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# Effects of Mutations of *Torpedo* Acetylcholine Receptor α1 Subunit Residues 184–200 on α-Bungarotoxin Binding in a Recombinant Fusion Protein†

Vijaya Chaturvedi, Diana L. Donnelly-Roberts, and Thomas L. Lentz*

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510*

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ABSTRACT: Residues between positions 184 and 200 of the *Torpedo* acetylcholine receptor α1 subunit were changed by oligonucleotide-directed mutagenesis in a recombinant fusion protein containing residues 166–211. Amino acids were substituted with residues present in the snake α subunit, with an alanine, or with a functionally dissimilar residue. The competitive antagonist α-bungarotoxin bound to the fusion protein with high apparent affinity ($IC_{50} = 3.2 \times 10^{-8}$ M), and binding was competed by agonists and antagonists. Mutation of His-186, Tyr-189, Tyr-190, Cys-192, Cys-193, Pro-194, and Asp-195 greatly reduced or abolished α-bungarotoxin binding, while mutation of Tyr-198 reduced binding, indicating these residues play an important role in binding either through functional interaction with neurotoxin residues or by stabilizing the conformation of the binding site. Molecular modeling of acetylcholine receptor residues 184–200 and knowledge of both neurotoxin and receptor residues essential for binding allow analysis of possible structure–function relationships of the interaction of α-bungarotoxin with this region of the receptor.

The nicotinic acetylcholine receptor (AChR)[1] belongs to a superfamily of ligand-gated ion channels (Betz, 1990; Stroud et al., 1990). The AChR on the postsynaptic surface of the neuromuscular junction transduces a chemical signal, the neurotransmitter acetylcholine (ACh), into an electrical event leading to contraction of the muscle cell. Upon binding of ACh, the AChR undergoes a conformational change in which a channel is opened, allowing sodium ions to enter and depolarize the cell. The AChR from *Torpedo californica* electric organ is a transmembrane glycoprotein composed of four subunits present in a stoichiometric ratio of 2α1, β1, γ, δ [see Stroud et al. (1990) and Galzi et al. (1991) for review]. This pentameric complex contains the binding sites for agonists, e.g., ACh, carbamylcholine, and nicotine, and for competitive antagonists, e.g. α-bungarotoxin and *d*-tubocurarine. In addition to the muscle-type AChR described above, there are homologous neuronal nicotinic AChRs which perform excitatory transmission (Sargent, 1993).

The snake venom neurotoxin α-bungarotoxin (α-Btx) has been utilized as a probe for the cholinergic-binding site because it binds specifically and with high affinity to the AChR and competitively blocks the depolarizing action of ACh. Studies investigating the binding of α-Btx to isolated α-subunit [see Lentz and Wilson (1988)], proteolytic peptide fragments [see Lentz and Wilson (1988)], synthetic peptides (Wilson et al., 1985, 1988; Mulac-Jericevic & Atassi, 1986; Neumann et al., 1986; Ralston et al., 1987; Gotti et al., 1988; Wilson & Lentz, 1988; Conti-Tronconi et al., 1990; Donnelly-Roberts & Lentz, 1991; Griesmann et al., 1990; Tzartos & Remoundos, 1990), and fusion proteins containing receptor sequences (Barkas et al., 1987; Gershoni, 1987) indicate a major neurotoxin-binding site is located between residues 173 and 204 of the α subunit. The affinity of toxin binding to peptides is reduced relative to the intact receptor, possibly as a result of a loss of the native conformation of the binding site in the denatured protein. However, the affinity of toxin binding to residues 173–204 ($4.2 \times 10^{-8}$ M) is comparable to that for intact, isolated α subunit ($4.6 \times 10^{-8}$ M) (Wilson et al., 1988), indicating this region represents the major toxin-binding determinant on the α subunit. In addition to this region, other regions of the α subunit (McCormick & Atassi, 1984; Cockcroft et al., 1990; Conti-Tronconi et al., 1990; Galzi et al., 1990; Cohen et al., 1991) and residues of other subunits (Czajkowski & Karlin, 1991; Chiara & Cohen, 1992) have been reported to contribute to the cholinergic-binding site.

Because a major component of the α-Btx-binding site is located within a defined amino acid sequence, it is possible to investigate structure–function relationships of this site. Fusion proteins containing the α-subunit sequence represent an efficient system for substitution of residues by site-directed mutagenesis. In this study, residues 184–200 of *Torpedo* α1 subunit have been systematically changed by means of site-directed mutagenesis in a recombinant fusion protein comprising residues 166–211 and effects on α-Btx binding measured. These studies show that seven residues, His-186, Tyr-189, Tyr-190, Cys-192, Cys-193, Pro-194, and Asp-195, are critical and an additional residue, Tyr-198, important for α-Btx binding. On the basis of this information and molecular modeling of residues 184–200, structure–function relationships of the neurotoxin-binding site are discussed.

## EXPERIMENTAL PROCEDURES

*Construction of Recombinant Plasmids.* A fusion protein consisting of the TrpE protein fused to residues 166–211 of the *Torpedo* α1 AChR subunit was produced in *Escherichia coli*. A construct containing full-length cDNA encoding the *Torpedo* α1 subunit in pBR 322 (gift of Dr. Toni Claudio) was digested with *Eco*RI to obtain the complete α-subunit cDNA. The *Eco*RI fragment was digested with *Bst*YI to produce a 446 base pair fragment encoding residues 166–313. This fragment was then digested with *Bbv*I to yield a 138 base pair fragment encoding residues 166–211, and the ends were

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* Address correspondence to this author at the Department of Cell Biology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510 [telephone (203) 785-4565].

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; BSA, bovine serum albumin; α-Btx, α-bungarotoxin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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filled by treatment with the Klenow fragment of DNA polymerase I. The DNA fragment was inserted into the *Sma*I site of a pATH10 expression vector (Koerner et al., 1991) and used to transform *E. coli* strain XL1-Blue.

*Site-Directed Mutagenesis.* Mutations have been performed on all of the residues between Trp-184 and Asp-200 except Leu-199 (Table I). Five mutations were performed in the following manner. The pATH10–*Torpedo* 138 base pair construct was digested with *Hin*dIII and *Eco*RI and the fragment subcloned into the *Hin*dIII and *Eco*RI sites of an M13mp19 vector. Mutagenesis was performed by means of oligonucleotide-directed mutagenesis using an Amersham (Arlington Heights, IL) kit. Thirty-six-residue oligonucleotides with the desired conversions were synthesized and purified by the Department of Pathology DNA Synthesizing Service, Yale University. A plasmid containing the mutant cDNA fragment was digested with *Hin*dIII and *Eco*RI and the resulting fragment subcloned into the corresponding sites of the pATH10 expression vector. All other mutations were performed using a polymerase chain reaction (PCR)-based method of site-directed mutagenesis termed the "megaprimer" method (Sarkar & Sommer, 1990). Three rounds of PCR were performed. The 138 base pair fragment was subcloned into a pBluescript SK +/− vector. The first PCR was performed on this construct using T3 and an oligonucleotide with the desired substitution to produce the megaprimer. The second PCR was performed using T7 and megaprimer to form the complete fragment. The mutated segment was electrophoresed, eluted from agarose, and purified by Geneclean (Bio 101, LaJolla, CA). The final round of PCR was performed to amplify the mutated DNA, which was then digested with *Hin*dIII and *Eco*RI, and the resulting fragment subcloned into the corresponding sites of pATH10. All cDNA fragments were sequenced to verify that the desired mutations were achieved.

*Preparation of TrpE Fusion Proteins.* Bacterial clones containing cDNA fragments were induced and harvested as previously described (Koerner et al., 1991). Briefly, starter cultures were cultured overnight in M9 medium supplemented with Casamino acids (0.5%), tryptophan (10 μg/mL), and ampicillin (50 μg/mL). Cells were then diluted 1:100 in the same M9 medium but lacking tryptophan and cultured for 2 h at 37 °C. Then 5 μg/mL of 3-β-indoleacrylic acid was added, and cultures were grown for 4–6 h at 37 °C. The bacteria were pelleted and solubilized in sample buffer [50 mM Tris-HCl, pH 6.8/10% (w/v) glycerol/100 mM dithiothreitol/0.1% bromophenol blue/2% sodium dodecyl sulfate (SDS)] and boiled for 5 min. The samples were electrophoresed on 15% polyacrylamide gels. Gels were stained with Coomassie brilliant blue or blotted onto nitrocellulose and overlayed with $^{125}$I α-Btx (Gershoni et al., 1983) and autoradiographed.

Positive clones were identified by virtue of a shift in the apparent $M_r$ of the TrpE protein to the approximate size expected for the fusion protein. Bacteria were pelleted and washed with 10 mM phosphate buffer/0.15 M NaCl, pH 7.4 (PBS), repelleted, and resuspended in 0.05 M phosphate buffer, pH 7.4, containing 500 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. The suspension was sonicated on ice for 1 min and then centrifuged for 10 min at 3000 rpm. The supernatant was discarded and the pellet resuspended in PBS containing phenylmethanesulfonyl fluoride. The fractions were stored at −70 °C.

*Assay of $^{125}$I α-Btx Binding to Fusion Proteins.* α-Btx was obtained from the Miami Serpentarium (Salt Lake City, UT) and iodinated as described (Wilson & Lentz, 1988). The specific activity of the labeled toxin was 300–600 cpm/fmol. Binding of $^{125}$I α-Btx to fusion proteins was tested in a solid-phase binding assay as described for synthetic peptides (Wilson & Lentz, 1988). Briefly, 100 μL of distilled water containing 3 μg of fusion protein was added to microtiter wells (Immulon Removawell Strips, Dynatech Labs, Chantilly, VA), and the wells were dried overnight at 45 °C. Wells were washed with PBS and quenched for 1 h with 10% bovine serum albumin (BSA). Labeled toxin (~200 000 cpm/100 μL of 10 mM phosphate buffer, pH 7.4/0.2% BSA/0.03% SDS) was added to wells and incubated for 2 h. Binding of α-Btx to the mutated fusion proteins was compared in this manner. The ability of α-Btx, cobrotoxin, *d*-tubocurarine, suberyldicholine, nicotine, carbamylcholine, and NaCl to inhibit $^{125}$I α-Btx binding to fusion proteins was tested. For competition experiments, increasing concentrations of competitor (50 μL) were added to wells followed immediately by 50 μL of $^{125}$I α-Btx (~100 000 cpm) in 10 mM phosphate buffer, pH 7.4/0.2% BSA/0.03% SDS and incubated for 1 h. Measurement of the rate of binding of $^{125}$I α-Btx to the nonmutated fusion protein showed that binding was linear up to 2 h (data not shown). Background binding to wells coated with TrpE protein was determined (~400 cpm) and subtracted from total binding to fusion proteins (~4000 cpm). All determinations were performed in triplicate. After incubation, wells were washed five times with PBS. Wells were separated and placed in vials, and radioactivity was measured in a gamma counter. Affinities of binding were approximated by determination of the concentration of ligand that resulted in a 50% reduction in the binding of $^{125}$I α-Btx ($IC_{50}$ value). Because the assays are not performed under equilibrium binding conditions, the $IC_{50}$ values obtained represent apparent affinities. Competition curves are graphically represented by fitted curves derived from a polynomial least-squares fit as performed by the computer program KaleidaGraph (Synergy Software, Reading, PA).

*Molecular Modeling.* Molecular modeling of residues 184–200 was performed using SYBYL molecular modeling software (Tripos Associates, St. Louis, MO) on a VAX 8800 computer and an Evans and Southerland PS300 graphic workstation.

## RESULTS

To characterize the pharmacological characteristics of the fusion protein comprising residues 166–211 of the α1 subunit, competition experiments were performed with cholinergic agonists and antagonists. These experiments yielded $IC_{50}$ values for α-Btx, cobrotoxin (*Naja naja atra*), *d*-tubocurarine, suberyldicholine, nicotine, NaCl, and carbamylcholine of $3.2 \times 10^{-8}$, $8.9 \times 10^{-6}$, $2.9 \times 10^{-5}$, $4.1 \times 10^{-4}$, $5.5 \times 10^{-3}$, $1.8 \times 10^{-2}$, and $4.0 \times 10^{-2}$ M, respectively (Figure 1).

Individual amino acids between positions 184 and 200 of the fusion protein were mutated and the effects on the binding of the competitive antagonist α-Btx measured. The distribution and characteristics of residues and the mutations performed are shown in Table I. Some mutations (W184F, K185W, W187S, Y189N, T191S, and P194L) converted a *Torpedo* residue to a snake residue. Other mutations (H186A, Y190A, C192A, C193A, D195A, T196A, P197A, Y198A, and D200A) substituted a *Torpedo* residue with an alanine. Alanine is considered a suitable replacement for most residues because it has a small side chain and no charged or hydrogen-bonding groups (Ward et al., 1990). The remaining mutations substituted a *Torpedo* residue with an amino acid bearing a

Table I: Mutations of *Torpedo* AChR α1 Subunit

| | 166 | 184 | 192 | 200 | 211 |
|---|---|---|---|---|---|
| *Torpedo* α1 sequence | DLSTFMESGEWVMKDYRGWKHWVYYTCCPDTPYLDITYHFIMQRIP | | | | |
| mutations | | FWASTNGSGALKAAA A | | | |
| | | A A A | | | |

| mutation | residue distribution | characteristics of mutation | Btx binding[a] |
|---|---|---|---|
| W184F | W184 conserved or conservatively substituted in muscle α subunits | conversion to residue in snake receptor; conservative substitution | 102 |
| K185W | K185 conserved or conservatively substituted in toxin-binding α subunits | conversion to snake residue; converts positively charged side chain to hydrophobic aromatic | 104 |
| H186A | H186 conserved in muscle α subunits | converts polar side chain to nonpolar hydrophobic | 10 |
| W187S | W187 not conserved | conversion to residue present in snake α, human α1, rat α2, chicken α2, and chicken α4; converts hydrophobic side chain to polar hydrophilic | 161 |
| V188T | V188 conserved in muscle α subunits | converts hydrophobic side chain to polar hydrophilic | 151 |
| Y189N | Y189 not conserved | conversion to snake residue; converts polar aromatic side chain to polar amide group | 0 |
| Y190G | Y190 conserved in α subunits | converts polar aromatic side chain to residue lacking side chain | 6 |
| Y190A | | converts polar aromatic side chain to nonpolar hydrophobic | 15 |
| T191S | T191 not conserved | conversion to residue present in snake α, mouse α1, rat α1, human α1, rat α5, and *Drosophila* α1 subunits; conservative substitution | 96 |
| C192G | C192 conserved in α subunits | converts thiol moiety to residue lacking side chain | 9 |
| C192A | | converts thiol moiety to nonpolar hydrophobic | 83 |
| C193A | C193 conserved in α subunits | converts thiol moiety to nonpolar hydrophobic | 3 |
| P194L | P194 conserved in toxin-binding, muscle α subunits | conversion to snake residue; converts cyclic imino acid to nonpolar aliphatic side chain | 2 |
| D195K | D195 conserved or conservatively substituted in α subunits | converts negatively charged side chain to positively charged | 8 |
| D195A | | converts negatively charged side chain to nonpolar hydrophobic | 18 |
| T196A | T196 conserved in muscle α subunits | converts polar, hydrophilic side chain to nonpolar hydrophobic | 90 |
| P197A | P197 conserved in muscle α subunits | converts cyclic imino acid to nonpolar hydrophobic | 98 |
| Y198A | Y198 conserved in α subunits | converts polar aromatic side chain to nonpolar aliphatic | 43 |
| D200A | D200 conserved in α subunits | converts negatively charged side chain to nonpolar hydrophobic | 125 |

[a] Percent of binding to nonmutated sequence.

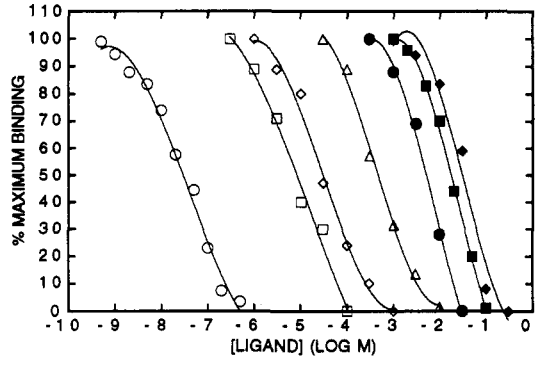


FIGURE 1: Competition of the binding of $^{125}$I α-Btx to *Torpedo* α1 166–211 fusion protein. Competition with unlabeled α-Btx (open circles), cobrotoxin (open squares), *d*-tubocurarine (open diamonds), suberyldicholine (open triangles), nicotine (solid circles), NaCl (solid squares), and carbamylcholine (solid diamonds) was performed in a solid-phase assay as described under Experimental Procedures. Values are the mean of two experiments with three replicates each.

functionally dissimilar side chain (V188T and D195K) or lacking a side chain (C192G).

Partially purified bacterial lysates were separated by polyacrylamide gel electrophoresis after transformation of *E. coli* with pATH10 vectors and growth under inductive conditions. After staining with Coomassie blue, the fusion proteins showed a shift in mobility from the molecular weight of the TrpE gene product (~37 000) to an apparent molecular weight of ~43 000 (Figure 2). A qualitative estimate of the ability of the fusion proteins to bind α-Btx was obtained by electrophoretically transferring the proteins to nitrocellulose and incubating the filter with $^{125}$I α-Btx. The results of this experiment showed that the Y189N, Y190A, Y190G, C192G, C193A, P194L, and D195K mutants did not bind toxin on protein blots, while the H186A, D195A, P197A, and Y198A mutants showed reduced binding relative to the nonmutated *Torpedo* α1 166–211 fusion protein (Figure 2).

To more precisely compare binding of the toxin to the fusion proteins, wells of microtiter plates were coated with 3 μg of fusion protein and incubated with ~200 000 cpm of $^{125}$I α-Btx. These experiments showed that some mutant fusion proteins, W187S, V188T, and D200A, bound somewhat more toxin than the nonmutated protein. Other mutations, W184F, K185W, T191S, T196A, and P197A, had no effect on binding. Binding was reduced in the Y198A mutant, while little or no binding was observed to the H186A, Y189N, Y190G, C192G, C193A, P194L, and D195K mutants (Figure 3; Table I). Binding was also reduced to Y190A and D195A mutant fusion proteins and was not significantly reduced to a C192A mutant (Table I).

Molecular modeling of residues 184–200 was performed to predict the conformation of this region of the α subunit. Because X-ray crystallographic data are not available for the receptor, certain structural estimations were made. First, the toxin-binding region of the receptor is predicted to be a β-barrel structure (Finer-Moore & Stroud, 1984; McCarthy & Stroud, 1989; Stroud et al., 1990). In addition, circular dichroism spectroscopy of a 181–200 α1 synthetic peptide revealed a structure of 50–60% β-pleated sheet and 25–30% random coil (Conti-Tronconi et al., 1991), and that of a 173–204 α1 peptide showed 80.3% β sheet, 18.9% random coil, and 0.8% α helix (Donnelly-Roberts & Lentz, 1993). Thus, this region of the receptor is predicted to be largely β structure. Second, a Cys–Cys disulfide requires a cis peptide bond [see Kao and Karlin (1986)]. The peptide bond between Cys-193 and Pro-194 is also estimated to be in the cis form because proline residues have a higher probability of having the cis isomer of

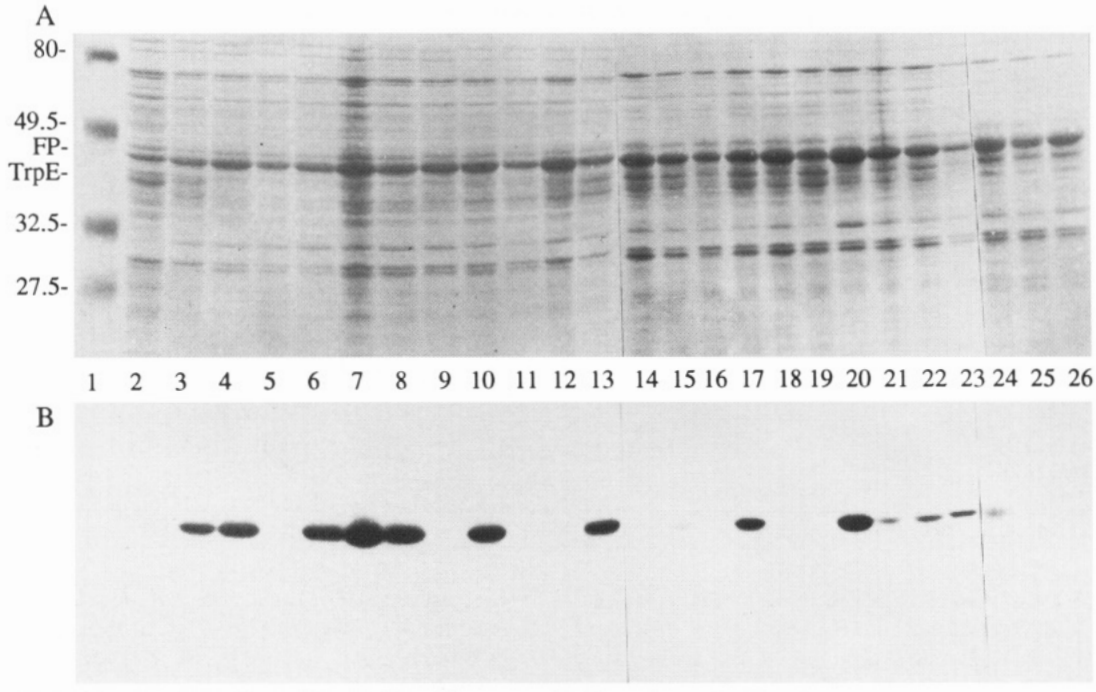


FIGURE 2: Analysis of lysates of transformed *E. coli*. Samples were resolved on polyacrylamide gels and either stained with Coomassie brilliant blue (A) or transferred onto nitrocellulose filters, overlayed with $^{125}$I α-Btx, and autoradiographed (B). (Lane 1) molecular weight standards; (lane 2) *E. coli* transformed with pATH10; (lane 3) *E. coli* transformed with *Torpedo* α1 166–211 cDNA fragment; (lane 6) W184F mutation; (lane 7) K185W mutation; (lane 8) W187S mutation; (lane 9) Y189N mutation; (lane 10) T191S mutation; (lane 11) P194L mutation; (lane 13) V188T mutation; (lane 14) D195K mutation; (lane 15) H186A mutation; (lane 16) Y190A mutation; (lane 17) C192A mutation; (lane 19) C193A mutation; (lane 20) T196A mutation; (lane 21) P197A mutation; (lane 22) Y198A mutation; (lane 23) D200A mutation; (lane 24) D195A mutation; (lane 25) Y190G mutation; (lane 26) C192G mutation; (lanes 4, 5, 12, and 18) disregard. Positions of molecular weight standards (MW × $10^{-3}$), fusion proteins (FP), and TrpE protein (TrpE) are shown on the left.

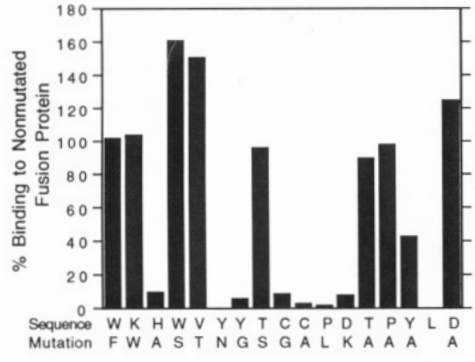


FIGURE 3: Binding of $^{125}$I α-Btx to fusion proteins. Wells of microtiter plates were coated with 3 μg of fusion proteins and incubated with ~200 000 cpm of $^{125}$I α-Btx for 2 h. Binding to TrpE protein alone was subtracted from experimental values. Values are the mean of at least four experiments with three replicates each.

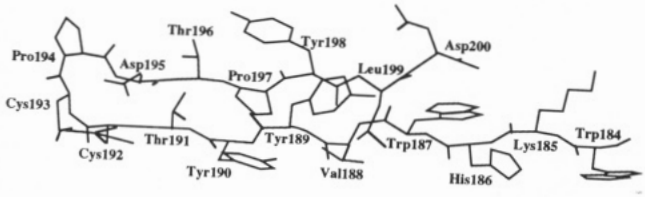


FIGURE 4: Energy-minimized model of residues 184–200 of *Torpedo* AChR α subunit. Structure is β sheet with cis peptide bonds between Cys-192 and Cys-193 and Cys-193 and Pro-194 and a disulfide bond between Cys-192 and Cys-193.

the preceding peptide bond than other residues (MacArthur & Thornton, 1991). When modeled on the basis of these estimations and energy minimization is performed, residues 184–200 form a loop with Cys-193 and Pro-194 at the tip of the loop (Figure 4). Minimized energy for the peptide is 70 kcal/mol.

## DISCUSSION

The fusion protein containing residues 166–211 of Torpedo AChR α subunit bound α-Btx with an apparent affinity of 3.2 × $10^{-8}$ M as determined from $IC_{50}$ values. This compares with $IC_{50}$ values of 3.5 × $10^{-10}$ M for α-Btx binding to native receptor (Wilson et al., 1988), 4.6 × $10^{-8}$ M for isolated α subunit (Wilson et al., 1988), 5.0 × $10^{-8}$ M for an α1 172–227 synthetic peptide (Donnelly-Roberts & Lentz, 1991), 4.2 × $10^{-8}$ M for an α1 173–204 synthetic peptide (Wilson et al., 1988), and 4.3 × $10^{-8}$ M for an α1 189–198 synthetic peptide (Tzartos & Remoundos, 1990). Thus, the affinity of toxin for the fusion protein is comparable to that for isolated α subunit and synthetic peptides encompassing Cys-192 and Cys-193. Competition of toxin binding with cholinergic agonists and NaCl indicates the absence of agonist-specific ability to compete α-Btx binding. The ability of NaCl to compete toxin binding is consistent with the cation sensitivity of the AChR (Schmidt & Raftery, 1974).

The results of this study indicate that residues His-186, Tyr-189, Tyr-190, Cys-192, Cys-193, Pro-194, Asp-195, and Tyr-198 are important for neurotoxin binding in the *Torpedo* AChR α1 subunit, since mutation of these residues reduced or abolished α-Btx binding. Sequence comparison (Table II), substitution of amino acids, and identification of residues covalently labeled by cholinergic ligands provide considerable information on the possible functions of residues in cholinergic binding.

In other studies, amino acids have been substituted in synthetic peptides and fusion proteins and effects on toxin binding measured. Tzartos and Remoundos (1990) substituted residues with an alanine or a glycine in a *Torpedo* α1 188–197 synthetic peptide. They found that substitution of Tyr-189, Tyr-190, and Asp-195 markedly reduced α-Btx binding, while substitution of Pro-194 partially reduced

Table II: Amino Acid Sequences of Residues 184–200 of AChR $\alpha$ Subunits

| | 184 200 | Btx[a] | ref |
|---|---|---|---|
| muscle | WKHWVYYTCCPDTPYLD | | |
| *Torpedo* $\alpha$1 | K | + | Noda et al. (1983) |
| *Xenopus* $\alpha$1 | FW S N S L | + | Baldwin et al. (1988) |
| cobra $\alpha$ | A | – | Neumann et al. (1989) |
| chicken $\alpha$1 | F S T | + | Nef et al. (1988) |
| rat $\alpha$1 | F A S | + | Boulter et al. (1990) |
| bovine $\alpha$1 | S T S | + | Noda et al. (1983) |
| human $\alpha$1 | | + | Noda et al. (1983) |
| neuronal | | | |
| rat $\alpha$2 | TYNSKK D AEI- P | – | Boulter et al. (1990) |
| rat $\alpha$3 | Y EIK N EEI- Q | – | Boulter et al. (1990) |
| rat $\alpha$4 | TYNTRK E AEI- P | – | Boulter et al. (1990) |
| rat $\alpha$5 | S GNRTDS W--- PY | +? | Boulter et al. (1990) |
| chicken $\alpha$7 | KRTESF E KE- P | + | Couturier et al. (1990); Schoepfer et al. (1990) |
| invertebrate | | | |
| dro ALS ($\alpha$1) | VRNEKF S EE- | + | Bossy et al. (1988) |
| dro SAD ($\alpha$2) | ER EK P AE- P | ? | Jonas et al. (1990); Sawruk et al. (1990) |
| locust $\alpha$ | ER EK P AE- P | + | Marshall et al. (1990) |

[a] Ability to bind $\alpha$-bungarotoxin.

binding. Substitution of the other residues in this region had no effect. Conti-Tronconi et al. (1991) substituted residues of a *Torpedo* $\alpha$1 181–200 synthetic peptide with glycine. They found that Val-188, Tyr-189, Tyr-190, Cys-192, Cys-193, and Pro-194 were necessary for $\alpha$-Btx binding, while the other residues were not essential. Ohana et al. (1991) substituted six *Torpedo* residues with residues of snake $\alpha$ subunit, which does not bind $\alpha$-Btx, in a fusion protein containing the sequence $\alpha$1 183–204. Y189N and P194L mutations reduced the affinity of binding. Chaturvedi et al. (1992) similarly substituted some *Torpedo* residues with snake residues in an $\alpha$1 166–211 fusion protein and concluded that Tyr-189, Cys-192, Cys-193, and Pro-194 are important in $\alpha$-Btx binding.

Site-directed mutagenesis and expression of AChR in *Xenopus* oocytes has been used to identify residues important in ligand binding and channel activation. Mutation of Cys-192 and Cys-193 to serine abolished the response of the receptor to ACh and reduced but did not completely eliminate $\alpha$-Btx binding (Mishina et al., 1985). Tomaselli et al. (1991) found that a Y190F mutation had a dramatic effect on ACh binding and receptor activation, while a Y198F mutation had a smaller effect. These mutations did not affect $\alpha$-Btx binding. On the basis of responses of the receptor to the partial agonists phenyltrimethylammonium and tetramethylammonium, O'Leary and White (1992) concluded that Y190F, Y198F, and D200N mutations do not alter the affinity of the ligand-binding site but instead interfere with the coupling of ligand binding to channel opening.

Affinity alkylating agents such as [4-(*N*-maleimido)benzyl]-trimethylammonium iodide labeled Cys-192 and Cys-193 (Kao et al., 1984; Dennis et al., 1986) and the cholinergic photoaffinity ligand *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate labeled Tyr-93, Trp-149, Tyr-190, Cys-192, Cys-193, and possibly Trp-86, Tyr-151, and Tyr-198 (Dennis et al., 1988; Galzi et al., 1990) of the $\alpha$ subunit. Lophotoxin, a small cyclic diterpene neurotoxin, reacted covalently with Tyr-190 (Abramson et al., 1991). [$^3$H]Nicotine labeled Tyr-198 primarily and, to a lesser extent, Tyr-190 and Cys-192 of the $\alpha$ subunit (Middleton & Cohen, 1991). *d*-Tubocurarine labeled Tyr-190, Cys-192, and Tyr-198 of the $\alpha$ subunit and also Trp-55 of the $\gamma$ subunit and Trp-57 of the $\delta$ subunit (Chiara & Cohen, 1992).

These studies point most strongly to a major function of Tyr-189, Tyr-190, Cys-192, Cys-193, Pro-194, and Asp-195 in $\alpha$-Btx binding. A role is also suggested for His-186, Val-188, and Tyr-198, although the evidence is less clear for these residues. Tyr-190, Cys-192, Cys-193, and Tyr-198 are involved in agonist binding, so that there appears to be at least partial overlap of residues playing a role in binding of agonists and competitive antagonists. Some of these residues may interact directly with cholinergic ligands, while others may be involved in stabilizing the structure of the binding site.

The abundance of aromatic residues in the binding site suggests a major function for these residues in ligand binding. Tyr-189 is present in muscle receptors that bind $\alpha$-Btx with high affinity and appears to be involved in neurotoxin but not agonist binding. Tyr-190 is present in all $\alpha$ subunits except neuronal $\alpha$5, which has not been demonstrated to form a functional receptor (Boulter et al., 1990), and it is not conserved in the homologous $\beta$, $\gamma$, and $\delta$ subunits. Tyr-198 is invariant in $\alpha$ subunits. It has been pointed out that aromatic residues could form an electronegative subsite (Dennis et al., 1988; Wilson & Lentz, 1988; Abramson et al., 1989; Galzi et al., 1990; Pearce et al., 1990; Cohen et al., 1991), e.g., through the formation of a nucleophilic tyrosinate anion (Abramson et al., 1989) or the $\pi$ electrons of electron-rich aromatic systems (Dougherty & Stauffer, 1990). The importance of aromatic rings in the binding of ACh is supported by determination of the three-dimensional structure of acetylcholinesterase, which reveals the active site to be lined by 14 aromatic residues and only a small number of negative charges (Sussman et al., 1991).

All nicotinic AChRs possess cysteine residues at positions 192 and 193 of the $\alpha$ subunit. These residues lie within 1 nm of the ACh-binding site (Karlin, 1969; Kao et al., 1984). The presence of an intact disulfide between Cys-192 and Cys-193 is necessary for the action of agonists (Karlin, 1969; Kao & Karlin, 1986). McLane et al. (1991) concluded that the vicinal disulfide is not required for $\alpha$-Btx binding, although Cys-192 and Cys-193 are part of the peptide–toxin interface. However, the cis peptide bond between these residues and the vicinal disulfide will exert constraints on the conformation of this region. Thus, it is possible the significant effects of substitution of these residues on $\alpha$-Btx binding are due to an alteration of conformation to one not conducive to binding.

A proline residue is present at position 194 in $\alpha$ subunits that bind $\alpha$-Btx with high affinity. It is probable that Pro-194, and possibly Pro-197, play an important role in determining the conformation of this region. Multiple proline patterns inhibit the formation of classical secondary structures

and are associated with surface loops or turns (MacArthur & Thornton, 1991). The effect of mutation of Pro-194 on α-Btx binding is most likely due to alteration of the conformation of this region. Asp-195 (glutamate in neuronal α subunits except α5) is a candidate for a negative subsite (Ohana & Gershoni, 1990), although it is substituted in some species and is present in the γ subunit. The lack of reaction of [$^3$H]-ACh mustard with acidic side chains indicates Asp-195 (and Asp-200) is not involved in cation binding (Cohen et al., 1991). Negative receptor residues, however, could play a role in steering and orientation of cationic neurotoxins.

There is considerable information on structure–function relationships of curaremimetic neurotoxins based on sequence comparisons and chemical modification studies (Karlsson, 1979; Dufton & Hider, 1983; Martin et al., 1983; Endo & Tamiya, 1987; Lentz & Wilson, 1988). X-ray crystallographic studies reveal the neurotoxins to be hand-shaped molecules consisting of a long central loop flanked by two shorter loops extending from a disulfide-bonded core (Love & Stroud, 1986; Low & Corfield, 1986). The concave binding surface of the toxin molecule contains clusters or bands of charged and hydrophobic residues alternating every 5–8 Å (Love & Stroud, 1986; Lentz & Wilson, 1988). Certain residues, termed functionally invariant residues, are conserved in neurotoxins but not in nonneurotoxic venom proteins. These residues are Trp-29, Asp-31, Phe-, His-, or Trp-33, Arg-37, and Gly-38 (Karlsson homology alignment positions). Lys-27 and Lys-53 are highly conserved, and position 42 is aspartate or glutamate. Most of these residues are located in the central loop (loop 2, residues 25–44). Synthetic peptides of loop 2 inhibit carbachol-induced $^{22}Na^+$ flux into BC3H1 cells, indicating they interact with the ACh-binding site on the receptor (Donnelly-Roberts & Lentz, 1989). The guanidinium group of Arg-37 is the only cationic group common to all of the neurotoxins and has been proposed to be the counterpart of the quaternary ammonium group of ACh (Karlsson, 1979). It has been suggested that Asp-31 and Arg-37 form an ion pair that stereochemically resembles ACh and interacts with the ACh site on the receptor (Tsernoglou et al., 1978; Low, 1979). Phe-33 could correspond to the hydrophobic acetyl methyl group of ACh (Lentz, 1991).

The recognition event between toxin and receptor may depend on electrostatic interactions between structurally and chemically complementary surfaces. After recognition, the toxin–receptor interface is subsequently stabilized by an induced fit involving multiple hydrogen-bonding and hydrophobic interactions (Love & Stroud, 1986; Lentz & Wilson, 1988). Mutation of single residues involved in the multipoint stabilization would not be likely to have a major effect on toxin binding. Thus, the finding that single mutations in the receptor binding region dramatically affect binding indicates these residues are involved directly in the recognition event or are necessary to maintain a conformation conducive to recognition.

Since critical groups on both the neurotoxin and the receptor have been identified, it should be possible to identify interacting sites. It can be expected that the binding domain on the receptor will be chemically complementary to the three-dimensional arrangement of the essential groups of the ligand (Taylor et al., 1990). However, because the three-dimensional structure of the AChR is not known, it is necessary to predict the conformation of the receptor binding domain. Computer modeling of residues 184–200 predicts a loop with Cys-193 and Pro-194 at the tip (Figure 4). Similar models for this region have been proposed (Finer-Moore & Stroud, 1984; Gotti et al., 1988; Ohana & Gershoni, 1990). On the basis of this model, adjacent residues in the antiparallel strands may form functional domains. Lys-185, His-186, and Asp-200 could form a charged domain. A hydrophobic domain is formed by Trp-187, Val-188, and Leu-199. Tyr-189, Tyr-190, and Tyr-198 may form a cluster of aromatic rings with the properties of hydrophobicity and electronegativity. Thr-191, Asp-195, and Thr-196 form a charged/polar domain. The Cys-192–Cys-193 disulfide and Pro-194 are hydrophobic. These receptor domains may interact with the alternating hydrophobic and ionic domains on the toxin's surface in a complementary manner.

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