0026-895X/89/050593-06$02.00/0

MOLECULAR PHARMACOLOGY, 35:593-598

Structural Characterization of α-Bungarotoxin-Binding Proteins from *Aplysia californica*

JAMES T. McLAUGHLIN and EDWARD HAWROT
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510
Received September 28, 1988; Accepted February 21, 1989

SUMMARY

Structural features of α-bungarotoxin-binding proteins from the marine mollusc *Aplysia californica* have been examined as a first step toward delineating their potential role in cholinergic neurotransmission. Protein blotting with ^{125}I-α-bungarotoxin was used to identify binding proteins in membranes prepared from *Aplysia* muscle and nervous tissue. Binding proteins from both tissues exhibited similar physical characteristics, which distinguish them from the prototypical α-bungarotoxin-binding protein, the nicotinic acetylcholine receptor obtained from *Torpedo californica* electric organ membranes. *Aplysia* binding activities migrate with an apparent molecular weight of 250 kDa on sodium dodecyl sulfate (SDS) gels in the presence of reducing agents. Binding of α-bungarotoxin to blots of *Aplysia* membranes is abolished by exposure of samples to heat or to low pH but is unaffected by reduction-alkylation treatment. In contrast, the α-bungarotoxin-binding subunit of the acetylcholine receptor from *Torpedo* membranes migrates on SDS gels at 40 kDa. It retains binding activity following exposure to heat or to low pH, but binding is substantially diminished by reduction-alkylation treatments. Another distinguishing characteristic of the *Aplysia* binding activities is revealed by examining recovery of membrane α-bungarotoxin-binding on protein blots; the high recovery of *Aplysia* binding contrasts sharply with the low recovery of *Torpedo* binding activity. The high apparent molecular weight of the *Aplysia* α-bungarotoxin-binding activities, their most distinguishing feature, is similar to an α-bungarotoxin-binding activity recently identified in lower vertebrate brain. Covalent cross-linking with ^{125}I-α-bungarotoxin demonstrates, however, that the mobility of both *Aplysia* binding activities is due to a multimeric protein that is unusually resistant to dissociation in SDS. The covalently radiolabeled *Aplysia* α-bungarotoxin-binding activity migrates at approximately 260 kDa on SDS gels when solubilized at room temperature. When it was boiled before electrophoresis, the mobility of the radiolabeled protein shifts to approximately 70 kDa. Resistance to dissociation in the absence of boiling may explain both the high recovery of activity on blots and the insensitivity to reductive alkylation. Conversely, dissociation of the multimeric complex upon boiling may explain the observed loss of binding activity. Our results demonstrate structural similarities and differences between *Aplysia* α-bungarotoxin-binding proteins and the *Torpedo* acetylcholine receptor. They also are consistent with the inclusion of α-bungarotoxin-binding proteins such as those of *Aplysia* in the proposed ligand-gated ion channel "gene family." This group of structurally and functionally related proteins includes acetylcholine-gated cation channels as well as anion selective γ-aminobutyric acid$_A$ and glycine receptors. Because there is evidence for an acetylcholine-gated chloride channel that is blocked by α-bungarotoxin in *Aplysia*, the binding proteins characterized in this report may be members of a distinct lineage within this receptor family.

α-BTX, a polypeptide component of elapid snake venom, is a high affinity competitive antagonist of nAChR found in vertebrate muscle and elasmobranch electric organ. This neurotoxin has been a valuable tool in the characterization and purification of the nAChR from various sources, including *Torpedo* electric organ (1, 2). The nAChR is a pentameric protein composed of four subunits (stoichiometry $\alpha_2\beta\gamma\delta$) that functions as an ACh-gated, cation-selective ion channel. Binding sites for ACh and α-BTX are located on an extracellular region of each of the two α-subunits (3, 4). High affinity α-BTX binding sites are also present in peripheral and central nervous system tissue from both vertebrates and invertebrates (5), but their designation as nAChRs is controversial.

Functional antagonism of neuronal nAChRs by α-BTX varies with different species and tissues. For example, in the rat peripheral and central nervous system, α-BTX does not block nicotinic cholinergic responses (reviewed in Ref. 6). Clarke *et al.* (7) showed the anatomical distribution of ^{125}I-α-BTX binding sites in the rat central nervous system is distinct from binding sites of either [^{3}H]ACh or [^{3}H]nicotine. This indicates

J.T.M. was supported by National Institutes of Health Predoctoral Training Grant GM 07324. E.H. is an Established Investigator of the American Heart Association. Additional support was provided in part by the Muscular Dystrophy Association and the National Institutes of Health (Grant GM32629).

ABBREVIATIONS: α-BTX, α-bungarotoxin; ACh, acetylcholine; AChR, acetylcholine receptor; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; NEM, *N*-ethyl maleimide; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; TEMED, *N,N,N,N*-tetramethylethylenediamine; EGF, epidermal growth factor; GABA, γ-aminobutyric acid; PVDF, polyvinylidine difluoride.

that the neuronal α-BTX binding sites in this species are proteins distinct from nAChRs. Similarly, differences in distribution of α-BTX binding sites and neuronal nAChRs have been demonstrated in chick sympathetic ganglia using monoclonal antibodies to nAChRs (8, 9). In general, neuronal nAChRs in vertebrates are distinct from neuronal α-BTX binding sites, the function of which remains unknown. Despite this, neuronal α-BTX-binding proteins are assumed to be members of the AChR "gene family" (10). This assumption is based on the shared ability to bind α-BTX, on monoclonal antibody cross-reactivity, and on limited sequence homology between nAChR subunits and an α-BTX binding protein from chick brain.

In contrast to these findings, α-BTX does block cholinergic responses in several lower vertebrate and invertebrate species. For example, α-BTX blocks some neuronal nAChRs in goldfish, toad (11), and the marine mollusc *Aplysia californica* (12, 13). In *Aplysia* neurons, ACh activates at least three distinct membrane conductances, a slow K^+ conductance, a fast Na^+ conductance and a fast Cl^- conductance (12, 14). Only the Cl^--mediated response is inhibited by α-BTX (12, 13). This last observation is particularly interesting in light of the recent demonstration of homology between nAChRs and the vertebrate receptors for $GABA_A$ and glycine, both of which are Cl^--selective, ligand-gated ion channels.

Protein blotting with ^{125}I-α-BTX has been valuable in mapping the α-BTX binding region of the *Torpedo* nAChR (15). It has also been used to identify α-BTX binding proteins in membranes prepared from lower vertebrate neural tissue (16). We have now applied protein-blotting techniques to the characterization of α-BTX binding proteins from the marine mollusc *A. californica*. Two factors make *Aplysia* an attractive system for examining the relationship between α-BTX binding and nAChR, the previously demonstrated antagonistic effect of α-BTX on an ACh-evoked response, and a high level of binding sites in membranes prepared from both muscle and nerve tissue. In this report, we describe the biochemical characterization of α-BTX-binding proteins from both of these *Aplysia* tissues. Protein blotting was used to determine the molecular weight of the binding activities, their sensitivities to heat and to low pH, and the effect of reduction and reductive alkylation on α-BTX binding. Competition of ^{125}I-α-BTX binding by cholinergic and noncholinergic drugs to both *Aplysia* tissues was used as another means to compare the two binding proteins. Finally, covalent cross-linking with ^{125}I-α-BTX was used to determine whether the *Aplysia* binding proteins are multimeric. In all of these studies, *Torpedo* membranes were used as a basis for identifying the similarities between the *Aplysia* α-BTX-binding proteins and the well characterized nAChR from *Torpedo*.

Materials and Methods

A. californica were from Alacrity (Redondo Beach, CA); frozen *Torpedo californica* electric organ was obtained from Pacific Bio-Marine (Venice, CA). Materials suppliers were as follows: PVDF (Immobilon) blotting membrane, Millipore (Bedford, MA); Tris buffer, ICN (Plainview, NY); molecular weight standards, Boehringer Mannheim (Indianapolis, IN); $Na^{125}I$, Amersham (Arlington Heights, IL); DSS, Pierce (Rockford, IL); DTT, NEM, and protease inhibitors, Sigma Chemical Co. (St. Louis, MO); and SDS, 2-mercaptoethanol, ammonium persulfate, and TEMED, Bio-Rad (Richmond, CA). Acrylamide was from Kodak (Rochester, NY) and was decolorized before use. Purified α-BTX was from Miami Serpentarium (Salt Lake City, UT). Mono-iodo ^{125}I-labeled α-bungarotoxin (specific activity, 400–800 cpm/fmol) was prepared as described previously (4). Protein concentration was estimated using the bicinchoninic acid assay system (Pierce), using BSA as a standard.

Tissue preparation. *Aplysia* cerebral, buccal, pedal, and pleural ganglia were collected, frozen in liquid nitrogen, and stored at −80°. Muscle (foot pad), hepatopancreas, and buccal muscle samples were similarly obtained. The body wall of *Aplysia* (including the foot) consists of thick musculature innervated with elaborate neural arborizations and plexi. No attempt was made to dissect these from the musculature of the body wall proper. Membranes from all tissues, including *Torpedo* electric organ, were prepared using an identical procedure. All steps were carried out at 4°. Tissues were homogenized in 10 volumes of 0.3 M sucrose, 5 mM sodium phosphate, pH 7.3, 5 mM EDTA, 5 mM EGTA, 0.5 mM iodoacetamide, plus protease inhibitors (1.0 mM PMSF, 1 mM 1,10-phenanthroline, 0.25 mM benzamidine, 25 μM bestatin, 25 μM leupeptin, 10 μM pepstatin A, and 1 μM aprotinin). The homogenate was centrifuged 10 min at 2000 × *g*, and the supernatant was then collected and centrifuged at 100,000 × *g*. Pellets from the high speed spin were resuspended in the same buffer at 2–3 mg of protein/ml and were stored at −80°.

Gel electrophoresis and protein blotting. Membranes were solubilized for 1 hr at 22° in 50 mM Tris·HCl, pH 6.8, 2% SDS, 2.5% 2-mercaptoethanol, 5% glycerol, and 0.1% bromophenol blue (SDS sample buffer). Some samples were heated 3 min at 100° before electrophoresis. For acid treatments, the pH of SDS-solubilized samples was adjusted to 4.0 with 0.1 M HCl, and samples were incubated 10 min at room temperature. Following this, pH was adjusted to 6.8 with 0.1 M NaOH. Samples (20–150 μg of protein/lane) were resolved on 5–15% gradient polyacrylamide gels, then electrophoretically transferred to PVDF membrane filters for 2 hr at constant current (0.4 A). Transfer was performed at 4° in 16 mM Tris base, 120 mM glycine, and 10% methanol. Transfers were quenched for 1 hr at 45° in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 0.02% NaN_3, and 2% casein (quench buffer) and then incubated overnight at 22° in quench buffer plus 10^5 cpm/ml ^{125}I-labeled α-BTX. Following three 10-min washes in ice-cold 25 mM Tris·HCl, pH 7.5, 125 mM NaCl, the transfers were autoradiographed using Kodak XAR-5 film and an intensifying screen (DuPont, Newark, DE). Films were generally exposed for 24–72 hr at −80°.

To estimate recovery of membrane binding on blots, samples were processed, transferred, and quenched as described and were then incubated 12–14 hr in 2 nM ^{125}I-labeled α-BTX. The amounts of each of the membrane preparations used for these blots was previously shown to be in the linear binding range. Filters were washed and autoradiographed, and the radioactive bands were localized and counted directly. Percent recovery was calculated by comparing mol of α-BTX bound to blots with values obtained from membrane binding assays. Values obtained for *Torpedo* binding recovery were similar to those reported previously (17).

For alkylation treatment, membranes were suspended in SDS sample buffer containing 10 mM DTT (without 2-mercaptoethanol) and incubated 30 min at 22°. NEM (final concentration, 25 mM) was then added to aliquots of each sample and incubated 60 min at 22°. Alkylation was terminated by addition of 2-mercaptoethanol to a final concentration of 2.5%.

Membrane binding. For membrane binding assays, samples were diluted into 50 mM Na sodium phosphate, pH 7.5, 1 mM EDTA, and 0.5 mg/ml BSA. The reaction was initiated by addition of ^{125}I-labeled α-BTX (final concentration, 2.0 nM), to triplicate samples, in a total volume of 1 ml. Membrane suspensions were incubated 6–8 hr at 22° and then collected on Whatman GF/C filters that were presoaked in 1% BSA, using a Millipore filtration manifold. Each filter was washed six times with 3 ml of ice-cold 25 mM Tris·HCl, pH 7.5, plus 125 mM NaCl; nonspecific binding was determined by addition of 1 μM unlabeled α-BTX.

Covalent cross-linking. For cross-linking experiments, membrane samples (1 mg of protein/ml) were suspended in 50 mM sodium phos-

phate, pH 7.5, 1 mM EDTA, and 0.5 mg/ml BSA. Following a 6–8-hr incubation with 2 nM ^{125}I-labeled α-BTX (with or without drug additions as indicated), membranes were washed by centrifugation three times in 2 volumes of phosphate-buffered saline (10 mM sodium phosphate, pH 7.5, 120 mM NaCl). Samples were resuspended to 1 mg of membrane protein/ml in phosphate-buffered saline, and DSS was added to a final concentration of 75 μM. After 15 min at 22°, the reaction was terminated by addition of SDS sample buffer. Cross-linked membrane samples were resolved on 5–15% gradient gels. The gels were fixed, stained with 0.2% Coomassie brilliant blue, and destained in 10% acetic acid/40% methanol. Destained gels were dried and autoradiographed as previously described.

Results and Discussion

Our objective in these studies was a biochemical description of *Aplysia* α-BTX-binding proteins based on comparison of the physical characteristics of *Aplysia* α-BTX binding and the nAChR from *Torpedo*. α-BTX binding is present in both ganglia and muscle tissues from *Aplysia*; total binding is approximately 1.0 pmol/g of tissue. This value is comparable to that found in other tissues, such as mammalian central nervous system (5), and is sufficient to allow biochemical characterization.

We began these studies using protein blotting, a technique uniquely suited for the examination of ligand-receptor interactions (17, 18). As previously reported (15), when *Torpedo* membranes are analyzed by protein blotting using ^{125}I-α-BTX as a probe, a 40-kDa band corresponding to the α-subunit of the AChR is labeled (Fig. 1A, *lane 4*). In contrast, protein blotting of *Aplysia* ganglia membranes reveals only a single band at 250 kDa that binds ^{125}I-α-BTX (Fig. 1A, *lane 2*). Protein blots of *Aplysia* muscle membranes reveal a similar 250-kDa α-BTX binding activity (Fig. 1A, *lane 3*). No α-BTX-binding activity is detectable in membranes prepared from *Aplysia* hepatopancreatic tissue (Fig. 1A, *lane 1*). This latter result demonstrates that the α-BTX-binding proteins in *Aplysia* are tissue specific (consistent with a role as a neurotransmitter receptor).

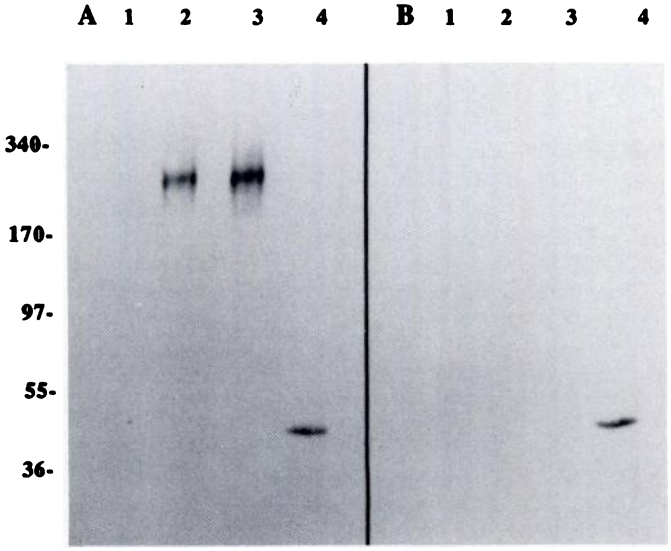

Fig. 1. Binding of ^{125}I-α-BTX to *Aplysia* and *Torpedo* membrane protein blots. A, Samples solubilized in SDS sample buffer (containing 2.5% 2-mercaptoethanol) 1 hr at 22°. B, Samples solubilized 3 min at 100°. A and B, *lane 1*, 100 μg of *Aplysia* hepatopancreas membranes; *lane 2*, 100 μg of *Aplysia* ganglia membranes; *lane 3*, 100 μg of *Aplysia* muscle membranes; *lane 4*, 100 μg of Torpedo electric organ membranes.

Another distinguishing feature of the *Aplysia* binding activities is thermal sensitivity. *Aplysia* α-BTX binding is abolished if samples are boiled before electrophoresis, whereas *Torpedo* binding is unchanged by boiling (Fig. 1B, *lane 4*). Similar loss of binding activity occurs when *Aplysia* membranes are exposed to acidic conditions (10 min at pH < 5) before electrophoresis (data not shown). Low pH treatment has no effect on *Torpedo* membrane α-BTX binding. The observed disparity in apparent molecular weights and the differential sensitivity to temperature and pH suggest underlying structural differences between *Aplysia* α-BTX-binding proteins and the *Torpedo* AChR.

In membrane binding assays, *Torpedo* membranes typically contain 1–10 pmol of α-BTX binding/mg of protein, whereas *Aplysia* membranes contain 100–200 fmol/mg. On protein blots, however, we found approximately the same specific binding activity in membranes from *Torpedo* electric organ, *Aplysia* muscle, and *Aplysia* ganglia. Recovery of membrane binding activity is presented in Table 1. The high recovery of *Aplysia* binding relative to *Torpedo* binding is another indication of a structural difference between the *Aplysia* and *Torpedo* α-BTX binding proteins. In particular, it suggests that the *Aplysia* α-BTX-binding proteins are unusually resistant to denaturation by SDS or, alternatively, they are more readily renatured on protein blots.

A common feature of vertebrate nAChRs is a readily reducible disulfide near the ACh binding site (1, 3); reduction or alkylation of this disulfide alters the functional properties of the receptor. We have previously shown that binding of α-BTX to the *Torpedo* α-subunit on protein blots is sensitive to reduction-alkylation (17), presumably due to the disulfide bond near the agonist (and α-BTX) binding site. Similarly, the susceptibility of *Aplysia* α-BTX-binding proteins to disulfide reduction and alkylation with NEM was examined by protein blotting. Fig. 2 shows that, after reduction-alkylation treatment, the binding of α-BTX to *Torpedo* α-subunit is significantly decreased (Fig. 2, *lanes 5* and *6*), but α-BTX binding to *Aplysia* muscle or ganglia membranes is unchanged (Fig. 2, *lanes 1–4*). This result indicates that either an analogous disulfide is absent in *Aplysia* binding proteins or, if it is present, the disulfide may be inaccessible to alkylation under the conditions used. It is also possible that alkylation of the analogous disulfide in *Aplysia* occurs without affecting α-BTX binding. In contrast to the large decrease in binding seen when the isolated *Torpedo* α-subunit is alkylated with NEM, similar treatment of the native AChR does not significantly affect the level of α-BTX binding (19). Therefore, with respect to NEM sensitivity, the *Aplysia* α-BTX-binding proteins are more similar to native *Torpedo* AChR than to isolated α-subunit on protein blots.

The characteristics of both *Aplysia* α-BTX-binding activities on protein blots are similar to those of high molecular weight α-BTX-binding proteins recently identified in lower vertebrate

TABLE 1

Recovery of ^{125}I-α-BTX-binding activity on protein blots

	Aplysia muscle	*Aplysia* ganglia	*Torpedo*
Membrane binding, fmol/mg	152 ± 8	110 ± 1	7932 ± 142
Protein blot binding (fmol/mg)	99 ± 14	31 ± 4	37 ± 4
Recovery (%)	65	28	0.5

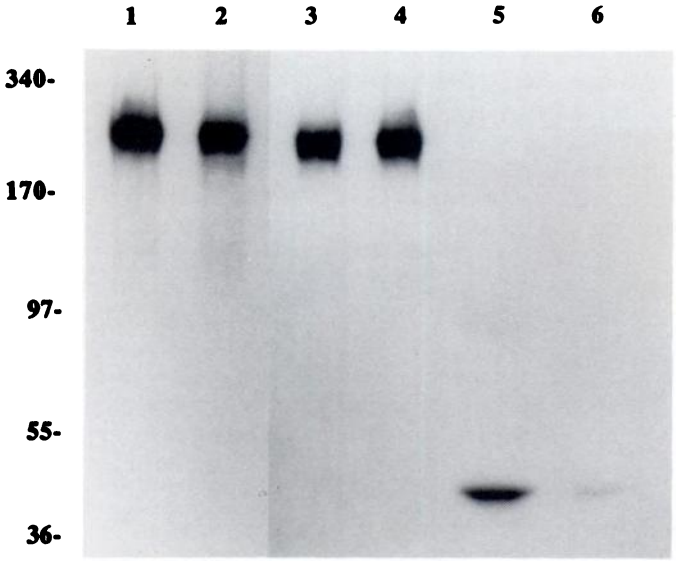

Fig. 2. Effect of NEM on binding of ^{125}I-α-bungarotoxin to *Aplysia* and *Torpedo* membrane protein blots. Membrane samples (150 μg of protein/lane) were exposed to DTT or DTT followed by NEM before electrophoresis and blotting, as described in Materials and Methods. *Lane 1, Aplysia* ganglia, DTT treated; *lane 2, Aplysia* ganglia, DTT/NEM treated; *lane 3, Aplysia* muscle, DTT treated; *lane 4, Aplysia* muscle, DTT/NEM treated; *lane 5, Torpedo*, DTT treated; *lane 6, Torpedo*, DTT/NEM treated.

TABLE 2

Equilibrium membrane binding of ^{125}I-α-BTX: competition studies with cholinergic and noncholinergic drugs

	IC_{50}		
	Aplysia muscle	*Aplysia* ganglia	*Torpedo*
		μM	
Carbamylcholine	60	>1000	95
Suberyldicholine	5.8	3.5	6.7
Hexamethonium	590	>1000	>1000
Decamethonium	87	540	90
Tubocurarine	7.6	3.3	5.7
Strychnine	0.94	0.44	>1000
Atropine	69	70	>1000

central nervous system (16), which also display sensitivity to boiling and resistance to reduction-alkylation treatments. These properties are, thus, not unique to the *Aplysia* binding proteins. The similar characteristics of the *Aplysia* and lower vertebrate α-BTX-binding activities suggest that this class of binding proteins may be phylogenetically widespread.

To determine whether the *Aplysia* ganglia and *Aplysia* muscle binding activities were distinct, we examined the pharmacology of α-BTX binding to membranes using competition assays with cholinergic and noncholinergic drugs. A comparison of these results with those seen for *Torpedo* AChR is presented in Table 2. Suberyldicholine and tubocurarine are effective competitors of α-BTX binding in all three tissues and provide evidence for the nicotinic character of the *Aplysia* α-BTX binding activities. Carbamylcholine and decamethonium are effective competitors with *Aplysia* muscle and with *Torpedo* AChR, but not with *Aplysia* ganglia. Conversely, atropine and strychnine compete effectively in *Aplysia*, both muscle and ganglia, but are ineffective with *Torpedo* membranes. Ono and Salvaterra (13) conducted similar studies on α-BTX binding to *Aplysia* ganglia membranes and found similar IC_{50} values for the drugs tested, with the exception of carbamylcholine. It is unclear why, in our hands, carbamylcholine was an ineffective competitor in ganglia, especially considering its effectiveness on both *Aplysia* muscle and *Torpedo* membranes. In any case, the unique pharmacology of *Aplysia* α-BTX binding activities provides further evidence that the *Aplysia* binding proteins differ structurally from the *Torpedo* AChR. Notably, both *Aplysia* binding sites exhibit a pharmacologic profile similar to that seen for the ACh-evoked Cl^- conductance [i.e., inhibition by atropine and strychnine but not by hexamethonium (12, 14)], an observation that is consistent with involvement of the α-BTX binding protein in the ACh-gated chloride conductance. These studies are also important because they show that, despite physical similarities of muscle and ganglia binding in protein blot analyses (Figs. 1 and 2), the two activities are pharmacologically distinct.

As a first step towards examination of the subunit structure of the *Aplysia* α-BTX-binding proteins, we used the bifunctional cross-linking reagent DSS to covalently attach radiolabeled toxin to the *Aplysia* and *Torpedo* α-BTX-binding polypeptides. This approach has been used in the past to examine the subunit structure of *Torpedo* AChR (19, 20), rat muscle AChR (21), and neuronal AchRs (22, 23). Cross-linking studies with other neurotoxins such as bungarotoxin 3.1 have been similarly used to identify neuronal nAChRs in invertebrate tissue (24). We incubated membrane samples in ^{125}I-α-BTX and then washed the samples to remove unbound ^{125}I-α-BTX. After exposure to DSS, the cross-linked membranes were solubilized in SDS, resolved on polyacrylamide gels, and autoradiographed to identify α-BTX-binding polypeptides. In both *Aplysia* preparations and in *Torpedo*, cross-linking with ^{125}I-α-BTX labels proteins with mobilities similar to those of proteins identified with the protein blotting technique, a 250- to 260-kDa band in the *Aplysia* membranes (Fig. 3, *lanes A1* and *B1)* and a 48-kDa band in the *Torpedo* membranes (Fig. 3, *lane C1*). Additional labeling of *Torpedo* membranes seen in the region from 55 to 70 kDa represents cross-linking to the β-, γ-, and δ-subunits. The pattern of labeling seen with *Torpedo* membranes is similar to that previously reported for DSS cross-linking of [^{3}H]-α-cobratoxin (19). Specificity of cross-linking to *Aplysia* membranes is demonstrated by the ability of unlabeled α-BTX (1 μM) to prevent labeling (Fig. 3, A–C, *lane 4*) and the selective ability of cholinergic drugs to prevent labeling. For example, 100 μM suberyldicholine effectively prevents

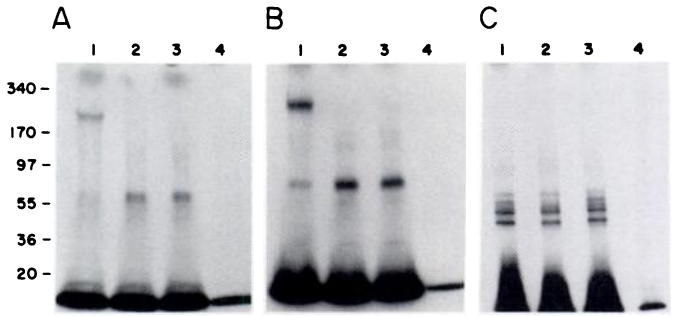

Fig. 3. Covalent cross-linking of ^{125}I-α-BTX to α-BTX-binding proteins of *Aplysia* and *Torpedo*. Membrane samples were labeled with ^{125}I-α-BTX by covalent cross-linking with DSS as described in Materials and Methods. A, *Aplysia* ganglia; B, *Aplysia* muscle; C, *Torpedo*. A–C, *lane 1*, samples solubilized in SDS 1 hr at 22°; *lane 2*, samples solubilized in SDS 3 min at 100°; *lane 3*, samples incubated 10 min at pH 5 before solubilization in SDS 1 hr at 22°; *lane 4*, samples cross-linked in the presence of 1 μM unlabeled α-BTX, solubilized in SDS at 22°.

cross-linking, whereas the same concentration of hexamethonium does not (data not shown).

Covalently labeled polypeptides were used to determine whether treatments previously shown to abolish α-BTX binding on protein blots did so by causing subunit dissociation. When *Aplysia* samples are solubilized at room temperature the major cross-linked toxin-receptor complex migrates at approximately 245–255 kDa (Fig. 3, *lanes A1* and *B1*). Less prominent labeled species at 60–70 kDa and 130–160 kDa are also seen in some preparations. The high molecular weight of the major *Aplysia* binding proteins labeled by cross-linking is approximately the same as that of the undenatured AChR and is also similar to that reported for the locust neuronal AChR (23). After the sample is heated, however, the major band cross-linked to ^{125}I-α-BTX migrates at 60–70 kDa (Fig 3, *lanes A2* and *B2*), similar to the minor band observed in samples solubilized at room temperature. Exposure of cross-linked *Aplysia* samples to low pH results in the same pattern of labeling as that produced by boiling (Fig. 3, *lanes A3* and *B3*). Similar heat and pH treatments have no major effect on the pattern of labeling seen with cross-linked *Torpedo* membranes (Fig. 3C). No evidence of cross-linking between subunits, yielding labeled polypeptides larger than 70 kDa, is seen in *Torpedo* samples under any conditions. This suggests that the high molecular weight α-BTX-binding polypeptide observed in *Aplysia* is not due to cross-linked subunits. Thus, the high molecular weight α-BTX-binding proteins of *Aplysia* appear to have a multimeric structure.

Data from the protein blot experiments were consistent with either of two possibilities. The *Aplysia* α-BTX-binding protein could be a monomer that maintains its tertiary structure and ability to bind α-BTX in the presence of SDS but is denatured in SDS when heated or exposed to low pH. Similar sensitivity to thermal denaturation of binding activity has been reported for the EGF receptor, a 170-kDa monomeric protein (18). Binding of ^{125}I-labeled EGF to EGF receptor on protein blots is abolished if receptor samples are boiled before electrophoresis. Alternatively, the α-BTX-binding protein could be a multimer that is resistant to SDS-induced subunit dissociation. Similar resistance to SDS-induced dissociation has been reported for other proteins, including matrix porin from *Escherichia coli* (25) and a neuronal AChR (23). Porin is a trimeric protein that is resistant to dissociation in chaotropic agents and in SDS but can be dissociated if the protein is heated or exposed to acidic pH in the presence of SDS. Breer *et al.* (23) reported that the locust nAChR migrated on SDS gels at 250–300 kDa when samples were solubilized at room temperature without reducing agents. Addition of DTT shifted the receptor polypeptide mobility to 130 kDa; boiled, reduced receptor migrated at 65 kDa.

Although we have not seen similar effects of reducing agents on *Aplysia* α-BTX-binding proteins, the cross-linking experiment reveals faint but reproducible labeled proteins between 130 and 160 kDa following boiling or low pH treatment (Fig. 3, A and B), in addition to the more prominent α-BTX-binding protein at 70 kDa. These results also provide strong evidence that the loss of binding activity on protein blots following heat or low pH treatments is due to subunit dissociation. Resistance to SDS-induced dissociation by the *Aplysia* α-BTX-binding proteins and the locust neuronal AChR may reflect a general property of invertebrate AChR-like proteins. A similar resistance to SDS-induced dissociation could also explain the properties of lower vertebrate α-BTX binding proteins discussed above.

The molecular weights of the *Aplysia* α-BTX-binding subunits (approximately 64 kDa) fall within the range reported for other neuronal toxin-binding subunits [chick retina, 57 kDa (22); locust, 65 kDa (23)]. The vertebrate α-BTX-binding proteins appear to be structurally similar to the *Torpedo* AChR in that they are heterooligomers (22, 26). In contrast to this, the locust α-BTX-binding protein has been reported to be a homooligomer, a structure predicted for ancestral AChRs based on the high degree of homology between the AChR subunits (27, 28). Although we cannot at this time distinguish between a homo- and heterooligomeric structure for the *Aplysia* α-BTX-binding proteins, it is clear that they are structurally more similar to the locust receptor than they are to muscle-type receptors from vertebrates.

One interesting aspect of our results is the similarity between muscle and neural binding proteins in *Aplysia*. The question arises, if the ganglia α-BTX-binding protein is a gated chloride channel, what is the functional role of the muscle α-BTX-binding protein? One possible answer is that the α-BTX binding detected in the muscle membrane preparations is derived from "contaminating" neural tissue. The specific transmitter or transmitters responsible for control of the foot muscle of *Aplysia* have not been clearly defined, but it is known that ACh provides excitatory input to at least some muscle groups in *Aplysia* (29). We were unable to detect any α-BTX binding on protein blots of membranes prepared from one of these, the buccal mass (data not shown). In other invertebrates, movement is controlled by the interplay of both excitatory and inhibitory inputs to the musculature (30), and so involvement of multiple transmitters in *Aplysia* is likely. It is possible that a subset of motor neurons provide inhibitory input to the foot pad musculature via ACh-gated chloride channels similar to those seen in ganglia. Alternatively, the muscle α-BTX-binding protein either may be presynaptic or could be a receptor for a different transmitter. Ono and Salvaterra (13) reported that, in ganglia, α-BTX could block some chloride-dependent responses to histamine and glutamate. Preliminary purification experiments indicate, however, that in addition to binding to α-BTX affinity columns, the muscle α-BTX binding activity also binds to ACh affinity columns.[1] This result supports the hypothesis that the α-BTX-binding protein is involved in mediating a response to ACh.

Recently published reports have demonstrated sequence homology among nAChRs, the $GABA_A$ receptor (31), and the strychnine-binding subunit of the glycine receptor (32). These findings have provided evidence for a family of structurally related ligand-gated ion channels (33). Both the $GABA_A$ and glycine receptors are ligand-gated channels but, unlike most nAChRs, they are selective for anions. The structural similarity between other AChRs and the *Aplysia* α-BTX-binding proteins demonstrated in this report, together with earlier electrophysiological studies, are consistent with the hypothesis that the *Aplysia* α-BTX binding proteins function as ACh-gated chloride channels. The combination of ACh gating and chloride selectivity suggest, in turn, that the *Aplysia* α-BTX-binding

[1] J. T. McLaughlin and E. Hawrot. Manuscript in preparation.

proteins may represent an evolutionary link between the cation and anion selective branches of this proposed receptor family.

References

1. Karlin, A. Molecular properties of nicotinic acetylcholine receptors, in *The Cell Surface and Neuronal Function* (C. W. Cotman, G. Poste, and G. L. Nicolson, eds). Elsevier/North Holland, New York, 191–260 (1980).
2. Stroud, R. M., and J. Finer-Moore. Acetylcholine receptor structure, function, and evolution. *Annu. Rev. Cell Biol.* **1**:317–351 (1985).
3. Kao, P. N., A. J. Dwork, R. J. Kaldany, M. L. Silver, J. Wideman, S. Stein, and A. Karlin. Identification of the half-cysteine specifically labeled by an affinity alkylating reagent for the acetylcholine receptor binding site. *J. Biol. Chem.* **259**:11662–11665 (1984).
4. Wilson, P. T., J. M. Gershoni, E. Hawrot, and T. L. Lentz. Binding of α-bungarotoxin to proteolytic fragments of the *alpha*-subunit of *Torpedo* acetylcholine receptor analyzed by protein transfer on positively charged membrane filters. *Proc. Natl. Acad. Sci. USA* **81**:2553–2557 (1984).
5. Oswald, R. E., and J. A. Freeman. *alpha*-bungarotoxin binding and central nervous system nicotinic acetylcholine receptors. *Neuroscience* **6**:1–14 (1981).
6. Clarke, P. B. S. Recent progress in identifying nicotinic cholinoceptors in mammalian brain. *Trends Pharm Sci.* **8**:32–35 (1987).
7. Clarke, P. B. S., R. D. Schwartz, S. M. Paul, C. B. Pert, and A. Pert. Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H] acetylcholine, [^{3}H] nicotine, and [^{125}I]α-bungarotoxin. *J. Neurosci.* **5**:1307–1315 (1985).
8. Jacob, M. H., and D. K. Berg. Ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. *J. Neurosci.* **3**:260–271 (1983).
9. Jacob, M. H., J. M. Lindstrom, and D. K. Berg. Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *J. Cell Biol.* **103**:205–214 (1986).
10. Lindstrom, J., R. Schoepfer, and P. Whiting. Molecular studies of the neuronal nicotinic acetylcholine receptor family. *Mol. Neurobiol.* **1**:281–337 (1987).
11. Freeman, J. A., J. T. Schmidt, and R. E. Oswald. Effect of α-bungarotoxin on retinotectal transmission in the goldfish and the toad. *Neuroscience* **5**:929–942 (1980).
12. Kehoe, J., R. Sealock, and C. Bon. Effects of α-toxins from *Bungarus multicinctus* and *Bungarus caeruleus* on cholinergic responses in *Aplysia* neurones. *Brain Res.* **107**:527–540 (1976).
13. Ono, J. K., and P. M. Salvaterra. Snake α-toxin effects on cholinergic and noncholinergic responses of *Aplysia californica* neurons. *J. Neurosci.* **1**:259–270 (1981).
14. Kehoe, J. Acetylcholine receptors in molluscan neurons. *Adv. Pharmacol. Ther.* **8**:285–298 (1979).
15. Wilson, P. T., T. L. Lentz, and E. Hawrot. Determination of the primary amino acid sequence specifying the α-bungarotoxin binding site on the α subunit of the acetylcholine receptor from *Torpedo californica*. *Proc. Natl. Acad. Sci. USA* **85**:8790–8794 (1985).
16. Hawrot, E., P. T. Wilson, J. M. Gershoni, J. H. Reese, and T. L. Lentz. *alpha*-Bungarotoxin binding to a high molecular weight component from lower vertebrate brain identified on dodecyl sulfate protein blots. *Brain Res.* **373**:227–234 (1986).
17. Gershoni, J. M., E. Hawrot, and T. L. Lentz. Binding of α-bungarotoxin to isolated α subunit of the acetylcholine receptor of *Torpedo californica:* quantitative analysis with protein blots. *Proc. Natl. Acad. Sci. USA* **80**:4973–4977 (1983).
18. Lin, P. H., R. Seinfreund, and W. Wharton. Western blot detection of epidermal growth factor receptor from plasmalemma of culture cells using ^{125}I-labeled epidermal growth factor. *Anal. Biochem.* **167**:128–139 (1987).
19. Hamilton, S. L., D. R. Pratt, and D. C. Eaton. Arrangement of the subunits of the nicotinic acetylcholine receptor of *Torpedo californica* as determined by α-neurotoxin cross-linking. *Biochemistry* **24**:2210–2219 (1985).
20. Raftery, M., S. Blanchard, J. Elliot, P. Hartig, H. Moore, V. Quast, M. Schimerlik, V. Witzemann, and W. Wu. Properties of *Torpedo californica* acetylcholine receptor. *Adv. Cytopharmacol.* **3**:159–182 (1979).
21. Nathanson, N. M., and Z. W. Hall. *In situ* labeling of *Torpedo* and rat muscle acetylcholine receptor by a photoaffinity derivative of α-bungarotoxin. *J. Biol. Chem.* **255**:1698–1703 (1980).
22. Betz, H., D. Graham, and H. Rehm. Identification of polypeptides associated with a putative neuronal nicotinic acetylcholine receptor. *J. Biol. Chem.* **257**:11390–11394 (1982).
23. Breer, H., R. Kleene, and G. Hinz. Molecular forms and subunit structures of the acetylcholine receptor in the central nervous system of insects. *J. Neurosci.* **5**:3386–3392 (1985).
24. Halvorsen, S. W., and D. K. Berg. Affinity labeling of neuronal acetylcholine receptor subunits with an α-neurotoxin that blocks receptor function. *J. Neurosci.* **7**:2547–2555 (1987).
25. Markovic-Housley, Z., and R. M. Garavito. Effect of temperature and low pH on structure and stability of matrix porin in micellar detergent solutions. *Biochem. Biophys. Acta* **869**:158–170 (1986).
26. Whiting, P. J., and J. M. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc. Natl. Acad. Sci. USA* **84**:595–599 (1987).
27. Hille, B. *Ionic Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA, 354–383 (1984).
28. Lindstrom, J. Probing nicotinic acetylcholine receptors with monoclonal antibodies. *Trends Neurosci.* **9**:401–407 (1986).
29. Cohen, J. L., K. R. Weiss, and I. Kupfermann. Motor control of buccal muscles in *Aplysia*. *J. Neurophysiol.* **41**:157–180 (1978).
30. Gershenfeld, H. M. Transmitters in invertebrate synapses. *Physiol. Rev.* **53**:1–119 (1973).
31. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriquez, L. M. Rhee, J. Ramachondron, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature (Lond.)* **328**:221–227 (1987).
32. Grenningloh, G., A. Rienitz, B. Schmitt, C. Methfessel, M. Zensen, K. Beyneuther, E. D. Gundelfinger, and H. Betz. The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. *Nature (Lond.)* **328**:215–220 (1987).
33. Barnard, E. A., M. G. Darlison, and P. Seeburg. Molecular biology of the $GABA_A$ receptor: the receptor/channel superfamily. *Trends Neurosci.* **10**:502–509 (1987).

Send reprint requests to: E. Hawrot, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510