Photoaffinity labeling and quaternary structure of the acetylcholine receptor from *Torpedo californica*

(subunit function/toxin binding site/molecular weight/postsynaptic membrane structure)

F. Hucho*, P. Layer*, H. R. Kiefer†, and G. Bandini*

* Fachbereich Biologie der Universität, 775 Konstanz, Germany; and † Basel Institute for Immunology, 487 Grenzacherstrasse, Postfach 4005 Basel 5, Switzerland

Communicated by N. K. Jerne, May 14, 1976

**ABSTRACT** Membrane fragments from electric tissue of *Torpedo californica* containing nicotinic acetylcholine receptor are composed of four different polypeptide chains with molecular weights of 40,000 (α), 48,000 (β), 62,000 (γ), and 66,000 (δ). The α and β chains are still present in all α and δ in some of the receptor preparations after Triton X-100 extraction and purification by affinity chromatography. All components of the receptor react covalently with the photoaffinity label 4-azido-2-nitrobenzyltrimethylammonium fluoroborate, the δ chain incorporating less of the reagent as compared to the α and β chains. Agonists and antagonists containing a quaternary ammonium group protect all chains against the label; the principal neurotoxin from Naja naja siamensis protects the α chain only. We conclude that the α chain binds the neurotoxin from Naja naja, the α and β chains are involved in the binding of ligands with quaternary ammonium groups, and the function of the γ and δ chains remains to be determined.

The quaternary structure of the nicotinic acetylcholine receptor appears to be complex. The protein extracted from the electric tissue of *Electrophorus electricus* has been shown to be an oligomer (1-5), possibly a pentamer (2) consisting of two different types of polypeptide chains. Two to four different polypeptide chains have been reported for receptor purified from *Torpedo californica* (6-8). The function of the different chains is unclear, because only one of them binds the neurotoxin from Naja naja siamensis (9). After reduction with dithiothreitol only the component having a molecular weight of 38,000 binds the affinity label 4-(N-maleimido)benzyltrimethylammonium iodide (6, 10). In this paper experiments with the photoaffinity label 4-azido-2-nitrobenzyltrimethylammonium fluoroborate are reported. This arylazide inactivates membrane-bound erythrocyte acetylcholine esterase and frog sartorius muscle acetylcholine receptor after irradiation (11). Since nonspecific reaction with other proteins of the membrane occurred and could not be selectively prevented by appropriate nitrene scavengers, it was concluded that the reaction might not represent a true photoaffinity labeling but an ordinary affinity labeling by a long-lived intermediate generated by photolysis (12). In our experiments reported here with detergent-solubilized and purified receptor the arylazide turned out to be a valuable tool for the investigation of the receptor structure.

A central question remains: what is the structure of the acetylcholine receptor in the postsynaptic membrane? Does the molecule extracted by detergent and purified by affinity chromatography represent the true entity or is it a preparative artifact? Finally, what is the structure of the functional postsynaptic membrane? It has been shown by electron microscopy of negative stained and freeze-etched membranes that the postsynaptic membrane contains doughnut-like particles believed to represent receptor molecules (13, 14). Receptor-rich membrane fragments can be purified (15, 7, 8), revealing a lattice organization of similar particles and containing acetylcholine receptor up to 20-40% of the total protein (14). By refining the purification procedures and by analyzing the receptor-rich membrane fragments we come to the conclusion that possibly up to 75% of the total protein is receptor protein and that the structure of the membrane is relatively simple.

**MATERIALS AND METHODS**

The synthesis of 4-azido-2-nitrobenzyltrimethylammonium fluoroborate (arylazide) will be described elsewhere. All chemicals were of the purest commercially available grade. All intermediates were characterized by nuclear magnetic resonance. The tritiated compound had a specific activity of 59.5 and 221 mCi/mmol. Starting from an intermediate, the radioactive compound was synthesized by NEN Chemicals, Dreieichenhain, Germany. Flaxedil was purchased from C. H. Boehringer Sohn, Ingelheim, Germany; hexamethonium bromide from Sigma, St. Louis, Mo.; and carbamoylcholine from Merck, Darmstadt, Germany. The principal neurotoxin of *Naja naja siamensis* was purified according to ref. 16.

Purification of the receptor protein from the electric organ of *Torpedo californica* has been described elsewhere (17, 9). The purified protein from *Torpedo* electric tissue contains toxin from the affinity column (18).

Receptor-rich membrane fragments were prepared from *Torpedo californica* electric tissue frozen with liquid nitrogen immediately after the animal was killed. We slightly modified the procedure described in ref. 19 by applying a continuous sucrose density gradient (25-50% sucrose) for the fractionation of the membrane vesicles. Furthermore, centrifugation time was only 6 hr.

Photoaffinity labeling was performed by the following procedure: 100 μl of the protein solution containing 0.01 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.1% Triton X-100, and varying amounts of the arylazide (see legends) were placed in small test tubes. Irradiation was performed at room temperature with an UV lamp (type "Sterisol") F 1140, 5241, Quarzlampen GmbH, Hanau, Germany) mounted 5.5 cm above the surface of the protein solution.

Receptor activity was determined by the Millipore assay according to Meunier et al. (4) using 125I-labeled *Naja naja* toxin. Acetylcholine esterase activity was determined according to Ellman et al. (20).

**RESULTS**

Peak fractions of receptor-rich membrane fragments purified by continuous sucrose gradient centrifugation have been analyzed by sodium dodecyl sulfate (NaDodSO4)-polyacrylamide gel electrophoresis of the total membrane. The specific receptor activity was 4200 nmol of 125I-labeled *Naja naja siamensis* toxin binding sites per g of protein, or one binding site per

Abbreviation: NaDodSO4, sodium dodecyl sulfate.
238,000 dalton. In accordance with ref. 8, the NaDdSO₄ gel (Fig. 1a) revealed four components which we name (bottom to top) α, β, γ, and δ. A fifth band was present to various degrees in different preparations, but never exceeded 5% of the total stain in the gel. Molecular weights represented by the bands were 40,000, 48,000, 62,000, 66,000, and 100,000, respectively. The α component was the predominant band, whereas γ was less than 10% in some preparations. Acetylcholine receptor purified from Torpedo electric tissue showed in some preparations a very similar band pattern (Fig. 1b); in others mainly the α and β component were present (Fig. 1c). Protease inhibitor (phenylmethylsulfonylfluoride) added throughout the preparation had no effect on the band pattern.

The receptor-rich membrane fragments appear to be composed of a complex of at least four different polypeptide chains. We investigated the function of the various chains by means of a photoaffinity label. First we had to assure the specificity of the label for cholinergic agonist and antagonist binding proteins.

Incubation of Triton-solubilized crude protein fractions containing acetylcholine receptor with 4-azido-2-nitrobenzyltrimethylammonium fluoroborate (arylazide) and subsequent irradiation resulted in significant reduction of the Naja naja toxin binding capacity. Acetylcholine esterase present in these fractions was inactivated simultaneously. The inactivation could not be reversed by extensive dialysis.

Inactivation was accompanied by incorporation of radioactivity into the protein when tritium-labeled photoaffinity label was used (Fig. 2). Extensive dialysis even under denaturing conditions did not remove the radioactivity. Various agonists and antagonists protected against incorporation of radioactivity, with flaxedil showing the largest protective effect (Fig. 2). Since we used, for these experiments, crude Triton X-100 extracts from Torpedo electric tissue with only about 1% of the protein being acetylcholine receptor, the size of the protective effect may indicate a certain selectivity of the photoaffinity label for cholinergic effector binding sites. Carbamoylcholine shows the least, flaxedil the strongest protective effect. This is in accordance with the different affinities of the various ligands for the receptor (21). An exception is Naja naja toxin.

The snake venom neurotoxins in vivo and in vitro show the highest affinity for the receptor. The relatively poor protection by Naja naja toxin, therefore, can be only partly explained by the fact that it binds only to acetylcholine receptor but does not affect the acetylcholine esterase also present in our crude protein extracts. Further experiments showed that highly purified receptor is also only partly protected by the toxin against the photoaffinity label. The reason became apparent by the following experiment: NaDdSO₄-polyacrylamide gel electrophoresis of purified receptor after photoaffinity labeling with the radioactive arylazide shows that both of the major protein bands (α and β) on the gel contain radioactivity (Fig. 3). Flaxedil, a powerful antagonist for cholinergic receptors, protects both kinds of polypeptide chains of the receptor molecule. Decamethonium and hexamethonium also protect the α and β chains (Fig. 3). (Small radioactivity peaks, about 100 cpn above background, appeared, for example, around slices number 42 and 21 of the gel and disappear in the presence of cholinergic ligands. Their significance is not clear, although they might represent, e.g., oligomers of the receptor subunits.)
**Biochemistry: Hucho et al.**

*Naja naja* toxin protects mainly the low-molecular-weight \( \alpha \) component (Fig. 4). Photoaffinity labeling of purified receptor containing the four components \( \alpha, \beta, \gamma, \) and \( \delta \) and subsequent NaDodSO\(_4\)-polyacrylamide gel electrophoresis show that all components reacted with the label but that the \( \delta \) component reacted less as compared to the \( \alpha \) and \( \beta \) component (Fig. 5). Again, only the \( \alpha \) component is protected by *Naja naja* toxin.

**DISCUSSION**

If the receptor-rich membrane fragments prepared as described (15, 8) originate unaltered from the postsynaptic membrane, the structure of this membrane appears to be relatively simple. Possibly only four different types of polypeptide chains comprise its integral proteins and are responsible for the functions of the membrane, e.g., transmitter binding and ion translocation. These four components appear to form a protein complex.

Different binding sites appear to exist for *Naja naja* toxin and for small-molecular-weight agonists and antagonists containing a quaternary ammonium group. We have previously shown that the neurotoxin probably binds only to one of the polypeptide chains of the receptor molecule (9). Our photoaffinity label, on the other hand, containing, like most agonists and antagonists, a quaternary ammonium group, reacts with both the \( \alpha \) and \( \beta \) chains and (to a smaller extent) with the \( \gamma \) and \( \delta \) chains. All can be protected against the label by the antagonist flaxedil. *Naja naja siamensis* toxin also protects more than one of the chains, but not to the same extent. Only the low-molecular-weight \( \alpha \) component is completely protected. This result, together with the well-known competition between the neurotoxin and small-molecular-weight agonists and antagonists, suggest that the toxin binds to the \( \alpha \) chain but partly also blocks, e.g., the flaxedil binding site of the \( \beta \) chain either by overlapping and steric hindrance or by a conformational change mediated by interactions between the polypeptide chains.

The result that several components, despite their different molecular properties, react with the photoaffinity label and are protected by flaxedil, decamethonium, and hexamethonium is somewhat surprising. The simplest explanation would be that the photoactivated intermediate of the label exists long enough to react with polypeptide chains in the neighborhood of the initial binding site. One could further speculate that \( \alpha \) and \( \beta \) chains together form a single ligand binding site (in analogy to the active site of antibodies, which is formed by the H and the L chains). However, for such a model one has to take into account that there does not appear to be a one to one ratio between \( \alpha \) and \( \beta \) chains.

Carbamoylcholine had only a relatively low protective effect. Partially different binding sites have been postulated for ligands with one or with several quaternary ammonium groups (7), but further experiments are necessary to confirm this.

The \( \delta \) component observed in the receptor-rich membrane fragments and in some purified receptor preparations apparently reacted to a smaller extent with our photoaffinity label than the \( \alpha \) and \( \beta \) components (Fig. 5). This could indicate that the component does not contain a binding site for quaternary ammonium groups or that this site is less accessible. The function of the \( \gamma \) and \( \delta \) components remains obscure. (The preparations contain virtually no acetylcholine esterase, ATPase, or adenylate or guanylate cyclase.) Perhaps they are more directly

---

*Fig. 3.* (Top) The electrophoretic distribution, on 5% acrylamide gels in 0.1% NaDodSO\(_4\) of \(^3\)H activity in a purified fraction of acetylcholine receptor of *Torpedo californica*, labeled with \(^3\)H[arylazide (●) without protection, (○) with flaxedil protection (4.6 × 10\(^{-3}\) M), (●) with hexamethonium protection, (△) with decamethonium protection. Purified receptor protein (100 \(\mu\)l) containing about 0.1 mg of protein was incubated with the effectors and then irradiated for 10 min (concentration of photoaffinity label, 5 × 10\(^{-4}\) M; net cpm in each sample, 2.5 × 10\(^3\) cpm). Electrophoresis was performed as described in ref. 1. (Bottom) Scan of the gel after it was stained with Coomassie blue.
involved in the ion translocation process through the membrane.

The various purification procedures developed by workers in different laboratories do not result in identical band patterns.

**Fig. 4.** (Top) Protection by *Naja naja* toxin against photolabeling. Experimental conditions same as for Fig. 3, but with *Naja naja* (0.1 mg/ml) protection against incorporation of the photolabel (O). (Bottom) Scan of the gel after it was stained with Coomassie blue.

**Fig. 5.** Photoaffinity labeling of acetylcholine receptor containing α, β, γ, and δ polypeptide chains. Experimental conditions same as for Fig. 3, but with 100 µl of receptor protein solution (0.1 mg/ml of protein, 1% Triton, Ringer's solution) showing a band pattern very similar to the total receptor membrane. Of the bands shown in the scan of the stained gel (below), δ is labeled less than α and β; only the α band is protected by the neurotoxin (O).
obtained by NaDodSO₄-polyacrylamide gel electrophoresis of the purified receptor (3-8). Even when the same procedure is used, the number and relative intensity of the bands on the gels of our preparations are not always the same. Furthermore, in some preparations the two bands (α and β) seem to be actually two double bands with slightly different electrophoretic mobilities. The possibility exists that some of the patterns observed represent degradation products caused by proteases or glycosidases. If the γ and δ chains are integral parts of the acetylcholine receptor molecule in the membrane, under some still unknown conditions they may get lost during the purification or they may be interconverted to the α and β chains.

The photoaffinity label, 4-azido-2-nitrobenzyltrimethylammonium fluoroborate, proved to be a valuable tool in these investigations. It reacts with the native receptor protein and differs in this respect from Karlin’s label (6, 10), which reacts only after reductive cleavage of a disulfide bridge of the receptor. Since our label reacts with more than just the α chains, it allows us to investigate the influence of cholinergic ligands on other components of the receptor complex as well. This way the exclusive binding of *Naja naja siamensis* toxin to the α chain could be demonstrated.

The photoaffinity label is reasonably specific for the acetylcholine binding proteins acetylcholine esterase and receptor. As previously mentioned, experiments to be published elsewhere indicate that it reacts with the anionic subsites (both peripheral and active site) of the esterase because only ligands of these sites inhibit the covalent incorporation of the label, whereas blockers of the esteratic subsite have little effect on this reaction. It will be interesting to isolate the labeled peptides and to compare them to the corresponding peptide from the acetylcholine receptor.

We thank Drs. A. S. Gordon and H. Sund for support and helpful discussions and Ms. Jutta Birner for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 138, and the Fonds der Chemischen Industrie.