Binding of α-Bungarotoxin to Synthetic Peptides Corresponding to Residues 173–204 of the α Subunit of *Torpedo*, Calf, and Human Acetylcholine Receptor and Restoration of High-Affinity Binding by Sodium Dodecyl Sulfate†

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ABSTRACT: In order to investigate structure–function relationships of a segment of the acetylcholine receptor α subunit, binding of α-bungarotoxin to synthetic peptides corresponding to residues 173–204 of *Torpedo*, calf, and human α subunits was compared using a solid-phase radioassay. The affinities of ^{125}I-α-bungarotoxin for the calf and human peptides were 15- and 150-fold less, respectively, than for the *Torpedo* peptide. On the basis of nonconservative substitutions in the calf and human sequences, aromatic residues (Tyr-181, Trp-187, and Tyr-189) are important for the higher affinity binding of the *Torpedo* peptide. Substitution of negatively charged Glu-180 with uncharged Gln in the calf peptide did not significantly affect toxin binding, indicating Glu-180 alone does not comprise the anionic subsite on the receptor to which the cationic quaternary ammonium groups of cholinergic agents bind. *d*-Tubocurarine competed toxin binding to the modified calf 32-mer which lacks Glu-180 and Asp-195 present in *Torpedo*. Thus, the negative subsite could be formed by another negatively charged residue or by more than one amino acid side chain. It is possible that the positive charges on cholinergic ligands are countered by a negative electrostatic potential provided by polar groups, such as the hydroxyl group of tyrosine, present on several residues in this region, and the negative charges present on any of residues 175, 180, 195, or 200. Equilibrium saturation binding of α-bungarotoxin to *Torpedo* peptide 173–204 revealed a minor binding component with an apparent K_D of 4.2 nM and a major component with a K_D of 63 nM. In the presence of 0.01% sodium dodecyl sulfate, one binding component with a K_D of 7.8 nM was detected. This compares with an affinity of K_D = 0.41 nM for toxin binding to native acetylcholine receptor in the solid-phase assay. Sodium dodecyl sulfate may stabilize a conformation of the peptide that is conducive to high-affinity binding.

Synthetic peptides have proven useful in localizing functional domains on the primary sequences of the subunits of the nicotinic acetylcholine receptor (AChR).[1] Several laboratories have investigated the binding of the snake venom neurotoxin α-bungarotoxin (BTX) to synthetic peptides of the α subunit of the AChR. The neurotoxin binding site appears to include the acetylcholine binding site because affinity alkylating agents such as [4-(*N*-maleimido)benzyl]trimethylammonium iodide (MBTA) and bromoacetylcholine that act as either cholinergic agonists or competitive antagonists are competitive with the binding of neurotoxins (Karlin, 1980). α-Bungarotoxin has been reported to bind to synthetic peptides corresponding to α-subunit residues 173–204 (Wilson et al., 1985), 182–198 (Mulac-Jericevic & Atassi, 1986), 185–196 (Neumann et al., 1986a,b), and 172–205 and 185–199 (Ralston et al., 1987). In addition, a cyanogen bromide fragment comprising residues 179–207 is labeled by affinity alkylating agents (Kao et al., 1984; Dennis et al., 1986) and the cholinergic photoaffinity ligand *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate (Dennis et al., 1986). Both MBTA (Kao et al., 1984) and [4-(*N*-maleimido)phenyl]trimethylammonium iodide (Dennis et al., 1986) labeled Cys-192 and Cys-193 within this fragment. Thus, these findings indicate that a major determinant for neurotoxin binding is located on the linear sequence of the α subunit in proximity to Cys-192 and -193. The possibility that toxin comes into contact with other regions in the native, folded α subunit is not ruled out (Mulac-Jericevic & Atassi, 1987).

In a previous report (Wilson et al., 1985), it was demonstrated that a 32 amino acid synthetic peptide comprising residues 173–204 of *Torpedo* AChR α subunit (Tα173 32-mer) bound BTX in a fashion that was indistinguishable from that seen with the intact, denatured α subunit. This region of the α subunit contains an unusually high number of hydrophobic and aromatic residues as well as many charged and polar residues. Comparison of the toxin binding activity of receptor peptides in which modifications have been introduced, either by synthetical methods or by site-directed mutagenesis, should allow structure–function relationships of this region to be elucidated.

In this paper, we report the results of studies employing synthetic peptides representing the sequences of the human and calf α subunits corresponding to the Tα173 32-mer. Both sequences show six substitutions between the *Torpedo* sequence. In the calf sequence, two of the changes are nonconservative, and in the human, three are nonconservative. Thus, any differences in the toxin binding activity of these

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[1] Abbreviations: AChR, acetylcholine receptor; ACN, acetonitrile; BSA, bovine serum albumin; BTX, α-bungarotoxin; CMC, critical micelle concentration; dTC, *d*-tubocurarine; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium iodide; PB, phosphate buffer; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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peptides will allow the function of the substituted residues in binding to be assessed. In addition, a modified calf peptide in which negatively charged Glu-180 was substituted with uncharged glutamine was synthesized. The rationale behind this synthesis was to test the possibility that Glu-180 represents the anionic subsite on the receptor to which the quaternary ammonium group of acetylcholine or the guanidinium group of neurotoxin Arg-37 binds (Wilson et al., 1985). Of the four negatively charged residues in the Tα173 32-mer, the charge at residue 195 is not conserved in α subunits from other species (Lentz & Wilson, 1988). The negatively charged residues at positions 175 and 200 are also present on at least two of the three other subunits of *Torpedo* AChR. Thus, only the negative charge at residue 180 is unique to the α subunit in this region. The results of toxin binding to the 32-mers indicate that aromatic residues are important in BTX binding and that Glu-180 by itself does not comprise the anionic subsite.

In addition, we report the effects of sodium dodecyl sulfate (SDS) on the binding of BTX to these peptides as well as to synthetic peptides representing sequences from the *Torpedo* α subunit. The affinity of toxin binding to the Tα173 32-mer is enhanced by SDS. The increased affinity of BTX for the 32-mer in the presence of SDS (7.8 nM) further supports the functional significance of this region of the α subunit in neurotoxin binding.

Materials and Methods

Iodination of BTX. α-Bungarotoxin (Miami Serpentarium, Salt Lake City, UT) was iodinated by the chloramine T method as described by Wang and Schmidt (1980). The monoiodinated BTX was separated from the diiodinated BTX by ion-exchange chromatography on a CM-25 column. The initial specific activity of the monoiodinated BTX was greater than 600 cpm/fmol.

Binding of ^{125}I-BTX to Purified AChR. The AChR was affinity purified from frozen electric organ tissue of *Torpedo californica* (Pacific Biomarine, Venice, CA) on a cobratoxin–Sepharose column as described (Gershoni et al., 1983). The receptor preparations routinely bound 7.5 nmol of ^{125}I-BTX/mg of protein and contained the four receptor subunits on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Binding of ^{125}I-BTX to the receptor was measured in a solid-phase radioassay in which the receptor was adsorbed to the wells of 96-well, polystyrene microtiter plates (Nunc, USA/Scientific Plastics). Receptor was diluted into 0.015 M Na_2CO_3/0.035 M $NaHCO_3$, pH 9.6 (coating buffer), to a concentration of 1 μg/mL. Wells were then inoculated with 100 μL of receptor solution (0.1 μg), and the receptor was allowed to adsorb to the plastic overnight at 4 °C. Wells were then washed 3 times with 10 mM phosphate buffer (PB)/0.15 M NaCl, pH 7.4 (PBS), to remove unadsorbed material. To reduce nonspecific binding of toxin to plastic, wells were quenched with 2% bovine serum albumin (BSA, fraction V; Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. To measure equilibrium binding of BTX to the receptor, 100 μL of increasing concentrations of ^{125}I-BTX in 10 mM PB, pH 7.4, containing 0.2% BSA (PB/BSA) was added and incubated for 5 h at room temperature. Other wells were incubated with the same concentrations of ^{125}I-BTX and a 200-fold excess of cold BTX. At the end of the incubation period, wells were washed 4 times with PBS. Bound ^{125}I-BTX was removed from the wells by adding 200 μL of 0.25 N NaOH containing 2.5% SDS (NaOH/SDS). After 2–5 min, the solution was removed from each well with a pipet and placed in a γ vial or tube. This procedure was repeated twice. Alternatively, labeled material can be removed by adding 100 μL of NaOH/SDS and swabbing each well twice with cotton-tipped applicators and placing them in a tube. Radioactivity was measured in a γ counter.

Table I: Amino Acid Sequences of the *Torpedo* (T), Human (H), Calf (C), Modified Calf (mC), and Rat Nerve (RN) Synthetic Peptides[a]

	175	180	185	190	195	200
Tα173 32mer	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					
Hα173 32mer	S G E W V I K E **S** R G W K H **S** V **T** Y S C C P D T P Y L D I T Y H					
Cα173 32mer	S G E W V I K E **S** R G W K H W V F Y A C C P **S** T P Y L D I T Y H					
mCα173 32mer	S G E W V I K **Q** **S** R G W K H W V F Y A C C P **S** T P Y L D I T Y H					
Tα181 18mer	Y R G W K H W V Y Y T C C P D T P Y					
Tα179 14mer	K D Y R G W K H W V Y Y T C					
Tα185 12mer	K H W V Y Y T C C P D T					
Tα186 11mer	H W V Y Y T C C P D T					
Tα194 11mer	P D T P Y L D I T Y H					
RNα179 14mer	I K A P G Y K H E I K Y N C					

[a] Amino acid residues in the calf and human sequences that represent nonconservative changes from the *Torpedo* sequence and the modification in the mCα173 32-mer sequence (glutamate-180 changed to glutamine-180) are indicated in bold, underlined script.

Synthetic Peptides. All synthetic 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 are designated by their species, position on the α subunit of the first residue, and the total number of residues (Table I). The integrity of the peptide sequences was determined by amino acid composition analysis and by reverse-phase, high-pressure liquid chromatography. Peptides were suspended to 5 mg/mL in 50% acetonitrile (ACN)/water containing 0.005% trifluoroacetic acid (TFA) before use.

Binding of ^{125}I-BTX to Synthetic Peptides. α-Bungarotoxin binding to synthetic peptides was measured by using the solid-phase assay. Peptides in ACN/water and 0.005% TFA were diluted into coating buffer to a concentration of 50 μg/mL. Wells were then incubated with 100 μL (5 μg) of peptide solution overnight at room temperature. The solution was then aspirated out of the wells. Alternatively, wells can be coated by evaporation of a solution containing peptide. Peptides 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 °C oven until all liquid had evaporated. This procedure results in a greater amount of peptide adsorbed to the plastic. Wells were then quenched with 200 μL of 2% BSA for 1 h at room temperature. Following removal of the BSA solution, the wells were incubated with ^{125}I-BTX in PB/BSA. For some experiments, SDS was included in the incubation medium. Wells were washed 4 times with 200 μL of PB/BSA. Bound ^{125}I-BTX was removed from the wells and radioactivity measured. In competition experiments, the competitor was added to the ^{125}I-BTX solution prior to incubation with the 32-mer. The incubation time for all competition experiments was 20 min. Measurement of the rate of binding of ^{125}I-BTX to the 32-mer indicated that binding was linear to times up to 3 h (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 (Rodbard & Frazier, 1975). Competition curves are graphically represented by fitted curves derived from a nonlinear polynomial least-squares fit as determined by the computer

Table II: Competition of ^{125}I-BTX Binding to *Torpedo*, Human, and Calf 32-mers[a]

peptide	IC_{50} (M) BTX	dTC	NaCl
Tα173 32-mer	4.2×10^{-8} (1.3)	8.6×10^{-5} (0.5)	16×10^{-3}
Cα173 32-mer	6.4×10^{-7} (0.9)	2.5×10^{-4} (1.1)	48×10^{-3}
mCα173 32-mer	4.0×10^{-7} (2.0)	5.8×10^{-4} (1.0)	83×10^{-3}
Hα173 32-mer	6.4×10^{-6} (1.2)	4.8×10^{-5} (0.5)	24×10^{-3}

[a] Wells were coated with 5 μg of the various 32-mers. After quenching, the wells were incubated with 3–4 nM ^{125}I-BTX and various amounts of unlabeled BTX, dTC, or NaCl for 20 min. The wells were then washed, and bound radioactivity was determined. Each IC_{50} value with a standard deviation represents the average of two experiments with three replicates each. Standard deviations are in parentheses.

program Sigmaplot (Jandel Scientific, Sausalito, CA). Both ^{125}I-BTX and unlabeled competitor were added simultaneously.

Equilibrium Binding. Equilibrium binding experiments were performed in the absence or presence of 0.01% SDS in PB/BSA in the solid-phase assay system described above. Wells of a 96-well polystyrene microtiter plate coated with peptide were incubated with various concentrations of ^{125}I-BTX for 10 h. After incubation, an aliquot of the ^{125}I-BTX solution was removed and counted in a γ counter to determine the amount of free ^{125}I-BTX. The wells were then washed 4 times with 10 mM PB, pH 7.4, and the amount of bound ^{125}I-BTX was determined as described. Equilibrium binding data were analyzed in Scatchard plots. Binding curves were fitted by linear least-squares analysis. Nonlinear curves were resolved into two linear binding curves by the limiting slope method of Hunston (1975). In the case of the Tα181 18-mer and Tα185 12-mer where high concentrations of BTX were required to achieve saturation, the curves were corrected for nonspecific binding of ^{125}I-BTX as described by Chamness and McGuire (1975).

Results

A solid-phase radioassay was used to measure binding of ^{125}I-BTX to synthetic peptides adsorbed to wells of polystyrene microtiter plates. The solid-phase assay is a useful and convenient method for performing binding assays because it requires little material and several replicates are easily performed simultaneously. In the case of peptides, it is not necessary to utilize a residue that might be important for toxin binding in order to covalently couple the peptide to a supporting matrix. Labeled toxin apparently has little affinity for the plastic as background binding to quenched wells lacking receptor is negligible (~100 cpm).

In order to directly compare the affinity of BTX for the synthetic peptides with that for native receptor, the equilibrium binding of ^{125}I-BTX to affinity-purified *Torpedo* AChR was measured by using the solid-phase radioassay. Saturable binding of ^{125}I-BTX to receptor adsorbed to plastic was obtained (Figure 1). Scatchard analysis of the equilibrium binding data yielded a dissociation constant (K_D) of 4.1×10^{-10} M (Figure 1).

In order to compare BTX binding to the *Torpedo* toxin binding peptide with binding to mammalian peptides, the sequences in the human (Hα173 32-mer), calf (Cα173 32-mer), and a modified calf (mCα173 32-mer) α subunits corresponding to the Tα173 32-mer were synthesized (see Table I for sequences). These peptides were examined for their ability to bind ^{125}I-BTX in the solid-phase assay. Affinity of toxin binding, as determined from IC_{50} values, was greatest for the Tα173 32-mer. The binding of ^{125}I-BTX could be

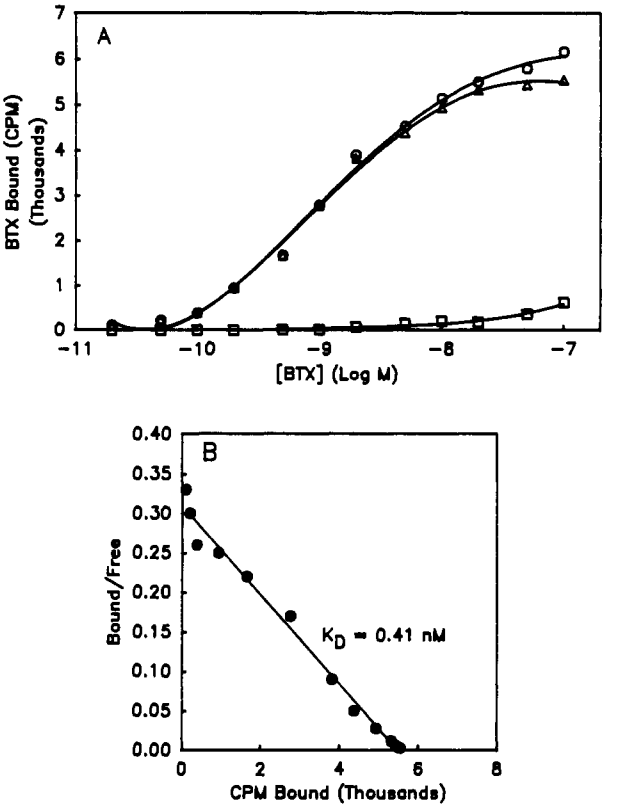

FIGURE 1: Saturation of ^{125}I-BTX binding to AChR using a solid-phase assay. (A) Binding as a function of BTX concentration. Wells of microtiter plates were coated with 0.1 μg of purified AChR in coating buffer overnight at 4 °C. Wells were washed, quenched with 2% BSA for 1 h, washed, and incubated with increasing concentrations (2×10^{-11} M to 1×10^{-7} M) of ^{125}I-BTX for 5 h. Wells were washed, and bound radioactivity was removed and counted in a γ counter. Nonspecific binding (squares) is binding of ^{125}I-BTX in the presence of a 200-fold excess of cold BTX. This value was subtracted from the total radioactivity bound (circles) to determine the amount specifically bound (triangles). Values are the mean of three replicates. (B) Scatchard analysis of the equilibrium binding data. cpm bound toxin/free toxin is plotted versus cpm bound toxin. The specific activity of ^{125}I-BTX was 154 cpm/fmol.

competed by unlabeled BTX to all 32-mers with IC_{50} values of 0.04 μM for the Tα173 32-mer, 0.64 μM for the Cα173 32-mer, 0.40 μM for the mCα173 32-mer, and 6.4 μM for the Hα173 32-mer (Figure 2, Table II). In addition, *d*-tubocurarine (dTC) competed the binding of ^{125}I-BTX to these peptides with IC_{50} values of 86 μM for the Tα173 32-mer, 250 μM for the Cα173 32-mer, 580 μM for the mCα173 32-mer, and 48 μM for the Hα173 32-mer (Figure 2, Table II). NaCl also competed the binding of BTX to all of the 32-mers at millimolar concentrations.

It had been previously reported that low concentrations of SDS enhanced the binding of BTX to isolated α subunit (Tzartos & Changeux, 1983). Since the Tα173 32-mer bound BTX in a fashion indistinguishable from that observed with the intact isolated α subunit (Wilson et al., 1985), it was of interest to determine if SDS had a similar effect on the binding of BTX to Tα173 32-mer. Therefore, the effect of SDS on the binding of ^{125}I-BTX to the Tα173 32-mer in the solid-phase assay was tested. SDS enhanced the binding of BTX to Tα173 32-mer (Figure 3). The effect was strongly dependent on the concentration of SDS and was maximal between 0.01% and 0.015% SDS. Background binding of ^{125}I-BTX to the polystyrene wells was also enhanced (Figure 3). This enhancement, however, was not as great as that seen with specific binding, nor was it as dependent on SDS concentration, being relatively constant at concentrations above 0.01%.

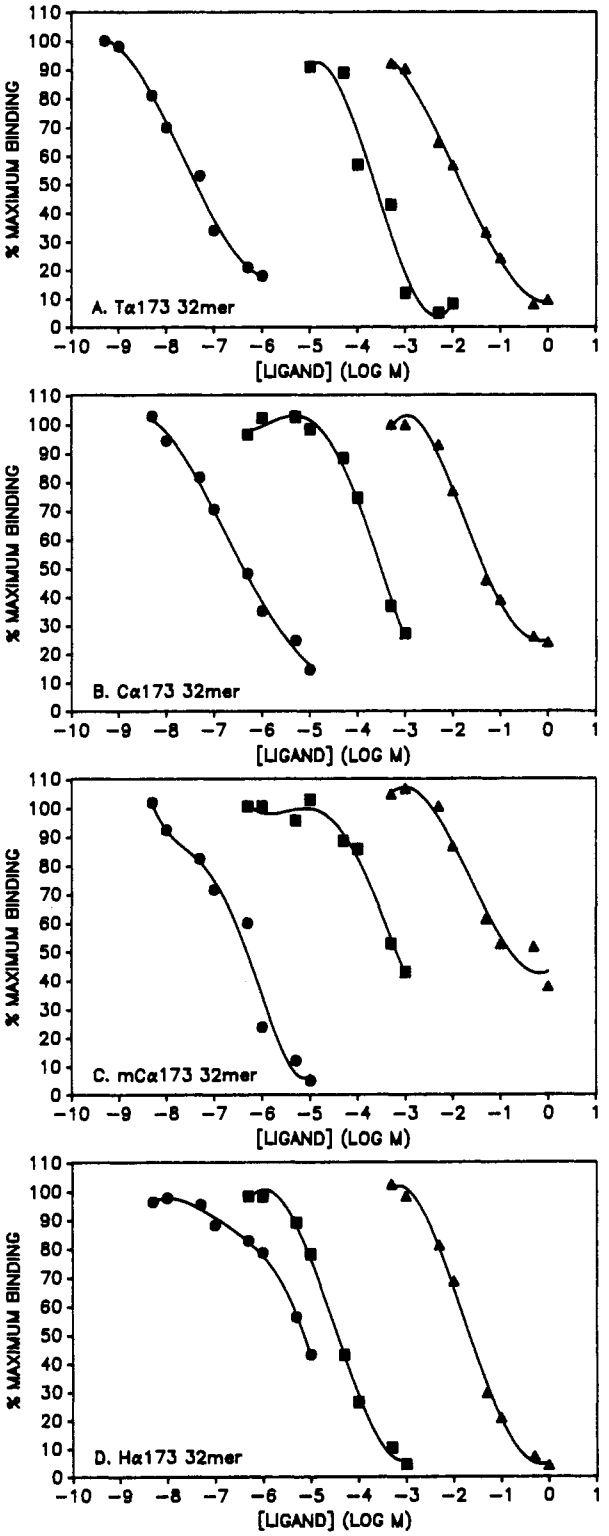

FIGURE 2: Competition of ^{125}I-BTX binding to Tα173 32-mer (A), Cα173 32-mer (B), mCα173 32-mer (C), and Hα173 32-mer (D). Wells of microtiter plates were coated with 5 μg of peptide by evaporation. The wells were then incubated with 3–4 nM ^{125}I-BTX and various amounts of either unlabeled BTX (circles), dTC (squares), or NaCl (triangles) for 20 min. The wells were washed, and bound radioactivity was determined. Each point represents the average of triplicate determinations.

The effect of 0.01% SDS on the binding of ^{125}I-BTX was screened for several peptides (Table III). For three peptides, ERA 13-mer, RN179 14-mer, and Tα194 11-mer, little or no binding was detected either in the absence or in the presence of 0.01% SDS. The ERA 13-mer corresponds to residues 187–199 of rabies virus ERA strain glycoprotein (Lentz et al., 1987). The RN179 14-mer is the portion of a rat neuronal PC12 cell protein (Boulter et al., 1986) corresponding to the Tα179 14-mer. All other peptides tested, however, showed enhanced binding in the presence of 0.01% SDS. The degree of enhancement depended on the peptide and ranged from 1.8-fold for Hα173 32-mer to 40-fold for Tα181 18-mer. Binding was increased 10-fold for Tα173 32-mer and Cα173 32-mer.

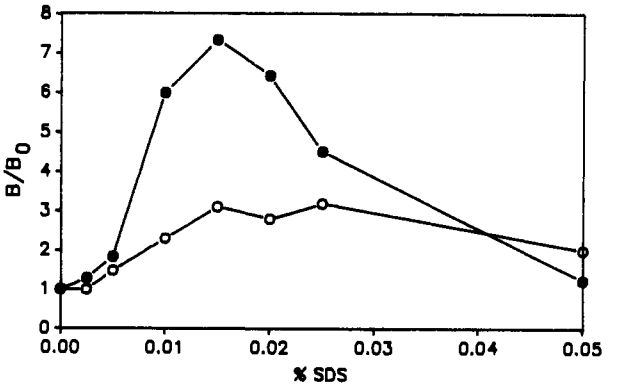

FIGURE 3: Effect of SDS concentration on the binding of ^{125}I-BTX to Tα173 32-mer. Wells of a microtiter plate were coated with 5 μg of Tα173 32-mer overnight. Following a 1-h quench in 2% BSA, the wells were incubated with 1 nM ^{125}I-BTX in 10 mM PB, pH 7.4, containing 0.1% BSA and various amounts of SDS. After 8 h at room temperature, the wells were washed 4 times with 10 mM PB/BSA. The net amount of bound ^{125}I-BTX (closed circles) was determined as described. Background binding (open circles) represents the amount of ^{125}I-BTX bound to wells at the same SDS concentration in the absence of Tα173 32-mer.

Table III: Effect of 0.01% SDS on the Binding of ^{125}I-BTX to Synthetic Peptides[a]

peptide	cpm, no SDS	cpm, 0.01% SDS	0.01% SDS/no SDS
Tα173 32-mer	7121 ± 296	73141 ± 5738	10.3
Tα181 18-mer	833 ± 210	33211 ± 2151	39.9
Tα179 14-mer	887 ± 232	3644 ± 1578	4.1
Tα186 11-mer	1731 ± 12	3049 ± 82	1.8
Tα194 11-mer	153 ± 158	<0	
Cα173 32-mer	2461 ± 182	24939 ± 4733	10.1
Hα173 32-mer	24157 ± 871	42434 ± 63	1.8
ERA 13-mer	18 ± 11	<0	
RN179 14-mer	87 ± 106	<0	

[a] Wells of polystyrene microtiter plates were coated with 5 μg of peptide. Following a 1-h quench with 2% BSA, a solution of ^{125}I-BTX (4.7 nM) in PB/BSA with or without 0.01% SDS was added to each well. After 3 h, the wells were washed 4 times with PB. Bound ^{125}I-BTX was removed as described. Results are presented as net cpm of ^{125}I-BTX bound. Net cpm were calculated by subtracting cpm of ^{125}I-BTX bound in the absence of peptide from cpm of ^{125}I-BTX bound in the presence of peptide.

The effect of 0.01% SDS on the affinity of BTX binding to peptides was measured by calculating the apparent K_D from equilibrium saturation binding curves in the absence and presence of 0.01% SDS. Scatchard analysis of BTX binding to Tα173 32-mer revealed the presence of two binding components in the absence of SDS: a minor component comprising 4% of the total binding sites with an apparent K_D of 4.2 nM and a major component comprising 96% of the total sites with an apparent K_D of 63 nM (Figure 4A). In the presence of 0.01% SDS, a Scatchard plot revealed one class of binding sites with an apparent K_D of 7.8 nM (Figure 4B).

Equilibrium saturation binding studies in the absence and presence of 0.01% SDS were performed for Cα173 32-mer (Figure 4C,D), Hα173 32-mer (Figure 4E,F), Tα181 18-mer (Figure 5A,B), and Tα185 12-mer (Figure 5C,D). SDS in-

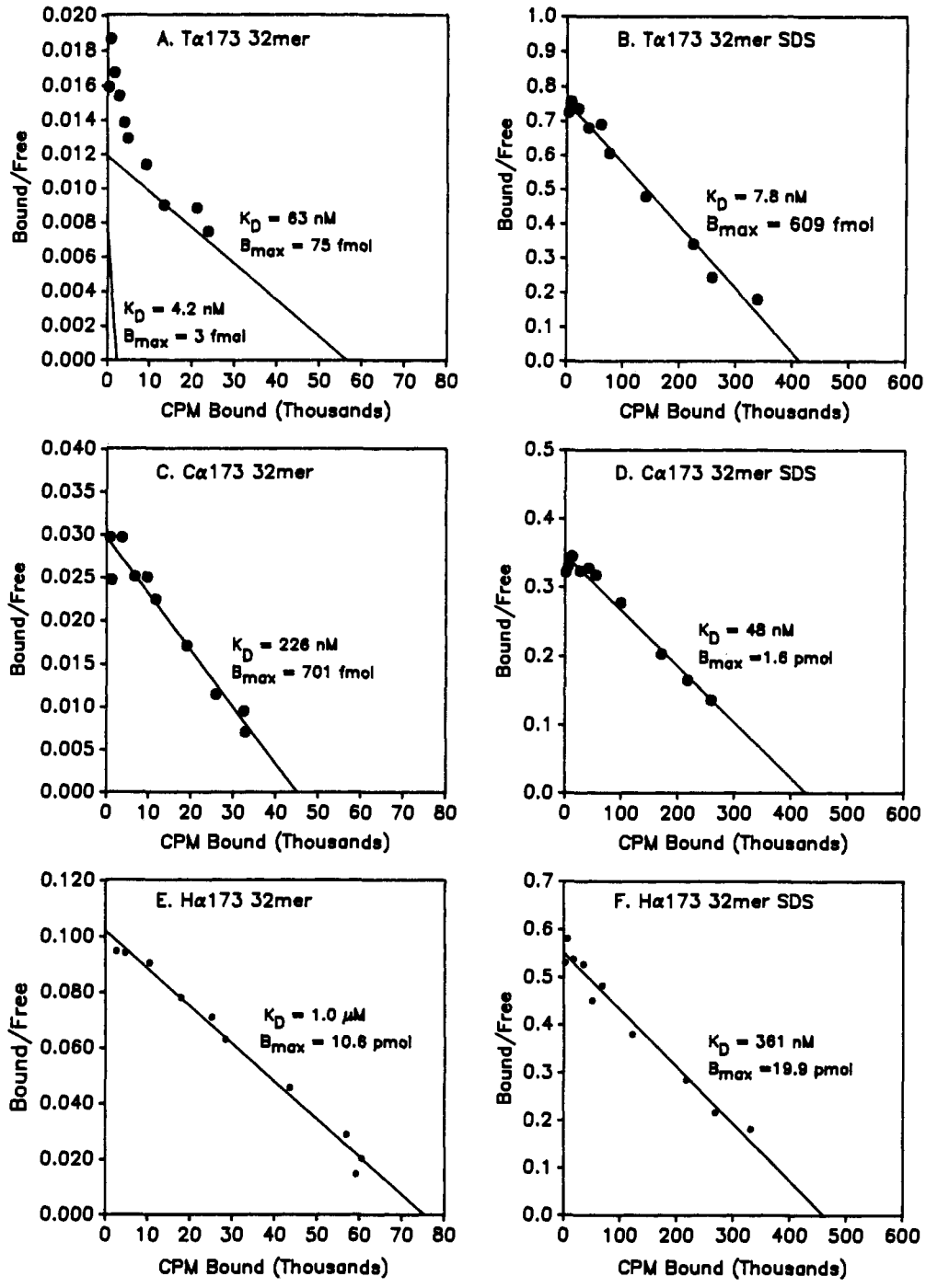

FIGURE 4: Scatchard analysis of the binding of ^{125}I-BTX to α173 32-mers in the presence and absence of SDS. Wells of a microtiter plate were coated with 5 μg of peptide. Following a 1-h quench in 2% BSA, the wells were incubated with various amounts of ^{125}I-BTX for 10 h at room temperature. An aliquot was then removed to determine the free ^{125}I-BTX concentration. The wells were immediately washed 4 times with PB/BSA, and the bound ^{125}I-BTX was determined. ^{125}I-BTX bound to quenched wells in the absence of peptide was subtracted from the amount bound in the presence of peptide. The data are presented as cpm bound/free vs cpm bound. ^{125}I-BTX binding to Tα173 32-mer in the absence of SDS. Specific activity of ^{125}I-BTX equals 751.8 cpm/fmol. (B) ^{125}I-BTX binding to Tα173 32-mer in the presence of 0.01% SDS. Specific activity of ^{125}I-BTX equals 704 cpm/fmol. (C) ^{125}I-BTX binding to Cα173 32-mer in the absence of SDS. Specific activity of ^{125}I-BTX equals 62.35 cpm/fmol. (D) ^{125}I-BTX binding to Cα173 32-mer in the presence of 0.01% SDS. Specific activity of ^{125}I-BTX equals 259.6 cpm/fmol. (E) ^{125}I-BTX binding to Hα173 32-mer in the absence of SDS. Specific activity of ^{125}I-BTX equals 7.13 cpm/fmol. (F) ^{125}I-BTX binding to Hα173 32-mer in the presence of 0.01% SDS. Specific activity of ^{125}I-BTX equals 23.2 cpm/fmol.

creased the affinity of ^{125}I-BTX binding for all four peptides (Table IV). The effect was greatest for Tα181 18-mer and least for Hα173 32-mer.

Discussion

Synthetic peptides corresponding to residues 173–204 of *Torpedo*, calf, and human AChR α subunits were synthesized and tested for their ability to bind ^{125}I-BTX in a solid-phase assay. The affinity of toxin binding to purified, detergent-solubilized AChR was measured with the solid-phase assay so that affinities of binding to native receptor and synthetic peptides could be compared directly. The equilibrium K_D for native, *Torpedo* AChR was 4.1×10^{-10} M, in close agreement with the values of $(2.8–4.2) \times 10^{-10}$ M reported by Lukas et al. (1981). The affinity of the Tα173 32-mer for toxin was 6.3×10^{-8} M, approximately 2 orders of magnitude less than that of the native receptor, but comparable to values determined for the intact, denatured α subunit (Haggerty &

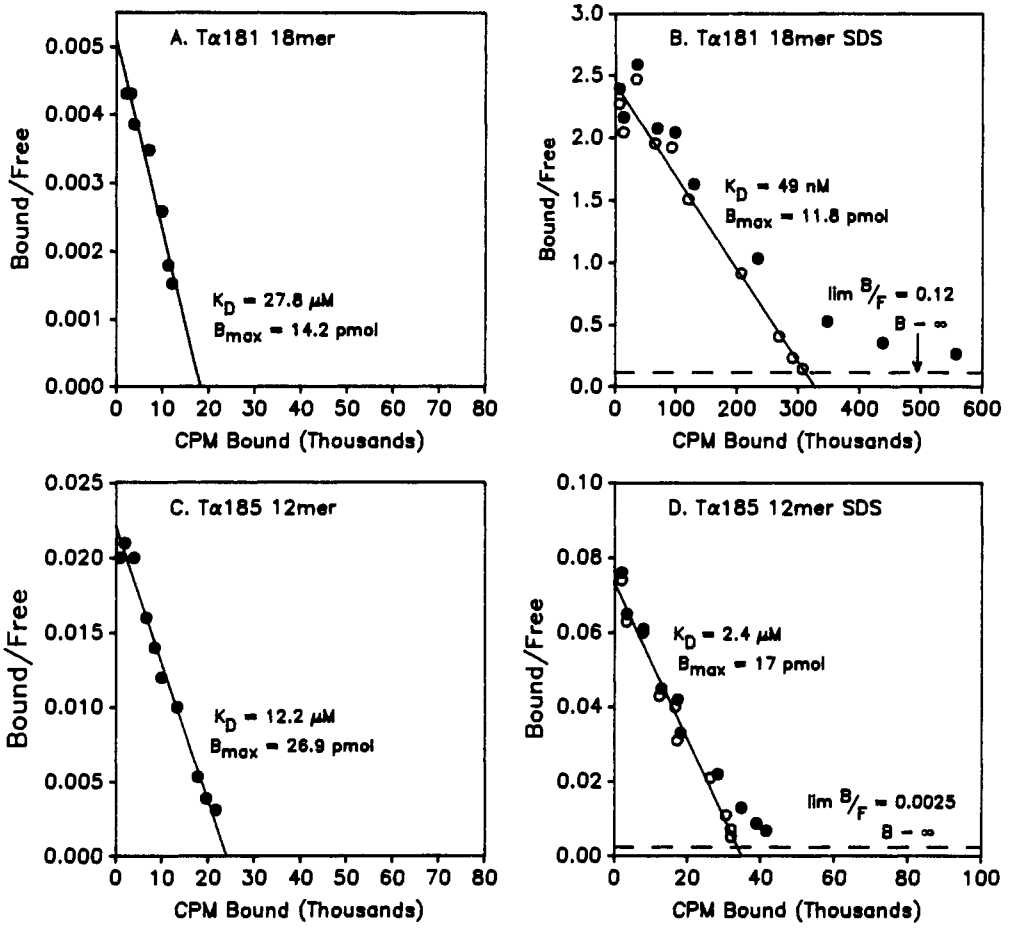

FIGURE 5: Scatchard analysis of the binding of ^{125}I-BTX to Tα181 18-mer and Tα185 12-mer in the presence and absence of SDS. Binding was measured as described in Figure 4. (A) ^{125}I-BTX binding to Tα181 18-mer in the absence of SDS. Specific activity of ^{125}I-BTX equals 1.29 cpm/fmol. (B) ^{125}I-BTX binding to Tα181 18-mer in the presence of 0.01% SDS. Specific activity of ^{125}I-BTX equals 27.9 cpm/fmol. (C) ^{125}I-BTX binding to Tα185 12-mer in the absence of SDS. Specific activity of ^{125}I-BTX equals 0.89 cpm/fmol. (D) ^{125}I-BTX binding to Tα185 12-mer in the presence of 0.01% SDS. Specific activity of ^{125}I-BTX equals 1.99 cpm/fmol. The dashed lines in panels B and D represent the limiting B/F ratio that is achieved after saturation of the high-affinity binding component.

Table IV: Apparent K_D Values (nM) for ^{125}I-BTX Binding from Scatchard Analysis[a]

peptide	no SDS	0.01% SDS
Tα173 32-mer	63	8
Cα173 32-mer	226	48
Hα173 32-mer	1035	361
Tα181 18-mer	27800	49
Tα185 12-mer	12200	2400

[a] Peptides were incubated with ^{125}I-BTX in the presence or absence of 0.01% SDS as described in Figures 4 and 5. K_D values were taken from Scatchard plots of the saturation binding curves. In the case of Tα173 32-mer, the K_D value in the absence of SDS represents the value for the major binding component.

Froehner, 1981; Gershoni et al., 1983; Oblas et al., 1983; Tzartos & Changeux, 1983). The decreased affinity of the α subunit and 32-mer could be the result of a loss of conformation of the binding site. However, the fact that the 32-mer retains considerable affinity for toxin and that this can be enhanced by SDS, as discussed below, indicates a major determinant of neurotoxin binding is located in this region of the α subunit.

Comparison of toxin binding to *Torpedo*, calf, and human 32-mers revealed that both the Cα173 32-mer and the Hα173 32-mer bound ^{125}I-BTX but the affinities were 15- and 150-fold less, respectively, as measured by IC_{50} values, than that of the Tα173 32-mer. The differences in affinity of BTX for the peptides may be the result of differences in their amino acid sequences. Sequence comparisons reveal six residue changes between both the Tα173 32-mer/Cα173 32-mer pair and the Tα173 32-mer/Hα173 32-mer pair (Table I). In the Cα173 32-mer, two changes represent nonconservative changes (Tyr-181 to Ser-181 and Asp-195 to Ser-195). In the Hα173 32-mer, three nonconservative changes occur (Tyr-181 to Ser-181, Trp-187 to Ser-187, and Tyr-189 to Thr-189). In this instance, all three changes involve the replacement of aromatic residues with polar residues. The effect on BTX affinity is striking (Tables II and IV). The large reduction in affinity would indicate that the aromatic residues, Trp-187, Tyr-181, and Tyr-189, are important for the higher affinity binding seen with the Tα173 32-mer.

Neumann et al. (1986b) suggested that Trp-187 is important in BTX binding. After modification of Trp-187 of the Tα185 12-mer with sulfonyl halide reagents, the absolute amount of BTX bound was reduced although the affinity was not measured. In addition, the human counterpart to this peptide, in which the Trp residue is replaced by a Ser residue, was synthesized (Neumann et al., 1986b). This peptide bound very low levels of ^{125}I-BTX. It was concluded that Trp-187 is an important determinant of BTX binding in the *Torpedo* peptide. Low and Corfield (1986) proposed that a receptor Trp interacts with invariant neurotoxin Trp-29.

The sequence changes between the Tα173 32-mer and Hα173 32-mer include the substitution of two Tyr residues (181, 189) in addition to the Trp residue. A Tyr residue (189) is also replaced in the human 12-mer of Neumann et al. (1986). Thus, the difference in affinity seen between the

Torpedo and human peptides could also be the result of the substitution of Tyr residues. The fact that toxin affinity for the Cα173 32-mer, in which Tyr-181 is substituted by Ser, Tyr-189 is substituted by Phe, and Trp-187 is present, is intermediate between affinity for *Torpedo* and human peptides indicates both Tyr and Trp residues are important in binding.

The effect of dTC on BTX binding to the various 32-mers was also investigated. ^{125}I-BTX binding was competed by dTC in all cases. Somewhat surprisingly, unlabeled BTX was only 7.5 times more effective than dTC in competing ^{125}I-BTX binding to the Hα173 32-mer. This stands in contrast to the binding of ^{125}I-BTX to the Tα173 32-mer, where BTX is at least 2000 times more effective than dTC. This finding indicates that some determinants of BTX binding in the Tα173 32-mer sequence may be distinct from dTC binding determinants, since the residue changes between the Tα173 32-mer and Hα173 32-mer greatly affect the IC_{50} value for BTX without substantially affecting the IC_{50} value for dTC (Table II). Alternatively, the polar residues substituted for the aromatic residues may interact effectively with dTC but less well with BTX. At high concentration, NaCl inhibited BTX binding to the 32-mers, consistent with the cation sensitivity of the receptor (Schmidt & Raftery, 1974).

A calf 32-mer in which negatively charged Glu-180 was replaced by Gln-180 was also synthesized (mCα173 32-mer). The abolition of this charge, however, had little discernible effect on BTX affinity, indicating that it is not crucial for BTX binding. It is interesting that the loss of two negative charges in the mCα173 32-mer present in *Torpedo* (Asp-180 and -195) does not abolish the ability of dTC to compete ^{125}I-BTX binding. Although dTC is slightly less effective in competing BTX binding to the mCα173 32-mer as compared to the Cα173 32-mer (Table II), determinants of dTC binding are still clearly preserved. It has been assumed that the presence, and often necessity, of the positively charged quaternary ammonium groups of cholinergic agonists implied the existence of a negative subsite in the ACh binding site (Taylor, 1980). The data presented here raise the possibility that the structural composition of the negative subsite may encompass more than one amino acid residue side chain. The results with the mCα173 32-mer indicate that negative charges on Glu-180 and Asp-195 are not essential for the binding of dTC. It is possible that, in addition to negatively charged residues, partial electronegative charges on the side chains of polar amino acid residues, such as the hydroxyl group of tyrosine, contribute to the anionic subsite. Thus, the positive charges on cholinergic antagonists and agonists could be countered by a negative electrostatic potential provided by polar groups, present on several residues in this region, acting together or in conjunction with the negative charge present on any of residues 175, 180, 195, or 200.

SDS had been previously shown to increase the affinity of BTX binding to the isolated α subunit (Tzartos & Changeux, 1983). Since the Tα173 32-mer behaved like the intact α subunit, it was of interest to determine whether SDS had a similar effect on ^{125}I-BTX binding to Tα173 32-mer. SDS was found to enhance binding of BTX to Tα173 32-mer with a maximum effect at an SDS concentration between 0.01% and 0.015% SDS. This concentration dependence is similar to that reported by Tzartos and Changeux (1983) for the α subunit. The maximal effect of SDS occurred at a concentration that is less than the critical micelle concentration (CMC) of SDS at the ionic strength used in the binding assay. Thus, the effect of SDS would seem to be the result of the binding of SDS monomers to both BTX and Tα173 32-mer and not the result of stabilization of α-helical structures which seems to require concentrations of SDS greater than the CMC (Steele & Reynolds, 1979; Huang et al., 1981). Direct binding of SDS to BTX is suggested by the observation that SDS increased the background binding of BTX in the absence of Tα173 32-mer, although this could also be explained by the binding of SDS to the polystyrene wells, making the wells "stickier". SDS binding to Tα173 32-mer is suggested by the observation that the enhancement in binding and affinity is dependent on the synthetic peptide used. SDS could enhance binding of the positively charged toxin by adding negative charges to the peptides. However, the enhancement of binding appears to be specific in that three peptides, including the Tα194 11-mer which possesses weak BTX binding determinants, show no specific binding in the presence of 0.01% SDS.

The effect of 0.01% SDS on the affinity of BTX binding to synthetic peptides was also determined. The apparent K_D values obtained from equilibrium binding experiments in the absence of SDS are in close agreement with the affinities as indicated by IC_{50} values from the competition experiments employing unlabeled BTX (Tables II and IV). SDS increased the affinity of BTX binding to all peptides studied. In the case of the Tα173 32-mer, the resultant K_D (7.8 nM) was close to the K_D reported by Tzartos and Changeux (1983) for the intact α subunit (3 nM). This compares with the K_D of 0.41 nM for intact receptor as measured in the solid-phase assay. The enhanced affinity further strengthens the conclusion that the Tα173 32-mer contains nearly all the determinants of the BTX binding site expressed on the isolated α subunit. Scatchard analysis also suggests a partial explanation for the nature of the effect of SDS on the affinity of BTX binding. In the absence of SDS, two binding components were noted: a prominent low-affinity component (K_D = 63 nM) and a minor high-affinity one (K_D = 4 nM). At least two explanations can be offered to explain this observation. First, either the BTX or the Tα173 32-mer may be structurally heterogeneous and the Scatchard plot reflects two structurally independent binding events. Second, either or both the BTX and Tα173 32-mer molecule may exist in multiple conformations, and the Scatchard plot reflects one binding event but in two interconvertible conformational states. The second explanation seems more likely because only one binding component is detected in the presence of SDS with an affinity close to the minor high-affinity component in the absence of SDS. This suggests that SDS is stabilizing a conformation of the Tα173 32-mer that is conducive to high-affinity binding.

Of the other peptides tested, the effect of SDS on the affinity of BTX binding was greatest for the Tα181 18-mer, where the apparent K_D decreased from 28 μM to 49 nM. To the extent that the effect of SDS represents a "renaturation" process, this result indicates that the 18-mer possesses significantly more BTX binding determinants than the Tα185 12-mer but not as many as the Tα173 32-mer. Furthermore, the magnitude of the enhancement (40-fold) also suggests that the residues of the Tα181 18-mer that flank the sequence of the Tα185 12-mer may be important for the maintenance of conformation in this region. These findings support the conclusion that BTX binding determinants are present throughout the length of the Tα173 32-mer. Even the regions near the ends of the Tα173 32-mer (173–180, 199–204) apparently contribute to the high affinity of BTX binding in that the K_D of ^{125}I-BTX binding to the Tα173 32-mer is 6-fold higher than to the Tα181 18-mer in the presence of 0.01% SDS (Table IV).

BTX did not bind to peptide 179–192 of a rat nerve protein. This protein from the rat pheochromocytoma cell line PC12 is homologous to the α subunit of the nicotinic AChR and may represent a neural nicotinic AChR α subunit (Boulter et al., 1986). The neuronal nicotinic AChR, however, does not appear to bind BTX (Boulter et al., 1986). The neuronal 14-mer has only five residues in common with the Tα179 14-mer. Trp-187, Tyr-181, and Tyr-189, which appear to have important functions in toxin binding to the α subunit 32-mers, are substituted in the rat nerve protein.

These studies demonstrate the potential utility of synthetic peptides in elucidating structure–function relationships. In this case, a 32-residue synthetic peptide binds BTX with the same affinity as the entire 437-residue, denatured α subunit and, in the presence of SDS, binds toxin with an affinity only about 1 order of magnitude less than that of the native, intact receptor. Thus, the 32-mer constitutes a major determinant of toxin binding, and the effects on binding of modification of individual residues should further characterize the nature of the toxin–receptor interaction and of the agonist binding site.

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