Identification of Polypeptides Associated with a Putative Neuronal Nicotinic Acetylcholine Receptor*

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Polypeptides involved in the binding of the nicotinic acetylcholine receptor ligand α-bungarotoxin ($M_\text{r} = 8,000$) to neuronal membranes were identified by three independent methods: (i) $^{125}\text{I}$-α-bungarotoxin bound to membrane fractions or to monolayer cultures of chick retina was cross-linked to its binding site by using glutaraldehyde, or the photoaffinity bifunctional reagent N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate. Electrophoretic analysis of the cross-linked membrane proteins revealed $^{125}\text{I}$-α-bungarotoxin-polypeptide adducts of apparent $M_\text{r}$ = 63,000, 43,000, and 35,000. (ii) Affinity purification of the α-bungarotoxin binding protein from detergent extracts of $[^35\text{S}]$methionine-labeled retina cultures identified one major polypeptide with an $M_\text{r}$ = 37,000. (iii) Indirect immunoprecipitation from detergent extracts of $[^35\text{S}]$methionine-labeled rat pheochromocytoma cells (PC 12) gave evidence for a specific co-precipitation of α-bungarotoxin with three polypeptides ($M_\text{r}$ = 57,000, 34,000, and 25,000). The data suggest that polypeptides of $M_\text{r}$ = 57,000, 35,000, and 25,000 (±3,000) are located at or close to the α-bungarotoxin binding domain of the putative neuronal nicotinic acetylcholine receptor.

The snake venom polypeptide α-bungarotoxin binds with high selectivity and in an almost irreversible fashion to the cholinergic ligand binding site of the nicotinic acetylcholine receptor in fish electric organ and skeletal muscle (1, 2). α-Bungarotoxin binding sites with nicotinic-cholinergic specificity are also present in the peripheral and central nervous systems of vertebrates and invertebrates (reviewed in Refs. 3 and 4). The identification of these neuronal α-bungarotoxin binding sites as nicotinic acetylcholine receptors is still controversial, since in most vertebrate preparations α-bungarotoxin fails to inhibit neuronal nicotinic-cholinergic responses (3, 4). In the goldfish, toad, and chick visual system, however, α-bungarotoxin can block cholinergic receptor function (5). The high molecular weight α-bungarotoxin binding protein of these tissues thus can be classified as a putative neuronal nicotinic acetylcholine receptor (3-9).

In a preceding publication, we have presented a detailed characterization of the α-bungarotoxin binding protein of chick retina neurons maintained in tissue culture (9). Here we report an attempt to identify the polypeptide(s) involved in this α-bungarotoxin binding site by using cross-linking techniques and affinity purification. Immunoprecipitation data on the subunit composition of the α-bungarotoxin binding protein of the rat pheochromocytoma cell line PC 12 are also included.

EXPERIMENTAL PROCEDURES

Materials—α-Bungarotoxin was purchased from Miami Serpentarium, Miami, FL; this toxin preparation was ≥98% pure as judged by polycrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate. Glutaraldehyde, benzamidine, nicotine, and aprotinin were from Sigma; N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate was from Pierce Chemical Co.; d-tubocurarine, benzethonium chloride, and phenylmethylsulfonyl fluoride were from Serva, Heidelberg, GFR. $^{125}\text{I}$-α-Bungarotoxin (initial specific activity, 100-150 Ci/mmol) and $[^35\text{S}]$methionine (initial specific activity, 1000-1200 Ci/mmol) were from New England Nuclear. Formalin-fixed Staphylococcus aureus cells (Pansorbin) were obtained from Calbi-chem-Behring Co. X-Omat XR-5 film and X-Omatic intensifying screens were from Eastman Kodak, Dulbecco's modified Eagle's medium, fetal calf serum, and horse serum were from Gibco Biscuit, Karlsruhe, GFR, and DEAE-Sephadex was from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

α-Bungarotoxin-derivatized agarose beads were prepared by reacting Affi-Gel 10 (Bio-Rad Laboratories) with α-bungarotoxin to a final substitution of 0.1-0.3 mg of toxin bound/ml of bead volume (10). Antibodies to α-bungarotoxin were obtained by immunizing rabbits repeatedly with sublethal doses (50-100 μg) of the toxin emulsified in Freund's complete adjuvant (11). α-Bungarotoxin antibodies were then purified from the rabbit antisera by sequential ammonium sulfate precipitation, DEAE-Sephadex chromatography, and affinity chromatography on α-bungarotoxin-derivatized agarose (11, 12).

In several experiments, buffers contained a mixture of protease-inhibiting reagents (“anti-protease mixture”) at the following final concentrations: EDTA, 5 mM; EGTA, 2 mM; benzethonium chloride, 100 μM; benzamidine, 1 mM; phenylmethylsulfonyl fluoride, 100 μM; aprotinin, 16 milliunits/ml; mercaptoethanol, 5 μM.

The pheochromocytoma cell line PC 12 (13) was kindly donated by Dr. L. Greene, New York University.

Crude Synaptic Membranes—P2 membrane fractions were prepared from the retina of newly hatched chickens as described previously (9). These membranes were further purified by a modification of the procedure described in Ref. 14. The P2 pellet was resuspended in 5 mM Tris-Cl, pH 7.4, and 60% (w/v) sucrose was added to a final concentration of 15% (w/v). Aliquots of this membrane suspension were layered on top of 10 ml of a 35%/5% (w/v) two-step sucrose gradient. The gradients were centrifuged in a Beckman SW 27 rotor at 26,000 rpm and 4 °C for 60 min. Material obtained from the 15%/35% sucrose interphase was diluted with 3 volumes of 25 mM potassium phosphate buffer, pH 7.4. The purified membranes were then collected by centrifugation at 100,000 × g for 20 min, frozen in liquid nitrogen, and stored at −70 °C.

Tissue Culture—Monolayer cultures of retina cells from 6-day-old chick embryos were prepared as described previously (9, 15) with the following modifications: 3 × 10⁷ dissociated cells were plated per polylysine-coated (16) 100-mm plastic dish (Nunc, Roskilde, Denmark). After 1 day in Dulbecco's modified Eagle's medium containing

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5% (v/v) fetal calf serum and 5% (v/v) horse serum, the cultures received the serum-free N1 medium of Bottenstein et al. (17) in order to test the neuronal cells for their ability to grow in tissue culture flasks (Nunc) as described by Greene and Tischler (13).

Metabolic labeling of cell proteins was achieved by incubating 4- to 7-day-old cultures for 24-48 h in 4-8 ml of N1 medium containing 20 μM methionine, 40-120 μCi/ml of [35S]methionine. In order to economize on label, the radioactive medium was re-used for up to four experiments. Usually, 10-20% of the radioactivity present in the medium was incorporated during one labeling period.

Cross-linking Experiments—Crude synaptic membrane fragments from chick retina were suspended in 50 mM sodium phosphate buffer, pH 7.4, containing 0.62% (w/v) Triton X-100 and the anti-protease mixture (buffer A). The membranes were incubated with 4 mM [125I]-α-bungarotoxin at 23 °C for 2 h in the presence or absence of 0.1 mM d-tubocurarine (total volume, 2.5 ml; 3.3 mg/ml of protein). The incubations were then diluted with 10 volumes of cold buffer A and centrifuged at 4 °C and 42,000 X g for 20 min. This washing step was repeated once.

For cross-linking with glutaraldehyde, the [125I]-Toxin-labeled membranes were suspended in half their original volume of 50 mM sodium phosphate buffer, pH 9.0, containing 5 mM EDTA, 3 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride. Glutaraldehyde was added to 0.2-ml aliquots of these membranes at final concentrations of 0.1-10 mM. After 30 min at 23 °C, 1 ml of 50 mM Tris-Cl, pH 7.4, was added, and the membranes were sedimented for 4 min in an Eppendorf 3200 centrifuge. After washing with the Tris-Cl buffer, the membranes were collected for electrophoresis.

In cross-linking experiments with N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, the [35S]-toxin-labeled membranes were resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, 3 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. Aliquots (0.2 ml) were transferred to glass tubes, and N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate dissolved in dimethyl sulfoxide in the reaction mixture was below 5% (v/v). After 10 min at 4 °C, the tubes were illuminated for 15 min at 4 °C with an Osram HNS 15-watt OFR mercury arc lamp from a distance of 3 cm. Then 1 ml of 50 mM Tris-Cl, pH 7.4, was added, and the contents of the tubes were transferred to 1.5-ml Eppendorf vials and processed as described for glutaraldehyde-cross-linked membranes.

In cross-linking experiments with intact cells, 7-day-old retina cultures were incubated for 1 h at 37 °C with 8.3 nmol [125I]-l-a-bungarotoxin in the presence and absence of 0.1 mM d-tubocurarine (9). After three washes with phosphate-buffered saline, the cultures were covered with 10 mM glutaraldehyde in phosphate-buffered saline adjusted to pH 8.0 (4 °C, 30 min). After washing with phosphate-buffered saline, the cells were incubated with 60 μM nicotine in phosphate-buffered saline for 30 min at 4 °C in order to remove most noncovalently bound [125I]-l-a-bungarotoxin (18). The cells were harvested in 0.1 M Tris-Cl buffer, pH 8.8, containing the anti-protease mixture and incubated for 30 min at 4 °C in 1 ml of 50 mM NaNH, dissolved in 0.1 M Tris-Cl, pH 9.6. After two more washes with 0.1 M Tris-Cl, pH 8.8, the cells were collected for electrophoresis.

Electrophoresis—Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed using the discontinuous buffer system of Laemmli (19). Immediately after preparation, all samples were heated for 5 min at 95 °C in sample buffer (19) containing 2% (w/v) sodium dodecyl sulfate, 5% (v/v) mercaptoethanol. The samples were stored at −20 °C overnight and electrophoresed on 1.5-mm separating gels containing 10% (w/v) acrylamide. After staining with Coomassie brilliant blue R-250, destaining, and washing in water, the gel slabs were dried on filter paper under vacuum. Kodak X-Omat XR-5 film was exposed to the dried gels at −70 °C for 1-5 weeks using an intensifying screen. For the determination of incorporation efficiency in cross-linking experiments, radioactive bands localized by autoradiography were cut out from the stained gels and counted. Slab gels containing [35S]methionine-labeled proteins were processed for fluorography using the sodium salicylate impregnation procedure (20). Densitometric tracings of autoradiograms were made with a Vitatron TLD 100 densitometer. The following molecular weight standards (Bio-Rad) were used to estimate the apparent molecular weights of the labeled proteins: phosphorylase B (97,400); bovine serum albumin (68,000); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); and lysozyme (14,400).

Affinity Purification—Affinity purification of the detergent-solubilized a-bungarotoxin binding protein from [35S]methionine-labeled cell cultures followed procedures previously reported for the muscle acetylcholine receptor (21). The [35S]methionine-labeled cells from 2-6 cultures were harvested and washed in phosphate-buffered saline. The cells were then homogenized in 0.5-2 ml of 10 mM Tris-Cl, pH 7.4, containing 1% (w/v) Triton X-100, 130 mM NaCl, and the anti-protease mixture. After 30 min on ice, the homogenate was centrifuged at 150,000 g for 30 min. The supernatant was incubated with 20-100 μl of a-bungarotoxin derivatized agarose on a roller shaker at 4 °C for 3 h. The agarose beads were then collected by centrifugation and extensively washed (four washes with 2 ml of 10 mM Tris-Cl, pH 7.4, containing 1% (w/v) Triton X-100, 1 mM NaCl, and the anti-protease mixture, and two washes with the same buffer without NaCl). The beads were resuspended in 80 μl of 100 μM d-tubocurarine dissolved in 10 mM Tris-Cl, pH 7.4, containing 1% (w/v) Triton X-100 and the anti-protease mixture, and kept at 4 °C for 30 min. After centrifugation, the supernatants were collected for electrophoresis.

Immunoprecipitation—Detergent extracts from [35S]methionine-labeled cultures of retina or PC-12 were prepared as detailed under affinity purification. Indirect immunoprecipitation of the solubilized a-bungarotoxin-tagged toxin binding site with anti-a-bungarotoxin and fixed S. aureus cells was performed as described by Merlie and Sebba (11). The immunoprecipitates were then washed with buffer as described in the affinity purification procedure and collected for electrophoresis.

RESULTS

Different methods were used to identify polypeptides associated with the a-bungarotoxin binding site of neuronal membranes.

Cross-linking of [125I]-l-a-Bungarotoxin to Membrane Fractions from Chick Retina—In initial experiments, [125I]-a-bungarotoxin was bound to membrane preparations from retina which were then treated with different concentrations of dimethylubemidate and glutaraldehyde. Only with the latter reagent, and at concentrations greater than 2 mM, was a significant (10% of the specifically bound toxin) covalent radiolabeling of membrane polypeptides obtained. As shown in Fig. 1, radioactive polypeptide species with apparent M, 64,000, 43,000, and 33,000 were detected upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the glutaraldehyde-cross-linked membranes. Incubation of the membranes with [125I]-l-a-bungarotoxin in the presence of an excess of the

![Fig. 1. Membrane fractions from retina were labeled with [125I]-l-a-bungarotoxin, cross-linked with 2.5 mM glutaraldehyde and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and autoradiography as described under "Experimental Procedures". Left, densitometric tracings of autoradiograms. Labeling in the absence (a) and presence (b) of 0.1 mM d-tubocurarine. Right, Coomassie blue staining of untreated membranes (c) and of membranes cross-linked with 2.5 mM glutaraldehyde (d). All lanes contained protein solubilized from equivalent amounts of membranes.](image-url)
nicotinic antagonist d-tubocurarine prevented the labeling of these polypeptides.

As glutaraldehyde at concentrations of >1 mM altered the protein staining of the electrophoresed membrane samples (Fig. 1, lanes c and d), cross-linking experiments were also performed with a photoactivatable cross-linker, N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate. Up to 2 mM, this agent had no apparent effect on the polypeptide pattern of the membranes (Fig. 2). Using 2 mM of N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, radiolabeled polypeptides of Mᵦ = 64,000 and 36,000 were found after incubation of the membranes with 125I-o-bungarotoxin. Incubation of the membranes with 125I-o-bungarotoxin in the presence of 0.1 mM d-tubocurarine prevented the labeling of these polypeptides. As with glutaraldehyde, the efficiency of cross-linking of N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate was low (8% of the specifically bound 125I-toxin).

Cross-linking of 125I-o-Bungarotoxin to Monolayer Cultures of Chick Embryo Retina—The findings obtained with membrane fractions were extended in cross-linking experiments using intact cultured retinal neurons. After binding 125I-o-bungarotoxin to cell surface receptors, glutaraldehyde treatment and subsequent reduction with NaBH₄ yielded radioactive polypeptides of Mᵦ = 64,000 and 36,000 upon electrophoresis of the total cellular proteins (Fig. 4, right). These radioactively labeled bands were absent from cells which had been preincubated with 125I-o-bungarotoxin in the presence of 0.1 mM d-tubocurarine.

Affinity Purification of the α-Bungarotoxin Binding Protein from Detergent Extracts of 14C/Methionine-labeled Retina Cultures—α-Toxin affinity columns are a powerful tool to purify nicotinic acetylcholine receptor sites from various sources (1, 4, 10, 21–25). We used α-bungarotoxin-derivatized agarose to purify the retinal α-bungarotoxin binding protein. In control experiments, it was checked that this affinity support isolated the detergent-solubilized acetylcholine receptor from Torpedo californica in its typical four-subunit composition (Mᵦ = 40,000, 50,000, 39,000, and 65,000). Furthermore, the toxin beads were found to adsorb >90% of the Triton X-100-solubilized high affinity α-bungarotoxin binding sites present in detergent extracts from chick retina membranes (data not shown). Up to 50% of these toxin binding sites could be recovered by ligand-specific elution of the beads with nicotine or d-tubocurarine, both of which accelerate dissociation of α-bungarotoxin from its neuronal binding site (18). Removal of the eluting ligand by dialysis or by chromatography on wheat germ agglutinin-Sepharose gave a >1000-fold purification of the α-bungarotoxin binding protein without significantly changing its 125I-o-bungarotoxin and cholinergic ligand binding properties (Ref. 9 and data not shown). The very low quantities of receptor protein which could be isolated from retina prevented, however, the accurate determination of specific binding activities and the analysis of polypeptide composition by protein staining. The receptor therefore was purified from detergent extracts of 14C/methionine-labeled retina cultures using batchwise adsorption on α-bungarotoxin-derivatized agarose. After thorough washing, the resin was briefly incubated with a high concentration of d-tubocurarine. Polyacrylamide gel electrophoresis of the d-tubocurarine eluate in the presence of sodium dodecyl sulfate revealed that >90% of the protein radioactivity was contained in a polypeptide of Mᵦ = 37,000 (Fig. 3). The isolation of this polypeptide was prevented by preincubating the detergent extracts with 10 μM α-bungarotoxin before adsorption on the affinity resin.

*H. Betz, unpublished experiments.*
S. aureus contained several polypeptides, three of which were absent when the precipitation was performed in the presence of a large excess of α-bungarotoxin over antitoxin (Fig. 4, left). The apparent molecular weights of these bands were 57,000, 34,000, and 25,000. The \( M_r = 57,000 \) band co-migrated with the polypeptide isolated from retina cultures by affinity purification.

**Discussion**

In this paper, several different techniques were used to identify the polypeptide(s) involved in the binding of α-bungarotoxin to the putative nicotinic acetylcholine receptor of retina. Using affinity purification on α-bungarotoxin-derivatized agarose, a polypeptide of \( M_r = 57,000 \) was isolated from detergent extracts of \(^{125}\)I-α-bungarotoxin in retina cultures. Different lines of evidence indicate that this polypeptide contains the α-bungarotoxin and cholinergic ligand binding site (6, 7, 9) of the retinal α-bungarotoxin receptor: (i) >90% of the protein radioactivity in the eluate from the affinity resin was contained in this polypeptide, and its isolation was prevented when the purification was performed in the presence of an excess of α-bungarotoxin; (ii) the α-toxin and cholinergic ligand binding functions of the receptor were recovered in the eluates from α-bungarotoxin-derivatized agarose after removal of the eluting ligand d-tubocurarine.

The participation of a \( M_r = 57,000 \, (±3,000) \) polypeptide in α-toxin binding to chick retina was confirmed by cross-linking of \(^{125}\)I-labeled α-bungarotoxin to synaptosomal membrane preparations or cultured cells. With glutaraldehyde and N-succinimidyl-6-[(4'-azido-2'-nitrophenylamino)hexanoate as cross-linkers, labeled polypeptide species of about \( M_r = 63,000 \), 43,000, and 33,000 were found upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Assuming that each radiolabeled polypeptide contained one covalently bound toxin molecule, these molecular weights correspond to receptor polypeptides of \( M_r = 55,000 \), 35,000, and 25,000 (±3,000). The band labeled by cross-linking of \( M_r = 63,000 \) thus corroborates the relevance of the \( M_r = 57,000 \) polypeptide isolated by affinity purification. The origin of the two other polypeptides identified by cross-linking is not clear. These polypeptides may represent receptor subunits which were lost during the incubation and washing procedures used in the affinity purification. Alternatively, they may not be integral subunits of the putative neuronal acetylcholine receptor, or they may have resulted from proteolysis of the polypeptide of \( M_r = 57,000 \). In view of the fact that cross-linking was performed within a few hours and that protease inhibitors and chelating agents were included, the last possibility appears, however, less likely. Also, a \( M_r = 35,000 \) polypeptide was detected in the cross-linking experiments with intact retinal cells in culture. We therefore suggest that the lower molecular weight bands described here are not artificial, but located in close proximity to the retinal α-bungarotoxin binding site.

This interpretation is also supported by the data obtained with another rapid isolation technique, the toxin-antitoxin immunoprecipitation method of Merlie and Sebbane (11), which has originally been developed for muscle acetylcholine receptor. With detergent extracts from \(^{125}\)I-labeled retina cultures, we were unable to recover sufficient radioactivity to reveal any specifically precipitated receptor polypeptides. This may be due to an accelerated dissociation of the receptor from the toxin-immunoglobulin complex. With the rat pheochromocytoma cell line PC 12 which possesses α-bungarotoxin binding sites very similar to those present in retina (20), a much higher metabolic labeling with radioactive amino acids of the cellular proteins could be achieved. Immunoprecipitates from detergent extracts of these cells contained in addition to several co-precipitated proteins three polypeptides of \( M_r = 57,000 \), 34,000, and 25,000 whose isolation was prevented by an excess of α-bungarotoxin over antitoxin.

Taken together, our results show that the α-bungarotoxin and cholinergic ligand binding site of the putative neuronal nicotinic acetylcholine receptor in chick and rat is contained in a polypeptide of \( M_r = 57,000 \, (±3,000) \). In addition, polypeptides of \( M_r = 35,000 \) and 25,000 (±3,000) may be associated with the α-bungarotoxin binding domain of the neuronal receptor. These molecular weights are different from those reported for the subunits of the nicotinic acetylcholine receptor of *Torpedo* electric organ (\( M_r = 60,000 \), 40,000, 30,000, and 20,000; reviewed in Ref. 1). In particular, both the α-toxin and the cholinergic ligand binding functions of this peripheral acetylcholine receptor have been localized on the smallest receptor subunit of \( M_r = 40,000 \) (1). Similar conclusions have been reached for the acetylcholine receptor of rat skeletal muscle (23). It should, however, be noted that photoaffinity labeling studies with \(^{125}\)I-α-bungarotoxin derivatives gave rather divergent results. For example, in membrane fractions of *Torpedo* electric organ, the receptor polypeptides of \( M_r = 40,000 \) and 65,000 were preferentially labeled (27–29). In *situ* photoaffinity labeling with an α-bungarotoxin derivative of the acetylcholine receptor of rat muscle gave in contrast a prominent radiolabeled band of \( M_r = 63,000 \) (28). Furthermore, purification of the acetylcholine receptor from rat and chick muscle has revealed a receptor polypeptide of \( M_r = 56,000 \) (23, 30). This subunit of the muscle receptor has a similar molecular weight to the large polypeptide of the neuronal receptor detected in this study; it is, however, not involved in cholinergic ligand binding (23). From immunological and sequence analysis, it has been suggested that the different subunits of the acetylcholine receptor from fish electric organ may have derived from a common ancestor polypeptide (31, 32). One may therefore speculate that during evolution the toxin and acetylcholine binding site of the peripheral and the neuronal receptor have been conserved on

**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radiolabeled α-bungarotoxin binding polypeptides. Left, fluorogram of \(^{125}\)I-methionine-labeled polypeptides separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Lane 1, affinity-purified \( M_r = 57,000 \) polypeptide isolated from \(^{125}\)I-methionine-labeled chick retina as detailed in Fig. 3. Lane 2, proteins immunoprecipitated from a detergent extract of a \(^{125}\)I-methionine-labeled culture of PC 12 cells by 10 μl of *S. aureus* suspension after incubation with 10 nm α-bungarotoxin and 3 μl of α-bungarotoxin antibodies. Lane 3, same as lane 2, but a 100-fold excess of α-bungarotoxin over antitoxin was used. About 3–4 × 10⁵ of the total counts/min present in the extract were recovered in the precipitates, A, actin. Right, autoradiograms of polypeptides cross-linked to \(^{125}\)I-α-bungarotoxin in retina cultures. 7-day-old cultures were incubated with \(^{125}\)I-α-bungarotoxin, washed, cross-linked with 10 μM glutaraldehyde, and processed for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and autoradiography as described under “Experimental Procedures.” Lanes 4 and 5, incubation with \(^{125}\)I-α-bungarotoxin in the absence and, lanes 7 and 8, in the presence of 0.1 mM d-tubocurarine.
polypeptides of different size. This hypothesis is supported by the low, but distinct immunological cross-reactivity of the Torpedo and the putative neuronal acetylcholine receptor (9, 33).

During the course of this work, attempts have been made by others to establish the subunit composition of the $\alpha$-bungarotoxin binding protein of rat brain (34, 35). So far, the results are conflicting. One group obtained only one polypeptide species of $M_r = 51,000$ (34), whereas another study described four subunits of $M_r = 44,000, 50,000, 56,000,$ and $62,000$ (35). Also, after submission of this manuscript, a report on the isolation of the putative nicotinic acetylcholine receptor from chick optic lobe was published (36). In that paper, only one receptor subunit of $M_r = 54,000$ was detected after chromatography on $\alpha$-bungarotoxin- and lentil lectin-derivatized Sepharose and shown to react with a cholinergic affinity reagent. This polypeptide appears to be similar to the $M_r = 57,000$ polypeptide isolated in our affinity purification procedure.

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