0026-895X/88/050643-08$02.00/0

MOLECULAR PHARMACOLOGY, 34:643-650

Distribution of α-Bungarotoxin Binding Sites over Residues 173–204 of the α Subunit of the Acetylcholine Receptor

PAUL T. WILSON, EDWARD HAWROT, and THOMAS L. LENTZ

Department of Cell Biology (P.T.W., T.L.L.) and Department of Pharmacology (E.H.), Yale University School of Medicine, New Haven, Connecticut 06510

Received April 27, 1988; Accepted August 1, 1988

SUMMARY

The binding of α-bungarotoxin to several synthetic peptides comprising different segments of the region 173–204 of the α subunit of the *Torpedo* acetylcholine receptor was investigated to further localize the neurotoxin-binding site on the primary sequence. When tested in a solid phase microwell assay system, a 32-amino acid peptide corresponding to residues 173–204 (32-mer) bound ^{125}I-α-bungarotoxin with the same affinity (4.2×10^{-8} M as determined from IC_{50} values) as the isolated α subunit (4.6×10^{-8} M). The relative affinities of other antagonists (α-cobratoxin, *d*-tubocurarine) maintained the same rank order in this assay system as has been demonstrated with the intact receptor. Agonists competed with binding of toxin at millimolar concentrations but lost all rank order of potency. These findings demonstrate that peptide 173–204 contains many of the antagonist-binding determinants present on denatured α subunit but has lost specificity of agonist binding. To further localize the toxin-binding site, α-bungarotoxin binding to seven shorter peptides corresponding to portions of the 32-mer was investigated. ^{125}I-α-Bungarotoxin bound to α subunit peptides 179–192, 181–198, 185–196, 186–196, and 193–204, but not to α subunit peptides 173–180 and 194–204. In a second assay, all of the peptides competed with binding of ^{125}I-acetylcholine receptor to immobilized α-bungarotoxin. The apparent affinity was highest for the 173–204 32-mer (1.4×10^{-7} M) and lowest for peptides 173–180 and 194–204 ($>10^{-4}$ M). The affinity of the other peptides was intermediate ($\approx 10^{-5}$ M) and about 100-fold less than that of the 32-mer. The affinity of α-bungarotoxin was 3.5×10^{-10} M, of isolated, native acetylcholine receptor, 3.2×10^{-9} M, and of isolated denatured subunit, 1.2×10^{-8} M, with this assay. The retention of some toxin-binding capacity by the shorter peptides indicates toxin-binding determinants are distributed over the entire length of the 32-mer. The determinants with higher affinity are located in the central region of the 32-mer between residues 179 and 196.

The nicotinic AChR mediates the initiation of the postsynaptic excitatory response at the neuromuscular junction by opening a cation-selective ion channel upon binding acetylcholine. The receptor is composed of four types of subunits forming a pentameric complex in the stoichiometry of $\alpha_2\beta\ \gamma\delta$ (1). The primary amino acid sequences of all of the subunits from several species have been elucidated from the nucleotide sequences of cDNA clones coding for the subunits (2). Complete understanding of receptor function depends on the localization of structural and functional domains, such as the acetylcholine-binding site, on the receptor molecule. Identification of the acetylcholine-binding site in the AChR would increase understanding of the mechanism by which binding of the ligand leads to changes in the receptor that result in opening of the channel. Localization of this site is greatly facilitated by the use of snake venom curarimimetic neurotoxins, such as BTX, which bind to the receptor with considerably higher affinity than does acetylcholine (3). Most evidence indicates that the toxin-binding site includes the acetylcholine-binding site (1, 4), so use of the toxins as biological probes should permit localization on the primary sequence of the α subunit of the site involved in the binding of acetylcholine.

Affinity alkylating agents, such as MBTA and bromoacetylcholine, that act as either cholinergic agonists or competitive antagonists label only the α subunit (1). Because these agents are competitive with the binding of neurotoxins, the latter are considered to bind at or very near the same site on the α subunit. The affinity alkylating agents bind to a cysteine residue that is located within 10 Å of the acetylcholine-binding site (1). It has been determined that Cys 192 and Cys 193, which are linked by a disulfide (5), are labeled by these agents (6, 7). On the basis of peptide mapping, neurotoxins bind on the C-terminal side of Asn 141, the site of *N*-linked glycosylation (8–11). A cyanogen bromide fragment comprising residues 179–

This work was supported by National Institutes of Health Grant NS 21896, National Science Foundation Grant BNS-8506404, and United States Army Contract DAMD17-86-6043 (to T.L.L.) and National Institutes of Health Grant GM 32629 (to E.H.).

ABBREVIATIONS: AChR, acetylcholine receptor; ACN, acetonitrile; BSA, bovine serum albumin; BTX, α-bungarotoxin; CTX, α-cobratoxin; DDF, *p*-(*N*,*N*-dimethylamino) benzenediazonium fluoroborate; *d*TC, *d*-tubocurarine; HPLC, high pressure liquid chromatography; MBTA, 4-(*N*-maleimido) benzyltrimethylammonium iodide; PB, phosphate buffer; SDS, sodium dodecyl sulfate, TFA, trifluoroacetic acid.

207 is labeled by affinity alkylating agents (6, 7) and the cholinergic photoaffinity ligand DDF (7). [^{3}H]DDF was incorporated to a lesser extent into two additional fragments, indicating other regions of the α subunit chain may contribute to the cholinergic-binding site. Synthetic peptides corresponding to portions of the α subunit have been used to localize the toxin-binding site. Peptides composed of residues 173–204 (8), 182–198 (12), 185–196 (13, 14), 172–205, and 185–199 (15) have been reported to bind BTX. The affinity of binding to peptides 173–204, 182–198, and 172–205 was the same as that for the isolated α subunit. Finally, fusion proteins containing residues 160–216 (16) and 166–200 (17) of the α subunit bind toxin.

Together, these findings strongly indicate that a major determinant for neurotoxin binding is located on the sequence of the α subunit in proximity to Cys 192 and Cys 193. This region of the α subunit may represent a functional domain that is largely responsible for binding of agonists and competitive antagonists. Small segments of protein structure that play crucial roles in protein interactions and whose function can be mimicked by synthetic peptides have been referred to as prototopes (18). Because synthetic peptides may prove extremely useful as a means to isolate and reconstitute important functional properties of proteins, we have undertaken further systematic characterization of the binding characteristics of *Torpedo* α subunit residues 173–204 and compared the ability of cholinergic agonists and antagonists to compete toxin binding. In addition, in an effort to further localize the binding site within the sequence of the 32-mer, toxin binding to shorter peptides comprising portions of the 32-mer has been investigated. Although others have demonstrated toxin binding to shorter peptides within this region, it is useful to directly compare affinities of binding to long and short peptides using the same assays. In addition, before undertaking investigation of structure-function relationships by modification of individual residues, it is necessary to determine the extent of the toxin-binding regions on the linear sequence. These studies show that, although the shorter peptides bind BTX, the affinity of binding is considerably reduced in comparison with binding to the 32-mer. These results indicate that a number of determinants distributed along the length of the 173–204 segment contribute to the neurotoxin-binding site.

Experimental Procedures

AChR and α subunit. AChR from *Torpedo* electric organ was isolated on a cobratoxin-Sepharose column as previously described (19). The α subunit was excised and electroeluted from 10% lithium dodecyl sulfate/polyacrylamide gels as described (8).

Iodination. α-BTX was iodinated with ^{125}I using the procedure of Wang and Schmidt (20). α-BTX (50 μg) (Miami Serpentarium, Salt Lake City, UT) in 250 μl of 16 mM PB, pH 7.5, containing 5 mCi Na^{125}I (Amersham Corp., Arlington Heights, IL) was radiolabeled by the addition of 125 μg of chloramine-T (Eastman Kodak Co., Rochester, NY). Sodium bisulfite (Aldrich Chemical Co., Milwaukee, WI) (250 μg) was added to the solution 30 sec after the addition of chloramine T to stop the reaction. The monoiodinated BTX was separated from the diiodinated BTX by ion exchange chromatography on a CM-25 column. The BTX species were eluted with a gradient of NaCl (0–80 mM) in 3 mM PB, pH 7.5, containing 1 mg/ml BSA (Sigma Chemical Co., St. Louis, MO; fraction V). Initial specific activity of the monoiodinated BTX was determined as described (19) and was greater than 600 cpm/fmol in all preparations.

AChR was iodinated with iodogen (Pierce Chemical Co., Rockford, IL). Glass vials were coated with 50 μg of iodogen by evaporating a solution of iodogen in chloroform (250 μg/ml). Affinity-purified AChR (50 μg) in 200 μl of 20 mM PB, pH 7.4, containing 0.2% deoxycholate was then placed into the vial. The reaction was started by the addition of 20 μl of Na^{125}I (2 mCi). The reaction was stopped after 5 min by the removal of the solution into a vial containing 0.5 ml 10 mM PB/0.4% BSA/0.2% deoxycholate. The iodination vial was then washed with 0.5 ml of 10 mM PB/0.2% deoxycholate, and the wash was added to the transferred solution. A small amount (0.1 g) of AG 1–X8 resin (200–400 mesh, formate form; Bio-Rad Laboratories, Richmond, CA) was added to the vial to absorb any free iodide. After 4 hr, the solution was removed into a new vial and stored at 4° until use. Storage of the radiolabeled AChR for periods greater than 2–3 weeks resulted in the gradual loss of binding activity, presumably the result of the destructive effects of radiodecay.

Synthetic peptides. All receptor peptides used in these studies were synthesized by the Protein Chemistry Facility, Department of Molecular Biophysics and Biochemistry, Yale University (New Haven, CT). The peptides correspond to portions of the *Torpedo* α subunit and are designated by the position of the first residue and the total number of residues (Table 1). The integrity of the peptide sequences was determined by amino acid composition analysis and by reverse phase HPLC. Peptides were purified further by reverse phase HPLC using a Vydac C_4 column and a gradient of 0 to 80% ACN/0.05% TFA. The eluted peptides were then lyophilized and resuspended in distilled water and relyophilized and resuspended in water to a final concentration of 5 mg/ml. β-Asp-angiotensin II was obtained from Sigma.

Solid phase assays. Experiments investigating the binding of ^{125}I-BTX to the *Torpedo* 32-mer employed 32-mer that had been absorbed onto 96-well, flat-bottomed polystyrene microwell plates (Nunc A/S, Roskilde, Denmark). Crude 32-mer, resuspended in 50% ACN containing 0.005% TFA to a concentration of 5 mg/ml, was diluted into 0.015 M $NaCO_3$/0.035 M $NaHCO_3$, pH 9.6 (coating buffer), to a concentration of 50 μg/ml. Wells were then incubated with 100 μl of peptide solution overnight at room temperature. The solution was aspirated out of the wells, and the wells were then quenched with 200 μl of 2% BSA for 1 hr at room temperature. Following removal of the BSA solution, the wells were incubated with ^{125}I-BTX in 10 mM PB, pH 7.4, containing 0.2% BSA (PB/BSA). Wells were washed four times with 200 μl of PB/BSA. Bound ^{125}I-BTX was removed from the wells by adding 100 μl of 0.25 N NaOH containing 2.5% SDS (NaOH/SDS) and swabbing each well with two cotton tipped applicators. The applicators were then assayed for radioactivity in a γ counter. In competition experiments, the competitor was added to the ^{125}I-BTX solution before incubation with the 32-mer. The incubation time for all competition experiments was 2 hr. Measurement of the rate of binding of ^{125}I-BTX to the 32-mer indicated that binding was linear to times up to 3 hr (data not shown).

Affinities of binding were approximated by the measurement of the concentration of unlabeled ligand that resulted in a 50% reduction in the binding of ^{125}I-BTX (IC_{50} value). IC_{50} values were determined from logit-log plots of the competition data (21). Competition curves are graphically represented by fitted curves derived from a nonlinear polynomial least squares fit as determined by the computer program Sigmaplot (Jandel Scientific, Sausalito, CA). Both ^{125}I-BTX and an unlabeled competitor were added simultaneously. Preincubation for 1 hr with the competing agent had no effect on the IC_{50} value obtained (data not shown).

For synthetic peptides other than the *Torpedo* 32-mer, the protocol described above was modified to enhance the binding of the peptide to the microwell plate. Crude peptides resuspended to 5 mg/ml in 50% ACN/TFA were diluted into H_2O to a concentration of 100 μg/ml. Wells were then inoculated with 50 μl of peptide. The plate was placed in a 45° oven until all liquid evaporated. The wells were then quenched as described for the 32-mer and washed one time with PB/BSA before the addition of ^{125}I-BTX. After incubation with ^{125}I-BTX, wells were washed and the bound ^{125}I-BTX removed as described for the 32-mer.

TABLE 1

Amino acid sequences and relative positions of synthetic peptides corresponding to sequences from *Torpedo* α subunit

Peptides are designated by the position of the first residue and number of residues. All peptides were synthesized with their amino- and carboxyl-termini blocked with exception of the α185 12-mer and α186 11-mer.

	Peptide Sequence
	175 180 185 190 195 200
α173 32-mer	S G E W V M K D Y R G W K H W V Y Y T C C P D T P Y L D I T Y H
α173 8-mer	S G E W V M K D
α181 18-mer	Y R G W K H W V Y Y T C C P D T P Y
α179 14-mer	K D Y R G W K H W V Y Y T C
α185 12-mer	K H W V Y Y T C C P D T
α186 11-mer	H W V Y Y T C C P D T
α193 12-mer	C P D T P Y L D I T Y H
α194 11-mer	P D T P Y L D I T Y H

In competition experiments, ^{125}I-BTX was incubated in the wells for 20 min.

The ability of synthetic peptides to bind BTX was also measured by testing the ability of purified peptides to compete with the binding of ^{125}I-AChR to BTX that had been immobilized in the wells of microtiter plates. BTX, isolated AChR, and isolated denatured α subunit were also tested with this assay for comparison with peptides. In these experiments, 100 μl of BTX in coating buffer (10 μg/ml) was pipetted per well. After incubation overnight at room temperature, the contents of the wells were aspirated, and the wells were quenched with 2% BSA for 1 hr. The wells were then washed two times with 200 μl of 10 mM PB, pH 7.0, containing 0.2% Triton X-100 and 0.2% BSA (PB/Triton/BSA). Approximately 500,000 cpm of ^{125}I-AChR in 100 μl of PB/Triton/BSA and competitor was added to each well. After incubation, the wells were washed four or five times with 200 μl of PB/Triton/BSA and the bound radioactivity was removed by adding 100 μl of NaOH/SDS and swabbing the well with two cotton tipped applicators as described above for the 32-mer. Radioactivity was measured in a γ counter. Incubation time for competition experiments was 15 min. The binding of ^{125}I-AChR to BTX was linear for times up to 30 min (data not shown).

Results

Competition of BTX binding to 32-mer by cholinergic ligands. The ability of various cholinergic antagonists to inhibit the binding of ^{125}I-BTX to the α173 32-mer was tested in a solid phase assay (Fig. 1A and Table 2). BTX, CTX from *Naja naja siamensis*, and *d*TC were all effective competitors of ^{125}I-BTX binding. Unlabeled BTX was the most effective agent, competing with binding with an IC_{50} of 42 nM. CTX was slightly less effective, competing with binding with an IC_{50} of 440 nM. ^{125}I-BTX binding was competed with *d*TC with an IC_{50} of 86 μM. The affinity of ^{125}I-BTX binding to isolated α subunit adsorbed to microtiter plates was 46 nM (Fig. 1B and Table 2).

In contrast to the antagonists, cholinergic agonists exhibited little difference in their ability to compete with the binding of ^{125}I-BTX to the α173 32-mer (Fig. 2 and Table 2). Suberyldicholine, the most potent of the commonly used nicotinic agonists, was only marginally more effective in competing with the binding of ^{125}I-BTX than was choline chloride, an acetylcholine precursor that is among the weakest of agonists. The agonists nicotine and arecoline were as effective as choline but more effective than NaCl in competing binding.

The solid phase assay represents a convenient method for performing binding assays and competition experiments. It has the advantage of being simple, requiring little material, and allowing several replications. It is not necessary to utilize a residue that might be important in binding for coupling the peptide to a matrix such as Sepharose. Toxin binding to

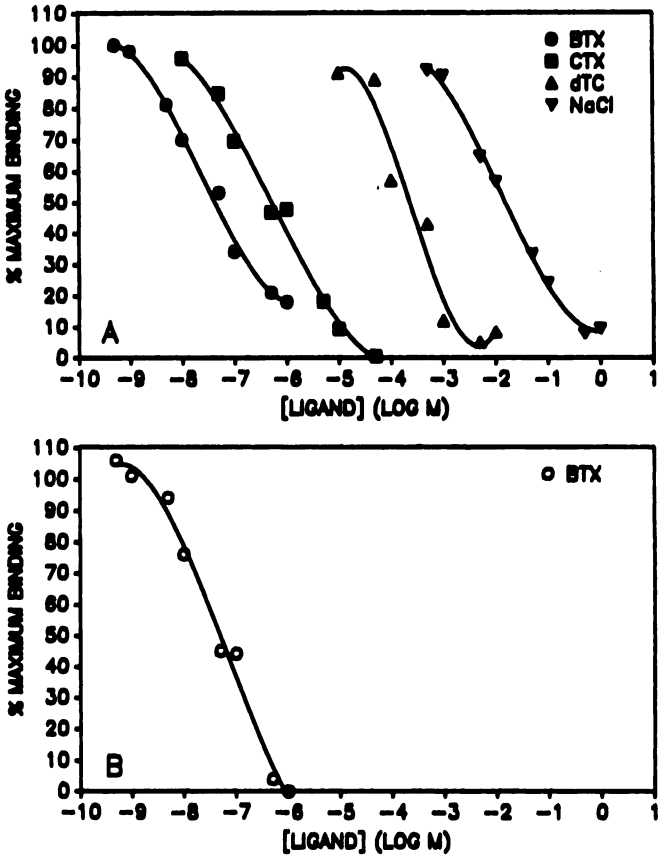

Fig. 1. Competition of ^{125}I-BTX binding to α173 32-mer with cholinergic antagonists (A) and of ^{125}I-BTX binding to isolated α subunit (B). Wells of microtiter plates were coated with 5 μg of α173 32-mer or 0.1 μg of α subunit and incubated with 3 nM ^{125}I-BTX and various amounts of unlabeled BTX (○, ●), CTX (■), *d*TC (▲), or NaCl (▼). After a 2-hr incubation for the 32-mer and 20 min for α subunit, the wells were washed and bound radioactivity was determined. For ^{125}I-BTX (A), 100% binding (binding in absence of ligand) equals 3666 cpm. Each point represents the average of triplicate determinations. Each *curve* represents one of two experiments performed.

quenched wells lacking peptide is negligible (~100 cpm). Possible disadvantages of this assay are that some peptides, particularly short ones, may not adhere to the plastic wells and that bound peptides may not be able to undergo the conformational changes that could take place upon binding to toxin. In addition, proteins may denature as they adsorb to the plastic of microtiter plates (22). These limitations are partially overcome by the assay in which peptides are tested for competition of ^{125}I-AChR binding to immobilized BTX (see below). Affinities

TABLE 2

Competition of binding of ^{125}I-BTX to α173 32-mer

Wells of microtiter plates were coated with 5 μg α173 32-mer. After quenching, the wells were incubated for 2 hr with 3–4 nM ^{125}I-BTX and various amounts of the listed competitors. The wells were then washed and bound radioactivity determined. IC_{50} values were determined from logit-log plots of the competition data. IC_{50} values are the result of one experiment with three replicates except for α-BTX and *d*TC which are the average of two experiments with three replicates.

Competitor	IC_{50}
	M
Antagonist	
α-Bungarotoxin	4.2×10^{-8}
α-Cobratoxin	4.4×10^{-7}
d-Tubocurarine	8.6×10^{-5}
Agonist	
Nicotine sulfate	8.6×10^{-3}
Suberyldicholine iodide	2.9×10^{-3}
Arecoline bromide	4.1×10^{-3}
Other	
Choline chloride	6.9×10^{-3}
Sodium chloride	1.6×10^{-2}

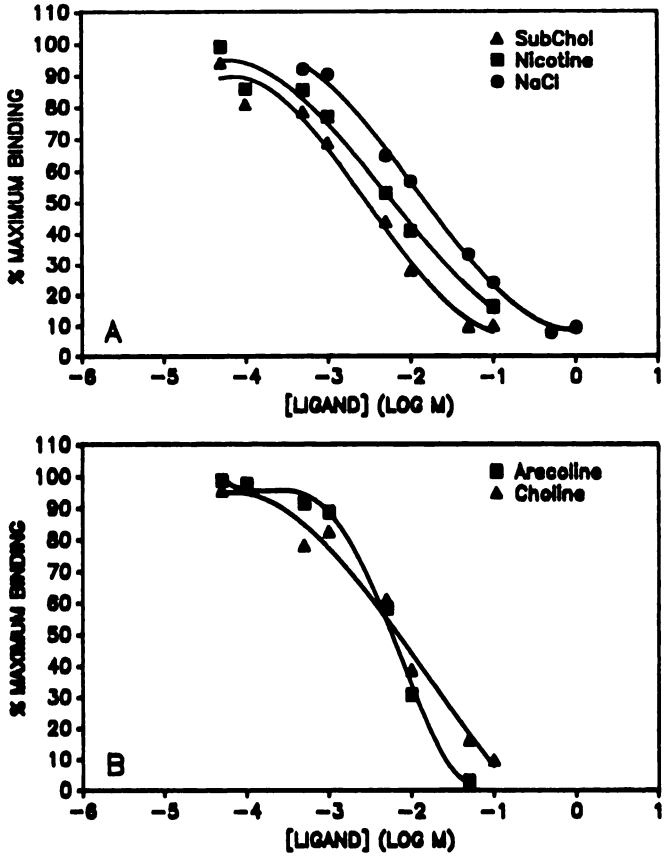

Fig. 2. Competition of ^{125}I-BTX binding to α173 32-mer with cholinergic agonists. Wells of microtiter plates were coated with 5 μg α173 32-mer and incubated with 3–4 nM ^{125}I-BTX and various amounts of suberyldicholine iodide (A; ▲), nicotine sulfate (A; ■), NaCl (A; ●) arecoline bromide (B; ■), or choline chloride (B; ▲). After a 2-hr incubation, the wells were washed and bound radioactivity was determined. Each *point* represents the average of triplicate determinations.

of cholinergic ligands as determined in the solid phase assay are generally 1 order of magnitude less than those determined from solution assays.

BTX binding to shorter peptides comprising portions of the 32-mer. To determine whether all of the toxin-binding determinants of the α173 32-mer are localized to a portion of the 32-mer, smaller peptides corresponding to portions of the α173 32-mer were synthesized and tested for their ability to bind ^{125}I-BTX (see Table 1 for sequences). All peptides tested, with the exception of the α173 8-mer and α194 11-mer, bound ^{125}I-BTX at levels above background, although the absolute amounts of ^{125}I-BTX bound varied markedly between peptides (Figs. 1 and 3). The ability of unlabeled BTX, *d*TC, and NaCl to compete with ^{125}I-BTX binding to three of the smaller

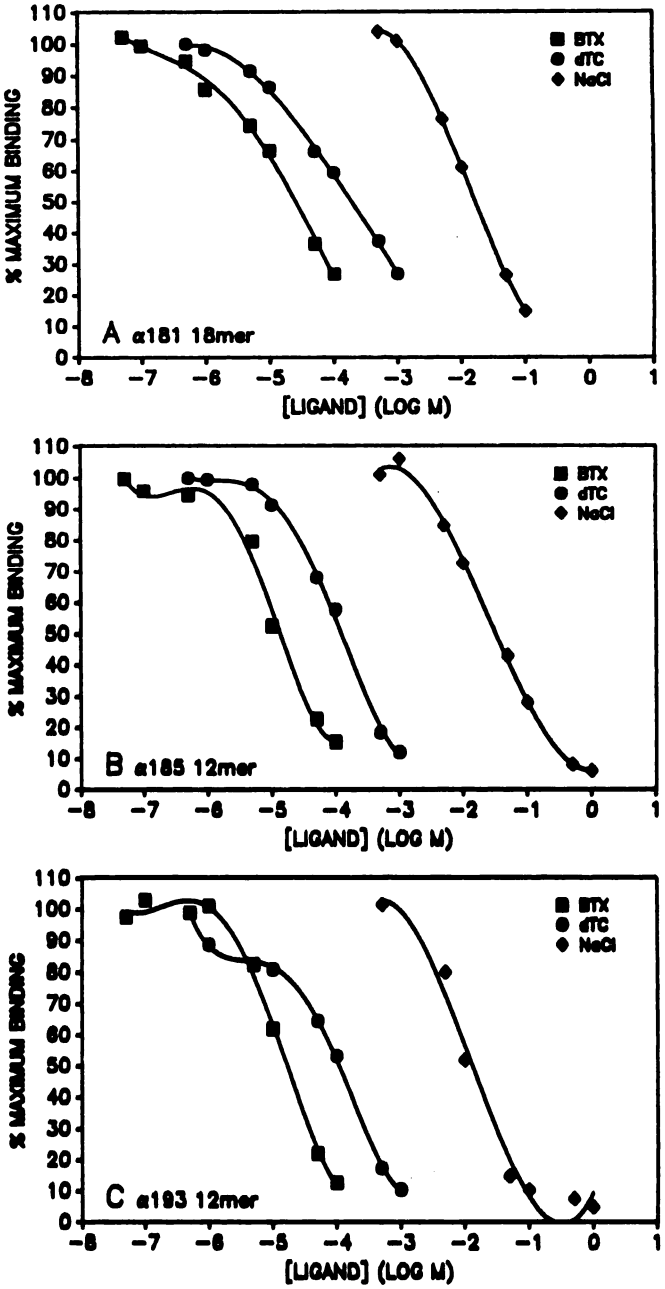

Fig. 3. Competition of ^{125}I-BTX binding to *Torpedo* α181 18-mer (A), α185 12-mer (B), and α193 12-mer (C). Wells of microtiter plates were coated with either 5 μg of α181 18-mer and α185 12-mer or 2 μg of α193 12-mer by evaporation. After quenching, the wells were incubated with 3–4 nM ^{125}I-BTX and various amounts of unlabeled BTX (■), *d*TC (●), or NaCl (◆) for 20 min. The wells were then washed and bound radioactivity was determined. One hundred percent binding of ^{125}I-BTX was 1384 cpm for the α181 18-mer, 2940 cpm for α185 12-mer, and 8873 cpm for α193 12-mer. Each *point* represents the average of triplicate determinations.

peptides, α181 18-mer, α185 12-mer, and α193 12-mer, was tested. Unlabeled BTX competed with ^{125}I-BTX binding to all three peptides. The IC_{50} values were 20 μM for α181 18-mer and 24 μM for both 12-mers (Fig. 3 and Table 3). Binding of BTX to all three peptides was also competed with *d*TC and high concentrations of NaCl.

Because of low signals or high variability of signal, the ability of unlabeled BTX to compete the binding of ^{125}I-BTX to the α173 8-mer, α179 14-mer, α186 11-mer, and α194 11-mer was not tested. Instead, another solid phase assay was developed to test whether these smaller peptides possessed BTX-binding determinants and to compare affinities of all peptides with the same assay. Peptides were tested for their ability to compete with the binding of ^{125}I-AChR to immobilized BTX. This assay is similar to one in which the ability of antibodies to block binding of ^{125}I-AChR to immobilized antibodies is tested (23). ^{125}I-AChR readily binds to BTX under these conditions and unlabeled AChR competes this binding with an IC_{50} value of 3.2 nM (Fig. 4 and Table 4). In contrast, no inhibition of ^{125}I-AChR binding occurs when β-Asp-angiotensin II, a 10-amino acid peptide, is used as a competitor, demonstrating that the inhibition seen with unlabeled AChR is specific.

All the synthetic peptides as well as BTX and isolated α subunit were tested for ability to inhibit ^{125}I-AChR binding. Because contaminating substances in the crude peptide mixtures were found to inhibit ^{125}I-AChR binding, the synthetic peptides used in these experiments were purified by reverse-phase HPLC, lyophilized, and resuspended in distilled water. BTX was found to compete with binding of labeled AChR with an IC_{50} value of 3.5×10^{-10} M, isolated α subunit with an IC_{50} of 1.2×10^{-8} M, and α173 32-mer with an IC_{50} of 1.4×10^{-7} M. Four of the peptides tested, the α181 18-mer, α185 12-mer, α186 11-mer, and α179 14-mer, were all nearly equipotent in competing with the binding of ^{125}I-AChR (IC_{50} 10–25 μM; Fig. 4 and Table 4), indicating that these peptides had determinants recognized by BTX. The α173 8-mer and α194 11-mer reduced binding of ^{125}I-AChR at very high concentrations (Fig. 4), indicating these peptides possess weak determinants. This finding is consistent with the observation that ^{125}I-BTX binding to these two peptides could not be detected in the solid phase assay.

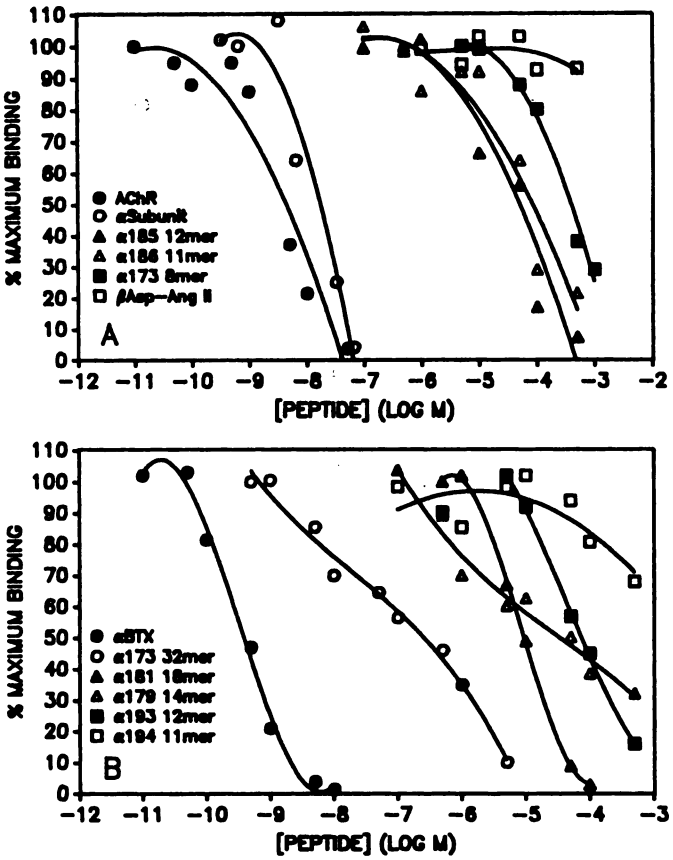

Fig. 4. Competition of ^{125}I-AChR binding to BTX. Wells of microtiter plates were coated with 1 μg of BTX. After quenching, the wells were incubated for 15 min with 350,000–600,000 cpm ^{125}I-AChR and various amounts of unlabeled AChR (A; ●), α subunit (A; ○), α185 12-mer (A; ▲), α186 11-mer (A; △), α173 8-mer (A; ■), β-Asp-angiotensin II (A; □), αBTX (B; ●), α173 32-mer (B; ○), α181 18-mer (B; ▲), α179 14-mer (B; △), α193 12-mer (B; ■), or α194 11-mer (B; □). The wells were then washed and bound radioactivity was determined. Each *point* represents the average of triplicate determinations. Each *curve* represents one of two experiments performed.

Discussion

These studies have characterized the binding of BTX to a synthetic peptide comprising residues 173–204 of the α subunit

TABLE 3

Competition of ^{125}I-BTX binding to peptides

Wells of microtiter plates were coated with 2 μg of Tα193 12-mer and 5 μg of the other peptides by evaporation. After quenching, the wells were incubated with 3–4 nM ^{125}I-BTX and various amounts of competitor for 20 min. The wells were then washed and bound radioactivity was determined. The IC_{50} values were calculated from logit-log plots of the competition data. Each IC_{50} value represents the average of one experiment with three replicates each, except for those where the standard deviation is shown in parentheses, which are the average of the two experiments.

Peptide	IC_{50}		
	BTX	*d*TC	NaCl
	M		
α Subunit	4.6×10^{-8}		
α173 32-mer	4.2×10^{-8} (1.3)	8.6×10^{-5} (0.5)	1.6×10^{-2}
α181 18-mer	2.0×10^{-5} (1.2)	1.6×10^{-4}	1.7×10^{-2}
α185 12-mer	2.4×10^{-5} (2.0)	1.3×10^{-4}	3.3×10^{-2}
α193 12-mer	2.4×10^{-5} (2.0)	1.0×10^{-4}	1.3×10^{-2}

TABLE 4

Competition of ^{125}I-AChR binding to BTX by peptides

Wells of microtiter plates were coated with 1 μg of BTX. After quenching, the wells were incubated for 15 min with 350,000–600,000 cpm ^{125}I-AChR and increasing concentrations of the listed proteins or peptides. The wells were then washed and bound radioactivity determined. The IC_{50} values were calculated from logit-log plots of the competition data. Each IC_{50} value represents the average of two experiments with three replicates.

Peptide	IC_{50}
	M
α-BTX	3.5×10^{-10}
AChR	3.2×10^{-9}
α Subunit	1.2×10^{-8}
α173 32-mer	1.4×10^{-7}
α181 18-mer	9.3×10^{-6}
α179 14-mer	1.7×10^{-5}
α185 12-mer	1.3×10^{-5}
α186 11-mer	2.2×10^{-5}
α193 12-mer	8.7×10^{-5}
α173 8-mer	4.0×10^{-4}
α194 11-mer	$>5.0 \times 10^{-4}$

of the AChR and suggest that this region represents a major determinant of toxin binding in the intact receptor. The competition studies with antagonists (Fig. 1 and Table 2) demonstrate that the antagonist site and the rank order of antagonist potency are preserved, albeit with affinities some 100- to 1000-fold less than observed for the intact AChR. These affinities are, however, comparable to previously reported values for the intact isolated α subunit (19, 24–26) and as determined for BTX with the two assays used in the present study (4.6×10^{-8} M and 1.2×10^{-8} M). The reduced affinity of isolated α subunit and the α173 32-mer could be the result of a loss of the native conformation of the binding region in the denatured protein. It is also possible that, in the native receptor, additional residues of the α subunit or even of other subunits contribute to the neurotoxin-binding site. These residues could be widely separated on the linear sequence of the α subunit but brought into proximity through folding of the native protein. It has been demonstrated that the isolated α subunit can achieve as high an affinity for BTX as the intact receptor, possibly as the result of a change in conformation (27).

The ability of the α173 32-mer to bind BTX is consistent with the findings of others that have localized BTX-binding determinants to the region encompassing Cys 192 and Cys 193 (12, 13, 15). McCormick and Atassi (28) reported that a synthetic peptide corresponding to residues 125–147 also bound ^{125}I-BTX. It is possible that this region binds BTX with low affinity and represents another portion of the BTX-binding site. Studies with antibodies to peptides comprising portions of this region, however, argue against this view (9, 29, 30). In addition, Neumann *et al.* (14) reported that BTX did not bind to a peptide corresponding to residues 126–143, and Ralston *et al.* (15) did not observe binding to peptide 127–143. Based on cobratoxin binding to overlapping α subunit synthetic peptides, Mulac-Jericevic and Atassi (31) localized five toxin-binding regions within residues 1–10, 32–41, 100–115, 122–150, and 182–198, with the latter exhibiting the highest binding activity. It is possible that the additional regions represent sites with which toxin comes into contact in the native, folded α subunit, but not in denatured α subunit.

Also in agreement with studies with isolated α subunit (26, 27) are the results with cholinergic agonists (Fig. 2 and Table 2) that demonstrate the lack of an agonist-specific ability to compete BTX binding. Agonists do compete the binding of ^{125}I-BTX with IC_{50} values in the expected millimolar concentration range, given the decrease in affinity observed with cholinergic antagonists. However, all rank order of potency is lost. Suberyldicholine is no more effective a competitor than the acetylcholine precursor choline, which is a poor agonist. The loss of agonist-specific competition of BTX binding could be explained by 1) the absence of elements of the agonist binding site in the α173 32-mer sequence or 2) the presence of the elements of the agonist binding site in the α173 32-mer sequence but in a conformation that is not capable of high affinity binding. The first explanation is unlikely because elements of the BTX and antagonist sites are present in the α173–204 32-mer. These sites are presumably much larger than the agonist site, and it is difficult to envision a small agonist site far removed from these larger sites that still display binding behavior consistent with direct competition. In addition, the sequence 179–207 is labeled by the affinity alkylating agents MBTA (6) and 4-(*N*-maleimido)phenyltrimethylammonium iodide and the cholinergic affinity ligand DDF (7). In the context of the second explanation, it is worth noting that both BTX and dTC are relatively large and rigid molecules and, therefore, might be able to induce renaturation of their respective binding sites on the α173 32-mer by stabilizing the appropriate conformation necessary for binding, whereas the small and relatively flexible agonists may not. This would explain why agonists exhibit no differences in their ability to compete the binding of ^{125}I-BTX even though the elements of the agonist site are most likely present in the α173 32-mer sequence.

It was of interest to determine whether smaller peptides with sequences representing portions of the α173 32-mer sequence would still possess toxin-binding determinants and to compare affinities of toxin binding with the 32-mer. The major finding from these experiments is that smaller peptides are competent to bind ^{125}I-BTX but at considerably lower affinities, indicating they do not contain all of the determinants of the BTX site detected on the α173 32-mer. When BTX affinity was measured by direct competition, two of the peptides, α185 12-mer and α193 12-mer, exhibited IC_{50} values of 24 μM, approximately 570-fold less than that measured for the α173 32-mer (42 nM). The affinity of BTX for the α185 12-mer agrees with that observed by Neumann *et al.* (14) (20 μM) for this peptide. When tested for direct binding of ^{125}I-BTX, all *Torpedo* α subunit peptides except the α173 8-mer and α194 11-mer exhibited binding above background levels. Ralston *et al.* (15) also reported that peptide 194–212 did not bind BTX. The α193 12-mer, in particular, bound high amounts of BTX/μmol of peptide. Competition studies with unlabeled BTX, however, indicate that the magnitude of the signal is a poor indicator of affinity because the IC_{50} value for unlabeled BTX for the α193 12-mer was no different than that for the α185 12-mer, even though more ^{125}I-BTX bound to the α193 12-mer than to the α185 12-mer under these assay conditions. Because the amount of immobilized peptide is not known, one possibility for differences in signals is that some peptides are retained much better onto the microwell plates than others. Therefore, in order to accurately compare toxin-binding to different peptides, it is necessary to measure affinities.

Not all peptides with BTX-binding determinants can be examined by direct binding studies. Four of the peptides reported here, the α173 8-mer, α179 14-mer, α186 11-mer, and α194 11-mer, gave signals that were either too low or too variable for the accurate determination of IC_{50} values of competition of ^{125}I-BTX binding. Because the lack of signal could be due to insufficient immobilization of peptides, another assay was developed to measure their affinity for BTX. In this assay, ^{125}I-AChR binding to immobilized BTX was measured, and the ability of BTX, AChR, α subunit, and synthetic peptides to compete with ^{125}I-AChR binding was tested. Four of the peptides, the α179 14-mer, α181 18-mer, α185 12-mer, and α186 11-mer competed with labeled receptor binding at similar concentrations ($\sim 10^{-5}$ M compared with 1.4×10^{-7} M for the α173 32-mer), indicating these peptides possess some but not all of the determinants of the BTX binding site. Two of the peptides, the α173 8-mer and α194 11-mer, however, competed with ^{125}I-AChR binding partially, and only at high concentrations, indicating the presence of weak BTX-binding determinants. This is consistent with the lack of signal seen in the direct binding assay and serves to further validate the utility of the ^{125}I-AChR binding assay in detecting BTX-binding determinants on the

synthetic peptides that might otherwise be missed. These findings indicate that toxin binding determinants are distributed throughout the 32-mer but that those with the highest affinity are located in the central region of the sequence between residues 179 and 196.

Similar affinities were observed for most substances that could be tested with both solid phase assays. The observed affinity of BTX was higher in the assay in which ^{125}I-AChR binding to BTX is competed (3.5×10^{-10} M) than in an assay in which ^{125}I-BTX binding to immobilized AChR was competed (4 nM; Ref. 32). This difference could be because in the former assay undenatured AChR and BTX can interact in solution, whereas in the latter labeled and unlabeled BTX compete for binding to AChR that may be partially denatured as a result of adsorption to plastic. The affinity of BTX for the 32-mer was lower (1.4×10^{-7} M) in the assay in which ^{125}I-AChR binding to BTX is competed than the assay in which ^{125}I-BTX binding to the 32-mer is competed (4.2×10^{-8} M), although this difference may not be significant.

It is interesting that the addition of Cys 193 to the sequence of the α194 11-mer, which makes the α193 12-mer, changes a peptide with weak determinants into one with a measurable affinity for BTX. In the intact AChR, the cysteine residues seem relatively unimportant for BTX binding. Reduction with dithiothreitol did not change the number of BTX sites or the initial rate of toxin binding to the AChR (33). Reduction followed by alkylation with *N*-ethylmaleimide reduced the initial rate but not the number of BTX molecules bound. These findings suggest that Cys 193 itself is not recognized by BTX. On the other hand, reduction of the α185–196 peptide with 2-mercaptoethanol followed by carboxymethylation by iodoacetamide reduced the toxin binding capacity of the peptide (14). Similarly, dithiothreitol reduction and alkylation by iodoacetamide of an α172–205 peptide abolished toxin binding (15). These findings indicate that an intact disulfide between Cys 192 and 193 is necessary for toxin binding to peptides, although a disulfide bond between these residues has not been demonstrated in the synthetic peptides. Alternatively, it is possible that Cys 192 and 193 stabilize a conformation of the peptide that is conducive to BTX binding. The peptides used in the present study were isolated in the reduced form. Investigation of shift in motility by HPLC of the α185 12-mer after alkylation with *N*-ethylmaleimide demonstrated that the peptide exists primarily (90%) in the reduced state (data not shown).

These studies have documented the utility of synthetic peptides as a means of reconstituting interesting functional domains of proteins, in this instance, a ligand-binding site. The results reported here demonstrate that a 32-amino acid peptide, the α173 32-mer, binds BTX in a fashion very similar to that observed with a 437-amino acid protein, the α subunit. The results with the shorter synthetic peptides indicate that determinants of BTX binding are scattered throughout the sequence of the α173 32-mer. Although higher affinity determinants are located in the central region of the 32-mer, no single high affinity "core" of BTX-binding determinants was identified. This is not surprising, considering that the surface of the α subunit that comes into contact with BTX must be to a good extent structurally and chemically complementary to the surface of the BTX molecule (4). It has been shown repeatedly that no one area of the neurotoxin molecule is essential for binding or toxicity, indicating that more global characteristics of the neurotoxin molecule are important in the binding event. It is, therefore, to be expected that the toxin-binding region of the α subunit should, to some extent, share the same characteristic.

References

1. Karlin, A. Molecular properties of the nicotinic acetylcholine receptor, in *The Cell Surface and Neuronal Function* (C. W. Cotman, G. Poste, and G. L. Nicholson, eds.) Elsevier/North Holland, New York, 191–260 (1980).
2. Kubo, T., M. Noda, T. Takai, T. Tanabe, T. Kayano, S. Shimizu, K.-I. Tanaka, H. Takahashi, T. Hirose, S. Inayama, R. Kikuno, T. Miyata, and S. Numa. Primary structure of δ subunit precursor of calf muscle acetylcholine receptor deduced from cDNA sequence. *Eur. J. Biochem.* **149**:5–13 (1985).
3. Karlsson, E. Chemistry of protein toxins in snake venoms. *Handb. Exp. Pharmacol.* **52**:159–212 (1979).
4. Lentz, T. L., and P. T. Wilson. Neurotoxin-binding site on the acetylcholine receptor. *Int. Rev. Neurobiol.* **29**:117–160 (1988).
5. Kao, P. N., and A. Karlin. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *J. Biol. Chem.* **261**:8085–8088 (1986).
6. Kao, P. N., A. J. Dwork, R.-R. J. Kaldany, M. L. Silver, J. Wideman, S. Stein, and A. Karlin. Identification of the α subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site. *J. Biol. Chem.* **259**:11662–11665 (1984).
7. Dennis, M., J. Giraudat, F. Kotzyba-Hibert, M. Goeldner, C. Hirth, J.-Y. Chang, and J.-P. Changeux. A photoaffinity ligand of the acetylcholine-binding site predominantly labels the region 179–207 of the α-subunit on native acetylcholine receptor from *Torpedo marmorata*. *FEBS Lett.* **207**:243–249 (1986).
8. 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* **82**:8790–8794 (1985).
9. Neumann, D., J. M. Gershoni, M. Fridkin, and S. Fuchs. Antibodies to synthetic peptides as probes for the binding site on the α subunit of the acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **82**:3490–3493 (1985).
10. Oblas, B., R. H. Singer, and N. D. Boyd. Location of a polypeptide sequence within the α-subunit of the acetylcholine receptor containing the cholinergic binding site. *Mol. Pharmacol.* **29**:649–656 (1986).
11. Pedersen, S. E., E. B. Dreyer, and J. B. Cohen. Location of ligand-binding sites on the nicotinic acetylcholine receptor α subunit. *J. Biol. Chem.* **261**:13735–13743 (1986).
12. Mulac-Jericevic, B., and M. Z. Atassi. Segment α182–198 of *Torpedo californica* acetylcholine receptor contains a second toxin-binding region and binds anti-receptor antibodies. *FEBS Lett.* **199**:68–74 (1986).
13. Neumann, D., D. Barchan, A. Safran, J. M. Gershoni, and S. Fuchs. Mapping of the α-bungarotoxin binding site within the α subunit of the acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **83**:3008–3011 (1986).
14. Neumann, D., D. Barchan, M. Fridkin, and S. Fuchs. Analysis of ligand binding to the synthetic dodecapeptide 185–196 of the acetylcholine receptor α subunit. *Proc. Natl. Acad. Sci. USA* **83**:9250–9253 (1986).
15. Ralston, S., V. Sarin, H. L. Thanh, J. Rivier, J. L. Fox, and J. Lindstrom. Synthetic peptides used to locate the α-bungarotoxin binding site and immunogenic regions on α subunits of the nicotinic acetylcholine receptor. *Biochemistry* **26**:3261–3266 (1987).
16. Barkas, T., A. Mauron, B. Roth, C. Alliod, S. J. Tzartos, and M. Ballivet. Mapping the main immunogenic region and toxin-binding site of the nicotinic acetylcholine receptor. *Science (Wash., D. C.)* **235**:77–80 (1987).
17. Gershoni, J. M. Expression of the α-bungarotoxin binding site of the nicotinic acetylcholine receptor by *Escherichia coli* transformants. *Proc. Natl. Acad. Sci. USA* **84**:4318–4321 (1987).
18. House, C., and B. E. Kemp. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science (Wash., D. C.)* **238**:1726–1728 (1987).
19. 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).
20. Wang, G.-K., and J. Schmidt. Primary structure and binding properties of iodinated derivatives of α-bungarotoxin. *J. Biol. Chem.* **255**:11156–11162 (1980).
21. Rodbard, D., and G. R. Frazier. Statistical analysis of radioligand assay data. *Methods Enzymol.* **37**:3–22 (1975).
22. Jemmerson, R. Antigenicity and native structure of globular proteins: low frequency of peptide reactive antibodies. *Proc. Natl. Acad. Sci. USA* **84**:9180–9184 (1987).
23. Dowding, A. J., and Z. W. Hall. Monoclonal antibodies specific for each of the two toxin-binding sites of *Torpedo* acetylcholine receptor. *Biochemistry* **26**:6372–6381 (1987).
24. Haggerty, J. C., and S. C. Froehner. Restoration of ^{125}I-α-bungarotoxin binding activity to the α subunit of *Torpedo* acetylcholine receptor isolated by gel electrophoresis in sodium dodecyl sulfate. *J. Biol. Chem.* **256**:8294–8297 (1981).

25. Oblas, B., N. D. Boyd, and R. H. Singer. Analysis of receptor-ligand interactions using nitrocellulose gel transfer: application to *Torpedo* acetylcholine receptor and alpha-bungarotoxin. *Anal. Biochem.* **130**:1–8 (1983).
26. Tzartos, S. J., and J.-P. Changeux. High affinity binding of α-bungarotoxin to the purified α-subunit and to its 27000-dalton proteolytic peptide from *Torpedo marmorata* acetylcholine receptor. Requirement for sodium dodecyl sulfate. *EMBO J.* **2**:381–387 (1983).
27. Blount, P., and J. P. Merlie. Native folding of an acetylcholine receptor α subunit expressed in the absence of other receptor subunits. *J. Biol. Chem.* **263**:1072–1080 (1988).
28. McCormick, D. J., and M. Z. Atassi. Localization and synthesis of the acetylcholine-binding site in the α-chain of the *Torpedo californica* acetylcholine receptor. *Biochem. J.* **224**:995–1000 (1984).
29. Plumer, R., G. Fels, and A. Maelicke. Antibodies against preselected peptides to map functional sites on the acetylcholine receptor. *FEBS Lett.* **178**:204–208 (1984).
30. Criado, M., V. Sarin, J. L. Fox, and J. Lindstrom. Evidence that the acetylcholine binding site is not formed by the sequence α127–143 of the acetylcholine receptor. *Biochemistry* **25**:2839–2846 (1986).
31. Mulac-Jericevic, B., and M. Z. Atassi. α-Neurotoxin binding to acetylcholine receptor: localization of the full profile of the cobratoxin-binding regions on the α-chain of *Torpedo californica* acetylcholine receptor by a comprehensive synthetic strategy. *J. Protein Chem.* **6**:365–373 (1987).
32. Lentz, T. L., E. Hawrot, and P. T. Wilson. Synthetic peptides corresponding to sequences of snake venom neurotoxins and rabies virus glycoprotein bind to the nicotinic acetylcholine receptor. *Proteins Struct. Funct. Genet.* **2**:298–307 (1987).
33. Walker, J. W., R. J. Lukas, and M. G. McNamee. Effects of thio-group modifications on the ion permeability control and ligand binding properties of *Torpedo californica* acetylcholine receptor. *Biochemistry* **20**:2191–2199 (1981).

Send reprint requests to: Dr. Thomas L. Lentz, Department of Cell Biology, Yale University School of Medicine, 333 Cedar St., P. O. Box 3333, New Haven, CT 06510.