg protein/ml. Alkaline extracts (19) were prepared from *Torpedo californica* membranes isolated as described by Sobel et al. (28).

Production of Monoclonal Antibodies: The methods for maintenance of cell lines, fusion of spleen cells with the NS-1 myelomas, selection and cloning of hybridomas, production of ascites fluids, and purification and radioiodination of IgG were essentially the same as previously described (10). Balb/c mice were injected with LIS or alkaline extracts according to the protocol described for AChR (10). A solid-phase assay was used for the initial screening of the cultures (10). The antigen used in these assays was partially purified to remove residual AChR by the following method: Membranes (6-10 mg/ml) were extracted with 10 mM LIS, 10 mM Tris, pH 8.5 (21), and dithiothreitol was added to the extract to a final concentration of 10 mM. The extract was then applied to a 5-20% sucrose gradient (in 10 mM Tris, pH 8.5, 1 mM dithiothreitol) and centrifuged for 15-17 h at 38,000 rpm in an SW 41 rotor. The gradient was fractionated into 20 aliquots and the fractions were analyzed by SDS microgel electrophoresis (18). Those fractions containing 43K protein (as well as other less prominent proteins) were pooled and diluted to 20 ml with PBS. Aliquots (25 μl) were immediately applied to wells of flexible microtiter plates (Linbro Chemical Co., Hamden, CT) and the plates were incubated for 15-20 h at room temperature. The protein solution was removed and the wells were filled with PBS containing 40 mg/ml BSA. After 15-30 min, the solutions were removed and the plates were stored at 4°C for up to several months.

Analysis of mab Specificity by Immunoblots: Immunoblots of SDS microgels were prepared as previously described (10). For two-dimensional immunoblots, membrane samples were separated on two-dimensional microgels as described by Porter and Froehner (21) with the following modifications. Membranes (35 μg) were dissolved in 0.1% Triton-0.02% SDS before the addition of sample buffer lacking SDS. Isoelectric focusing was performed for 17-18 h. Preparation of the replicas of the two-dimensional gels and their incubation with mabs was performed as described for the one-dimensional SDS gels.

For comparison of the amounts of 43K protein in membranes and cytosol, electric organ (5 g) was homogenized in a Virtis homogenizer in 4 ml of 10 mM NaPO₄, pH 7.4, 1 mM EDTA, 1 mM EGTA, 20 U/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide. Insoluble material was collected by centrifugation for 30 min at 35,000 g and resuspended in 4 ml of homogenization buffer (membrane fraction). The supernatant was centrifuged again for 30 min at 250,000 g and used as the cytosolic fraction. Aliquots of both fractions were subjected to SDS microgel electrophoresis and immunoblotting as above. For quantitative analysis, bands corresponding to the 43K protein were cut out of the blots and counted in a gamma counter. Under conditions of excess mab and ¹²⁵I-anti-mouse IgG, the amount of radioactivity bound to the blot is linearly dependent on the volume of sample applied to the gel and reflects the amount of antigen present.

Immunofluorescence Procedures: Preparation of cryostat sections of *Torpedo* electric organ (11) and frog and rat muscle (10) and their incubation with antibodies have been described.

Quantitative Assay for Antigens in Sucrose Gradient Fractions: A solid-phase well assay similar to that used for assay of hybridoma supernatants (10) was utilized with the following modifications. Wells of microtiter plates (Corning 25860, Corning Glassworks, Corning, NY) were incubated with 50 μl of sample from the sucrose gradient for 3 h at room temperature. For wells to be assayed with mab 1043A, the fractions were diluted 1/10 in PBS-NaN₃. For wells to be assayed with mab 139A, mab 1132E, or mab 1127A, the fractions were diluted 1/200, 1/10, and 1/10, respectively, in PBS-NaN₃ containing 0.1% Triton. These conditions were found to be optimal for binding of the corresponding antigen to the wells. The plates were then incubated with a 4% BSA solution as described and then incubated for 15 h with 50 μl of mab ascites fluid diluted 1/100 for mabs 139A, 1132E, 1127A or

hybridoma culture supernatant diluted 1/8 for mab 1043A. Washings and incubation with ¹²⁵I-anti-mouse IgG were performed as described (10). Radioactivity was eluted from the wells with 100 μl of 2% SDS and counted in a gamma counter. Binding of ¹²⁵I-α-bungarotoxin (αBuTx) was measured as described previously (9).

Binding of Creatine Kinase to Anti-43K mabs: Cytosolic fractions were prepared by homogenizing 5 g of electric organ in 3 ml PBS. The homogenate was centrifuged at 35,000 g for 30 min and the supernatant was used. Creatine kinase (CK) activity was measured with Sigma 46-UV diagnostic kit according to the instructions provided in the manufacturer's technical bulletin (Sigma Chemical Co., St. Louis, MO). To test for binding of creatine kinase by mabs, affinity columns (Affigel 10; Bio-Rad Laboratories, Richmond, CA) were prepared with either MPC-11 IgG or with 1132E according to the manufacturer's instructions. Columns of ~1 ml containing 12.7 nmol of MPC-11 IgG (control) or 15.3 nmol of 1132E IgG (anti-43K protein) were equilibrated with PBS containing 1 mg BSA/ml (PBS-BSA) and 300 μl of cytosolic fraction containing 4.6 U of CK activity (equivalent to 0.8 nmol assuming a molecular weight of 40,000 and a specific activity of the purified enzyme of 150 U/mg [see reference 1]) was applied. After a 30-min incubation at room temperature, the columns were washed with PBS-BSA and the material eluted from the columns was assayed for CK activity.

RESULTS

Characterization of Monoclonal Antibodies

Alkaline and LIS extracts of AChR-rich membranes were used as antigens for the production of mabs. After initial screening for antibody activity against the antigens using a solid-phase well assay, supernatants from positive cells were analyzed by immunoblots of membranes separated on SDS gels. Hybridomas that were positive in this test were recloned and analyzed again. Seven mabs to a protein(s) of apparent molecular weight of 43,000 were obtained in this manner (Fig. 1). In some experiments, a faint band of ~85,000 mol wt was also seen in the autoradiograms. This is most apparent in the experiment shown here with mabs 1114A, 1120A, and 1201C, antibodies that give a particularly strong signal on immunoblots. This component probably represents a dimeric form of the 43,000-mol-wt protein and an even fainter band of ~120,000 mol wt, visible only after longer exposure of the autoradiogram, may be an oligomer. All of these mabs are of the IgG₁ isotype, except 1120A which is an IgM.

Initially, several hybridomas were obtained with specificities for proteins in the 50,000-60,000-mol-wt range, but only one of these was successfully cloned and characterized. Mab 1127A, an IgM, recognized a 58,000-mol-wt component (58K protein) of the purified membranes (Fig. 1). Finally, two mabs (1027C and 1043A) reacted with a 90K protein. Both of these mabs are IgG₁ isotypes and were obtained from mice injected with alkaline extracts. Membranes subjected to alkaline extraction often lack the 90K band but this component is generally not recovered intact in the alkaline extract (11, 19). It seems possible that the alkaline treatment alters the mobility of the 90K protein on SDS gels, possibly converting it to small fragments. This altered form may have served as the immunogenic component that produced these two hybridomas.

At least three different proteins of molecular weights near 43,000 have been described as components of AChR-rich membranes. One of these, termed 43K protein (21) or v_1 (12), appears to be found in all preparations of membranes. This component is composed of isoelectric variants (pI = 7.0-8.0), all of which have identical peptide mabs. It is tightly associated with the membrane and can be removed by pH 11 treatment or with LIS (12, 21). A second component is found in some but not all preparations and has been called v_2 (12). This protein is a major cytoplasmic component comprised of
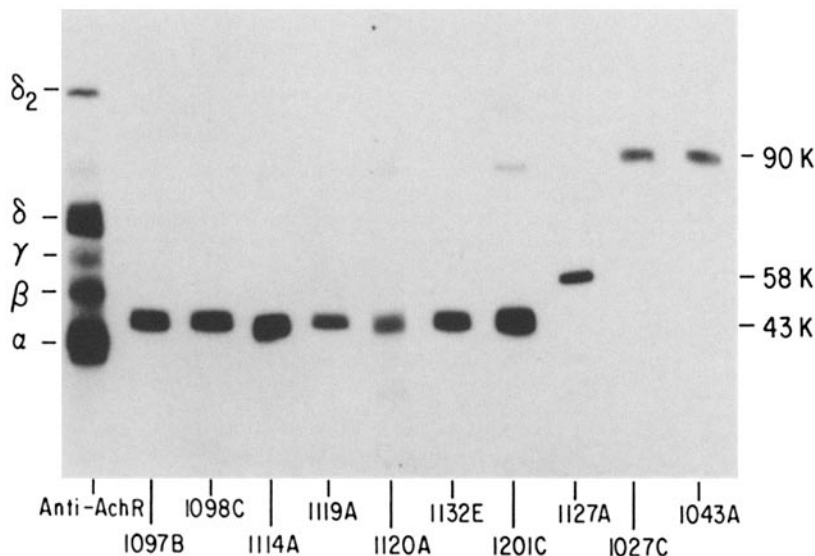
FIGURE 1 Autoradiogram of one-dimensional immunoblots. AChR-rich membranes were subjected to SDS microslab gel electrophoresis and immunoblots were prepared as described in Materials and Methods. Ascites fluid diluted 1/100 was used in each case except 1027C and 1043A which were undiluted culture supernatant. Greek letters to the left indicate the positions of the AChR subunits: α (40,000 mol wt), β (50,000), γ (60,000), δ (65,000). δ_2 is dimeric delta subunit. Numbers to the right of the figure indicate the apparent molecular weights. Numbers assigned to the mabs are indicated along the bottom.

isoelectric variants (pI = 6.8–7.2) and is depleted from membranes subjected to affinity purification. A membrane component with very similar properties has been identified as creatine kinase (1). The third component is found in the membranes in relatively small quantities, has an isoelectric point of ~5.5, and yields peptide mabs similar to that of actin (21). In view of this heterogeneity, it was necessary to determine which of these proteins reacts with the anti-43K mabs.

Two-dimensional gel electrophoresis demonstrates that there are two or three basic 43,000-mol wt proteins present in the membranes used for the production of the mabs (Fig. 2A; also see reference 21). These spots probably represent a single protein since they yield indistinguishable peptide maps and the extent of heterogeneity is influenced by the method of sample preparation (21). Immunoblots of membranes separated by 2-dimensional gels show that all of the anti-43K mabs reacted with these basic proteins (Fig. 2, B–F). They did not recognize proteins with the properties of actin nor did they recognize any other components in the membranes. Furthermore, the anti-43K mabs were unreactive with purified AChR as determined either by a solid-phase well assay (10) or in detergent solution after labeling the receptors with radioactive α-BuTx (9) (data not shown). No evidence for reactivity with the 85,000-mol-wt component noted in the one-dimensional immunoblots was seen in the two-dimensional blots, probably because the conditions for reduction of the sample were more stringent in the latter.

Several lines of evidence demonstrate that the anti-43K mabs did not recognize either the ν_2 protein described by Gysin et al. (12) or Torpedo CK. First, the membranes used for injection appear to lack the ν_2 component (21). Second, the 43K protein recognized by these mabs is only a minor component of the cytosol. This was determined by quantitative immunoblots of soluble and insoluble fractions of electric organ. As shown in Fig. 3, at least 90% of the 43K protein recognized by mab 1201C is in a particulate, presumably membrane-bound form. The small amount of cytosolic 43K protein has the same mobility on two-dimensional gels as that found in purified membranes (data not shown). Finally, an immuno-affinity column prepared with mab 1132E is not able to bind cytosolic Torpedo CK specifically. Recovery of CK activity from passage through immuno-affinity columns

was 74% for both MPC-11 and 1132E (see Materials and Methods for details). Thus, the anti-43K mabs described here are specific for the 43K protein described by Porter and Froehner (21) which is probably identical to ν_1 (12).

Mab 1127A recognizes a group of spots with isoelectric points of ~6.4–6.7 and apparent molecular weight of 58,000 (Fig. 2G). These components are possibly isoelectric variants of a single protein and are minor constituents of both the membranes and LIS extracts. Unpurified or receptor-depleted membranes analyzed by one-dimensional immunoblots contain a 58K protein as well as other components of ~75,000, 115,000, and 150,000 mol wt that are recognized by mab 1127A. The relationship between these higher molecular weight proteins and the 58K protein found in the receptor-rich membranes has not yet been determined.

Co-purification of Receptor and Nonreceptor Proteins on Sucrose Gradients

Proteins that are specific components of the postsynaptic membrane should co-purify with receptor-rich membranes throughout the purification procedure. We compared the distribution of the 43K, 58K, and 90K proteins with that of the AChR on a linear sucrose gradient (25–42%). The amount of each component was measured using a solid-phase assay and compared with the distribution of total protein and ^{125}I-αBuTx binding activity (Fig. 4). AChR-rich membranes were found at a density equivalent to 36% sucrose in this gradient as determined by either ^{125}I-αBuTx binding activity or binding of mab 139A, an mab specific for the alpha subunit of AChR (10). The distributions of both the 43K and the 58K proteins were essentially coincident with the receptor. The 90K protein was distributed more broadly across the gradient with a peak at a lower density than that of the receptor-rich membranes.

Immunofluorescence Studies on Electrocytes and Muscle

The distribution of the 43K, 58K, and 90K proteins in electric organ and muscle was also examined by immunofluorescence. In these experiments, simultaneous labeling of these proteins and AChRs was accomplished with mabs plus
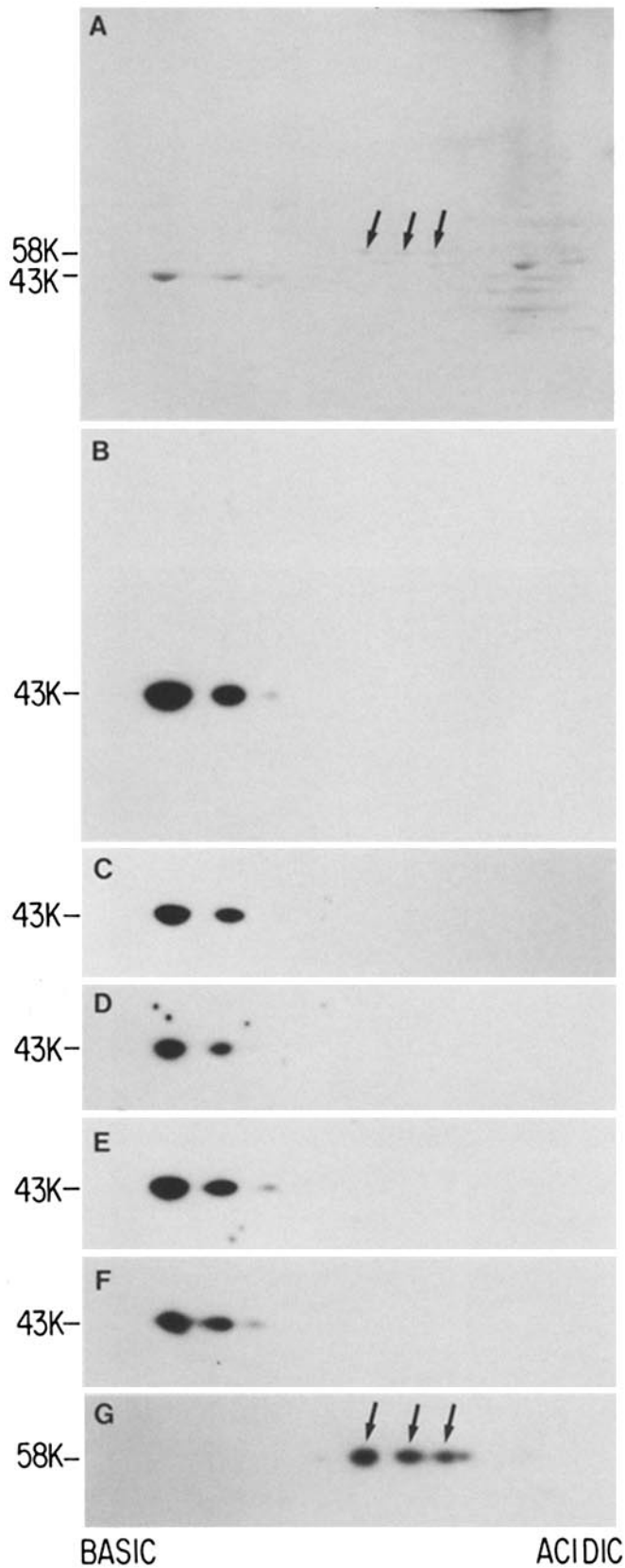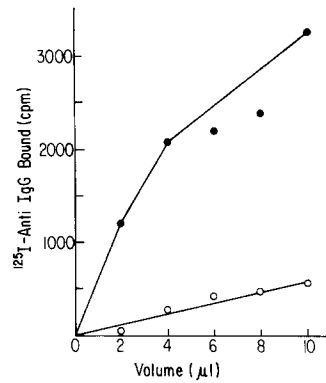
FIGURE 3   Comparison of the amounts of membrane-bound and cytosolic 43K protein. Quantitative one-dimensional immunoblots were prepared with 1201C ascites fluid (diluted 1/100) and counted as described in Materials and Methods. Volumes indicated on the ordinate are amounts of cytosol (O) or membrane fraction (●) applied to the gel. 10 μl equals 0.25% of the cytosolic or membrane fraction derived from 5 g of tissue.
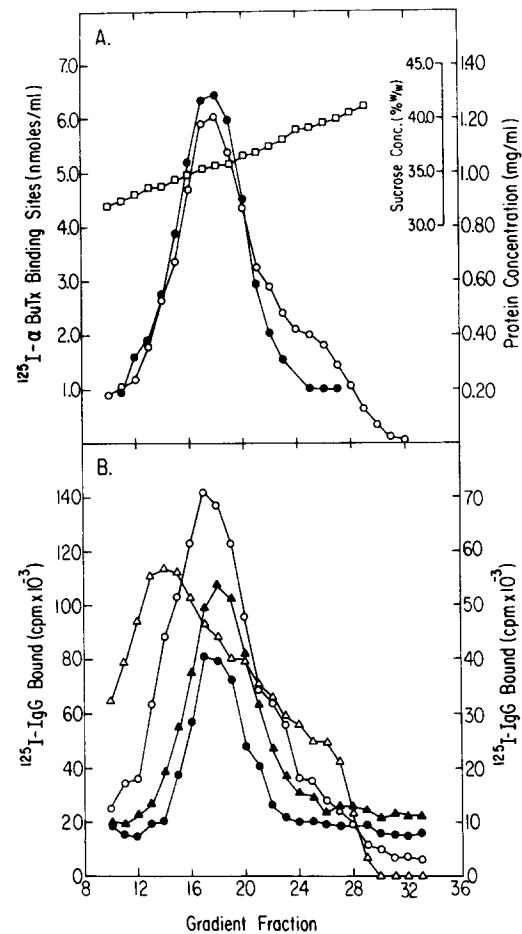
FIGURE 4   Distribution of AChR and nonreceptor proteins on sucrose gradient of Torpedo membranes. Partially purified Torpedo membranes were subjected to equilibrium density gradient centrifugation and fractions of the gradient were analyzed. In A, the concentrations of total protein (O) and ^{125}I-αBuTx binding sites (●) are shown. The sucrose concentration (□) was measured by refractometry. The binding of mabs was measured by a solid-phase assay (B). The scale for mab 139A (anti-AChR, O) is shown on the left and the scale for mab 1132E (anti-43K, ●), mab 1127A (anti-58K, ▲), and mab 1043A (anti-90K, △) is shown on the right.

FIGURE 2   Two-dimensional immunoblots of mabs. AChR-rich membranes were subjected to two-dimensional gel electrophoresis and immunoblots (using ascites fluid diluted 1/100) were prepared as described in Materials and Methods. (A) Coomassie Blue-stained gel of membranes. Autoradiograms of immunoblots are shown in (B) mab 1097B, (C) mab 1114A, (D) mab 1119A, (E) mab 1132E, (F)

mab 1201C, and (G) mab 1127A. Only the part of the immunoblots having reactivity are shown in C–G. Numbers to the left indicate the apparent molecular weight. The arrows in A point to the protein spots reactive with mab 1127A shown by arrows in G.
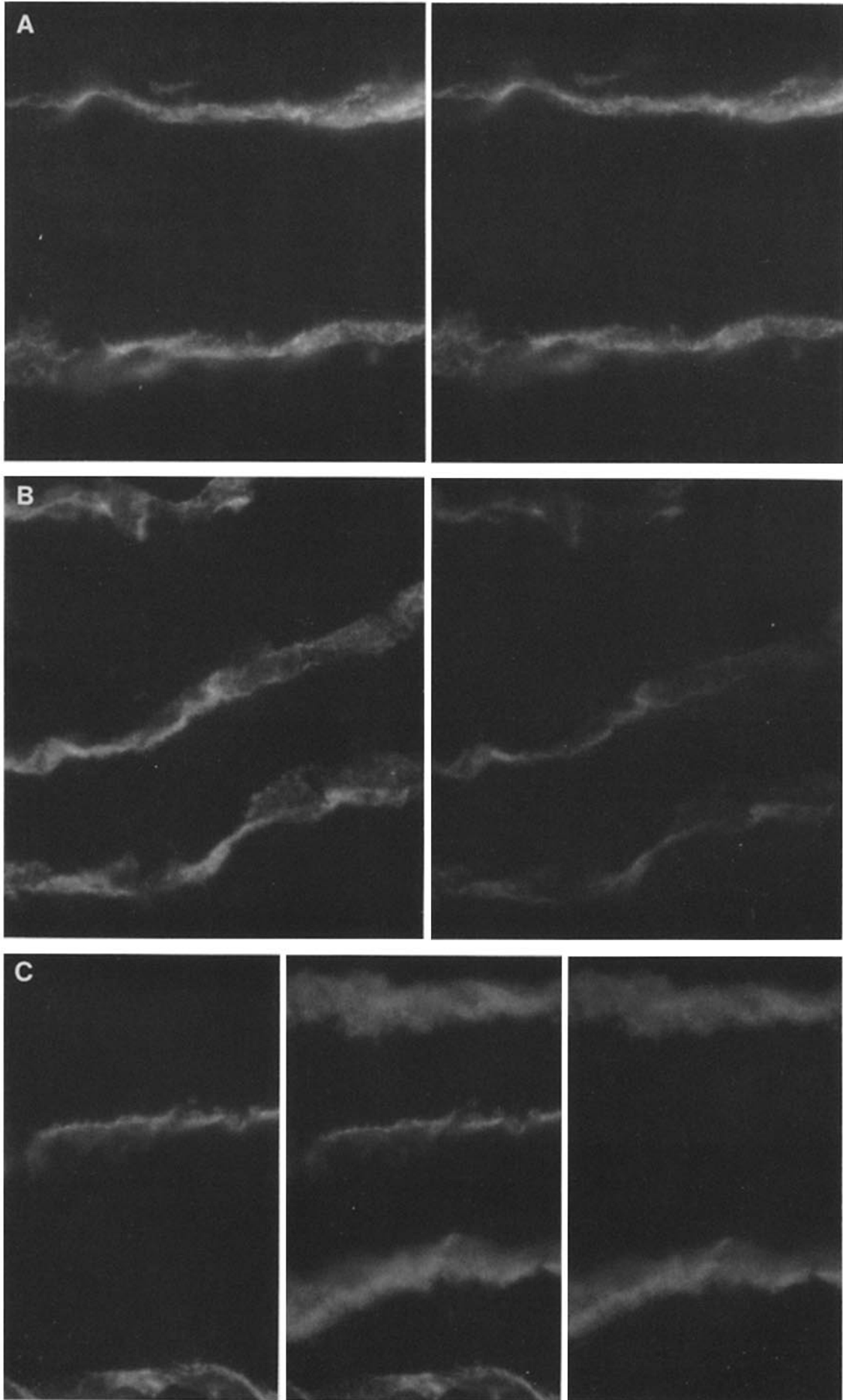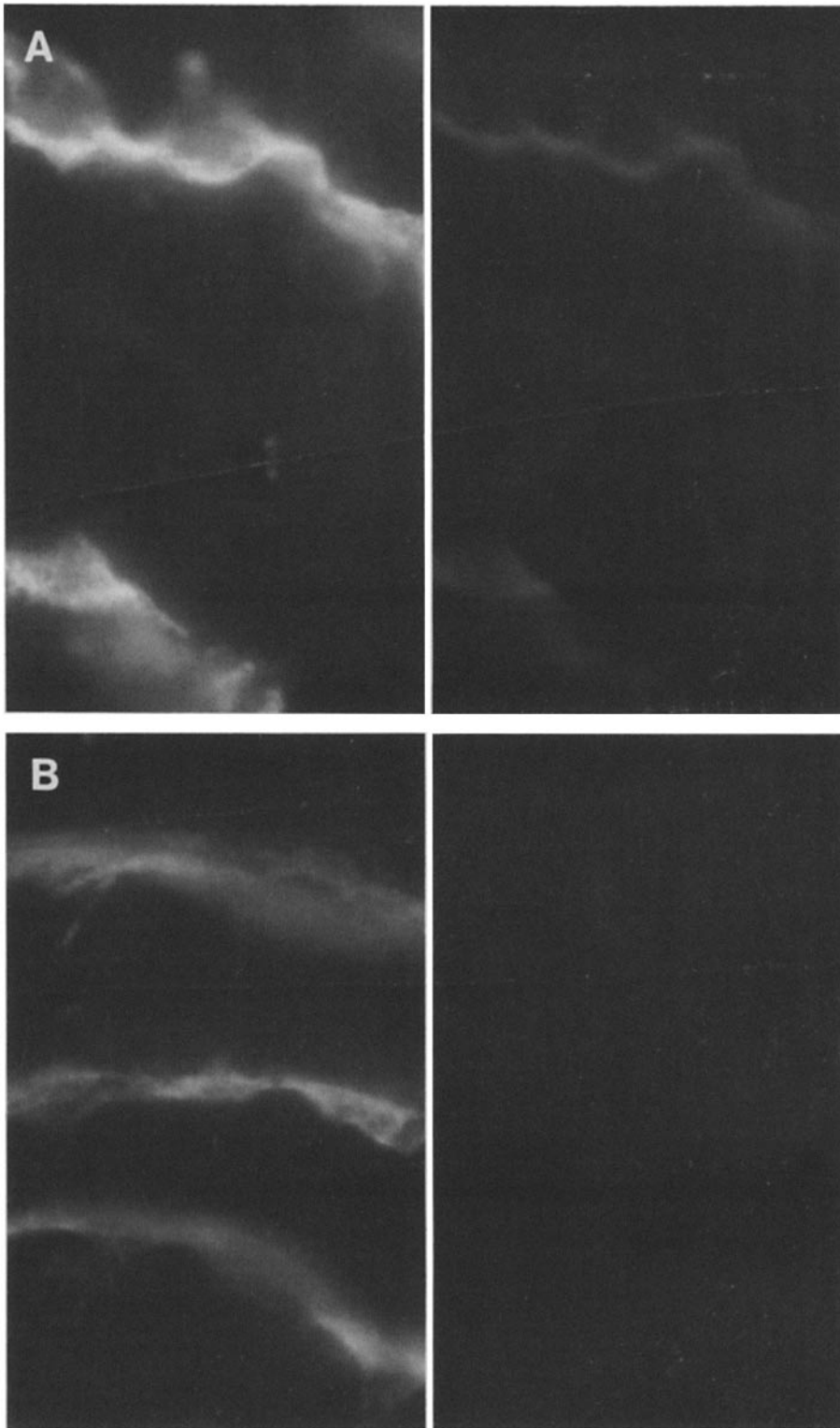
FIGURE 6 Immunofluorescence images of *Torpedo* electric tissue reacted with mabs. Cryostat sections of electric organ were incubated with (A) mab 1127A, specific for the 58K protein and with (B) mab MPC-11, a control cell that secretes IgG of unknown specificity. Ascites fluid diluted 1/200 was used in both cases. Rhodamine-αBuTx staining is shown on the left and fluorescein-antibody staining is shown on the right. × 400.

fluorescein-conjugated anti-mouse IgG and rhodamine-αBuTx, respectively. The ventral surface of electrolyte cells is innervated and contains high concentrations of receptors while the dorsal membrane is uninnervated and virtually devoid of receptors (reference 26 and references therein). The utility of immunofluorescence localization in this tissue is

FIGURE 5 Immunofluorescence images of *Torpedo* electric tissue reacted with mabs. Cryostat sections of electric organ were incubated with (A) mab 139A, specific for the alpha subunit of AChR, (B) mab 1098C, specific for the 43K protein, and (C) mab 1043A, specific for the 90K protein. Ascites fluid (diluted 1/200) was used in A and B and undiluted culture fluid was used in C. Rhodamine-αBuTx staining is shown on the left and fluorescein-antibody staining is shown on the right. The center panel in C is a double exposure of rhodamine and fluorescein staining. × 400.

demonstrated by experiments using anti-AChR mab 139A. The distributions of rhodamine-αBuTx labeling and mab 139A labeling are restricted to one face of the cells and are virtually indistinguishable from each other (Fig. 5A). In contrast, rhodamine-αBuTx and an anti-90K mab label different membranes. This is most readily seen in photographs of double exposures of rhodamine and fluorescein images (Fig. 5C, center). Thus, the 90K protein seems to be restricted primarily to non-innervated membrane and may be absent from the innervated membrane.

Anti-43K mab 1098C reacted with the innervated face of electrocytes producing a labeling pattern that is very similar if not identical to that of rhodamine-αBuTx (Fig. 5B). In general, the intensity of the fluorescence was weaker than that produced by anti-AChR mabs. Like the AChR, the 43K protein appeared to be highly concentrated in and possibly restricted to the innervated membrane of these cells. Similar results were obtained with the anti-58K mab (1127A). The

Figure 8 Competition between anti-43K mabs for binding to 43K protein. Microtiter plate wells coated with LIS extract were incubated first with unlabeled IgG from mabs 1097B (O), 1098C (●), 1114A (□), or 1132E (■). The plates were then washed and incubated with either ^{125}I-1098C IgG or with ^{125}I-1114A IgG as described in Materials and Methods.
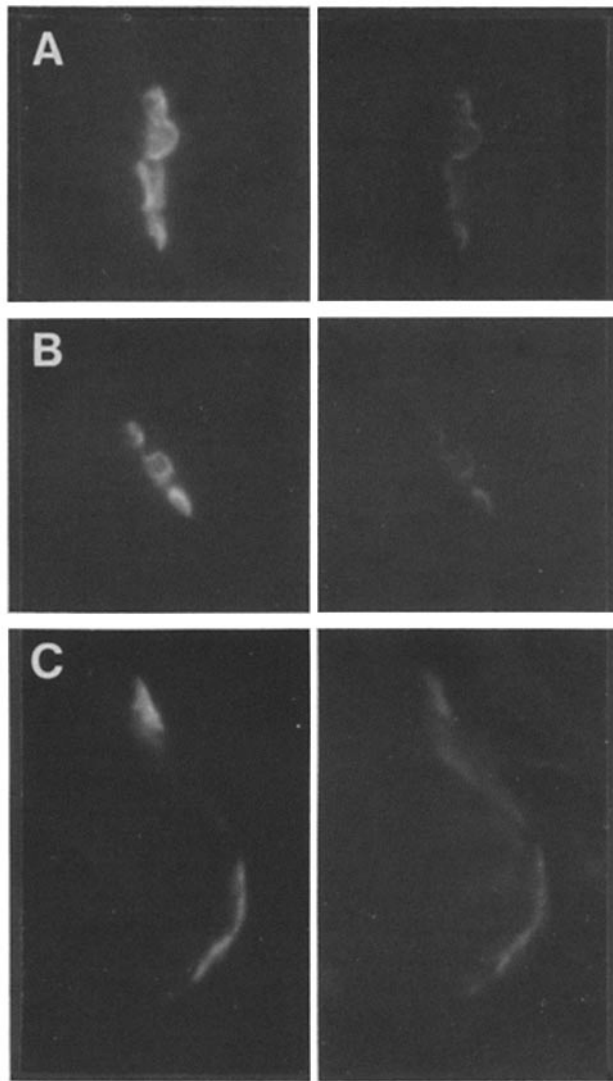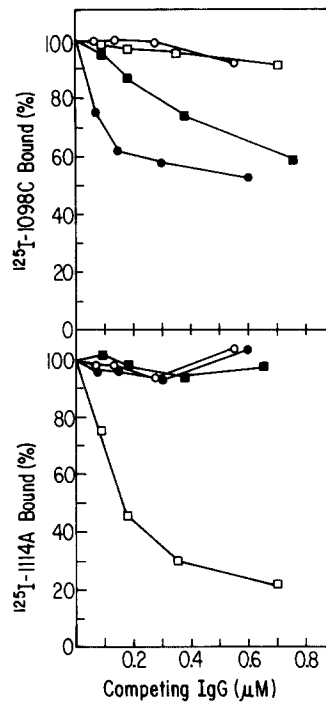

Figure 7 Immunofluorescence images of muscle reacted with mab 1201C. Cryostat sections of rat diaphragm muscle (A and B) or frog cutaneous pectoralis muscle (C) were incubated with mab 1201C ascites fluid (diluted 1/100). Rhodamine-αBuTx staining is shown on the left and fluorescein-antibody staining is shown on the right. × 400.

staining with this mab was quite weak (Fig. 6A) but clearly brighter than in controls (Fig. 6B).

The synaptic localization of the 43K protein was further substantiated by immunofluorescence experiments on frog and rat muscle. In these tissues, receptors are found at concentrations at least 1,000-fold higher in the subsynaptic membrane than in extrasynaptic areas. Since the postsynaptic membrane occupies <0.1% of the surface area of a muscle fiber, a precise comparison of the distributions of receptors and other membrane proteins can be made. One of the mabs described here, 1201C, was found to react with both rat and frog muscle. In both cases, the rhodamine-αBuTx labeling and the anti-43K mab labeling were coincident (Fig. 7).

Comparison of the Anti-43K mabs

The specificities of the anti-43K mabs have been compared by two methods to determine whether they recognize the same or different sites on the protein. The first procedure involved competition between mabs for binding sites. As shown in Fig. 8, binding of mab 1098C to 43K protein in a solid-phase well assay was unaffected by prior incubation of the wells with 1097B IgG or 1114A IgG but was inhibited by 1132E IgG. Binding of mab 1114A was unaffected by any of the mabs tested (1097B, 1098C, and 1132E), indicating that the epitope for 1114A is in a different part of the molecule from the others.

This result was confirmed by experiments in which each of the mabs was reacted with proteolytic fragments of the 43K protein. LIS extracts or membrane vesicles permeabilized with saponin (30) were digested with Staphylococcus aureus SAV8 protease, immunoblots were prepared, and mabs were reacted with them. Major protein fragments of apparent molecular weights 35,000, 29,000, and 18,000 were generated by digestion of LIS extracts (Fig. 9). This pattern was obtained after 20 min of incubation with protease (4 μg/ml) and was stable for at least 60 min. Most of the mabs, including 1097B, 1132E, 1098C, 1120A, and 1119A, reacted with the 35-mol-
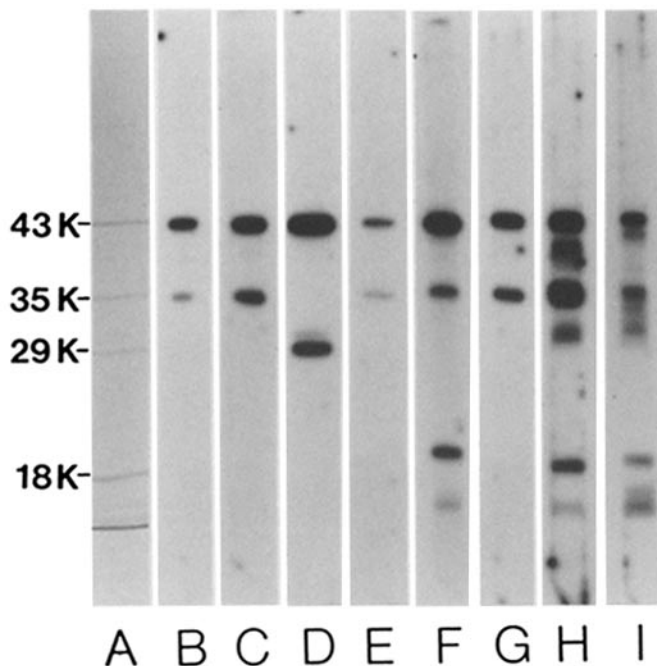
FIGURE 9   Autoradiograms of immunoblots of proteolytic fragments of 43K protein. LIS extract (A–H) or saponin-permeabilized membrane vesicles (I) were digested for 30 min at 37°C with 4 or 100 μg/ml SVA8 protease, respectively. The digests were applied to 12% SDS microgels and immunoblots were prepared as described in Materials and Methods. The Coomasie Blue-stained gel of digested LIS extract is shown in A. The following mab ascites fluids diluted 1/100 were used: (B) mab 1097B, (C) mab 1098C, (D) mab 1114A, (E) 1119A, (F) mab 1201C, (G) mab 1132E, (H and I) mab 1120A. Numbers of the left indicate the apparent molecular weights of the polypeptides.

wt fragment but not with the 29-mol-wt peptide. In contrast, the epitope for 1114A was located on the 29-mol-wt fragment but not the 35-mol-wt fragment. Thus, the binding site for 1114A is located relatively near one end of the molecule in a region lacking epitopes for the other mabs.

In addition to the 35-mol-wt fragment, mab 1120A also recognized peptides of ~40,000, 30,000, and 16,000 mol wt. Since these three fragments are not recognized by any of the other mabs, 1120A must be unique. Furthermore, 1201C is the only anti-43K mab that recognized the mammalian counterpart of the 43K protein, indicating that its specificity is also different from the others. Thus, the anti-43K mabs recognize at least five different epitopes.

Proteolysis of saponin-permeabilized membrane vesicles caused the disappearance of the 43K protein but since it represents only a small portion of the total protein, it is not possible to discern which fragments are derived from it in SDS gels stained for protein (data not shown). Therefore, we analyzed proteolyzed membranes by immunoblots with anti-43K mabs. As shown for mab 1120A, the pattern of fragments generated from LIS extract or from permeabilized membranes was nearly the same (Fig. 9). Similar results were obtained with the other anti-43K mabs (data not shown).

DISCUSSION

The nicotinic postsynaptic membrane and the cytoplasmic and extracellular structures associated with it undoubtedly contain proteins other than those directly involved in inter-

actions with acetylcholine. In the absence of known enzymatic activities or specific probes such as αBuTx, alternative approaches must be used to identify these components. One approach is to use antibodies to known proteins to determine if they are present at the neuromuscular junction. Studies of this kind have shown that cytoplasmic actin (13), α-actinin (4), filamin (4), and vinculin (4) are concentrated on the cytoplasmic side of the postsynaptic membrane. Another approach is to identify those components of membrane preparations enriched in postsynaptic membranes that are authentic synaptic components. By preparing mabs to nonreceptor components of Torpedo nicotinic membranes, we have shown that a 43K protein is localized at the synapse. This confirms our earlier suggestion, using an antiserum to alkaline extracts with predominant reactivity toward the 43K protein, that this protein is a synaptic component and that an immunologically related molecule is highly concentrated at rat endplates (11). Furthermore, ultrastructural studies using anti-43K mabs (20, 27) as well as the persistence of synaptic staining at denervated endplates (11) demonstrate that 43K protein is part of the postsynaptic membrane. Its localization to the cytoplasmic side of this membrane has been thoroughly documented (20, 21, 27, 30, 32).

The 43K protein, the cytoskeletal proteins mentioned above, and a 51,000-mol-wt protein with some similarities to intermediate filament protein (5) have all been identified as components of the postsynaptic cytoplasmic specialization. An attractive hypothesis is that these components, and possibly others yet to be identified, serve to anchor or stabilize the receptor in the membrane. If this proposal is correct, then at least one of these proteins must interact directly with the receptor in a stoichiometric manner. The 43K protein is the only protein present in purified Torpedo postsynaptic membranes in amounts sufficient to meet this criterion, although some proteins may be depleted during the isolation procedure. Currently, two lines of evidence support the notion that the 43K protein interacts with the receptor. First, ultrastructural studies using these anti-43K mabs on Torpedo electrocytes and membranes derived from this tissue indicate that the receptor and the 43K protein are essentially co-extensive in their distribution (27). Second, in isolated membrane preparations, the beta subunit of the receptor can be chemically cross-linked to a 43kd protein, presumably the same protein that we have shown is localized on the postsynaptic membrane (33).

Several conclusions can be drawn concerning the structure of the 43K protein from these studies. The inability of seven mabs to distinguish among the isoelectric variants seen on two-dimensional gels is consistent with our earlier evidence that these variants are not individual unique proteins (21). However, posttranslational modifications of the 43K protein may still occur (23). Secondly, since these mabs recognize at least five different determinants, the immunological response to the 43K protein is not dominated by one highly immunogenic region of the molecule. This is in contrast to the AChR which contains a main immunogenic region (31). Finally, virtually the same patterns of proteolytic fragments of 43K protein were generated from LIS extracts and from permeabilized membranes. This suggests that the membrane, once it is permeabilized, does not protect large portions of the protein from proteolytic digestion.

Some membrane preparations contain an alkaline-extractable protein of ~43,000 mol wt called ν_2 (12) that is not

recognized by these anti-43K mabs. One report indicates that this component, which is depleted from the most purified membranes (12), is creatine kinase (1). The presence of a protein in isolated membranes, even in highly purified ones, is not sufficient evidence to permit its classification as a synaptic protein, especially if it, like ν_2, is a major cytosolic component (12). It will be necessary to examine the in situ distribution of this protein to determine if it is a component of the postsynaptic membrane. Furthermore, in view of the clear distinctions in structure and presumably also function among the three 43,000-mol-wt proteins, the nomenclature ν_1, ν_2, and ν_3 (12) or "ν-proteins" (1) seems inappropriate since it implies some structural relationship among the components.

The 58K protein appears to be a component of the postsynaptic membrane, given its association with isolated AChR-rich membranes and with the innervated membrane in tissue sections. However, several considerations make this conclusion a tenative one. First, these studies have been performed with a single mab. Confirmation with others directed against different epitopes would be desirable. Second, the immunofluorescence staining seen with mab 1127A is quite weak so that occurrence of the 58K protein in other regions of the cell at lower concentrations may have gone undetected. Thus, the 58K protein may be a component of the postsynaptic membrane but not located there exclusively. Finally, the reactivity of mab 1127A with components in unpurified membrane fractions other than the 58K protein raises the possibility that the component identified by immunofluorescence is not the 58K protein. Further investigations, preferably with mabs that recognize the mammalian counterpart of the 58K protein, are necessary to clarify these points.

Most postsynaptic membrane preparations from *Torpedo* contain significant amounts of a 90K protein. Some evidence suggests that this component is a subunit of the Na^+,K^+-ATPase (7, 16), a protein found in high concentrations in the non-innervated membrane of electrocytes (3). Our finding that mabs to a 90K protein react intensely with the non-innervated membrane is consistent with this proposal. Its presence in preparations in AChR-rich membranes probably arises from contamination by membranes from nonsynaptic regions of the cell.

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