Structural Heterogeneity and Subcellular Distribution of Nicotinic Synapse-associated Proteins*

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Peripheral membrane proteins (M, = 43,000) are associated with Torpedo membranes highly enriched in nicotinic receptor. These 43,000-dalton proteins are not required for ion translocation or other known receptor functions, but they have been implicated in constraint of the nicotinic receptor within the plane of the membrane bilayer. Sodium dodecyl sulfate-polyacrylamide electrophoresis allows partial resolution of the 43,000-dalton band into a doublet. We have carried out further analysis using two-dimensional gel electrophoresis, which reveals the existence of at least seven Coomassie blue-staining spots in the isoelectric focusing dimension. Peptide maps of the individual spots serve to elucidate the observed electrophoretic complexity. Three different membrane-bound proteins, designated \( p_1 \), \( p_2 \), and \( p_3 \), were identified on the basis of their characteristic peptide maps which show apparent homology in amino acid sequence. Two of these proteins, \( p_1 \) and \( p_2 \), are resolved into multiple spots in the isoelectric focusing dimension, but each group of isolectric focusing variants has nearly identical peptide fingerprints. Of relevance to the putative role of these proteins in synaptic or receptor supramolecular structures is the observation that only \( p_1 \) is exclusively membrane bound, and co-purifies with receptor whereas both \( p_2 \) and \( p_3 \) are also prominent proteins of the cytoplasm and are depleted from membrane fractions most enriched in receptor. These proteins may interact in the formation or maintenance of synaptic and nicotinic receptor supramolecular structures.

Upon solubilization and purification by affinity chromatography, the nicotinic cholinergic receptor from Torpedo and Electrophorus electroplax consists of four subunits: \( \alpha \) (M, = 40,000), \( \beta \) (M, = 50,000), \( \gamma \) (M, = 60,000), and \( \delta \) (M, = 65,000), (1–3) with a stoichiometry \( \alpha \beta \gamma \delta \) (4). The receptor is known to span the membrane bilayer (5), and hence is an integral multisubunit membrane protein. The Torpedo receptor is localized primarily to subsynaptic membranes, whereas, in Electrophorus electroplax and denervated muscle, the nicotinic receptor exists in junctional and extrajunctional forms (6, 7). The junctional or subsynaptic receptor is constrained, while the extrajunctional receptor freely diffuses within the plane of the membrane (8, 9). The two forms have different isoelectric focusing points (10) and there is evidence for other differences in their functional and biochemical properties (7, 11–13). The molecular bases for these observed differences remain obscure. Biochemical studies of solubilized Torpedo nicotinic receptor, a dimer of the \( \alpha \beta \gamma \delta \) structure, have not yielded much information concerning the molecular basis for the subsynaptic membrane supramolecular structure, which must be formed from higher order structures. Thus, other mechanisms for the observed structures must be entertained. There are four major structural explanations for the formation of subsynaptic receptor-receptor complexes: 1) via covalent receptor-receptor bonds; e.g. trans-receptor dimer disulfide bonds which do not survive detergent extraction conditions; 2) via noncovalent receptor-receptor interactions, e.g. hydrophobic interactions which are displaced by amphipathic detergents; 3) via intervening proteins that contain multivalent binding sites for the \( \alpha, \beta, \gamma, \) or \( \delta \) subunits; or 4) via intervening multivalent proteins that interact with the highly structured lipid domains surrounding the receptor integral proteins. Such intervening structures could be localized to the external or cytoplasmic faces of the synapse. None of the above structural possibilities should be considered mutually exclusive. Morphological evidence supports the concept that cytoplasmic filaments located beneath and basal lamina structures above the postsynaptic membrane may play pivotal roles in the formation and maintenance of the neuroelectrocyte synapse. These structures are best observed with the quick freezing technique (14). On the other hand, the presently available evidence indicates that direct interactions among the \( \alpha, \beta, \gamma, \) or \( \delta \) subunits is limited to the formation of dimers.

Our approach to the differentiation of these possibilities is to purify the nicotinic receptor, without the use of detergents, in its native membrane environment by the application of improved affinity-partitioning techniques (15, 16). The resulting preparations contain between 5.1 and 7.8 nmol of \( \alpha \)-BuTx binding sites/mg of protein. The purified fractions are depleted of 90,000-dalton proteins observed in earlier subsynaptic membrane preparations (17–22) but contain considerable amounts of the 43,000-dalton species. The ratio of 43,000-dalton protein to the \( \alpha \) subunit varied by 33% among receptor-containing membrane subpopulations resolved by combining affinity partitioning with countercurrent distribution. Thus, the stoichiometry of the 43,000-dalton subunit is not as rigidly conserved as the \( \alpha, \beta, \gamma, \) and \( \delta \) subunits. This 43,000-dalton band had been observed by others (23) and shown to be a peripheral membrane protein in the sense that it is released from the membrane by incubation at pH 11.0 (19, 20). Removal of the 43,000-dalton protein results in no discernible change in receptor ion translocation and acetylcholine binding properties (19, 20); on the other hand, the receptor translational and rotational mobilities within the plane of the mem-

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brane are increased (24–26). Recently, Sealock (27), using a novel tannic acid-mediated negative contrasting technique, has observed a cytoplasmic structure co-extensive with the receptor-enriched domains of the membrane. This cytoplasmic structure is disrupted or removed by pH 11 treatment, suggesting that the 43,000-dalton protein (the predominant component in the alkali extract) is localized to the cytoplasmic surface of the subsynaptic membrane.

The 43,000-dalton band is sometimes resolved into a doublet upon Laemmli SDS-polyacrylamide gel electrophoresis (19, 26). By reducing the sample volume of our previous procedure 2-fold (16), we occasionally resolve the 43,000-dalton band. This encouraged us to analyze these proteins by utilizing two-dimensional electrophoresis which provides additional resolution of proteins on the basis of their isoelectric focusing $pI$ as well as molecular weight. We report that the 43,000-dalton band is considerably more heterogeneous than previously recognized and includes a putative synapse-specific protein as well as two 43,000-dalton proteins which are among the most prevalent cytoplasmic constituents. These identified proteins may play a structural role in the organization of the neuroelectrocyte synapse.

**MATERIALS AND METHODS**

Membrane Purification and Assay Procedures—Torpedo californica electric organs were dissected and used immediately or frozen and stored at $-56 \, ^\circ C$ until use. Homogenization and centrifugation procedures have been described previously (16, 21). The supernatant from the first 35,000-rpm centrifugation was stored frozen for subsequent studies of cytosol proteins. Highly purified membranes were obtained by a single affinity-partitioning step with 0.05% of the total poly(ethylene oxide) replaced with hexaethonium-poly(ethylene oxide) with a substitution grade intermediate to ligand polymers II and III described previously (16). Alkali extraction of affinity-purified membranes (15, 20), $^{14}$1-AbTx filter binding assay (28), and protein determinations (16, 29) were performed as described previously.

**SDS-Polyacrylamide Electrophoresis—**Gels containing 10% polyacrylamide were prepared according to Laemmli (30). Electrophoresis and staining with Coomassie brilliant blue R-250 were performed as described previously (16), using the following protein standards: β-galactosidase ($M_r = 130,000$), phosphorylase $a$ ($M_r = 94,000$), bovine serum albumin ($M_r = 68,000$), ovalbumin ($M_r = 45,000$), and carbonic anhydrase ($M_r = 30,000$).

**Two-dimensional Polyacrylamide Gels—**Isoelectric focusing separations were performed according to O'Farrell (31) with the modifications described by Wilson et al. (32) and the following additional modifications. Ampholines (Bio-Rad) were mixed in the following proportions: 3 parts pH 3–10, 1 part pH 5–7, and 1 part pH 7–9. Samples were prepared by solubilization in SDS and 2-mercaptoethanol as described by Rubin and Milikowski (33) and supplemented to contain the final concentrations of urea, Nonidet P-40, and ampholines (32). Separation on precoated cylindrical gels (5 mm x 11 cm) was as described, except that 0.5% ethanalamine was used as the cathode solute. Under these conditions, a gradient in the range from pH 4.5 to 8.0 was regularly established. Variations in the slope of the gradient and extension above pH 8.0 are sometimes obtained depending on the batches of amphophiles used. After focusing, the gels were equilibrated and separated in the second (SDS) dimension as described.

**Peptide Analysis—**Selected spots from Coomassie-stained two-dimensional gels were cut out and iodinated with 250–350 μCi of Na$^{131}$I according to the procedure of Elder et al. (34). Peptides were obtained by digestion with 1-tosylaldehyde-2-phenylethylchloromethyl ketone-treated trypsin (Worthington). The entire digest, containing 0.5 to 2 μl, was applied on cellulose plates (20 x 20 cm; EM Laboratories No. 5757) and separated by electrophoresis and ascending chromatography (34). Autoradiograms were obtained by exposing the plates for 6 to 48 h to Kodak XA-R x-ray film.

**RESULTS AND DISCUSSION**

**Identification of Nicotinic Cholinergic Receptor-associated Proteins—**The 43,000-dalton band (often observed as a doublet (19, 26) but not reproducibly so) is prominent in membrane fractions purified by centrifugation (Fig. 1A), after further purification by affinity partitioning (Fig. 1B) and in alkali extracts of membranes (Fig. 1D). High resolution Laemmli (30) SDS-polyacrylamide gel electrophoresis separates proteins primarily on the basis of molecular weight but is also influenced by charge group modification, e.g. post-translational modification such as phosphorylation or methylation. Two-dimensional systems provide greatly increased power of resolution through the addition of isoelectric focusing. Unfortunately, the integral membrane protein subunits of the receptor are poorly resolved, with evidence of complex formation between the various subunits in the isoelectric focusing dimension and subsequent “streaking” in the SDS dimension (Fig. 2, A and B), even at loading levels 10 times lower than shown here. This somewhat confusing picture is likely the result of reassociation between the integral receptor subunits during the first electrophoretic separation which is carried out in 9 M urea, 2% Nonidet P-40. Further support for this interpretation is obtained by re-electrophoresis of individual α, β, γ, and δ subunits first purified by preparative SDS gel electrophoresis. Upon subsequent two-dimensional electrophoresis, the integral membrane protein bands are poorly resolved as diffuse streaks. In contrast, the 43,000-dalton proteins, which are known to be peripheral membrane proteins, are well resolved in the isoelectric focusing dimension whether performed directly (Fig. 2, A-D) or after preparative SDS gel electrophoresis (data not shown).

In keeping with the previous nomenclature (18), we have designated these subunits $\alpha$, $\beta$, and $\gamma$. The closely spaced isoelectric focusing variants are designated $a$, $b$, and $c$. As described below, this nomenclature is consistent with the
Neuroelectrocyte Synapse-associated Proteins

ISOELECTRIC FOCUSING

FIG. 2. Two-dimensional gel electrophoresis. Torpedo membranes and cytosol were first separated by isoelectric focusing and then on 10% polyacrylamide gels in the second dimension and stained with Coomassie blue. A, membranes from sucrose density gradient (200 μg); B, affinity-purified membranes (100 μg); C, cytosol (80 μg); D, alkali extract of affinity-purified membranes (50 μg). The basic results of peptide mapping data. Since the $v_1$ proteins are enriched in affinity-purified membranes while $v_2$ and $v_3$ are depleted (cf. Fig. 2, A and B), $v_1$ proteins are designated "synapse-associated proteins."

Interestingly, the 43,000-dalton proteins are prominent in the cytosol (Fig. 1C) as well as in receptor-enriched membranes. While $v_2$ and $v_3$ are prominent cytosol components (Fig. 2C), no $v_1$ is observed in the cytosol. Two-dimensional analysis identifies the major cytosol protein as $v_2$. We estimate that there is over an order of magnitude more $v_2$ and $v_3$ combined than $v_1$ in the electroplax. All $v$ subunits are extracted from membranes by incubation at pH 11 (Figs. 1D and 2D).

Determination of the Interrelationships of 43,000-dalton Proteins by Peptide Mapping—The amino acid composition of a 43,000-dalton protein preparation derived from Torpedo marmorata contains 8 to 9 tyrosines/43,000-dalton unit and a 3-fold higher content of lysine and arginine residues (23). Thus, iodination of the proteins with $^{125}$I, proteolysis with trypsin, and analysis of the peptides (34) are likely to yield information on peptides from various regions of the amino acid sequences of $v_1$, $v_2$, and $v_3$. Should major sequence stretches of these proteins prove identical, we would expect to identify common labeled peptides.

The analysis of the individual protein spots from the two-dimensional gels provides evidence for three distinct gene products among the various isoelectric focusing species (Fig. 3). This data provides validation of the nomenclature described above because the independent gene products are designated with unique numerical subscripts, while the possibly trivial isoelectric focusing variants are further delineated by letter subscripts. This nomenclature is not meant to imply that $v_2$ and $v_3$ are uniquely subsynaptic membrane components, since these proteins are found in the cytosol as well as in receptor-enriched membrane fractions. Whether $v_2$ or $v_3$ are isolated from the cytosol or membrane does not affect their peptide mapping profiles (data not shown). Thus, any structural differences in the membrane-bound and soluble forms of these proteins are either labile to the urea and SDS solubilization conditions or too subtle to be discerned by the two-dimensional analysis. Peptide maps of $v_1$, $v_2$, and $v_3$ subunits demonstrate that $v_1$, $v_2$, and $v_3$ are related neither to any other receptor subunits nor to the putative proteolytic fragment ($M_r = 35,000$) shown in Fig. 2D.

Discovery of the 43,000 dalton proteins, which are associated with highly enriched receptor-containing membranes, raised the possibility that these proteins play a role in immobilizing the receptor within the plane of the membrane. This hypothesis is supported by studies which demonstrated that removal of the 43,000-dalton proteins by alkali treatment renders the receptor free to rotate within the plane of the membrane (24-26). However, the alkali treatment also results in saponification and release of free fatty acids. The increased receptor mobility could be due to the action of free fatty acids.
These studies have raised interesting questions concerning the role of 43,000-dalton proteins in the neuroelectric synapses in *Torpedo* electroplax and similar, but undiscovered, proteins in the neuromuscular endplate. A crucial first step, accomplished in our investigation, is the cataloging of proteins that are likely to play a role in synapse formation and maintenance. Of the three distinct proteins identified in this study, only \( r_n \) is uniquely associated with receptor structures. However, the tenacity of \( r_n \) and \( r_3 \) binding to all membrane fractions is remarkable, after the multiple homogenization and centrifugation washes during sucrose density gradient and affinity-partitioning procedures. Of the prominent cytoplasmic proteins resolved by the O’Farrell gel, none are nearly as prominent in purified membrane fractions as \( r_3 \) and \( r_4 \). The roles of actin (Mr = 43,000) (35), \( r_3 \), and \( r_4 \) in the 5- to 10-nm electroplax cytoplasmic filamentous structures remain to be elucidated.

It is apparent that care must be observed in studies of the 43,000-dalton proteins using one-dimensional polyacrylamide gel electrophoresis. For example, peptide maps derived from the 43,000-dalton bands of one-dimensional gels display much greater complexity than any of the patterns from two-dimensional gel spots (not shown). In particular, studies in which \( ^{14} \)Ciodoacetic acid or other labels are attached to the receptor and the incorporation monitored by one-dimensional analysis must be interpreted with caution, because the label may be distributed differentially among the various 43,000-dalton proteins. Also, the currently available amino acid analysis (23) probably reflects the composition of two or more of the \( \nu \) subunits that have been identified in the current study.

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