AN ACETYLCHOLINE RECEPTOR PREPARATION LACKING THE 42,000 DALTON COMPONENT

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SUMMARY: Acetylcholine receptor has been purified from Electrophorus in the presence of the serine protease inhibitor phenylmethyl sulfonyl flouride. The purified material has a specific toxin-binding capacity of 3.6 nmoles of toxin per mg of protein. Electrophoresis of reduced, dissociated receptor on acrylamide gels containing sodium dodecyl sulfate reveals components of 110,000, 60,000, 54,000, and 48,000 daltons. No component with an apparent molecular weight of less than 48,000 daltons is seen. Limited digestion of this preparation with trypsin results in the appearance of components of 44,000 and 42,000 daltons. Prolonged digestion with trypsin generates species with apparent molecular weights of less than 42,000 and has no effect on the specific protectable toxin-binding capacity of the preparation.

Acetylcholine receptor has been purified from the electric organs of Electrophorus and Torpedo in a number of laboratories (for reviews, see 1 and 2). In each case in which the purified receptor has been examined on SDS* acrylamide gel electrophoresis a component has been found with a molecular weight near 42,000 daltons (3-7). A variety of higher molecular weight species have also been found to make up a considerable portion of various receptor preparations. In particular, a discrete component of 54,000 daltons has been observed in at least two instances (2,8), and 47,000 and 60,000 dalton components have been recently reported (9). The relative proportions of these higher molecular weight

*Abbreviations: SDS, sodium dodecyl sulfate; αBT, alpha bungarotoxin; PMSF, phenylmethyl sulfonyl fluoride; PBS, 138 mM NaCl, 3 mM KCl, 0.9 mM $CaCl_2$, 0.05 mM $MgCl_2$, 8.15 mM Na_2HPO_4, 1.47 mM KH_2PO_4.

components vary from one preparation to the next. In our efforts to make a reproducible receptor preparation we have succeeded in generating a preparation which contains only the higher molecular weight components and is essentially devoid of components smaller than 48,000.

MATERIALS AND METHODS

NEUROTOXIN: Neurotoxin was purified from the venom of N. naja kaouthia (Biologicals Unlimited) and coupled to Agarose A-50m at a final concentration of 0.2 mg neurotoxin per ml of gel bed. Each affinity column was washed with 8 M urea immediately before use, used only once, and then discarded. The αBT was purified from the venom of Bungarus multicinctus and iodinated by the procedure of Vogel et al (10). The diiodo and monoiodo toxins were separated and the diiodo toxin was used for toxin binding assays.

ACETYLCHOLINE RECEPTOR: Electrophorus (2½ to 3 feet in length) was obtained from Aquarium Foods (Ardsley, N.Y.) and used within 24 hours of its arrival in the lab. The eel was killed by cutting the spinal cord and pithing. Electric organ tissue was removed in the cold, trimmed free of fat and muscle tissue, and washed three times in ice cold Ringers solution containing 0.1 mM PMSF. Unless indicated otherwise, all succeeding steps were carried out at 0-4°. The washed, chilled organ was minced on a cutting board with razor blades, suspended in an equal volume of Ringers solution containing 0.1 mM PMSF, and homogenized in a Virtis homogenizer for one minute at 75% maximum speed. The homogenate was sedimented at 105,000 x g for 30 minutes. The pellet fraction was recovered, homogenized in at least 10 volumes of 1 M NaCl containing 0.1 mM PMSF and sedimented at 105,000 x g for 60 minutes. This step was repeated and the resulting pellet suspended in 3% Triton X-100, 3 mM Tris (pH 7.4), 100 mM NaCl, 0.1 mM PMSF to give a final volume of about 450 ml. The suspension was achieved first by gentle homogenization in a Virtis and then by stirring overnight in the cold room. The next morning the suspension was sedimented at 105,000 x g and the supernatant fluid decanted through glass wool. This triton extract was applied to a 25 ml affinity column at a flow rate of about 45 ml per hour. The column was then washed for 24-36 hours with 4-6 liters of 0.5% Triton X-100, 1 mM Tris (pH 7.4), 100 mM NaCl.

Elution of receptor from the affinity column was carried out at room temperature. The column was removed from the cold room, washed first with two column volumes of the above washing buffer pre-warmed to room temperature, and then with two column volumes of 0.1% Triton X-100, 1 mM Tris (pH 7.4), 100 mM NaCl (0.1% TXB). The column was then loaded with 1 mM benzoquinonium chloride (a gift of Sterling-Winthrop Research Institute) in 0.1% TXB. After one hour incubation one column volume of eluate was removed and stored at 0-4°. A second column volume was removed after an additional hour of incubation. These two fractions were combined and concentrated in an Amicon Ultrafiltration Chamber using a new PM 10 membrane. This and all succeeding steps were carried out

at 0-4°. The concentrated eluate was applied to sucrose gradients (5-20%) in 0.1% TXB and sedimented for 21 hours at 37,000 RPM in a SW 41 rotor. Solutions for the gradients were always freshly made. The gradients were fractionated into a single set of tubes and each fraction assayed for protein by the Lowry method (11). The single protein peak was pooled, concentrated in an Amicon, again with a new PM 10 membrane, and dialyzed versus PBS containing 0.1% Triton X-100. The dialyzed receptor preparation was passed through a millipore filter into a sterile vial and stored in the cold. Toxin-binding activity was assayed by sucrose gradient centrifugation (2) and by radioimmunoassay (12).

PROTEOLYTIC DIGESTION: Trypsin and Soybean Trypsin inhibitor were obtained from Sigma. Trypsin digestion was carried out in the cold using 0.047 nmoles of toxin-binding sites in 25 µl of PBS plus 0.1% Triton X-100. Trypsin was added in a volume of 1 µl to give a final concentration of 8 µgr per ml. The reaction was stopped by addition of trypsin inhibitor to a final concentration of 16 µgr per ml. An aliquot was removed for assay of toxin-binding activity and to the remainder was added an equal volume of SDS gel electrophoresis sample preparation buffer (13). PMSF was then added to 0.5 mM and the preparation heated in a boiling water bath for three minutes. These samples were electrophoresed on 7½% acrylamide gels containing 0.1% SDS, fixed in 7½% trichloroacetic acid-50% methanol, stained with 0.05% Coomassie Blue, and destained by diffusion. The gels were scanned at 560 nm using a Gilford Linear Transport system. Molecular weight standards were run in parallel with each set of sample gels.

RESULTS AND DISCUSSION

Figure 1 shows a tracing of an SDS gel profile obtained after electrophoresis of freshly purified acetylcholine receptor with a specific activity of 3.6 nmoles of toxin-binding sites per mg of protein. Four principle components are present in this gel profile, all of which are larger than the 42,000 dalton species normally associated with purified acetylcholine receptor. Although there is essentially none of the 42,000 dalton component in fresh preparations, it does begin to appear after storage. Gels run periodically after purification show an increasing amount of material migrating in the 42-44,000 dalton region of the gels. For example, Figure 2A shows a profile of the same receptor preparation used for Figure 1 after two weeks storage. A shoulder with apparent molecular weight of 44,000 daltons is now apparent.

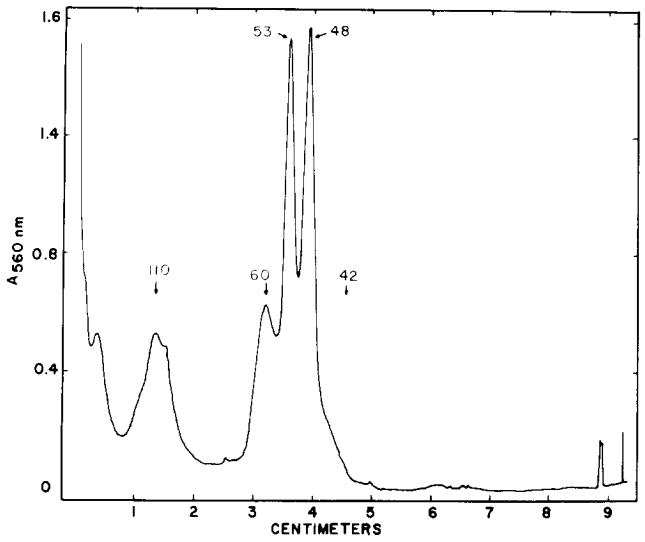

Figure 1. Tracings of an SDS acrylamide gel profile of acetylcholine receptor. Thirteen micrograms of acetylcholine receptor of a specific activity of 3.6 nmoles of toxin-binding sites per mg protein were dissociated and run as described in Materials and Methods. The numbers beside the principle peaks give the molecular weights in thousands.

The procedures used to obtain receptor preparations lacking the 42,000 dalton component were designed to remove and inhibit protease activity. Receptor obtained by these procedures can be converted, by limited proteolysis, into a preparation which contains a 42,000 dalton component. Tracings of SDS gel profiles of acetylcholine receptor before and after treatment with trypsin are shown in Figure 2. After five minutes of trypsin digestion there is nearly quantitative conversion to material of 44,000 and 42,000 daltons. Prolonged digestion generates additional lower molecular weight components. Addition of trypsin inhibitor to the receptor before the addition of trypsin yielded gel profiles indistinguishable from those of untreated controls.

Receptor was assayed for toxin-binding activity after trypsin digestion. The results in Figure 3 show that, under the conditions

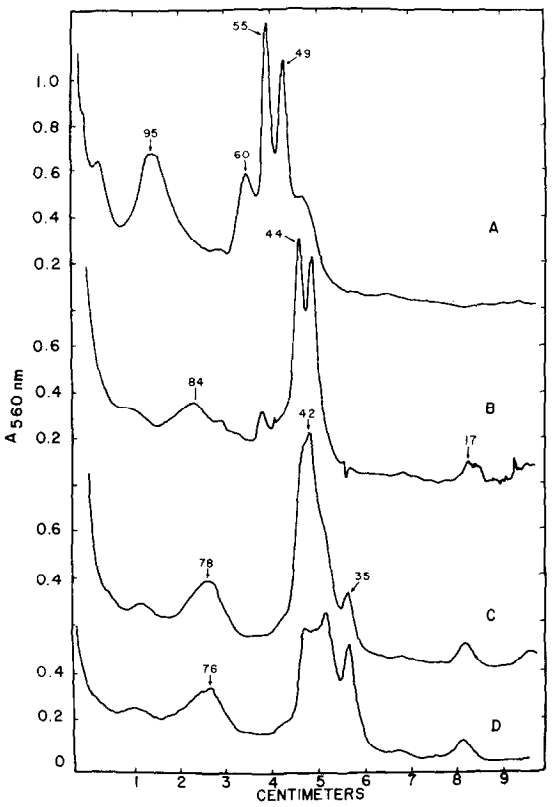

Figure 2. Tracings of SDS acrylamide gel profiles of acetylcholine receptor before trypsin digestion (A), after 5 minutes (B), one hour (C), and 6 hours (D) of trypsin digestion. Each gel received 13 micrograms of receptor. The numbers beside the principle peaks give the molecular weights in thousands.

employed here, trypsin has no effect on toxin-binding activity for at least six hours. Since the 44,000 dalton species is clearly less abundant after six hours digestion, it follows that species of less than 44,000 daltons bind toxin. In fact, others have reported isolation of acetylcholine receptor with toxin-binding activity in which the predominant component was less than 35,000 daltons (14), a species which we find after one hour digestion.

It is clearly possible to prepare acetylcholine receptor which is essentially devoid of components of less than 48,000 daltons.

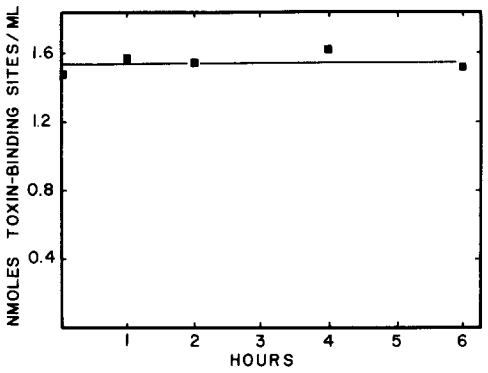

Figure 3. Acetylcholine receptor was treated with trypsin for the indicated times and the reaction stopped as described in Materials and Methods. Aliquots were removed, incubated with «BT, and the toxin-binding activity determined by sedimentation on sucrose gradients. The figure shows the number of toxin-binding sites remaining per ml as a function of time after addition of trypsin.

Lower molecular weight species that are generated by limited trypsin digestion are of about the same molecular weight as those routinely seen in preparations of purified acetylcholine receptor. Likewise, the changes seen on storage may be a consequence of protease activity retained in the purified receptor preparation. Thus the 42,000 dalton species previously found by ourselves and others may have been a product of proteolysis that occurred during purification.

The specific activity which we report here is lower than that found by others (1). Acetylcholine receptor of high specific activity may be a consequence of removal of a larger portion of contaminating proteins. It may also result from purification of receptor in which a higher portion of the receptor molecules exhibit maximal binding activity. Alternatively, high specific activity may be due to purification of a low molecular weight ligand binding protein after loss of much of the native receptor as proteolysis products.

The relationships between the components present in our more recent preparations are unclear. All those species present may be degradation products, with the native receptor still unidentified. On the other hand, one or more of these components may represent native receptor subunits.

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