AFFINITY-LABELING OF PURIFIED ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA

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SUMMARY: The receptor for acetylcholine purified from electric tissue of Torpedo californica has been assayed both by affinity-alkylation and by neurotoxin binding. The specific activity by the latter method is about twice that by the former. Four major components of apparent molecular weights of 39,000, 48,000, 58,000 and 64,000 are separated by dodecyl sulfate-acrylamide gel electrophoresis. Reduction and affinity-alkylation of the receptor with a tritiated quaternary ammonium maleimide derivative results in the exclusive labeling of the 39,000 dalton subunit. This subunit, it is concluded, contains all or part of the acetylcholine binding site.

Much progress has been made in the isolation and characterization of the acetylcholine receptor from Electrophorus and Torpedo electric tissue (see ref. 1 for review), but the mechanism of the transduction of the binding of acetylcholine into a change in membrane permeability is still unknown. It has yet to be shown that all of the components of the present receptor preparations are subunits or are either necessary or sufficient for permeability control. It is important, therefore, to try to determine the function of the putative subunits of the receptor.

It has been possible to infer that one component of the receptor preparation from Electrophorus is a subunit that contains all or part of the acetylcholine binding site. The receptor from Electrophorus has been reduced with dithiothreitol and then affinity-labeled with the quaternary ammonium alkylating agent

[^{3}H]MBTA* in intact electroplax (2), subcellular membrane fragments (3), and solubilized and purified preparations (4). In all cases, a single polypeptide subunit of approximately 40,000 daltons is specifically labeled. This component is not labeled in the presence of agonists or antagonists of the receptor. Two other components of the purified receptor preparation of apparent molecular weights of 47,000 and 53,000 are not labeled by [^{3}H]MBTA under conditions used to label the 40,000 dalton component completely.

We have purified the receptor from electric tissue of Torpedo californica, assaying it by affinity-labeling and by neurotoxin binding. Although we find some differences between the putative subunit composition of the receptor from Torpedo and that from Electrophorus, a striking similarity is that a 39,000 dalton subunit and only that subunit is labeled by [^{3}H]MBTA.

MATERIALS AND METHODS

Receptor extraction: Electric tissue was dissected from Torpedo californica and extracted immediately at 4° or stored in liquid nitrogen for later extraction. Typically, 600 g of tissue was minced and homogenized in 2200 ml of 1 mM EDTA (pH 7.4) using a 4 liter capacity Waring blendor (20 s at "low"). The homogenate was filtered through gauze and centrifuged in a Beckman 19 rotor at settings of 19,000 rpm and 45 min. The resulting pellet was rehomogenized (20 s at "high") in 1200 ml of 1 M NaCl-2 mM $NaPO_4$ - 1 mM EDTA (pH 7.0) and resedimented at settings of 19,000 rpm and 55 min. The pellet was suspended (10 s at "low") in 1200 ml of 1 mM EDTA (pH 7.4) and centrifuged as before. The pellet was resuspended (20 s at "medium") in 210 ml (final volume)

*[^{3}H]MBTA, 4-(N-maleimido)benzyltri[^{3}H]methylammonium iodide

of 3% Triton X-100 - 50 mM NaCl-10 mM $NaPO_4$ - 1 mM EDTA - 3 mM NaN_3 (pH 8.0) and stirred for 1 hr. The suspension was centrifuged in a 60 rotor at 60,000 rpm for 30 min and the supernatant collected. The pH was adjusted to 7.0 with 0.6 M NaH_2PO_4.

Affinity chromatography and sucrose density gradient centrifugation: The Triton-extract was applied at 4° to a column containing 16 ml of a p-carboxyphenyltrimethylammonium derivative of 4% agarose. The column was washed and the receptor was eluted with carbamylcholine as described for Electrophorus (4). Carbamylcholine was removed by dialysis before assays for receptor and acetylcholinesterase (EC 3.1.1.7). Further purification of receptor was achieved by centrifugation in a 5%-20% sucrose density gradient containing 0.2% Triton X-100 - 150 mM NaCl - 10 mM $NaPO_4$ - 1 mM EDTA - 3 mM NaN_3 (pH 7.0) in an SW 41 rotor at 40,000 rpm for 16 hr. Receptor was routinely stored at -196° without loss of activity measured by either assay below.

Assays: Protein was determined by the Lowry procedure (5) using bovine serum albumin (Sigma Type "F") as a standard. Receptor was assayed by the "quick assay"; i.e. by the difference in [^{3}H]MBTA labeling in the absence and presence of toxin 3 of Naja naja siamensis (4). Receptor was also assayed by the binding of [^{3}H]methyltoxin 3 of N. n. siamensis. The toxin was purified (6), labeled by reaction with formaldehyde and tritiated sodium borohydride (7,8), and repurified. The specific activity of the toxin is 4 Ci per mmole. The [^{3}H]methyltoxin has full biological activity and is completely bound by an excess of receptor (details to appear elsewhere). The binding of toxin was determined by incubation of receptor and toxin for 1 hr at 25° and separation of bound from unbound toxin on a small column of Bio Gel P-30. In the absence of receptor, no ^{3}H-activity appears in the void vol-

ume. Acetylcholinesterase was assayed using acetylthiocholine as a substrate (9).

RESULTS AND DISCUSSION

Purification: Typically from 600 g of Torpedo tissue we obtain in the Triton-extract 1.1 g of protein, 25 nmoles of active acetylcholinesterase catalytic sites, and 370 nmoles of specific MBTA-reactive sites. The fractions eluted from the affinity gel with carbamylcholine contain 26 mg of protein, 1.5 nmoles of acetylcholinesterase catalytic sites, and 75 nmoles of MBTA-reactive sites. Sucrose density gradient centrifugation of this preparation results in a peak with a specific activity of 4 nmoles of MBTA-reactive sites per mg of protein, and less than 2 pmoles of acetylcholinesterase catalytic sites per mg of protein. A similar procedure applied to Electrophorus typically results in specific activities of 4.5 nmoles of MBTA-reactive sites per mg and 2 pmoles of acetylcholinesterase catalytic sites per mg (10). Active acetylcholinesterase, at least, is not a significant contaminant of the purified receptor preparations.

[^{3}H]Methyltoxin 3 binding: The "quick" assay for receptor, the results of which are given above, is specific in three aspects: the reduction of the binding site disulfide, the affinity alkylation of a resultant sulfhydryl group, and the blocking of this alkylation by snake α-neurotoxins (11). Only the [^{3}H]MBTA labeling which is blocked by N. n. siamensis toxin 3 is considered specific; in fact, this is greater than 95% of the total labeling of purified receptor. Conversely, the binding of radioactively labeled α-neurotoxins, including toxin 3, is often used as an assay for the receptor. Recently published specific activities

for purified preparations of Torpedo receptor range from 3 to 12 nmoles of toxin bound per mg of protein (12-14) and of Electrophorus receptor, 4 to 11 nmoles of toxin bound per mg of protein (8, 15-18). We obtain about 6 nmoles [^{3}H]methyltoxin bound per mg of protein in preparations of Torpedo receptor. The average ratio of [^{3}H]methyltoxin binding to [^{3}H]MBTA labeling for six pairs of determinations is 1.6 $\pm$ 0.2. Similarly, a ratio of about 2 was found for purified receptor from Electrophorus (10). It has been reported that the ratio of moles of toxin bound to moles of acetylcholine bound in Torpedo (13) and Electrophorus receptor (17,18) is also close to 2. The reason for the greater binding of toxin is not known. Based on the [^{3}H]MBTA reaction, the weight of Torpedo receptor per acetylcholine binding site is 250,000 daltons.

Subunits: Dodecyl sulfate-gel electrophoresis of purified Torpedo receptor, both before and after sucrose density gradient centrifugation, results in the separation of four major components (Fig. 1). On the basis of three independent determinations, each the result of at least duplicate runs of receptor and of protein standards, the means and ranges of the apparent molecular weights of the components are 39,200 $\pm$ 500, 47,600 $\pm$ 700, 58,000 $\pm$ 1,000, and 64,000 $\pm$ 2,000. In addition, there are faintly staining bands in the vicinity of 100,000 daltons. The major components of Electrophorus receptor were found to have apparent molecular weights of 40,000, 47,000 and 53,000; thus components of apparent molecular weights of 39,000-40,000 and 47,000-48,000 are common to both Torpedo and Electrophorus receptor preparations and may form a common core structure. For neither receptor preparation does changing the gel system (19) result in significant changes in the characteristic electrophoretic pattern. Different compo-

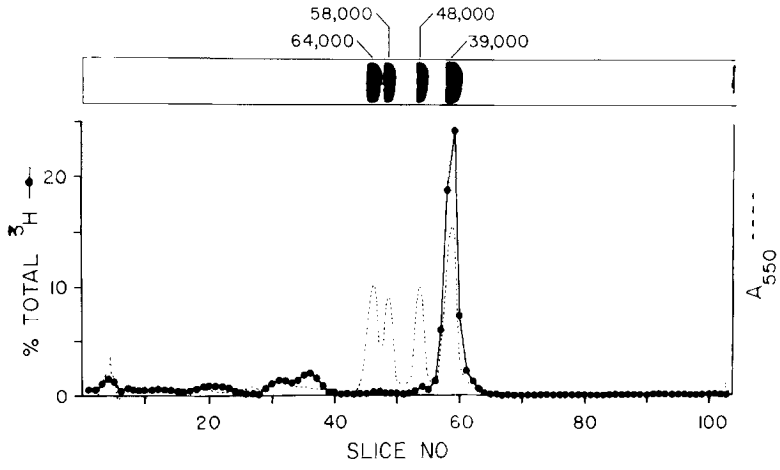

Fig. 1. Acrylamide gel electrophoresis in sodium dodecyl sulfate of purified Torpedo receptor affinity-labeled with [^{3}H]MBTA. Receptor of a specific activity by "quick assay" of 3.7 nmoles per mg of protein was "slow-labeled" (4) at final concentrations of 115 µg receptor per ml and of 560 nM [^{3}H]MBTA. The receptor incorporated 3.3 nmoles [^{3}H]MBTA per mg protein; thus 90% of the available sites was labeled. Sample preparation was as before (4): 120 µg of labeled receptor in 1.2 ml of 0.2% Triton X-100 - 150 mM NaCl - 10 mM $NaPO_4$ - 1 mM EDTA (pH 7.0) was dialyzed against 250 ml (changed twice) of 1% dodecyl sulfate - 2 mM triethanolamine (pH 8.0) for 2 hr. The protein was then precipitated and washed with 9 volumes of acetone, dried *in vacuo*, redissolved in 75 µl of 2% dodecyl sulfate - 10 mM Tris - 1 mM EDTA - 10 mM dithiothreitol - 10% sucrose (pH 8.0), and incubated at 50° for 2 hr. Samples containing about 18 µg of protein and 43,000 cpm were applied to 5.6% acrylamide gels prepared, run, stained, sliced, and counted as before (4). A photograph of a gel is shown at top. The average apparent molecular weights of the components were determined by comparison with myosin, β-galactosidase, phosphorylase, bovine serum albumin, γ-globulin, aldolase, and lysozyme run on parallel gels. The densitometer tracing (A_{550}) of the sample gel is superimposed on the distribution of radioactivity in 1 mm slices.

sitions for preparations of Torpedo receptor have been reported; these include two or three components of molecular weights less than 39,000 (13,14).

The 40,000 dalton component of Electrophorus receptor has been shown to be uniquely and specifically labeled by [^{3}H]MBTA (4). Remarkably, the 39,000 dalton component of Torpedo receptor is similarly uniquely labeled (Fig. 1). Thus the subunit forming all or part of the acetylcholine binding site appears to be

similar in Torpedo and Electrophorus. Both are reduced by dithiothreitol and affinity-alkylated by [^{3}H]MBTA. Both are polypeptide chains with approximately the same number of residues. A reducible disulfide group at the binding site of the "nicotinic" receptor has been demonstrated physiologically in several vertebrate species (11). This, together with the present evidence, supports the hypothesis that the subunit binding acetylcholine is a phylogenetically stable component of the receptor present at the vertebrate neuromuscular junction and homologous synapses.

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