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Interaction of the 43K Protein with Components of *Torpedo* Postsynaptic Membranes†

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Received July 17, 1984

ABSTRACT: Interactions of the major M_r 43 000 peripheral membrane protein (43K protein) with components of *Torpedo* postsynaptic membranes have been examined. Treatment of membranes with copper *o*-phenanthroline promotes the polymerization of 43K protein to dimers and higher oligomers. These high molecular weight forms of 43K protein can be converted to monomers by reduction with dithiothreitol and do not contain any of the other major proteins found in these membranes, including the subunits of the acetylcholine receptor, as shown by immunoblotting with monoclonal antibodies. To study directly its interactions with the membrane, the 43K protein was radioiodinated and purified by immunoaffinity chromatography. Purified 43K protein binds tightly to pure liposomes of various compositions in a manner that is not inhibited by KCl concentrations up to 0.75 M. The binding can be reversed by adjusting the pH of the reaction to 11, the same treatment that removes 43K protein from postsynaptic membranes. Unlabeled 43K protein solubilized from *Torpedo* membranes with cholate can be reconstituted with exogenously added lipids in the absence of the receptor. The results suggest that 43K protein molecules are amphipathic and that they may interact with each other and with the lipid bilayer. These interactions cannot explain the coextensive distribution of 43K proteins with acetylcholine receptors in situ. However, they could account for the association of the 43K protein with the postsynaptic membrane and may contribute to the maintenance of the structure of the cytoplasmic specialization of which this protein is a major component.

A peripheral membrane protein of M_r 43 000 (43K protein)[1] is a major component of purified postsynaptic membranes from *Torpedo* electric organ (Sobel et al., 1978; Neubig et al., 1979; Elliot et al., 1980). Immunocytochemical studies using monoclonal antibodies have shown that in situ the 43K protein and the acetylcholine receptor are coextensively distributed in the postsynaptic membrane (Sealock et al., 1984) and that a component related immunologically to the *Torpedo* 43K protein is found at the mammalian neuromuscular junction (Froehner et al., 1981; Porter & Froehner, 1983; Froehner, 1984). The 43K protein is located on the cytoplasmic side of the membrane (Wennogle & Changeux, 1980; St. John et al., 1982; Porter & Froehner, 1983; Nghiem et al., 1983; Sealock et al., 1984), is present in the membranes in concentrations similar to that of the receptor (Burden et al., 1983), and is biochemically and immunologically distinct from actin and creatine kinase, both of which are found in some *Torpedo* nicotinic membrane preparations (Barrantes et al., 1983; Gysin et al., 1983; Porter & Froehner, 1983).

Although the function of this synaptic protein has not been directly demonstrated, some evidence suggests that it may be involved in anchoring receptors in the postsynaptic membrane so as to maintain a high concentration of them directly beneath the sites of transmitter release. Removal of the 43K protein from *Torpedo* membranes can be accomplished with alkaline

† This work was supported by grants from the Muscular Dystrophy Association and the National Institutes of Health (NS-14871).
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[1] Abbreviations: 43K protein, the basic, membrane-bound M_r 43 000 protein of *Torpedo* postsynaptic membranes; AChR, nicotinic acetylcholine receptor; LIS, lithium diiodosalicylate; SDS, sodium dodecyl sulfate; PC, dioleoylphosphatidylcholine; PA, dioleoylphosphatidic acid; DTT, dithiothreitol; BSA, bovine serum albumin; CuP, copper *o*-phenanthroline; NEM, *N*-ethylmaleimide; EDTA, (ethylenedinitrilo)-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid; mab, monoclonal antibody; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid.

0006-2960/85/0424-0425$01.50/0

pH without detectable effects on the ligand binding or ion transport properties of the receptor (Neubig et al., 1979; Elliot et al., 1980). However, the receptors in such membranes are more mobile, both rotationally (Rousselet et al., 1979; Lo et al., 1980) and translationally (Barrantes et al., 1980; Cartaud et al., 1981; Rousselet et al., 1981) than when the 43K protein is present. Other proteins are also removed from the membranes by alkaline pH treatment, but the 43K protein is the only one present in a concentration sufficient to interact on a one-to-one basis with the receptor. Nevertheless, since neither reversal of the effects of alkaline extraction on receptor mobility nor direct binding to cytoplasmic domains of the receptor has been demonstrated with homogeneous 43K protein, the role of this protein in anchoring receptors remains to be determined.

We have investigated the interactions of the 43K protein with the components of *Torpedo* postsynaptic membrane by two methods: cross-linking via endogenous sulfhydryl groups and binding of purified 43K protein to membranes and liposomes. The results suggest that the 43K protein is an amphipathic molecule that, in its membrane-associated state, may interact with other 43K proteins and with the lipid bilayer.

Materials and Methods

Preparation of AChR-Rich Membranes. Membranes were prepared from 100 g of frozen *Torpedo nobiliana* electric tissue according to the method of Elliott et al. (1980) with the modifications described previously (Porter & Froehner, 1983). Alkaline extraction of the membranes was performed as described by Neubig et al. (1979). Extraction of the 43K protein with LIS was conducted as previously described (Porter & Froehner, 1983), except in some cases the membranes were buffered at pH 8.5 with 20 mM Tris-HCl.

Gel Electrophoresis. Proteins were separated by SDS gel electrophoresis in 9% slab (Laemmli, 1970) or microslab (Matsudaira & Burgess, 1978) gels run at 20 mA per gel, constant current. Two-dimensional electrophoresis was performed as described previously (Porter & Froehner, 1983).

Cross-Linking of Membrane Proteins with Copper o-Phenanthroline. Treatment of AChR-rich membranes with copper *o*-phenanthroline (CuP) was done by using the method of Hamilton et al. (1979). *Torpedo* membranes (stored in buffer lacking NEM) were suspended in MOPS buffer (10 mM MOPS, pH 7.4, 1 mM $CaCl_2$, 100 mM NaCl) and mixed with the reagent to give final concentrations of 0–40 μM CuP and 2.25 mg of protein/mL. After 3–60-min incubation on ice, the reaction was stopped by addition of NEM to a final concentration of 10 mM. Cross-linked products were resolved on SDS gels and immunoblotted as described previously (Froehner et al., 1983).

Characterization of Monoclonal Antibodies and Conjugation to Affi-Gel 10. Monoclonal antibodies against the acetylcholine receptor and the other proteins found in these membrane preparations have been described in previous publications from this laboratory (Froehner et al., 1983; Froehner, 1984). IgG was purified from ascites fluid by protein A–Sepharose chromatography (Ey et al., 1978).

Pooled fractions from a protein A–Sepharose column were dialyzed vs. 0.1 M $NaHCO_3$, pH 8.5, for 24 h. The dialyzate was centrifuged for 10 min at 10000 rpm in a JA-20 rotor to remove aggregates, and the protein concentration in the supernatant was determined by absorbance at 280 nm by using an extinction coefficient of 1.46 $(mg/mL)^{-1}$. The purified IgG (3–4 mL) was added to 3 mL of washed Affi-Gel 10 resin (Bio-Rad) and incubated on a rotary shaker at 4 °C for at least 5 h. Typically 85% or more of the IgG bound to the resin. Best results were obtained with an IgG concentration of 1 mg/mL or greater. The reaction was stopped by addition of 3 mL of 0.1 M ethanolamine hydrochloride, pH 8.0, and incubated for 1 h at 22 °C. The resin was washed extensively in 20 mM Tris-HCl, pH 8.5, 0.1 M NaCl, and 0.02% NaN_3 and poured into a 1 × 10 cm chromatography column.

Radioiodination of Torpedo Membranes with ^{125}I-Labeled Bolton–Hunter Reagent. Purified receptor-rich membranes were suspended to a protein concentration of 5.5 mg/mL in PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.02% NaN_3), and saponin was added to a final concentration of 0.2%. After 30 min on ice the suspension was diluted into 5 mL of 0.1 M sodium borate, pH 8.5, and centrifuged at 15000 rpm for 30 min in a JA-20 rotor. The pellet was suspended to a protein concentration of 10 mg/mL in borate buffer. The ^{125}I-labeled Bolton–Hunter reagent solution (Bolton & Hunter, 1973) in benzene (0.5 mCi; Amersham Corp.) was dried down with a gentle stream of N_2. After the vial was cooled on ice, the membrane suspension was added, and the reaction was allowed to proceed on ice with mild agitation every 10 min. After 1 h the mixture was transferred to 5 mL of 0.1 M borate and 0.2 M glycine to quench the reaction and centrifuged for 30 min at 15000 rpm in a JA-20 rotor. The pellet was washed with 5 mL of 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 1 mM EGTA and suspended to a protein concentration of 5 or 10 mg/mL. Incorporation of ^{125}I was quantitated by TCA precipitation (Hubbard & Cohen, 1975).

Purification of ^{125}I 43K Protein. Iodinated *Torpedo* membranes were extracted with LIS as described above. After the second centrifugation of the extract, diisopropyl fluorophosphate was added to a concentration of 5 mM to inhibit proteolysis. Before the extract was applied onto the mab affinity column, LIS, which interferes with the binding of 43K protein to the mab, was removed by using a small Bio-Gel P-6 desalting column. One milliliter of hydrated Bio-Gel P-6 was poured into a 3-mL plastic syringe and equilibrated with 20 mM Tris-HCl, pH 8.5, and 0.02% NaN_3. The column was spun dry by centrifugation in a tabletop centrifuge (IEC Model HN-S) at half speed for 2 min. The extract was adjusted to pH 8.5 by addition of 0.1 N NaOH, applied to the column, and spun at half speed for 2 min. The eluate was collected into a 1.5-mL microfuge tube fitted to the bottom of the syringe. In this way the LIS concentration was reduced from 10 mM to approximately 0.1 mM, a concentration that did not interfere with binding to the affinity column. The extract was made 0.10 M in NaCl and applied to the Affi-Gel 10 affinity column at 1 mL/h. The column was overlayed with 20 mM Tris-HCl, pH 8.5, and 0.1 M NaCl and incubated at 4 °C for 4 h. It was then washed sequentially with (1) 20 mM Tris-HCl, pH 8.5, (2) 20 mM Tris-HCl, pH 8.5, 2% Triton X-100, and 0.4% SDS, and (3) 20 mM Tris-HCl, pH 8.5, again to remove the detergent and then eluted with 50 mM ethylamine, pH 10.5, and 10% (w/w) sucrose. The sucrose minimizes sticking of eluted ^{125}I 43K protein to the collection tubes. Progress of washing and elution was monitored by quantitation of ^{125}I in the fractions. The pH of the 43K protein peak fractions was adjusted immediately to 8.5 by addition of 2 M Tris-HCl, pH 7.3.

Preparation of Liposomes. Solid dioleoylphosphatidylcholine (PC), dioleoylphosphatidic acid (PA) (Avanti Polar Lipids, Inc.), 5(6)-cholesten-3-ol (cholesterol), and asolectin (Sigma Chemical Co.) were dissolved in chloroform to a concentration of 20 mM and then combined in 1.5-mL microfuge tubes to give the desired proportion of each. Three

microliters of [^{14}C]phosphatidylcholine (0.06 μCi; 520 pmol) was added to 100 μL of final lipid–chloroform solution to give a final concentration of 0.03 mol % [^{14}C]PC. The chloroform was evaporated under a stream of N_2 and then under vacuum for at least 1.5 h. The lipids were suspended and vortexed in buffer (20 mM Tris-HCl, pH 8.5, 0.02% NaN_3) to a final concentration of 20 mM. Each suspension was sonicated in a Megason bath sonicator (Ultrasonic Instruments International, Inc.) for five 1-min treatments with at least 2 min on ice between each treatment. The tubes were microfuged briefly to bring down droplets formed during sonication and then sonicated once more, yielding a nearly transparent solution. Finally, each lipid solution was centrifuged for 30 min at 41000g in a Beckman SW-27 rotor to remove large vesicles.

Binding of ^{125}I 43K Protein to Membranes and Liposomes. Binding of [^{125}I]LIS extracts and ^{125}I 43K protein to membranes and liposomes was done in 20 mM Tris-HCl, pH 8.5, 5 mM DTT, and 1 mg/mL BSA (reaction buffer) containing the indicated concentration of KCl. Membrane binding experiments were carried out with native and alkali-extracted *Torpedo* membranes. *Torpedo* membranes containing 50 μg of protein and 100 pmol of AChR were combined in a final volume of 0.1 mL with [^{125}I]LIS extract containing approximately 10 pmol of 43K protein. Alternatively, *Torpedo* membranes containing 0.05–25 pmol of AChR were combined with 20–350 fmol of purified ^{125}I 43K protein in a final volume of 0.05 or 0.1 mL. Reaction mixtures were incubated at 22 °C in 0.4-mL microfuge tubes. After 2 h the tubes were microfuged for 15 min, the supernatants were aspirated, and the amount of radioactivity bound to the membranes was quantitated in a γ counter.

Binding of [^{125}I]LIS extracts and ^{125}I 43K protein to liposomes was done in a volume of 0.3 mL with 5–15 pmol of ^{125}I 43K protein and 4 mM lipid in sonicated liposomes containing a trace label of [^{14}C]phosphatidylcholine. After 2 h at 22 °C, 0.7 mL of 1.3 M sucrose in reaction buffer was added, which gave a final sucrose concentration of 0.91 M. A step gradient was then created by carefully overlayering with 2 mL of 0.7 M sucrose and 1.3 mL of 0.1 M sucrose. The gradients were centrifuged for 15 h at 100000g in a Beckman SW-60 rotor and were fractionated from the top. Fractions were analyzed for ^{125}I 43K protein in a γ counter and for ^{14}C-labeled lipid by scintillation counting. Corrections were made for overlap of the two isotopes.

Reconstitution of 43K Protein to Liposomes. A partially purified preparation of 43K protein was used for this procedure. Membranes (2 mg) were dissolved in 1 mL of 20 mM Tris-HCl, pH 8.5, 0.02% NaN_3, and 4% cholate, and insoluble material was removed by centrifugation. Dithiothreitol was added to a final concentration of 5 mM, and the sample was applied to a 5–20% sucrose gradient (20 mM Tris-HCl, pH 8.5, 5 mM DTT, 1% cholate, 0.02% NaN_3) and centrifuged for 20 h at 38000 rpm in an SW 41 rotor. The gradients were fractionated, and 50-μL aliquots (diluted 1/10) of each fraction were assayed for 43K protein. The assay was performed by a modification the solid-phase assay previously described (Froehner et al., 1983). Wells of a Corning microtiter plate (25860) were coated with 50 μL of sample (90 min), blocked with 4% BSA solution, and then incubated for 2 h with anti-43K mab ascites fluid (1201C diluted 1/50). After being washed, the wells were incubated for 2 h with a β-galactosidase conjugate of anti-mouse IgG (BRL Scientific), washed, and developed for enzyme activity. Fractions containing 43K protein were pooled and used for reconstitution. SDS gel electrophoresis showed that the 43K protein comprised about 75% of the protein in these fractions (the major contaminant was a protein of M_r 58000) and that AChR was absent.

Reconstitutions were carried out by dialysis of samples containing 43K protein and PC/PA/chol lipids trace labeled with [^{14}C]PC. Lipid solutions (20 mM) were prepared as described above, except that the buffer contained 4.16% cholate. Lipid solution (300 μL) and 43K protein (700 μL containing 55 μg of protein) were combined and dialyzed against four changes of 500 mL of 20 mM Tris-HCl, pH 8.5, 5 mM DTT, 0.02% NaN_3, and 0.15 M KCl for a total of 60 h. Control samples containing only lipids or 43K protein without lipids were prepared in exactly the same manner.

After reconstitution, lipids were separated from unbound protein by flotation on a sucrose gradient as described above except that reconstituted samples (1 mL) were made 0.91 M sucrose by addition of solid sucrose. After centrifugation, the gradients were fractionated and 43K protein was assayed by a "dot immunoassay" on nitrocellulose paper as described by Jahn et al. (1984) with the following modifications. Samples were made 5% Triton, and three aliquots of 6 μL were spotted on the paper. Preliminary experiments showed that lipids prevented the binding of 43K protein to the paper unless Triton was added and that concentrations of Triton up to 5% had no effect on binding in the absence of lipids. Triton (0.1%) was added to the washing buffer used after the mab incubation, and radioiodinated anti-mouse IgG was used as the second antibody (Froehner et al., 1983).

Gel Filtration Chromatography. A Sepharose CL-6B column (1 × 95 cm) was equilibrated with 20 mM Tris-HCl, pH 8.5, and 1 mM DTT and was calibrated with thyroglobulin (M_r 670000), ferritin (M_r 470000), and immunoglobulin G (M_r 150000). A sample containing ^{125}I 43K protein was applied, and fractions (1 mL) were collected at a flow rate of 7 mL/h. The elution profile was determined by analyzing the fractions for radioactivity.

Protein Determination. Quantitation of membrane protein was done by the method of Lowry et al. (1951). A microprotein assay for LIS extracts and affinity purified 43K protein was performed as described by Markwell et al. (1981) with the following modifications: LIS and ethylamine, which interfere with protein determination, were removed from extracts and purified 43K protein, respectively, by the spinning column method using 0.5–1.0 mL of Bio-Gel P-6 equilibrated with 10 mM sodium phosphate, pH 7.4, and 1% cholate. Samples were brought to a final volume of 0.25 mL with the same buffer.

Results

Cross-Linking of Membrane-Bound 43K Proteins. Since the M_r 43000 proteins are the components of AChR-rich membranes that are the most extensively alkylated with *N*-ethylmaleimide (NEM) (Hamilton et al., 1979), they must contain a substantial number of sulfhydryl groups. Hamilton et al. (1979) have shown that treatment of membranes with copper *o*-phenanthroline (CuP) promotes the formation of disulfide bonds and causes loss of the M_r 43000 band on an SDS gel. Pretreatment with NEM blocked the CuP effect. We have extended these results by examining the size and the composition of the cross-linked species.

Membranes treated with various concentrations of CuP were analyzed by SDS gel electrophoresis (Figure 1A). With increasing concentrations of CuP, the amount of protein that migrated as M_r 43000 decreased coincident with an increase in the amount of Coomassie Blue stained material that failed to enter the stacking gel. Reduction of the samples with dithiothreitol prior to electrophoresis reversed the cross-linking

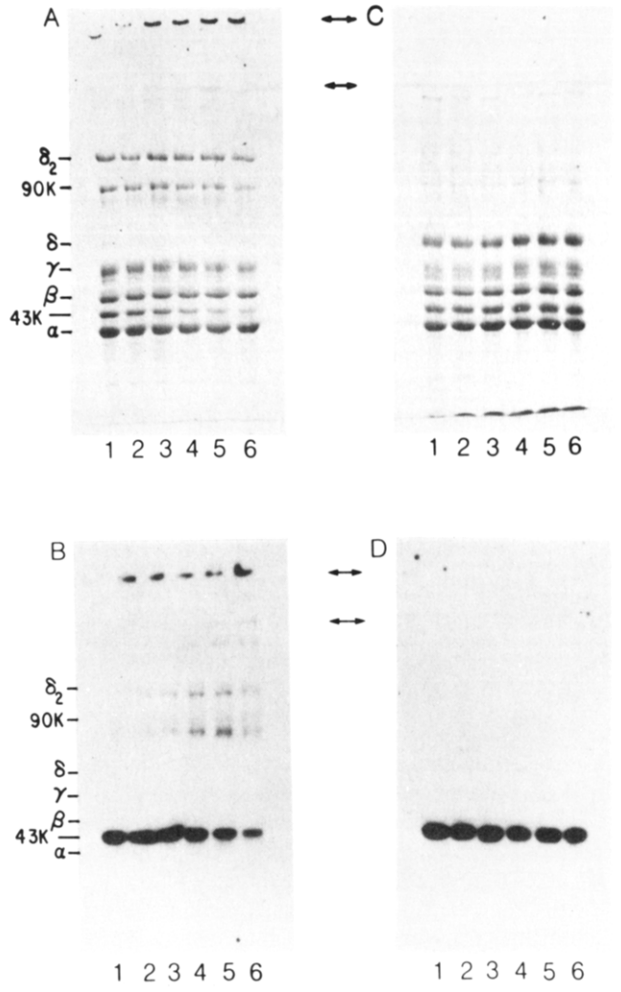


FIGURE 1: Cross-linking of membrane-bound 43K protein with copper *o*-phenanthroline. Membranes (stored in buffer lacking NEM) were treated with (1) 0, (2) 2.5, (3) 5, (4) 10, (5) 20, and (6) 40 μM copper *o*-phenanthroline and then analyzed by SDS gel electrophoresis either without (A, B) or with prior reduction with 25 mM DTT for 3 min at 100 °C (C, D). (A) and (C) were stained with Coomasie Blue. (B) and (D) are autoradiograms of immunoblots reacted with anti-43K mab 1201C. Gels identical with (A) and (C) were used to make (B) and (D), respectively. Arrows indicate the top of the stacking gel and the interface between stacking and resolving gels. The Greek letters indicate the positions of the AChR subunits (α, M_r 40 000; β, M_r 50 000; γ, M_r 60 000; δ, M_r 65 000). The 43K and 90K indicate the positions of the M_r 43 000 and M_r 90 000 proteins, respectively.

(Figure 1C). The 43K protein was identified in samples identical with those shown in Figure 1A,C by immunoblotting with a monoclonal antibody. As shown in Figure 1B, treatment with CuP at concentrations higher than 10 μM converts most of the 43K protein to components with apparent molecular weights of approximately 85 000 and 120 000 in addition to very large complexes that do not enter the gel. Reduction of the samples with dithiothreitol prior to electrophoresis eliminated these higher molecular weight forms (Figure 1D).

The cross-linked material could be composed of a 43K protein linked via disulfide bonds to other 43K protein molecules or to some other membrane component. To test this possibility, membranes were treated with 20 μM CuP and nitrocellulose replicas were prepared. The locations of each of the subunits of the receptor, the 43K protein, and two other *Torpedo* membrane proteins of M_r 58 000 and M_r 90 000 were determined with mabs. The higher molecular weight forms of the 43K protein were reactive only with the anti-43K mab (Figure 2A). Thus, the membrane-bound 43K proteins are

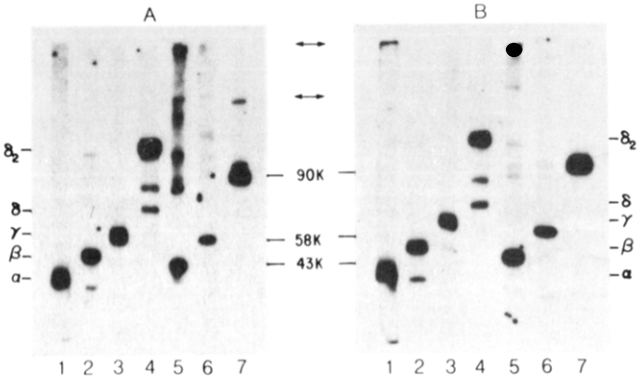


FIGURE 2: Reaction of monoclonal antibodies with membrane proteins treated with copper *o*-phenanthroline. Membranes treated with (A) 20 μM copper *o*-phenanthroline and (B) untreated, control membranes were alkylated with 10 mM NEM, separated on SDS gels without sample wells, and transferred onto nitrocellulose paper. Strips of each replica were incubated with mabs as described under Materials and Methods, and autoradiograms were prepared. (1) mab 139A, anti-AChR α subunit; (2) mab 265A, anti-AChR β subunit; (3) mab 274D, anti-AChR γ subunit; (4) mab 264E, anti-AChR δ subunit; (5) mab 1201C, anti-43K protein; (6) mab 1127A, anti-58K protein; (7) mab 1043A, anti-90K protein. The AChR δ subunit migrates primarily as a dimer. Arrows indicate the top of the stacking gel and the interface between stacking and resolving gels.

capable of forming disulfide-linked homopolymers. The presence of some of these homopolymers prior to CuP treatment (particularly in membrane preparations that had been stored at 4 °C for several days) indicates that they form spontaneously (compare lane 5 in Figure 2A,B). The conversion of the 43K protein to higher molecular weight forms was prevented by storage of the membranes in buffers containing 0.1 mM NEM (data not shown).

Purification of Radioiodinated 43K Protein by Immunoaffinity Chromatography. To study directly its association with the membrane, we have radiolabeled 43K protein and purified it using a monoclonal antibody column. Membranes were labeled with ^{125}I-labeled Bolton–Hunter reagent, and the 43K protein was extracted with LIS. This protocol was adopted for several reasons. First, the protein was labeled in its membrane-bound form in order to minimize the chances of incorporation of label at sites of interaction with other membrane components. Second, we observed that other iodination procedures, including those catalyzed by Chloramine T and lactoperoxidase, rendered the 43K protein resistant to solubilization with LIS. Labeling of the membranes with ^{125}I-labeled Bolton–Hunter reagent occurs preferentially in the 43K protein and maintains it in a form that can be removed from the membrane with LIS, although a minority of the labeled protein remains with the membrane (Figure 3). Finally, extraction of the 43K protein from the membrane with LIS accomplishes a substantial purification. Typically, about 50% of the radioactivity in the extract was associated with the M_r 43 000 proteins, 15-20% occurred in the M_r 58 000 protein, and the remainder was found in a number of minor proteins.

To utilize the monoclonal antibodies for purification of the 43K protein, it was necessary to establish conditions that were compatible with both 43K protein solubility and binding to the antibody. As shown in Figure 4, the pH has a dramatic effect on the solubility of the 43K protein. In a sucrose gradient buffered at pH 8.5, the protein sediments as a broad peak with a sedimentation coefficient of approximately 6 S. At pH 7.4, the 43K protein is insoluble and sediments to the bottom of the gradient. Attempts to solubilize the protein in the pellet with buffers lacking SDS were unsuccessful. Therefore, we adopted a procedure in which the LIS concentration was

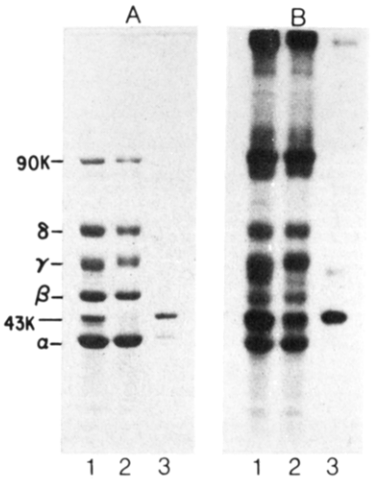


FIGURE 3: SDS–polyacrylamide gel electrophoresis of radioiodinated *Torpedo* membranes and LIS extract. Purified AChR membranes were labeled with ^{125}I-labeled Bolton–Hunter reagent and extracted with LIS as described under Materials and Methods. Samples were separated on a 9% microslab gel. (A) Coomassie blue stained gel; (B) autoradiogram. (1) Purified AChR membranes; (2) membranes extracted with 10 mM LIS; (3) LIS extract. The positions of the AChR subunits and 43K protein are indicated.

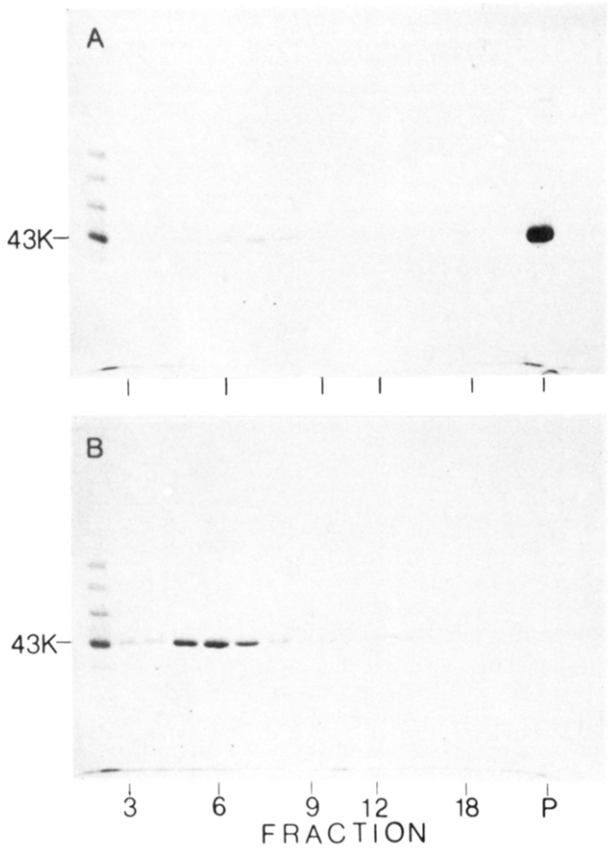
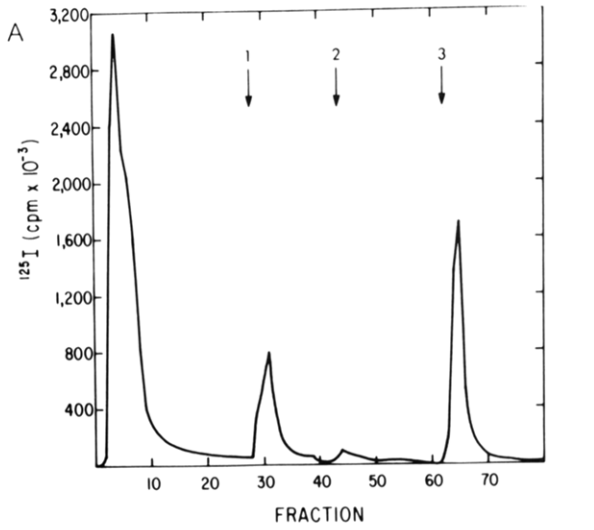
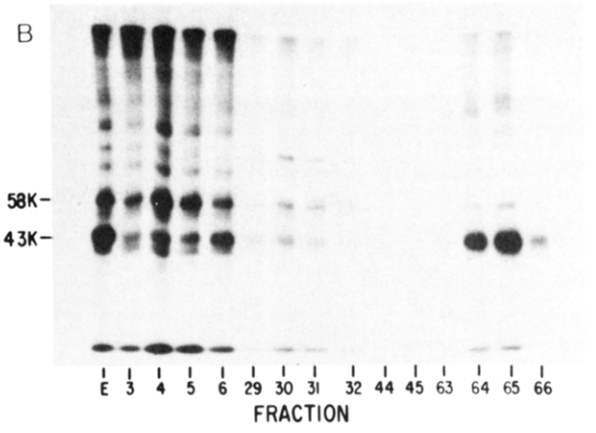


FIGURE 4: Velocity sedimentation analysis of 43K protein at pH 7.4 and pH 8.5. LIS extract (200 μL) was applied to a 4.3-mL 5–20% sucrose gradient containing 1 mM dithiothreitol. Gradients were centrifuged for 16.5 h at 119000*g*, and the gradient fractions were analyzed by SDS gel electrophoresis. Sucrose solutions were buffered with 10 mM Tris-HCl at (A) pH 7.4 or (B) pH 8.5. The peak of soluble 43K protein migrated with a sedimentation coefficient of approximately 6 S. In both gels, the first lane on the left is affinity-purified AChR. The lane marked "P" shows material solubilized from the pellet with SDS.

lowered either by dilution or more effectively by chromatography on Bio-Gel P-6 before applying the sample to the mab affinity column at pH 8.5. Nonspecific binding to the affinity





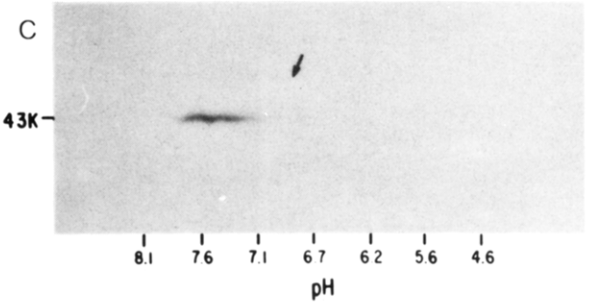


FIGURE 5: Immunoaffinity purification of 43K protein. Radiolabeled 43K protein was purified from LIS extract (0.37 mg of protein in 0.7 mL) on a 3-mL affinity column containing 21 nmol of mab 1098C IgG as described under Materials and Methods. (A) Elution profile. The arrows indicate washes with (1) 2% Triton X-100 and 0.4% SDS in buffer, (2) buffer, and (3) 50 mM ethylamine, pH 10.5, and 10% sucrose. A total of 80% of the input radioactivity was recovered from the column. (B) An autoradiograph of an SDS gel shows the LIS extract in lane E and fractions from the elution. (C) Fractions 64 and 65 were pooled and analyzed by two-dimensional electrophoresis and autoradiography. A small amount of 58K protein is indicated by the arrow.

column under these conditions was minimal since less than 1% of the radioactivity in an LIS extract bound to a control IgG column.

The results of one purification experiment are shown in Figure 5. A radiolabeled LIS extract was applied to an Affi-Gel column derivatized with mab 1098C IgG, shown previously to be specific for the 43K protein (Froehner, 1984). Many of the contaminating proteins were readily separated

from the 43K protein and appeared in the pH 8.5 buffer wash (fractions 3–6). Other proteins, in particular the 58K protein, were less easily removed and required a wash step with buffer containing 2% Triton–0.4% SDS (fractions 29–32). Buffers containing 1% Triton, 1 M NaCl, or a combination of the two were less effective. Finally, 43K protein was eluted with 50 mM ethylamine, pH 10.5 (fractions 64–66). Material purified in this way was contaminated with only a small amount of 58K protein. The higher molecular weight components present in these fractions were probably oligomers of 43K protein. Two-dimensional gel electrophoresis confirmed that the purified component had the properties of the 43K protein (Figure 5C).

Molecular exclusion chromatography of immunoaffinity-purified ^{125}I 43K protein on Sepharose CL-4B equilibrated with 20 mM Tris, pH 8.5, and 5 mM dithiothreitol showed that about 30% of the radioactivity was excluded from the column and the remainder occurred in a broad peak of approximately M_r 280000 (using globular proteins as standards). If the equilibration buffer contained 0.15 M KCl, a larger percentage of the radioactivity (46%) was excluded from the column (data not shown). Similar results were obtained with radiolabeled LIS extracts. Thus, the purified protein appears to associate into oligomeric and aggregated forms under these conditions.

Binding of 43K Protein to Membranes and Liposomes. In initial experiments, we examined the ability of purified ^{125}I 43K protein to bind to *Torpedo* receptor-rich membranes or alkaline-extracted *Torpedo* membranes. Under a variety of conditions, the radiolabeled 43K protein became associated with the membranes, but no reproducible difference in the binding to the two membrane preparations was observed. We considered the possibility that binding of 43K protein to the lipid bilayer could account for the lack of discrimination between native and depleted membranes.

To examine this possibility, the binding of the purified protein to liposomes was studied. Radiolabeled 43K protein was incubated with liposomes, and the bound and unbound protein were separated by flotation on a sucrose gradient of density greater than that of liposomes. As shown in Figure 6B, the 43K protein floated to a position in the gradient coincident with the phospholipid. In the absence of lipids, 43K protein remained near the bottom of the gradient. The association with liposomes does not occur through trapping of the protein within the lumen of the vesicles since neither radiolabeled α-bungarotoxin (Figure 6A) nor IgG (data not shown) floated with the lipids.

Binding of 43K protein to lipid vesicles was significantly enhanced by the presence of KCl. This effect was maximal at 0.15 M KCl and remained constant up to 0.75 M, the highest concentration tested (Figure 7). This contrasts with other basic proteins such as cytochrome *c* and lysozyme that bind electrostatically to negatively charged phospholipids in a manner that is inhibited by high salt concentrations (Kimelberg & Papahadjopoulos, 1971). This salt dependence of 43K binding to liposomes parallels that found with binding to *Torpedo* membranes (data not shown). When incubated at 0.15 M KCl, ^{125}I 43K protein bound comparably to vesicles of different phospholipid and cholesterol content (Table I). However, binding to PC/PA/cholesterol vesicles had a greater dependence on KCl concentration than did binding to pure PC vesicles, particularly at the lowest salt concentrations (Figure 7).

The 43K protein can be extracted from native *Torpedo* membranes by alkaline pH treatment (pH 11) (Neubig et al.,

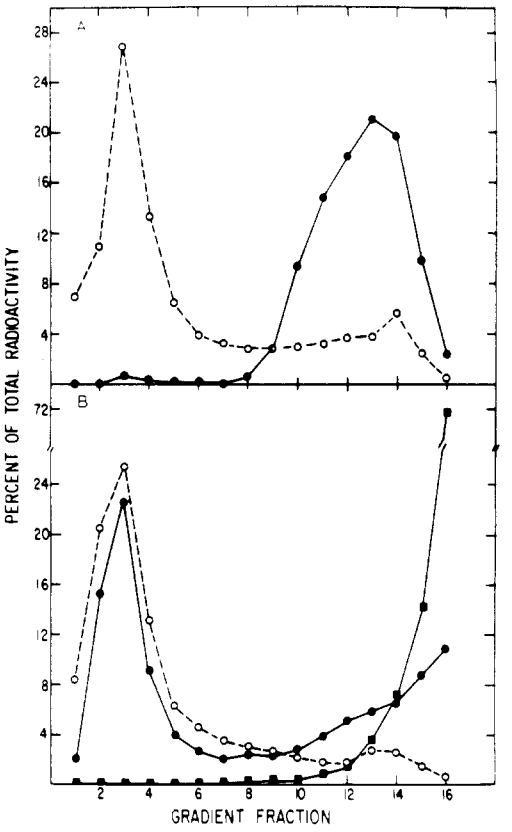


FIGURE 6: Binding of ^{125}I 43K protein and [^{125}I]-αBuTx to lipid vesicles. Radiolabeled proteins were incubated with liposomes (prepared with PC and trace labeled with [^{14}C]PC) in a reaction volume of 0.3 mL and then analyzed by the sucrose gradient flotation assay. Fractions were collected and analyzed for radioactivity. (A) [^{125}I]-αBuTx (17.5 pmol) (●) was incubated with ^{14}C lipids (○). (B) Approximately 14 pmol of ^{125}I 43K protein (●) was incubated with ^{14}C liposomes (○) or in reaction buffer without liposomes (■).

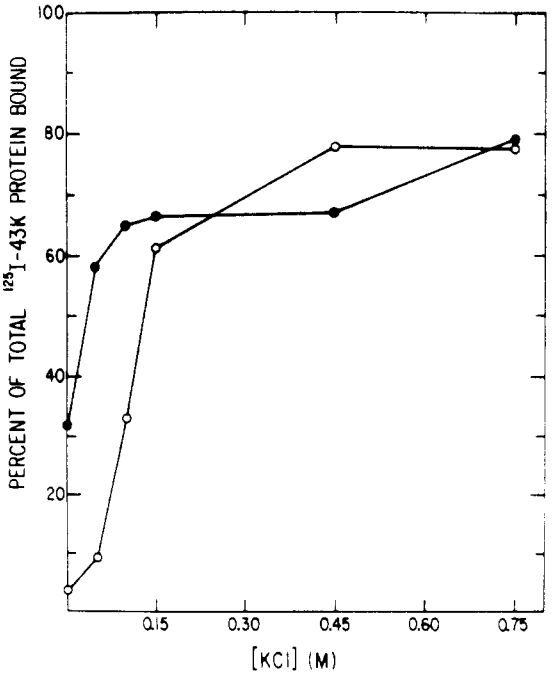


FIGURE 7: Effect of KCl concentration on ^{125}I 43K protein binding to lipid vesicles. ^{125}I 43K protein (approximately 14 pmol) was incubated with lipid vesicles containing PC (●) or PC/PA/cholesterol (○), both trace labeled with [^{14}C]PC, and assayed by the vesicle flotation assay. The percent of total ^{125}I radioactivity that coincided with the peak of ^{14}C radioactivity is shown as a function of KCl concentration.

Table I: Binding of ^{125}I 43K Protein to Lipids

lipid (mol %)					% of total radioactivity[a]		
PE	PS	PC	PA	chol	^{14}C	^{125}I	$^{125}I/^{14}C$
75	25				67.7	60.2	0.88
56	19			25	59.6	63.7	0.98
		100			90.7	79.3	0.87
		75	25		54.2	48.1	0.89
		71	24	5	55.3	39.9	0.72
		68	23	10	49.6	38.5	0.78
		64	21	15	66.8	45.4	0.68
		56	19	25	74.7	57.9	0.78
		38	12	50	73.6	64.2	0.87

[a] ^{125}I 43K protein (1 μM) was incubated at 0.15 M KCl with 4 mM lipid contained in vesicles made from different combinations of phospholipid and cholesterol and trace labeled with [^{14}C]PC. Binding was assayed as described under Materials and Methods. Radioactivity (^{125}I and ^{14}C) associated with floated vesicles is presented as a percentage of the total.

1979; Elliot et al., 1980). If the interaction of ^{125}I 43K protein with phospholipid vesicles is relevant to the mechanisms that maintain it at the postsynaptic membrane, then binding to lipid vesicles should be reversed by alkaline pH. Radiolabeled 43K protein was first bound to the vesicles under normal reactions conditions (0.05 or 0.15 M KCl), and then pH of the reaction was adjusted to 11. Vesicles were floated on a sucrose gradient buffered at pH 11. Alkaline extraction effectively reversed the binding to asolectin and PC/PA/cholesterol vesicles (Table II). However, the binding to PC vesicles was only slightly reduced (0.15 M KCl) or unaffected (0.05 M KCl) by alkaline pH treatment. At pH 11, charge repulsion between phosphatidic acid and negatively charged 43K protein may be sufficiently strong to promote dissociation. This may not occur with pure PC liposomes which are neutral at pH 11. Examination of the reversal of binding by LIS, which also removes 43K protein from *Torpedo* membranes (Elliot et al., 1980; Porter & Froehner, 1983), was precluded by the failure of LIS-treated vesicles to float in the gradients.

Since unpurified, radiolabeled 43K protein in alkaline and LIS extracts also binds to liposomes (data not shown), this property is not a consequence of denaturation that occurred during purification. However, since the labeling and extraction procedures may also alter the protein, we examined the liposome-binding characteristics of 43K protein prepared by a different procedure. Unlabeled membranes were solubilized with cholate, and 43K protein was partially purified by sucrose gradient centrifugation to separate it from AChR. Reconstitution of 43K protein with exogenously added lipids (PC/PA/chol) was then performed by dialysis to remove detergent. As shown in Figure 8, unlabeled 43K protein reconstituted with lipids floats on sucrose gradients in a distribution identical with that of the liposomes. The protein carried through the reconstitution process in the absence of lipids remains near the bottom of the gradient, well separated from the liposomes. Affinity-purified radiolabeled 43K protein can also be reconstituted to liposomes by this method (data not shown).

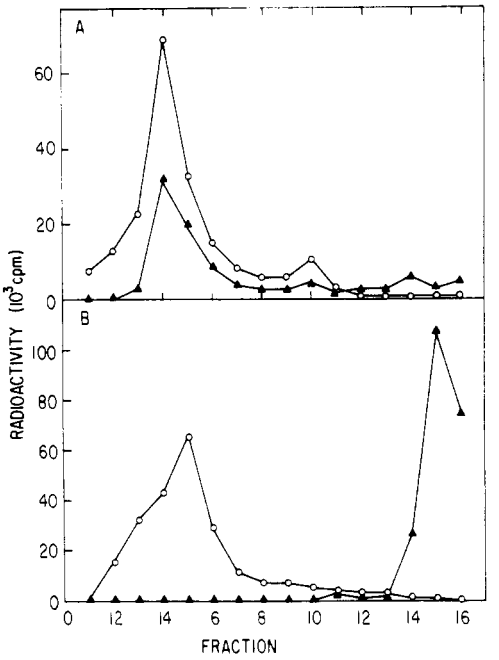


FIGURE 8: Reconstitution of 43K protein with lipid vesicles. Unlabeled membranes were solubilized with cholate, and 43K protein was purified by sucrose gradient centrifugation as described under Materials and Methods. Fractions containing 43K protein (55 μg of protein) were combined with PC/PA/cholesterol lipids in cholate and dialyzed to remove detergent. Samples were then floated on sucrose gradients, and fractions were analyzed for ^{14}C lipids (O) and for 43K protein (▲) by the dot immunoassay. (A) Lipids and 43K protein reconstituted together. (B) Lipids and 43K protein carried through the reconstitution process separately and analyzed on separate sucrose gradients.

Discussion

The results presented here suggest that the 43K protein may interact both with neighboring 43K proteins and with the lipid bilayer. Neither of these interactions appears to be sufficient to explain its restricted location at the postsynaptic membrane in a distribution virtually identical with that of the receptor. However, interactions between 43K protein molecules may contribute to maintenance of the structure of the postsynaptic cytoplasmic specialization of which this protein is a major component (Cartaud et al., 1981; Sealock, 1982). Furthermore, the binding of 43K protein to liposomes may represent

Table II: Alkaline Extraction of ^{125}I 43K Protein from Lipid Vesicles

		% of total radioactivity[b]						
		pH 8.5			pH 11.0			
lipid[a]	salt	^{14}C	^{125}I	$F_{8.5}$	^{14}C	^{125}I	F_{11}	$F_{11}/F_{8.5}$
PC	0.15 M KCl	92.4	70.2	0.76	93.5	56.9	0.61	0.80
PC/PA/chol	0.15 M KCl	71.2	33.7	0.47	77.5	7.3	0.09	0.19
asolectin	0.15 M KCl	76.7	64.3	0.84	79.2	11.4	0.14	0.17
PC	0.05 M KCl	83.4	53.4	0.64	88.7	53.8	0.61	0.95
PC/PA/chol	0.05 M KCl	64.8	11.6	0.17	91.9	6.9	0.08	0.47
asolectin	0.05 M KCl	65.7	21.3	0.32	72.8	6.0	0.08	0.25

[a] Vesicles made from the following: PC, dioleoylphosphatidylcholine; PC/PA/chol, dioleoylphosphatidylcholine, dioleoylphosphatidic acid, and cholesterol in a molar ratio of 56/19/25 respectively; asolectin, soybean phospholipids. [b] ^{125}I 43K protein was incubated with vesicles at pH 8.5. After 2 h the pH either was not altered (control) or was adjusted to pH 11 with 1 N NaOH, and sucrose solutions buffered at pH 8.5 or pH 11 were added. Lipid binding activity was assayed as described under Materials and Methods. Radioactivity (^{125}I and ^{14}C) associated with floated vesicles is presented as a percentage of the total. To compare the amount of lipid-associated ^{125}I 43K protein in two separate gradients where the amount of ^{14}C-labeled lipid recovered in vesicles may not be exactly the same, the ratio of $^{125}I/^{14}C$ at pH 8.5 and pH 11 is expressed as $F_{8.5}$ and F_{11}, respectively. If equal amounts of 43K protein remain lipid associated at pH 8.5 and pH 11, then $F_{11}/F_{8.5} = 1$.

an important mechanism for association of this peripheral membrane protein with the membrane.

The ability of membrane-bound 43K proteins to become cross-linked via disulfide bonds to form homopolymers indicates that the distance between sulfhydryl groups on adjacent molecules is about 2 Å. Although currently there is no evidence to suggest that disulfide bonds exist between 43K proteins in vivo, adjacent molecules are probably close enough together to interact in a noncovalent manner. This arrangement is not merely a consequence of treatment with the cross-linker since polymerization of 43K proteins occurs in its absence, though at a much slower rate. Cross-linking of 43K proteins with *p*-azidophenacyl bromide has also been reported, although neither the composition nor the size of the polymers was determined directly (Cartaud et al., 1982). Electron microscopic analysis of the meshwork that remains after Triton extraction of *p*-azidophenacyl cross-linked membranes revealed a reticulum of filamentous structures and globular entities. It should now be possible by using monoclonal antibodies to various components of isolated postsynaptic membranes to identify the structural elements of the network produced by these and other cross-linking agents.

The amphipathic nature of 43K protein may account for its ability to interact with liposomes in a manner that cannot be explained by trapping of the molecules within the lumen of the vesicles or by electrostatic interaction with the lipid head groups. Since cholate solubilized 43K protein can be reconstituted to liposomes, binding to the lipid bilayer is not solely a characteristic induced by extraction and purification of the protein. Extraction of 43K protein from both the postsynaptic membrane and liposomes occurs at alkaline pH, suggesting that the mechanisms that anchor the protein to them are similar. The 43K protein is isoelectric near pH 8 and, thus, would acquire a net negative charge as the pH is increased from near 7 to 11. The lipid component of the postsynaptic membrane and artificial liposomes containing PC and PA (but not pure PC liposomes) would also have increased negative charge at pH 11. Thus, disassociation may occur as a consequence of charge repulsion between the protein and the lipids.

How the precise coextensive distribution of 43K protein and AChR in the postsynaptic membrane is attained is not known. The most attractive possibility is that 43K proteins interact directly with the receptors. Although we were not able to obtain evidence for specific binding of purified 43K protein to AChR, the conditions may have been inappropriate or the interaction may have been too weak to be detectable by the protocols used. After solubilization of postsynaptic membranes with detergents such as Triton or cholate, AChR and 43K protein are readily separable by affinity chromatography or sucrose gradient centrifugation. Thus, any association between them is apparently much less stable than interactions between the subunits of the receptor and may require an intact membrane. Recently, Burden et al. (1983) have reported chemical cross-linking of an M_r 43 000 protein and the β subunit of the receptor. Although it was not shown which of the M_r 43 000 proteins found in nicotinic membranes is recognized by the mab used to identify the cross-linked products, their results suggest that a subsynaptic protein, possibly the 43K protein, lies in close proximity to the AChR. Whether the 43K protein is responsible for anchoring receptors beneath the nerve terminal and whether this function involves direct interaction between 43K protein and AChR remains to be determined.

Acknowledgments

We thank Susan Klink and Karen Douville for excellent technical assistance and Drs. Robert Jackson, Gus Lienhard, and Robert Sealock for helpful discussions and suggestions.

Registry No. PC, 10015-85-7; PA, 14268-17-8; cholesterol, 57-88-5.

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