An α-Bungarotoxin-Binding Sequence on the *Torpedo* Nicotinic Acetylcholine Receptor α-Subunit: Conservative Amino Acid Substitutions Reveal Side-Chain Specific Interactions†

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ABSTRACT: In the α subunit of the *Torpedo* nicotinic cholinergic receptor (AChR), a sequence region surrounding a pair of adjacent cysteinyl residues at positions 192 and 193 contributes to a binding site for cholinergic ligands, including the snake α-neurotoxins. Synthetic and biosynthetic peptides corresponding to this region bind α-bungarotoxin (α-BTX) in the absence of other structural components of the AChR and, therefore, represent a "prototope" for α-BTX. Using synthetic peptides corresponding to the complete AChR α subunits of *Torpedo* electroplax and mammalian muscle, we previously defined a sequence segment corresponding to a universal prototope for α-BTX binding between amino acid residues 181 and 200 [Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R. Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221–6230; McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 1537–1544]. To elucidate the structural requirements for α-BTX binding, we initially used nonconservative single amino acid substitution analogues of the parental α(181–200) sequence, and we found that residues at positions 188–190 (VYY), and 192–194 (CCP) and several flanking residues seemed to be involved in α-BTX binding [Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, A., & Maelicke, A. (1991) *Biochemistry 30*, 2575–2584]. In the present study, amino acid residues previously found to affect α-BTX binding were replaced by different conservative single amino acid substitutions, in order to determine the nature of the amino acid side-chain interactions with α-BTX. Whereas V188 could be replaced by Ile or Thr with minor effects on α-BTX binding, substitution of Phe, His, or Thr for Y189 and Y190 resulted in large to moderate decreases in α-BTX binding. Similarly, α-BTX binding activity was intolerant to substitutions of C192 or C193 with Ser, His, or Val. Structural changes of the peptide α(181–200) induced by substitution of P194 or P197 with two adjacent Gly residues, and insertion of a Gly between C192 and C193, were also incompatible with α-BTX binding. Conservative substitutions of other aliphatic and aromatic residues resulted in only minor effects on α-BTX binding, as did replacements of K185 and D195 that changed or maintained the charge distribution of peptide α(181–200). The recognition site for α-BTX formed by the prototope α(181–200), therefore, involves important interactions with Y189, Y190, C192, and C193 that are highly specific to the amino acid residue at that position. Furthermore, P194 and P197 appear to play important structural roles in maintaining the correct conformation of the peptide to display this binding motif.

The subunits of the nicotinic acetylcholine receptor (AChRs)[1] are a group of homologous transmembrane proteins whose different ligand binding properties and differential contribution to formation of AChR molecules confer pharmacological and functional diversity to this receptor family [reviewed in Stroud et al. (1990), Lindstrom et al. (1989), Connolly (1989), Deneris et al. (1991), and McLane et al. (1993a)]. The muscle-type AChR, of which the *Torpedo* AChR is the prototype, are pseudosymmetric pentamers formed by four different subunits (α, β, γ or ε, and δ) (Raftery et al., 1980; Conti-Tronconi et al., 1982). The neuronal AChRs are pentamers with alternative subunit stoichiometries: they consist of one (αx), two (αx, βy), or three (αx, αy, βz) different subunits, where α and β are one of at least eight or five different subtypes, respectively (Cooper et al., 1991; Anand et al., 1991; Listerud et al., 1991; Conroy et al., 1992).

The sensitivity of these different AChR subtypes to cholinergic neurotoxins is largely, although not entirely, dependent on the α subunit subtypes that contribute to forming the receptor complex (Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Luetje et al., 1990a; Couturier et al., 1990; Luetje & Patrick, 1991; Conroy et al., 1992; Bertrand et al., 1992). For example, two peptide neurotoxins from the venom of *Bungarus multicintus*, which compete with other nicotinic ligands for a cholinergic binding site, have been used extensively to distinguish different AChR subtypes [reviewed in Deneris et al. (1991), Chiappinelli (1991), and McLane et

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; κ-BTX, κ-bungarotoxin, also referred to as bungarotoxin 3.1 (Ravdin & Berg, 1979), toxin F (Loring et al., 1984), and neuronal bungarotoxin (Lindstrom et al., 1987); HPLC, high-pressure liquid chromatography; KP, 10 mM potassium phosphate, pH 7.4; TBS, 10 mM Tris-HCl, pH 7.4, 100 mM NaCl; PBS, 10 mM sodium phosphate, 100 mM NaCl, pH 7.2.

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al. (1993a)]. α-Bungarotoxin (α-BTX) is a potent inhibitor of AChRs containing the α subunit from *Torpedo* electroplax, the vertebrate muscle, and the neuronal α7 subunit, which can form functional homoliogmeric AChRs. κ-Bungarotoxin (κ-BTX) is a selective antagonist of the α3β2 AChR subtype, which is found in the vertebrate brain and sympathetic ganglia (Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Luetje et al., 1990b; Couturier et al., 1990; Bertrand et al., 1992).

In addition to the α subunit, the γ and δ subunits contribute to the binding site for several cholinergic ligands (Blount & Merlie, 1989; Blount et al., 1990; Pederson & Cohen, 1990; Middleton & Cohen, 1991; Sine & Claudio, 1991; Czajowski & Karlin, 1991). Other AChR subunits may also be involved in α-BTX and κ-BTX binding, as indicated by the finding that AChRs composed of α3 and β4 subunits are not inhibited by κ-BTX (Duvoisin et al., 1989; Luetje et al., 1990). However, the demonstration that isolated α subunits from *Torpedo* AChRs are able to bind α-BTX even after denaturation indicates that a continuous sequence segment of this subunit can form a binding site for α-BTX (Haggerty & Froehner, 1981). A number of laboratories have confirmed that isolated peptide sequences of the *Torpedo* AChR α subunit bind α-BTX using proteolytic fragments, bacterially expressed fusion proteins containing regions of α subunits, or synthetic peptides [reviewed in Lentz and Wilson (1988) and McLane et al. (1993a)]. The convergence of the results from several peptide mapping studies has defined a sequence region between residues 174–204 of the *Torpedo* α subunit that is able to form an α-BTX binding prototope[2] (Neumann et al., 1986; Ralston et al., 1987; Wilson et al., 1988; Wilson and Lentz, 1988; Lentz and Wilson, 1988; Conti-Tronconi et al., 1990, 1991; Greismann et al., 1990; McLane et al., 1991a; Barkas et al., 1987; Aronheim et al., 1988; Ohana & Gershoni, 1990; Ohana et al., 1991). Using synthetic peptides corresponding to overlapping sequence segments of the complete *Torpedo* electroplax AChR α subunit, our laboratory has defined an α-BTX binding prototope between amino acid residues 181–200 (Conti-Tronconi et al., 1990). Using the same approach, we have further demonstrated that the homologous region on the vertebrate muscle AChR α1 subunit of several different species, and on the avian brain AChR α7 and α8 subunits, contains a universal binding site for α-BTX (McLane et al., 1991a,c). This region differs from the principal prototope for κ-neurotoxins on the α3 subunit, which is within the sequence region α3(51–70), (McLane et al., 1990a,b) and is homologous to a secondary prototope for α-BTX, between residues 55 and 74 of the *Torpedo* α subunit (Conti-Tronconi et al., 1990).

A hallmark of the AChR α subunit contained within the α-BTX binding site is a pair of adjacent cysteine residues at positions 192 and 193. The Cys–Cys pair is believed to form a vicinal disulfide bond (Kao et al., 1984; Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris et al., 1988) which is unusually susceptible to reduction and requires a *cis* peptide bond. Several other residues are also highly conserved or conservatively substituted in α subunits that are able to bind α-BTX, particularly K185, Y189, Y190, P194, D195, P197, and Y198 (McLane et al., 1991a,b). These residues and several others are almost entirely conserved between the highly homologous AChR α1 subunit sequences from the muscle of different species (>75% amino acid identity). Given the highly divergent nature of the neuronal α subunits, however, sequence homology has not been a reliable predictor of α-BTX binding, for which there is no universal binding motif, nor is amino acid conservation indicative of residues functionally important for α-BTX binding (McLane et al., 1990b, 1991b,c, 1993a).

In order to determine the amino acid residues that are important in forming the interface with α-BTX, we have used synthetic single substitution peptide analogues of the parental sequence α(181–200) to study α-BTX binding. As a first approach, we sequentially substituted each position of the *Torpedo* α(181–200) sequence with glycine (Conti-Tronconi et al., 1991). We found that amino acid residues at positions 188–190 (VYY) and 192–194 (CCP) were critical to α-BTX binding. In addition, several other amino acids were implicated in forming the α-BTX binding prototope (W184, K185, W187, D195, T196, P197, and Y198).

In the present study, we have replaced each of the amino acid residues of the parental sequence *Torpedo* AChR α-(181–200), which were previously found to be important for α-BTX binding, with different conservative substitutions. For example, Y residues were substituted with (i) F, which lacks a hydroxyl group, (ii) T, which contains a hydrophobic side chain and a hydroxyl group, or (iii) H, which is both aromatic and capable of hydrogen-bonding interactions. Substitutions of other aromatic and aliphatic residues were designed to test for changes in size and polarity of the amino acid side chains, and charged residues were substituted to maintain, change, or neutralize the positive or negative charge of the side chain. In making this panel of mutants, we hoped to identify physicochemical attributes important in forming the interface with α-BTX. We were also interested in determining the effects of conformational changes in the peptide that occur upon replacement of important P residues (P194, P197) each with two adjacent glycine residues and insertion of a G between the adjacent C/C pair (C192, C193). The role of a vicinal disulfide bond in α-BTX binding activity of peptide α(181–200) was also tested by incorporating S, H, or V substitutions at positions 192 or 193.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Characterization. Peptides were synthesized by manual parallel synthesis (Houghten, 1985) and characterized as previously reported (Conti-Tronconi et al., 1991; McLane et al., 1991a,b). The purity was assessed by reverse-phase HPLC (high-pressure liquid chromatography) using a C18 column (Ultrasphere ODS) and an acetonitrile/water gradient (5–70%) containing 0.1% trifluoroacetic acid. The major peak consistently accounted for 65–85% of the total absorbance at 214 nm. The amino acid composition of selected peptides was determined by derivatization of amino acid residues released by acid hydrolysis with phenylisothiocyanate, followed by separation on a reverse-phase HPLC column (PICO.TAG) as described by Heinrickson and Meredith (1984). These results gave excellent correspondence between experimental and theoretical values. The sequence and codes of the peptides are reported in Figure 1.

Preparation and Calibration of Radiolabeled α-BTX. Commercial preparations of α-BTX (Biotoxins Inc.) were used in the present studies. α-BTX was radiolabeled with carrier-free $Na^{125}I$ (Lindstrom et al., 1981) and calibrated as

[2] "Prototope" is a term that was originally coined by House and Kemp (1987) for a peptide analogue of the protein kinase C regulatory domain and subsequently used by Lentz and co-workers (Wilson et al., 1988) to refer to toxin binding peptides. It refers to a continuous peptide sequence that is able to form an independent ligand-binding site in the absence of surrounding structural elements, by analogy with the continuous epitope recognized by antibodies.

PEPTIDE	SEQUENCES
NATIVE	YRGWKHWVYYTCCPDTPYLD
W184-F	YRG**F**KHWVYYTCCPDTPYLD
K185-E	YRGW**E**HWVYYTCCPDTPYLD
K185-R	YRGW**R**HWVYYTCCPDTPYLD
W187-F	YRGWKH**F**VYYTCCPDTPYLD
W187-M	YRGWKH**M**VYYTCCPDTPYLD
W187-Y	YRGWKH**Y**VYYTCCPDTPYLD
V188-I	YRGWKHW**I**YYTCCPDTPYLD
V188-T	YRGWKHW**T**YYTCCPDTPYLD
Y189-F	YRGWKHWV**F**YTCCPDTPYLD
Y189-H	YRGWKHWV**H**YTCCPDTPYLD
Y189-T	YRGWKHWV**T**YTCCPDTPYLD
Y190-F	YRGWKHWVY**F**TCCPDTPYLD
Y190-H	YRGWKHWVY**H**TCCPDTPYLD
Y190-T	YRGWKHWVY**T**TCCPDTPYLD
T191-S	YRGWKHWVYY**S**CCPDTPYLD
T191-V	YRGWKHWVYY**V**CCPDTPYLD
C192-S	YRGWKHWVYYT**S**CPDTPYLD
C192-H	YRGWKHWVYYT**H**CPDTPYLD
C192-V	YRGWKHWVYYT**V**CPDTPYLD
C192/G/C193	YRGWKHWVYYTC**G**CPDTPYLD
C193-S	YRGWKHWVYYTC**S**PDTPYLD
C193-H	YRGWKHWVYYTC**H**PDTPYLD
C193-V	YRGWKHWVYYTC**V**PDTPYLD
P194-GG	YRGWKHWVYYTCC**GG**DTPYLD
D195-E	YRGWKHWVYYTCCP**E**TPYLD
D195-N	YRGWKHWVYYTCCP**N**TPYLD
T196-S	YRGWKHWVYYTCCPD**S**PYLD
T196-V	YRGWKHWVYYTCCPD**V**PYLD
P196-GG	YRGWKHWVYYTCCPDT**GG**YLD
Y198-F	YRGWKHWVYYTCCPDTP**F**LD
Y198-H	YRGWKHWVYYTCCPDTP**H**LD
Y198-T	YRGWKHWVYYTCCPDTP**T**LD

FIGURE 1: Sequences of single amino acid substituted synthetic peptides corresponding to an α-BTX binding domain between positions 101–200 of the *Torpedo* electric organ AChR α subunit. Peptides corresponding to the sequence region 181–200 of the *Torpedo* AChR α subunit, and those containing single amino acid substitutions, were synthesized and characterized as described under Experimental Procedures. The sequence of the sequence segment *Torpedo* AChR α(181–200) (Noda et al., 1982) is underlined, and the synthetic peptide corresponding to the sequence is referred to as the unsubstituted or "native" peptide in the text, tables, and figures. The single amino acid substitution analogues are aligned with the native peptide, and the amino acid substitution indicated in boldface. The peptides are designated as follows: the single amino acid code of the native amino acid and its position in the native *Torpedo* AChR α(181–200) sequence, followed by a dash and the amino acid substituted. C192/G/C193 refers to the insertion of a Gly residue between C192 and C193. The single amino acid substitutions are referred throughout the text and figures using this nomenclature.

described by Blanchard et al. (1979) using membrane-bound AChR prepared from *Torpedo californica* electric organ. The specific activity of the ^{125}I-α-BTX used in experiments was 11.7–42 Ci/mmol, as specified in the figure and table legends.

Toxin Blots and Scatchard Analysis. Nitrocellulose strips were spotted with 1 μL of each peptide solution [1 mg/mL in 10 mM potassium phosphate buffer, pH 7.4 (KP buffer), containing 20% dimethylforamide and 0.01% acetic acid] and allowed to dry at room temperature. The strips were blocked with 5 mg/mL cytochrome *c* in 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 0.05% Tween 20 (TBS/Tween) overnight at 4 °C. Cytochrome *c* (5 mg/mL), which has charge properties similar to those of α-BTX, was added to solutions of ^{125}I-α-BTX to reduce the nonspecific binding. The blocked nitrocellulose strips were incubated with 0.1–10 μM ^{125}I-α-BTX in PBS (10 mM sodium phosphate, 100 mM NaCl, pH 7.2) for 3 h at room temperature, washed eight times with 3 mL of TBS/Tween (30 s/wash). In some experiments, ^{125}I-α-BTX binding was detected by autoradiography using Kodak X-Omat film and exposure at −70 °C for 16–24 h. In the experiments reported here ^{125}I-α-BTX binding was determined by counting triplicate samples (5-mm squares) in a γ counter. At each concentration of ^{125}I-α-BTX, nonspecific binding was determined by preincubation of replicate strips with 100 μM unlabeled α-BTX during the blocking step performed prior to addition of ^{125}I-α-BTX. Nonspecific binding was found to vary approximately linearly with the concentration of ^{125}I-α-BTX (5–25% of the total ^{125}I-α-BTX bound). The total amount of ^{125}I-α-BTX bound to peptides did not exceed 3% of the radioactivity added. The relative affinities of peptides bound to nitrocellulose were compared by calculating an apparent K_d using Scatchard analysis. Given the solid phase nature of these assays, the apparent K_d values reported here only approximate the true values. The B_{max} values (given in the legend of Table 2) simply reflect the amount of peptide adsorbed to nitrocellulose that is in the active conformation.

Competitive Inhibition of ^{125}I-α-BTX Binding by Peptides. Peptides (5–500 μg/mL) were preincubated with ^{125}I-α-BTX (1–2 pmol) in KP buffer containing 5 mg/mL cytochrome *c* overnight at 4 °C. To 100 μL of the peptide/toxin solution was added 1 pmol of membrane-bound *Torpedo* AChR (Neubig et al., 1979; Elliot et al., 1980). After incubation for 3 min at room tempertaure, the assay tubes were centrifuged at 14000*g* for 30 min, washed with PBS, and recentrifuged. The pellet was counted in a γ counter. Nonspecific binding (>5%) was determined by preincubation of *Torpedo* AChR with 20 nM unlabeled α-BTX for 10 min prior to addition to the radiolabeled toxin solutions. Binding of ^{125}I-α-BTX in the presence of peptides was compared with values obtained with toxin preincubated with only buffer. Assays were performed in triplicate, and the IC_{50} values were determined using linear regression of the Hofstee plot (%inhibition versus %inhibition/[peptide]), where the negative slope of the regression curve is equal to the apparent IC_{50} (Molinoff et al., 1981; McLane et al., 1991a–c).

RESULTS

Rationale. In the present studies, 33 synthetic peptides either corresponding to the *Torpedo* AChR α subunit sequence region, positions 181–200, or containing conservative substitutions of single amino acids within this sequence were tested for their ability to bind ^{125}I-α-BTX. The amino acid sequence of the native *Torpedo* AChR α(181–200) sequence and the single amino acid substitution analogues are given in Figure 1. The peptides are designated as follows: the single amino acid code of the native amino acid and its position in the native *Torpedo* AChR α(181–200) sequence, followed by a dash and the amino acid substituted. For example, the code W184-F refers to an analogue of the sequence α(181–200) where residue W at position 184 has been substituted by an F residue. Peptides P194-GG and P196-GG contain a substitution of P194 and P196 with two G residues; peptide C192/G/C193 represents the insertion of a glycine residue between C192 and C193. The single amino acid substitutions are referred throughout the text and figures using this nomenclature.

In previous studies, we found that α-BTX binding to certain peptides is affected by their adsorption to solid supports (McLane et al., 1990a,b, 1991a; Conti-Tronconi et al., 1991). For this reason, we have used two different types of assays to determine and verify the effects of different amino acid residue substitutions on α-BTX binding (Conti-Tronconi et al., 1991; McLane et al., 1991a,b): (i) a solid phase assay, referred to as a "dot blot assay", in which peptides were adsorbed onto nitrocellulose, and ^{125}I-α-BTX binding was directly determined by counting the strips in a γ counter or by autoradiography, and (ii) a solution phase assay, which we refer to as a "*Torpedo*

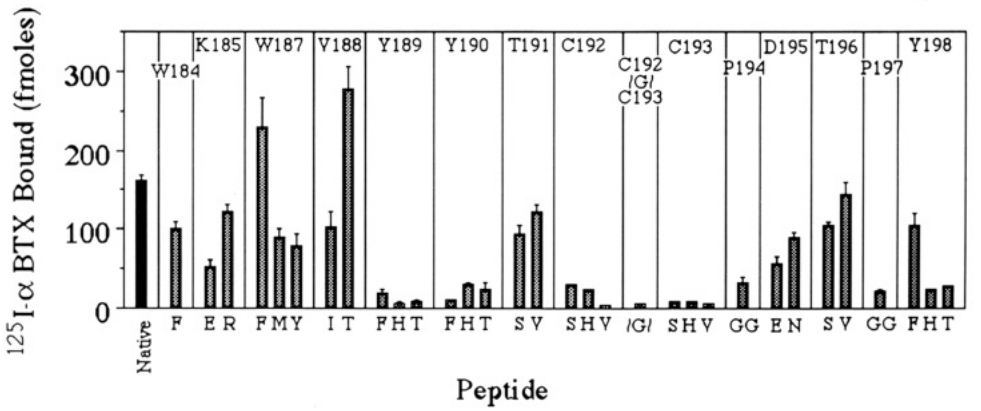

FIGURE 2: Dot blot assays of ^{125}I-α-BTX binding to synthetic peptides corresponding to *Torpedo* AChR α(181–200) and single substitution analogues. The synthetic peptides (1 μg) were spotted onto nitrocellulose strips and allowed to dry at room temperature. The strips were blocked overnight at 4 °C with 5 mg/mL cytochrome *c*, followed by addition of ^{125}I-α-BTX (0.4 μM; 13 Ci/mmol). The strips were incubated with ^{125}I-α-BTX for 4 h at room temperature with agitation, washed, and counted as described under Experimental Procedures. The peptides (Figure 1) are indicated as follows: the amino acid and its position in the native sequence are above the bar, and the amino acid substituted at that position is indicated on the horizontal axis. The results are expressed as the average of triplicate determinations, and the error bars are standard deviations.

AChR competition assay", in which peptides are preincubated with ^{125}I-α-BTX prior to addition of native electroplax AChR, and the ability of the peptides to sequester ^{125}I-α-BTX, and therefore compete for its binding to AChR, is determined. By using both assays we are able to make definitive comparisons with our previous studies using "glycine scanning mutagenesis" and naturally occurring amino acid substitutions in the muscle AChRs of different species (Conti-Tronconi et al., 1991; McLane et al., 1991a).

Qualitative Dot Blot Assays with ^{125}I-α-BTX. Dot blot assays assessed the direct binding of ^{125}I-α-BTX to peptides as described under Experimental Procedures. The relative levels of ^{125}I-α-BTX binding to peptides adsorbed to nitrocellulose were determined by cutting nitrocellulose strips and counting them in a γ counter (Figure 2 and Table 1)[3,4] and/or by autoradiography (data not shown). Three experiments were performed at a single concentration of ^{125}I-α-BTX (0.4 μM). Figure 2 depicts the results of a typical experiment. The amino acid and position of the unsubstituted peptide replaced is indicated above the columns, and the particular substitution administered is given along the horizontal axis. ^{125}I-α-BTX binding by the substituted peptides was linear within the concentration range 0.1–1 μM ^{125}I-α-BTX (data not shown). Table 1 summarizes the results from the dot blot experiments. The overall averages and standard deviations of the results of three experiments is reported for each peptide.

On the basis of the level of ^{125}I-α-BTX binding relative to the unsubstituted peptide, the substitutions can be grouped into three different categories: (i) Substitutions that essentially abolish ^{125}I-α-BTX binding (Y189-F, Y189-H, Y189-T, Y190-F, Y190-H, Y190-T, C192-S, C192-H, C192-V, C192/G/C193, C193-S, C193-H, C193-V, P194-GG, D195-E, P197-GG, Y198-H, and Y198-T); (ii) substitutions that result in ^{125}I-α-BTX binding at apparently higher levels than the unsubstituted peptide (V188-T and W187-F); and, (iii) substitutions that moderately affect the level of ^{125}I-α-BTX binding (W184-F, K185-E, K185-R, W187-M, W187-Y, V188-I, T191-S, T191-V, D195-N, T196-S, T196-V, and Y198-F). These results agree generally with our previous results using "glycine scanning mutagenesis," where we found that the critical residues for ^{125}I-α-BTX binding were V188, Y189, Y190, C192, C193, and P194. The differential effects of alternative substitutions at other positions are discussed below.

Quantitative Dot Blot Assays of ^{125}I-α-BTX Binding. We have previously found that peptides bound to solid supports can differ in the portion of the peptide that is an active conformation, thus affecting the level of α-BTX binding observed (McLane et al., 1991a–c). Thus, single concentration assays can be misleading by suggesting that lower levels of binding reflect lower binding affinity, when actually only the B_{max} is affected. For this reason it is advisable to determine the relative affinities of ^{125}I-α-BTX for the substituted peptides by determining the apparent relative K_ds for the different peptides that were able to bind ^{125}I-α-BTX in our qualitative dot blot assay (Figure 2). Dot blot assays were performed as described in Figure 2 using concentrations of ^{125}I-α-BTX between 0.1 and 6 μM. The results for Scatchard analyses for all of the single amino acid substitution analogues that bound ^{125}I-α-BTX are summarized in Table 2. The graphical analysis of the concentration dependency of ^{125}I-α-BTX binding to four representative peptides that varied in their apparent relative affitinities for α-BTX are illustrated in Figure 3. Given the solid phase nature of these assays and the nonsaturating conditions used, the apparent K_d values reported here only approximate the true values but are still useful for comparison among peptides and assessing the more subtle effects of the amino acid substitutions on the relative affinities for α-BTX.

The apparent K_d values ranged from 1.5 to 5.9 μM for those peptides that were able to bind ^{125}I-α-BTX, and the relative affinities agreed generally with the results of our qualitative assays (Figure 2). Peptides W184-F, W187-F, V188-I, and T196-S bind ^{125}I-α-BTX with an affinity close to that of the unsubstituted peptide. Reductions in the level of ^{125}I-α-BTX binding to substituted peptides were found to reflect an increase in their apparent K_ds, rather than an influence of their B_{max} values (see legend to Table 2), which merely reflect differences in the proportion of the peptide bound to nitrocellulose in the active conformation.

Qualitative Torpedo AChR Competition Assays with ^{125}I-α-BTX. Peptides can differ both qualitatively and quantitatively in their abilities to bind peptide neurotoxins in solution and solid phase assays (McLane et al., 1990a,b, 1991a,b; Conti-

[3] The results obtained for the dot blot and competition assays summarized in Table 1 are available upon request from the authors.

[4] The Scatchard and IC_{50} analyses for all of the peptides used in this study are available upon request from the authors.

Table 1: Summary of the Dot Blot and Competition Assay Data

peptide	dot blot assays average % binding[a]	competition assays average % inhibition[b]
native	100	74 ± 16
W184-F	72 ± 16	73 ± 18
K185-E	36 ± 9	11 ± 19
K185-R	64 ± 14	51 ± 14
W187-F	136 ± 12	76 ± 15
W187-M	50 ± 4	68 ± 23
W187-Y	49 ± 3	73 ± 19
V188-I	58 ± 16	44 ± 12
V188-T	143 ± 56	68 ± 21
Y189-F	17 ± 10	39 ± 11
Y189-H	9 ± 8	17 ± 20
Y189-T	12 ± 9	10 ± 9
Y190-F	11 ± 7	3 ± 2
Y190-H	24 ± 8	45 ± 11
Y190-T	15 ± 5	0 ± 0
T191-S	60 ± 2	67 ± 2
T191-V	64 ± 16	10 ± 9
C192-S	20 ± 2	18 ± 16
C192-H	16 ± 1	4 ± 6
C192-V	6 ± 6	1 ± 2
C192/G/C193	7 ± 5	5 ± 4
C193-S	10 ± 8	14 ± 8
C193-H	6 ± 2	19 ± 3
C193-V	6 ± 2	2 ± 4
P194-GG	17 ± 4	5 ± 5
D195-E	29 ± 6	53 ± 4
D195-N	51 ± 10	71 ± 15
T196-S	48 ± 29	65 ± 2
T196-V	70 ± 37	9 ± 8
P197-GG	14 ± 1	8 ± 13
Y198-F	52 ± 16	55 ± 22
Y198-H	14 ± 1	51 ± 35
Y198-T	17 ± 1	43 ± 36

[a] Three dot blot assay experiments were performed as described under Experimental Procedures and in the legend to Figure 2 using 0.4 μM ^{125}I-α-BTX. Two experiments were performed in triplicate, and a single determination for each peptide was performed in a third experiment. The specific activity of ^{125}I-α-BTX in the three experiments was 12.9, 19.7, and 42 Ci/mmol. The percent binding of ^{125}I-α-BTX to the single residue substituted peptide analogues, as compared to the binding to the unsubstituted synthetic sequence *Torpedo* α(181–200), was calculated. The average ± standard deviation of the percent binding obtained in the three experiments reported here.[3] [b] Three independent competition assay experiments were performed as described under Experimental Procedures and in the legend to Figure 4. The specific activity of ^{125}I-α-BTX for the experiments was 22.9, 23.5, and 22.0 Ci/mmol. Percent inhibition of the specific ^{125}I-α-BTX binding (total binding minus nonspecific binding obtained in the presence of excess unlabeled α-BTX) was calculated. The 0% inhibition levels were the average ^{125}I-α-BTX binding in the absence of any peptide and in the presence of peptides Y190-F, Y190-T, C192-V, C192/G/C193, C193-V, and P194-GG, which consistently failed to compete for ^{125}I-α-BTX binding to *Torpedo* AChR, i.e., in all experiments the specific ^{125}I-α-BTX binding obtained in their presence was equal to or larger than that obtained in the absence of any peptide, minus one standard deviation. The average inhibition of specific ^{125}I-α-BTX binding ± standard deviation for each peptide are reported.[3]

Tronconi et al., 1991). In order to determine if the ability to bind ^{125}I-α-BTX was obscured for any of the single substitution analogues by poor binding to a solid support, we were interested in verifying the results of our dot blot assays using a solution phase assay. We have previously found the results of *Torpedo* AChR competition assays, performed as described under Experimental Procedures, to provide a sensitive and relatively quantitative measure of the relative affinities of peptides for ^{125}I-α-BTX (McLane et al., 1990b, 1991a,b,c; Conti-Tronconi et al., 1991; Wahlsten et al., 1993).

In the *Torpedo* AChR competition assays summarized in Table 1 and presented in Figure 4, ^{125}I-α-BTX was preincubated with each of the single substitution analogues at a final concentration of 40 μM, and the ability of each of the peptides to inhibit the binding to native *Torpedo* AChR was tested. Table 1 summarizes the results of three independent competition experiments. On the basis of the level of inhibition of ^{125}I-α-BTX binding to *Torpedo* AChR relative to the unsubstituted peptide, the substitutions administered can be grouped into three different categories: (i) substitutions that strongly affected the ability of peptides to compete with native *Torpedo* AChR for ^{125}I-α-BTX binding (K185-E, Y189-H, Y189-T, Y190-F, Y190-T, T191-V, C192-S, C192-H, C192-V, C192/G/C193, C193-S, C193-H, C193-V, P194-GG, T196-V, and P197-GG); (ii) substitutions that moderately affect the ability of peptides to compete for ^{125}I-α-BTX binding (K185-R, V188-I, Y189-F, Y190-H, and D195-E); and (iii) substitutions that do not appear to affect α-BTX binding (W184-F, W187-F, W187-M, W187-Y, V188-T, T191-S, D195-N, T196-S, Y198-F, Y198-H, and Y198-T).

Quantitative Torpedo AChR Assays of ^{125}I-α-BTX Binding. In order to quantitatively compare the relative abilities of different peptides to bind ^{125}I-α-BTX in solution, we determined the dose-dependency of the inhibition of ^{125}I-α-BTX binding to *Torpedo* AChR and performed IC_{50} analyses for all peptides that were able to inhibit ^{125}I-α-BTX binding to *Torpedo* AChR as indicated in Figure 4. The assays were perfomed at concentrations between 2 and 100 μM. The IC_{50} values were determined from the negative slope of the Hofstee plots (%inhibition versus %inhibition/[peptide]) (Molinoff, 1981; McLane et al., 1991a,b,c). The Hofstee plots for five representative peptides that differ in their ability to inhibit ^{125}I-α-BTX binding are given in Figure 5, and the results obtained concurrently for all of the substitution analogues are summarized in Table 2. The IC_{50} obtained for the unsubstituted peptide α(181–200) was ~3 μM, which is comparable to values previously obtained (McLane et al., 1990b, 1991a,b,c,). The IC_{50} values obtained for the single amino acid substitution analogues varied from approximately 2-fold to a 100-fold increases over that obtained for the unsubstituted peptide. This broad range of values confirms the results of previous studies, which found that the IC_{50} values obtained from *Torpedo* competition assays give a broader range for the relative affinties of peptides for α-BTX than found from Scatchard analyses of peptides bound to solid supports (McLane et al., 1991a,b).

The relative IC_{50} values obtained for peptides that bound α-BTX agreed well with the results of the qualitative assays (Figure 4). Substitution analogues that competed for α-BTX binding at levels comparable to the native peptide had small increases in the IC_{50} values (the values in parentheses are increases in the IC_{50} relative to the unsubstituted peptide, which is considered equal to 1.0) [W184-F (1.7), W187-F (2.1), W187-M (1.7), W187-Y (1.8), V188-T (1.9), D195-N (2.5)]. Other substitutions had more profound effects on the relative affinities for ^{125}I-α-BTX: K185-E (34.4), K185-R (8.4), V188-I (7.1), Y190-H (5.5), T191-S (20), T196-S (5.6), Y198-F (4.9), Y198-H (3.3), Y198-T (4.0).

Comparison of Dot Blot and Competition Assays. We have previously found differences in the results of solid and solution phase binding assays and have attributed these discrepancies to differences in the ability of peptides to bind to solid supports in an active conformation or to the propensity of some peptides to dimerize in solution (Conti-Tronconi et al., 1991; McLane et al., 1991a–c). By comparing the results of these two different assays systems, however, we are able to reliably determine which amino acid side chain contacts are critical for α-BTX binding. The validity of this approach is also reflected in the agreement between our results using peptides and those of complementary studies using recom-

Table 2: Apparent Binding Affinities of Peptides to α-BTX Determined by Scatchard and IC_{50} Analyses

	scatchard analysis			IC_{50} analysis		
peptide	K_d (μM)[a]	correlation coefficient	relative to native[b]	IC_{50} (μM)[c]	correlation coefficient	relative to native[d]
native	1.7	(0.98)	1.0	3.0	(0.88)	1.0
W184-F	1.8	(0.95)	1.1	5.2	(0.91)	1.7
K185-E	5.9	(0.89)	3.5	103	(0.91)	34.4
K185-R	4.0	(0.85)	2.4	25.2	(0.98)	8.4
W187-F	1.8	(0.86)	1.1	6.3	(0.99)	2.1
W187-M	5.2	(0.84)	3.0	5.2	(0.94)	1.7
W187-Y	3.4	(0.90)	2.0	5.3	(0.94)	1.8
V188-I	3.9	(0.94)	2.3	21.3	(0.94)	7.1
V188-T	1.5	(0.91)	0.85	5.7	(0.96)	1.9
Y189-F	nc[e]			14.2	(0.99)	4.7
Y189-H	nc			21.3	(0.92)	7.1
Y189-T	nd[f]			nd		
Y190-F	nd			nd		
Y190-H	nd			16.6	(0.97)	5.5
Y190-T	nd			nd		
T191-S	4.3	(0.81)	2.5	60	(0.79)	20
T191-V	3.3	(0.95)	1.9	nd		
C192-S	nd			300	(0.76)	100
C192-H	nd			nd		
C192-V	nd			nd		
C192/G/C193	nd			nd		
C193-S	nd			nd		
C193-H	nc			nd		
C193-V	nc			nd		
P194-GG	nc			nd		
D195-E	nc			15.3	(0.98)	5.1
D195-N	5.5	(0.86)	3.2	7.4	(0.85)	2.5
T196-S	4.9	(0.88)	2.9	16.9	(0.97)	5.6
T196-V	2.2	(0.83)	1.3	nd		
P197-GG	nc			nd		
Y198-F	4.0	(0.85)	2.4	14.7	(0.91)	4.9
Y198-H	nc			9.9	(0.82)	3.3
Y198-T	nc			11.8	(0.99)	4.0

[a] The *apparent* K_d values were determined using Scatchard analysis of dot blot assays as described under Experimental Procedures and in the legend to Figure 3. The K_d values of the substituted peptides reported in this table are meant only to reflect their apparent affinities relative to the native peptide. Given the solid phase nature of the assay, the K_d values only approximate the true values of the dissociation constants. The K_d values are determined from the slope of the regression curves. The correlation coefficients of the linear regression are given in parentheses. The corresponding theoretical B_{max} values (in pmol), which reflect the amount of peptide bound in an active conformation to the nitrocellulose, were native (4.8), W184-F (3.5), K185-E (15.5), K185-R (9.0), W187-F (2.3), W187-M (8.3), W187-Y (9.2), V188-I (8.9), V188-T (3.9), T191-S (8.4), T191-V (5.3), D195-N (4.5), T196-S (9.6), T196-V (3.8), and Y198-F (9.0). [b] The K_d values for the single amino acid substituted peptides are divided by that obtained for the native peptide. [c] The IC_{50} values were obtained using the competition assay described under Experimental Procedures and in the legends to Figures 4 and 5. The IC_{50} values were obtained from linear regression of the Hofstee plots, as shown in Figure 5. The IC_{50} values of the substituted peptides are reported only to reflect their apparent affinities relative to the native peptide. The correlation coefficients of the linear regression are given in parentheses. [d] The IC_{50} values for the single amino acid substituted peptides are divided by that obtained for the native peptide. [e] "Not calculable", i.e., insufficient data were obtained for calculation of the K_d and B_{max} due to weak binding of ^{125}I-α-BTX by the peptide. [f] "Not done", i.e., the peptide does not detectably bind α-BTX using this assay system.

binant fusion proteins containing the same α subunit sequences. For example, agreement between these two approaches is observed for: (i) differences in α-BTX binding arising from naturally occurring substitutions in the sequences of muscle AChR α1 subunits of different species (McLane et al., 1991b; Ohana & Gershoni, 1990; Ohana et al., 1991; Chaturvedi et al., 1992); (ii) the demonstration of α-BTX binding to the chick AChR α7 and α8 subunits (McLane et al., 1991b; Couturier et al., 1990); and (iii) prediction of the critical amino acid residues for α-BTX binding (Tzartos & Remoundos, 1990; Conti-Tronconi et al., 1991; Tomaselli et al., 1991; Chaturvedi et al., 1992, 1993; and discussed below).

In the present study, the results of solid and solution phase were found to be in general agreement for most of the peptides used in these assays. Where the results of the two assays did not perfectly coincide, disagreement arose primarily in the extent (high versus moderate) of inhibition of α-BTX binding (discrepancies occurred for peptides K185-E, Y189-F, Y190-H, T191-V, and D-195-E). For two peptides Y198-H and Y198-T, solution phase competition assays did not demonstrate an effect of these substitutions on α-BTX binding, whereas in dot blot assays these peptides were unable to bind α-BTX. In our previous studies, substitution of K185, D195, and Y198 by glycine markedly affected α-BTX binding in dot blot assays and did not affect the ability to these peptides to bind α-BTX in solution phase competition assays (Conti-Tronconi et al., 1991). Therefore, the discrepancies observed in the present studies for solution and solid phase assays could be anticipated from our previous results.

DISCUSSION

We have previously demonstrated that the sequence segment α(181–200) of the *Torpedo* AChR α subunit forms an α-BTX binding prototope (Conti-Tronconi et al., 1990; McLane et al., 1991a,b). Using single amino acid substitution analogues, in which each amino acid residue of the native sequence of *Torpedo* α(181–200) were replaced by glycine, we identified several residues important for α-BTX binding (Conti-Tronconi et al., 1991). In particular, substitution of amino acid residues 188–190 (VVY) and 192–194 (CCP) were detrimental to α-BTX binding. In the present study, we selected amino acid residues of the sequence *Torpedo* α(181–200) for which glycine substitution reduced α-BTX binding, for more extensive mutational analysis. Amino acid substitutions were chosen

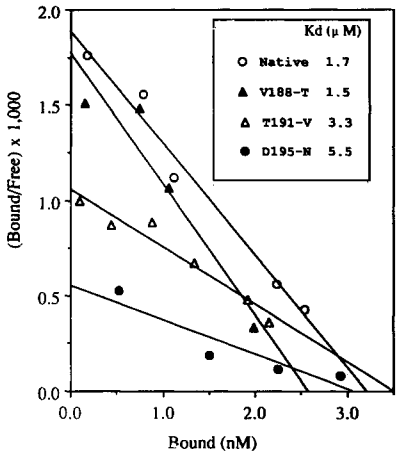

FIGURE 3: Scatchard analysis of ^{125}I-α-BTX binding to single substitution peptide analogues of *Torpedo* AChR α(181–200). Dot blot assays were performed using the following ^{125}I-α-BTX concentrations (11.7 Ci/mmol): 0.1, 0.5, 1, 2, 4, and 6 μM. Nonspecific binding (10–21% of the total cpm) was determined by preincubating the strips with unlabeled α-BTX (100 μM) prior to addition of ^{125}I-α-BTX. The data points were determined in triplicate and are presented as scatchard plots of the fmol of ^{125}I-α-BTX bound at each concentration. The *apparent* K_d and B_{max} values and the linear regression correlation coefficient of the scatchard analysis are reported for each peptide in Table 2. The K_ds of the peptides for which the scatchard plots are presented in the inset.

in order to determine: (i) physicochemical attributes of the amino acid side chains that mediate α-BTX binding (i.e., steric, hydrophobic, hydrogen-bonding, and/or electrostatic interactions) and (ii) how the secondary structural constraints imposed by Pro residues or a disulfide bond confer α-BTX binding activity to this prototope. Thirty-two synthetic peptides corresponding to single amino acid substitution analogues of *Torpedo* α(181–200) (Figure 1) were tested for their ability to bind α-BTX in solid phase dot blot assays and solution phase competition assays.

The conclusions of these studies, based on consensus of solid and solution phase assays, are summarized in Figure 6. The amino acid substitutions that had a profound effect on α-BTX binding are indicated above the native *Torpedo* α-(181–200) sequence ("not tolerated"), and those amino substitutions that either slightly or moderately affected α-BTX binding are indicated below the native sequence ("tolerated"). As indicated in Figure 6, several conservative substitutions of Y189, Y190, C192, and C193 essentially abolish α-BTX binding. These residues had previously been shown to form important contacts with α-BTX by substitution with glycine (Conti-Tronconi et al., 1991). The present results indicate α-BTX binding is specific to these amino acid side chains as they cannot be replaced by amino acids with related physicochemical characteristics.

Y189 and Y190 are highly conserved amino acid residues in homologous regions of the AChR α subunits of different subtypes and different species that bind α-BTX (McLane et al., 1991a,b). Y190 is found in all α subunits, with the exception of α5, and does not in itself confer α-BTX binding activity (McLane et al., 1990b, 1991c). However, replacements of Y190 by two aromatic amino acids (F and H), or T, which contains a polarizable -OH group, are not compatible substitutions for α-BTX binding. Y189, on the other hand, has been shown to be responsible for high-affinity α-BTX binding activity (Wilson & Lentz, 1988; Ohana & Gershoni, 1990; McLane et al., 1991a,b). In the homologous sequence segments of calf α1, mouse α1, and chick α7 AChR subunits, Y189 is replaced by F with a moderate decrease in affinity for α-BTX (Mclane et al., 1991a,b), and as would be predicted we observed a moderate increase in the IC_{50} for α-BTX binding for Y189-F. In the human α1 sequence, Y189 is replaced by T resulting in very low affinity α-BTX binding of the corresponding peptide (McLane et al., 1991a). In the present study, peptide Y189-T does not bind α-BTX detectably. The reduction in binding observed upon replacement of Y189 and Y190 with F or T indicates that both the aromatic ring and the hydroxyl group of Y provide important hydrogen-bonding, hydrophobic, and/or π-electron interactions with α-BTX. Substitution of several aromatic residues, in addition to Y189 and Y190, was shown to affect α-BTX binding moderately (Y198-H, Y198-F, Y198-T) or minimally (W184-F, W187-M, W187-Y, W187-F). These results are compatible with a recent report in which bacterially expressed mutants Y198-A and W184-F were found to bind α-BTX (Chaturvedi et al., 1993).

The role of several aliphatic amino acids was also reevaluated in the present study. Substitution of T191 and T196 with S or V resulted in a moderate and/or inconsistent decrease in α-BTX binding. These results agree with a study which used fusion proteins carrying mutations T191-S and T191-A, which retained a high level of α-BTX binding activity (Chaturvedi et al., 1993). α-BTX binding was previously found to be intolerant to glycine substitution of V188 (Conti-Tronconi et al., 1991), but it is only moderately affected by the conservative substitution V188-I. The substitution V188-T is tolerated by α-BTX in a synthetic peptide in the present study or a fusion protein (Chaturvedi et al., 1993). We conclude, therefore, that although V188 is a critical contact point with α-BTX, several amino acid residues are allowable at this position, with the notable exception of glycine (Conti-Tronconi et al., 1991).

In previous studies, glycine substitutions at position K185 and D195 were found to decrease α-BTX binding in dot blot assays (Conti-Tronconi et al., 1991). In the present study, moderate reduction in α-BTX binding activity were found with the following substitutions, which either preserve, neutralize, or reverse the polarity of the charge: K185-E, K185-R, D195-E, D195-N. Using fusion proteins Chaturvedi et al. (1993) demonstrated that K185 can be replaced by W without effect on α-BTX binding. On the other hand, their mutant proteins D195-K and D195-A exhibited lower α-BTX binding activity relative to the parental sequence. The lack of a predictable tendency of the charge change to affect α-BTX binding demonstrates that charge–charge interactions play only a minor role in conferring α-BTX binding properties to the peptide *Torpedo* α(181–200), suggesting the validity of a model of the interface between α-BTX and the AChR involving few electrostatic and primarily hydrophobic and hydrogen-bonding interactions (Love & Stroud, 1986).

On the basis of its structure in solution and in crystals (Love & Stroud, 1986; Basus & Scheek, 1988; Basus et al., 1988), α-BTX is believed to contact the AChR through a region of extensive β-sheet that forms a carapace, excluding water from the AChR/α-BTX interface (Love & Stroud, 1986). The guanidium group of R37 on α-BTX, which is common to all α-neurotoxins, has been proposed to be analogous to the quaternary ammonium group of acetylcholine and other nicotinic agonists and antagonists (Karlsson, 1979). A complementary negative subsite on the α subunit of the AChR was proposed to involve D195 (Ohana & Gershoni, 1990), but affinity labeling studies have indicated that this residue is not involved in cation stabilization of acetylcholine (Cohen et al., 1991). Our present studies also indicate that D195 can

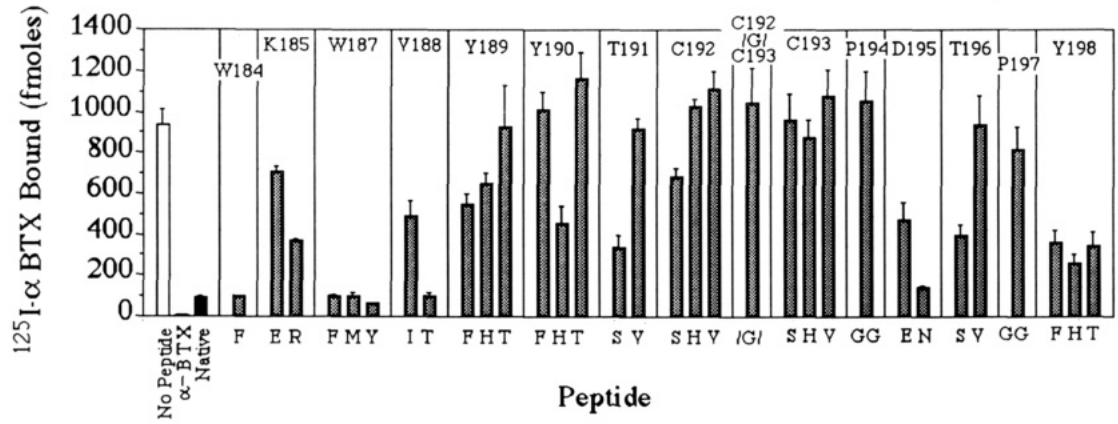

FIGURE 4: Competition of peptides for ^{125}I-α-BTX with native membrane bound *Torpedo* AChR. Peptides (40 μM) were incubated with 2 pmol of ^{125}I-α-BTX (22 Ci/mmol) overnight at 4 °C *Torpedo* AChR (6 pmol) was added, and, after 3 min at room temperature, the membrane-bound AChR was centrifuged and washed as described under Experimental Procedures. Assays were performed in triplicate for each peptide. The data are presented as the average fmol of ^{125}I-α-BTX bound to the AChR pellet after preincubation with each peptide. The error bars are standard deviations. The controls include (i) "No Peptide", in which ^{125}I-α-BTX is preincubated buffer rather than peptide, and (ii) "α-BGT", in which the *Torpedo* AChR is preincubated with unlabeled α-BTX prior to addition of ^{125}I-α-BTX that has been preincubated with buffer only.

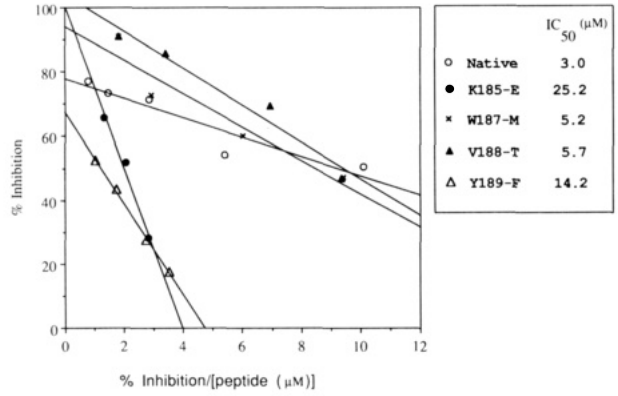

FIGURE 5: IC_{50} analysis of competitive inhibition of ^{125}I-α-BTX binding to *Torpedo* AChR. Competition assays were performed as described in the legend to Figure 4, except that the concentrations of peptides used as inhibitors of ^{125}I-α-BTX binding to *Torpedo* AChR ranged from 2 to 200 μM. The assays at each concentration were performed in triplicate, and the average ^{125}I-α-BTX binding at each concentration determined and the percent inhibition calculated using the "No Peptide" control values as 0% inhibition (refer to Experimental Procedures and Figure 4). The assays were performed over a period of several weeks, and the specific activity of ^{125}I-α-BTX used was 21–27 Ci/mmol. The results are presented as a Hofstee plot (Molinoff et al., 1981), where the IC_{50} values (inset) can be directly determined as the negative slopes of the regression lines. The IC_{50} values and the correlation coefficients for all peptides are listed in Table 2, and those values correspond to the IC_{50} determinations illustrated here.

FIGURE 6: Summary of the effect of conservative amino acid substitutions on the binding of ^{125}I-α-BTX to *Torpedo* AChR α-(181–200). Consistent results obtained for both the solution and solid phase assays are summarized. The native sequence of *Torpedo* α-(181–200) is indicated at the center of the figure, and the residue position numbers indicated under amino acids are relative to the *Torpedo* α subunit. The amino acid substitutions that are tolerated and result in equal or slightly lower α-BTX binding activity, are indicated by arrows from below the positions of the native sequence, which are replaced. Similiarly, those substitutions that are not tolerated and abolish α-BTX binding activity of the peptides are indicated by arrows above the native sequence.

be substituted by Asn with little effect on α-BTX binding.

An alternative model for a complementary binding subsite on the AChR α subunit for the quaternary ammonium of acetylcholine is suggested by two model systems, which indicate that cation stabilization can be provided by π-electrons from aromatic rings: (i) a synthetic acetylcholine binding site composed entirely of aromatic rings (Doughtery & Stauffer, 1990), and (ii) the X-ray crystal structure of acetylcholinesterase, which contains an active site formed by a gorge lined with aromatic amino acids (Sussman et al., 1991). Thus, it is likely that the subsite required for cation stabilization is provided by either the tyrosine anion or the π-electron system of Y189 and/or Y190.

C192 and C193 has been shown to form a highly reactive vicinal disulfide in the native *Torpedo* AChR (Kao et al., 1984; Kao & Karlin, 1986; Dennis et al., 1988; Mosckovitz & Gershoni, 1988; Kellaris et al., 1989). Several studies, however, indicate that an intact vicinal disulfide bond is not required for α-BTX binding. For instance, *Torpedo* AChR, reduction, or reduction and alkylation does not affect the number of α-BTX binding sites (Moore & Raftery, 1979; Walker et al., 1981). Expression studies in *Xenopus* oocytes of *Torpedo* α subunits carrying mutations of C192 or C193 also indicate that α-BTX binding can occur in the absence of an intact disulfide (Mishina et al., 1985). α-BTX binding studies using synthetic peptides that have been modified with sulfhydryl reagents indicate that α-BTX binding is not affected by the redox state of the C192/C193 pair and that formation of a vicinal disulfide is not a requirement (Conti-Tronconi et al., 1991; McLane et al., 1991a–c; 1993a).

In the present study, substitution of either C192 or C193 by residues with side chains of similar size (S and V) is not tolerated, nor is substitution by H, which shares polar and hydrophobic properties. Chaturvedi et al. (1993) have also demonstrated that substitution of C193 by A abolishes α-BTX binding. Therefore, it appears that the important attributes of C at these positions does not involve a propensity to form a vicinal disulfide but is rather a function of the size and hydrophobic nature of the C side chains. Formation of a disulfide bond results in a loss of hydrogen-bonding potential,

with an increase in hydrophobicity, and neither of these changes appear to be determinants for α-BTX binding. The C/C pair is an important structural component of the α-BTX binding site, however, as demonstrated by the inability of the peptide C192/G/C193 to bind α-BTX, where a glycine is inserted between C192 and C193. The introduction of conformational flexibility within this region, as discussed below for substitutions P194-GG and P197-GG, is deleterious for α-BTX binding.

The two P residues (P194 and P197) are conserved between the muscle-type α1 subunits of different species that bind α-BTX (McLane et al., 1991b). However, if one includes other neuronal α subunit subtypes that bind α-BTX (chick α7, chick α8, *Drosophila* ALS, *Drosophila* SAD), only P197 is predictive of α-BTX binding (McLane et al., 1991b). In the present study, we tested the involvement of these P residues in the formation of the α-BTX prototope by their replacement with two adjacent G residues (peptides P194-GG and P197-GG). This substitution results in a profound change in the propensity for a turn in the peptide backbone (Wilmot & Thornton, 1988). In both the solid phase and solution phase assays peptides P194-GG and P197-GG were unable to bind α-BTX, indicating that P residues are important structural elements of the α-BTX binding site.

Common to both the vicinal disulfide and proline imides is the propensity to form nonplanar, *cis* peptide bonds (Stewart et al., 1990; Creighton, 1993). It is possible that two adjacent *cis* peptide bonds could act together to cause a turn in the peptide backbone, providing an important element of secondary structure for the active α-BTX-binding conformation of this prototope. Molecular modeling of this region (Chatuverdi et al., 1993) indicates that the vicinal disulfide, P194, and P197 are likely to induce a β-turn (Wilmot & Thornton, 1988), which would allow the β-strand formed by this peptide to fold back on itself, providing stabilization of this conformation by β-sheet formation (Creighton, 1993). We have previously demonstrated that the peptide *Torpedo* α(181–200) is largely β-sheet in solution and that there is an increase in β-sheet secondary structure upon α-BTX binding, as revealed by the difference circular dichroism spectra (Conti-Tronconi et al., 1991). This increase in β-sheet could be due to an increase in secondary structure of the peptide or α-BTX, or both.

In conclusion, our present studies reconfirm our previous observations that several aliphatic and aromatic amino acid residues are involved in forming the α-BTX binding site. In particular, Y189 and Y190 play a critical role in this interaction, which is mediated by both the hydroxyl group and the phenyl ring. The adjacent C/C pair, C192 and C193, are also intimately involved in forming contacts with α-BTX, and even conservative substitutions (S or V) of either C residue are not tolerated. Although the consensus of previous C/C modification studies indicates that a vicinal cystine bond is not an absolute requirment for α-BTX binding (McLane et al., 1993a), such a structure is formed by the peptide *Torpedo* α(181–200) (Conti-Tronconi et al., 1991; McLane et al., 1991a), and this structure, in addition to adjacent P194, would tend to induce a turn at this position of the peptide. The importance of P194, P197, and the potential of a turn in the peptide are also demonstrated by the profound effect that replacement of either P with two G residues. The results indicate that the β-sheet structure observed for this peptide may reflect the folding back of the peptide backbone, as a result of a turn, and the stabilization of β-strand structure, as suggested by molecular modeling of this sequence segment (Chaturvedi et al., 1993). The present studies provide further information for a more refined working model of the α-BTX binding site, which also reflects the accessibility of amino acid side chains to other cholinergic ligands.

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