Sharom, F. J., & Grant, C. W. M. (1975) *Biochem. Biophys. Res. Commun. 67*, 1501–1506.
Strasberg, P., Grey, A., Warren, I., & Skomorowski, M.-A. (1989) *J. Lipid Res. 30*, 121–127.
Stromberg, N., Ryd, M., Linberg, A. A., & Karlsson, K. A. (1988) *FEBS Lett. 232*, 193–198.
Taylor, C. M., Milford, D. V., Rose, P. E., Roy, T. C., & Rowe, B. (1990) *Pediatr. Nephrol. 4*, 59–61.
Waddell, T., Head, S., Petric, M., Cohen, A., & Lingwood, C. (1988) *Biochem. Biophys. Res. Commun. 152*, 674–679.
Waddell, T., Cohen, A., & Lingwood, C. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7898–7901.

Substitution of *Torpedo* Acetylcholine Receptor α1-Subunit Residues with Snake α1- and Rat Nerve α3-Subunit Residues in Recombinant Fusion Proteins: Effect on α-Bungarotoxin Binding†

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: A fusion protein consisting of the TrpE protein and residues 166–211 of the *Torpedo* acetylcholine receptor α1 subunit was produced in *Escherichia coli* using a pATH10 expression vector. Residues in the *Torpedo* sequence were changed by means of oligonucleotide-directed mutagenesis to residues present in snake α1 subunit and rat nerve α3 subunit which do not bind α-bungarotoxin. The fusion protein of the *Torpedo* sequence bound ^{125}I-α-bungarotoxin with high affinity (IC_{50} = 2.5 × 10^{-8} M from competition with unlabeled toxin, K_D = 2.3 × 10^{-8} M from equilibrium saturation binding data). Mutation of three *Torpedo* residues to snake residues, W184F, K185W, and W187S, had no effect on binding. Conversion of two additional *Torpedo* residues to snake, T191S and P194L, reduced α-bungarotoxin binding to undetectable levels. The P194L mutation alone abolished toxin binding. Mutation of three *Torpedo* α1 residues to neuronal α3-subunit residues, W187E, Y189K, and T191N, also abolished detectable α-bungarotoxin binding. Conversion of Tyr-189 to Asn which is present in the snake sequence (Y189N) abolished toxin binding. It is concluded that in the sequence of the α subunit of *Torpedo* encompassing Cys-192 and Cys-193, Tyr-189 and Pro-194 are important determinants of α-bungarotoxin binding. Tyr-189 may interact directly with cationic groups or participate in aromatic–aromatic interactions while Pro-194 may be necessary to maintain a conformation conducive to neurotoxin binding.

The nicotinic acetylcholine receptor (AChR)[1] of skeletal muscle and fish electric organ is a pentamer composed of two α1 subunits and β, γ, and δ subunits [reviewed in Karlin et al. (1986), McCarthy et al. (1986), Stroud et al. (1990), and Galzi et al. (1991)]. Binding of the neurotransmitter acetylcholine (ACh) induces transient opening of a channel, allowing sodium ions to enter the cell. Identification of the agonist-binding site on the receptor has been facilitated by the use of snake venom curaremimetic neurotoxins, such as α-bungarotoxin (α-Btx), which bind specifically and with high affinity to the AChR and competitively block the binding of ACh [see Lentz and Wilson (1988)]. Studies of the binding of α-Btx to synthetic peptides of the α1 subunit have demonstrated toxin binding to residues 173–204 (Wilson et al., 1985, 1988; Wilson & Lentz, 1988), 182–198 (Mulac-Jericevic & Atassi, 1986), 185–196 (Neumann et al., 1986a,b), 172–205 and 185–199 (Ralston et al., 1987), 188–201 (Gotti et al., 1988), 183–200 (Takamori et al., 1988), 181–200 (Conti-Tronconi et al., 1990), 185–196 and 181–198 (Pearce & Hawrot, 1990), 185–199 (Griesmann et al., 1990), and 172–227 (Donnelly-Roberts & Lentz, 1991). These studies indicate a major neurotoxin determinant is located in a region of the primary sequence flanking Cys-192 and Cys-193. These cysteines are labeled by affinity alkylating agents and are considered to lie close to the ACh-binding site (Kao et al., 1984; Dennis et al., 1986). In addition to synthetic peptides, recombinant toxin-binding proteins have been utilized to investigate the toxin-binding site. Fusion proteins containing α1-subunit residues 160–216 (Barkas et al., 1987), 166–200 (Gershoni, 1987), 184–200 and 184–196 (Aronheim et al., 1988), and 183–204 (Ohana & Gershoni, 1990) bind α-Btx, confirming the findings with synthetic peptides.

As a first step in understanding how binding of agonists leads to opening of the ion channel, it is necessary to gain knowledge of structure–function relationships of the binding site. Some information can be obtained by comparison of sequences from species which bind neurotoxins with different affinities. For example, in a study of α-Btx binding to a synthetic peptide comprising residues 173–204 of the α1 subunit, the affinity for the corresponding calf peptide was 15-fold less than for the *Torpedo* peptide, and for the human peptide, 150-fold less (Wilson & Lentz, 1988). The rank order of decreasing affinity (K_D) for α-Btx binding to fusion proteins containing residues 183–204 is *Torpedo*, chick, *Xenopus*, *Drosophila*, mouse, calf, and human (Ohana & Gershoni, 1990). Some snakes and lizards are not sensitive to α-Btx

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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.

[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; α-Btx, α-bungarotoxin; κ-Btx, κ-bungarotoxin.

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(Burden et al., 1975). Comparison of the sequences of a cobra (*Naja naja atra*) (Neumann et al., 1989) and *Torpedo* (Noda et al., 1982) reveals six substitutions within residues 179–205 of the α1 subunit. Similarly, proteins homologous to muscle α1 subunit are present in the central nervous system (Wada et al., 1988). Most of these neuronal nicotinic AChRs do not bind α-Btx (Deneris et al., 1991). The rat nerve α3 subunit (Boulter et al., 1986) has a sequence which is homologous to the α1 subunit but contains several nonconservative substitutions in the sequence flanking Cys-192 and Cys-193. Since this region has been shown to represent a major toxin-binding determinant in *Torpedo* AChR, these substitutions in the snake and neuronal sequences may account for at least some and perhaps most of the reduced toxin-binding ability of these receptors. In order to test this possibility directly, residues in the *Torpedo* sequence have been converted to snake α1 and neuronal α3 residues in fusion proteins by site-directed mutagenesis and the effect on toxin binding measured. It is shown that conversion of two *Torpedo* residues to snake residues and three *Torpedo* residues to neuronal residues markedly decreases the affinity of toxin binding. The latter conversion, however, did not enhance the binding of κ-bungarotoxin (κ-Btx), an antagonist of neuronal nicotinic AChRs (Chiappinelli, 1985).

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 *E. coli*. A construct containing full-length cDNA encoding the *Torpedo* α1 subunit in pBR322 (gift of Dr. Toni Claudio) was digested with *Eco*RI to obtain the complete α-subunit cDNA. The *Eco*RI fragment was disgested 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 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.

Three residues in the *Torpedo* α1 166–211 sequence were mutated to residues present in cobra. To achieve this, 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. a 36-residue oligonucleotide was synthesized by the Protein and Nucleic Acid Chemistry Facility, Yale University. The oligonucleotide corresponds to amino acid residues 181–192 of the *Torpedo* sequence with the following conversions to snake residues: W184F, K185W, and W187S. The resulting fragment, containing three mutations, was further mutated to contain two additional substitutions: T191S and P194L. Similarly, three *Torpedo* residues were mutated to residues in the neuronal α3 subunit, W187E, Y189K, and T191N, and a single *Torpedo* residue was mutated to snake, Y189N, by the same procedures. A plasmid containing the mutant cDNA fragment was digested with *Hin*dIII and *Eco*RI and the resulting fragment subcloned into the *Hin*dIII and *Eco*RI sites of pATH10. Another *Torpedo* residue was converted to a snake residue, P194L, by a polymerase chain reaction based method of site-directed mutagenesis termed the "megaprimer" method (Sarkar & Sommer, 1990) after subcloning the 138 base pair fragment into a pBluescript SK +/− vector. All cDNA fragments were completely sequenced to verify that the desired mutations were achieved (Figure 1).

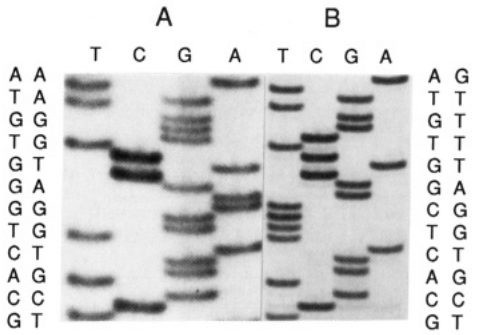

FIGURE 1: DNA sequence of a portion of the 138 base pair *Bst*YI–*Bbv*I *Torpedo* α1 fragment (A) and the W184F, K185W, W187S mutant (B) showing nucleotide substitutions in the mutant. Nucleotide sequences are shown at margins beginning at the bottom of right-hand columns.

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 3-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 molecular weight 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 and κ-Btx were obtained from the Miami Serpentarium (Salt Lake City, UT) and iodinated as described (Wilson & Lentz, 1988). The specific activity of the labeled toxins was 300–600 cpm/fmol. Binding of ^{125}I-α-Btx to fusion proteins was tested in a solid-phase binding assay as described previously for synthetic peptides (Wilson & Lentz, 1988). Briefly, 100 μL of distilled water containing 3 μg of fusion protein was added to wells of 96-well, polystyrene microtiter plates (Nunc, USA Scientific Plastics), and the plates were dried overnight at 45 °C. Alternatively, wells were coated by incubating fusion protein in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6) overnight at 4 °C. Wells were then washed with PBS and quenched for 1 h with 10% bovine serum albumin (BSA). For comparison of α-Btx and κ-Btx binding to fusion proteins, labeled toxin [~200 000 cpm/100 μL of 10 mM phosphate buffer (~5 nM), pH 7.4/0.2% BSA/0.2% BSA/0.03% SDS] was added to wells and incubated for 2 h. For competition experiments, 50 μL of unlabeled toxin was

Table I: Amino Acid Sequences of Residues 166–211 of *Torpedo* α1, Snake (*Naja naja atra*) α1, and Rat Nerve α3 Subunits[a]

	166 211
Torpedo α1	DLSTFMESGEWVMKDYRGWKHWVYYTCCPDTPYLDITYHFIMQRIP
Snake α1	NY Q TL **FW S N S L** LL L
Rat nerve α3	N KDYW AIIKAP Y **EIK N** EE-I Q SLYIR L

[a] In the snake and rat nerve sequences, substitutions in the *Torpedo* sequence are shown. Residues that were mutated in the fusion proteins are shown in boldface type.

added to wells followed immediately by 50 μL of ^{125}I-α-Btx (20 000–30 000 cpm) in 10 mM phosphate buffer (pH 7.4)/0.2% BSA/0.03% SDS and incubated for 1 h (final concentrations from 5.0×10^{-10} to 5.0×10^{-7} M). 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 and subtracted from the total binding to fusion proteins. To measure the equilibrium binding of α-Btx to the fusion proteins, 100 μL of increasing concentrations of ^{125}I-α-Btx in 10 mM phosphate buffer (pH 7.4)/0.2% BSA/0.03% SDS was added and incubated for 4 h. Cold toxin was added to ^{125}I-α-Btx to reduce the specific activity. All determinations were performed in triplicate. After incubation, wells were washed 5 times with PBS. Bound ^{125}I-α-Btx was removed from the wells by adding 75 μL of 0.25 N NaOH/2% SDS and swabbing each well with a cotton-tipped applicator and placing it in a tube. The procedure was repeated and radioactivity measured in a γ counter.

Affinities of binding were approximated by determination of the concentration of unlabeled toxin that resulted in a 50% reduction in the binding of ^{125}I-α-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 polynomial least-squares fit as performed by the computer program KaleidaGraph (Synergy Software, Reading, PA). Equilibrium binding data were analyzed in Scatchard plots.

Results

Residues 166–211 of *Torpedo californica* electric organ α1 subunit (Noda et al., 1982), snake muscle α1 subunit (Neumann et al., 1989), and rat nerve α3 subunit (Boulter et al., 1986) are shown in Table I. Within residues 184–199, a region encompassing Cys-192 and Cys-193, considered to be the major determinant of α-Btx binding, the snake subunit shows six substitutions and the neuronal subunit nine. Five *Torpedo* residues were substituted with snake residues in two mutants. The first contains W185F, K185W, and W187S substitutions. Two additional substitutions, T191S and P194L, were made in the second mutant. In a third mutant, three *Torpedo* residues were substituted with neuronal residues (W187E, Y189K, and T191N). Two point mutations were made in which a *Torpedo* residue was converted to a snake residue (Y189N and P194L).

After transformation of *E. coli* with pATH10 vectors and growth under inductive conditions, partially purified bacterial lysates were separated by polyacrylamide gel electrophoresis. In gels stained with Coomassie blue, the TrpE gene product is the major protein expressed and migrated at a molecular weight of ~37 000 (Figure 2). The fusion proteins show a shift in mobility to an apparent molecular weight of ~43 000. The proteins were electrophoretically transferred to nitrocellulose, and the filter was incubated with ^{125}I-α-Btx. The nonmutated *Torpedo* α1 166–211 fusion protein (α-subunit residues 166–211) and the W184F, K185W, W187S mutant bound toxin (Figure 2). The W184F, K185W, W187S, T191S, P194L mutant, W187E, Y189K, T191N mutant, Y189N mutant, and P194L mutant did not bind toxin on protein blots.

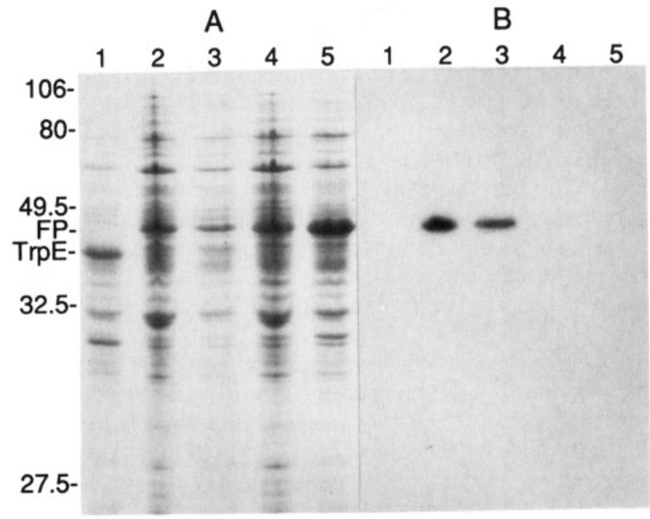

FIGURE 2: Analysis of lysates of transformed *E. coli*. Samples were resolved on polyacrylamide gels and either stained with Coomassie brilliant Blue (A) or transferred onto nitrocellulose filters, overlayered with ^{125}I-α-Btx, and autoradiographed (B). Lane 1, *E. coli* transformed with pATH10; lane 2, *E. coli* transformed with *Torpedo* α1 166–211 cDNA fragment; lane 3, W184F, K185W, W187S mutant; lane 4, W184F, K185W, W187S, T191S, P194L mutant; lane 5, W187E, Y189K,T191N mutant. Positions of molecular weight standards (MW $\times 10^{-3}$), fusion proteins (FP), and TrpE protein (TrpE) are shown on the left.

Wells of microtiter plates were coated with 3 μg of fusion protein and incubated with ~200 000 cpm of ^{125}I-α-Btx. Two experiments with four replicates each were performed. Background binding to the TrpE protein alone was subtracted from total binding to determine specific binding, and standard deviations were calculated. The W184F, K185W, W187S mutant fusion protein bound slightly more toxin (4355 ± 260 cpm) than the *Torpedo* α1 166–211 fusion protein (3464 ± 340 cpm). The signals obtained with the W184F, K185W, W187S, T191S, P194L mutant, W187E, Y189K, T191N mutant, Y189N mutant, and P194L mutant did not differ significantly from background levels. Similarly, ^{125}I-κ-Btx binding to the fusion proteins was compared, and no significant binding to any of the proteins could be detected using the solid-phase assay (data not shown).

The ability of unlabeled α-Btx to inhibit binding of ^{125}I-α-Btx to the *Torpedo* α1 166–211 fusion protein and to the W184F, K185W, W187S mutant fusion protein was tested in a solid-phase assay (Figure 3). Unlabeled α-Btx competed with labeled α-Btx binding with IC_{50} values of 2.5×10^{-8} and 2.3×10^{-8} M for the two fusion proteins, respectively. Equilibrium binding of ^{125}I-α-Btx to these two fusion proteins was measured. Scatchard analysis of the equilibrium binding data yielded dissociation constants (K_D) of 2.3×10^{-8} M for the α1 166–211 fusion protein and 1.1×10^{-8} M for the W184F, K185W, W187S mutant (mean of two experiments, data not shown). Binding studies could not be performed with the other mutated fusion proteins due to the very low signals.

Discussion

A fusion protein containing residues 166–211 of the *Torpedo* α1 subunit was constructed, and certain residues were converted to those of snake α1 subunit or rat nerve α3 subunit, neither of which bind α-Btx. The *Torpedo* α1 166–211 fusion

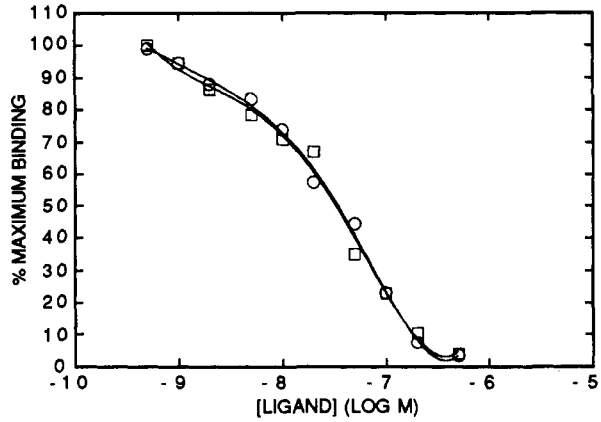

FIGURE 3: Competition of the binding of ^{125}I-α-Btx to *Torpedo* α1 166–211 fusion protein (circles) and W184F, K185W, W187S mutant fusion protein (squares). Competition with unlabeled α-Btx was performed in a solid-phase assay as described under Experimental Procedures. Values are the mean of three replicates.

protein bound α-Btx in a manner similar to a *Torpedo* α1 173–204 synthetic peptide (Wilson & Lentz, 1988) and a *Torpedo* α1 172–227 synthetic peptide (Donnelly-Roberts & Lentz, 1991). Unlabeled α-Btx competed with labeled α-Btx binding to the nonmutated fusion protein with an IC_{50} value of 2.5×10^{-8} M, compared to $IC_{50} = 4.2 \times 10^{-8}$ M for the 173–204 peptide and $IC_{50} = 5.0 \times 10^{-8}$ M for the 172–227 peptide. Equilibrium saturation binding studies yielded a K_D = 23 nM for the *Torpedo* α1 166–211 fusion protein compared to 7.8 nM for the 173–204 peptide and 3.5 nM for the 172–227 peptide. The K_D for the fusion protein was consistently somewhat lower than those of the peptides. This difference may be due to the attachment of the receptor sequence to the TrpE protein which could constrain conformational changes or interfere with binding.

Three substitutions, W184F, K185W, and W187S, in the *Torpedo* sequence did not significantly affect the affinity for α-Btx. Similarly, substitution of each of these residues individually with a glycine residue did not significantly affect α-Btx binding to a *Torpedo* α1 181–200 synthetic peptide (Conti-Tronconi et al., 1991). Trp-184 is not conserved among receptors that bind α-Btx, and Phe represents a conservative substitution for Trp. Replacement of Lys-185 with Trp represents a nonconservative substitution of a residue with a positively charged side chain for one with a hydrophobic, aromatic group. However, this substitution appears to have little effect on binding. Substitution of Ser for Trp-187 represents a second nonconservative substitution in which a residue with a hydrophilic side chain is substituted for one with a hydrophobic group. Ser is also present at this position in the human AChR sequence. Trp-187 has been proposed to play a role in α-Btx binding (Neumann et al., 1986b). The affinity of α-Btx binding to a human α1 peptide comprising residues 173–204 was 150-fold less than to the corresponding *Torpedo* peptide (Wilson & Lentz, 1988). Similarly, binding affinity was 100-fold less to a fusion protein containing human α1 residues 183–204 than to *Torpedo* (Ohana & Gershoni, 1990). However, the present results, as well as those of Conti-Tronconi et al. (1991), indicate substitution of Trp-187 has little effect on binding. Within residues 173–204, the human sequence has five other substitutions, two of which are nonconservative, which could be responsible for the lower affinity of binding to the human peptides (Wilson & Lentz, 1988).

When two additional substitutions, T191S and P194L, were introduced into the W184F, K185W, W187S mutant, toxin binding was reduced to undetectable levels. Because the direct binding assays cannot detect binding with an affinity lower than $K_D \cong 10^{-5}$ M, it is possible α-Btx binds to the mutant fusion protein with low affinity. The T191S substitution is conservative. Thr-191 is not highly conserved among α subunits and is not crucial for α-Btx binding to synthetic peptides (Tzartos & Remoundos, 1990; Conti-Tronconi et al., 1991). The P194L mutation alone abolished toxin binding, indicating this residue is crucial for binding. Pro-194 is conserved among vertebrate muscle α subunits. Pro-194 is substituted with a Glu residue in the neuronal α3 subunit. Substitution of Pro-194 with Gly in a synthetic peptide comprising residues 181–200 of the *Torpedo* α1 subunit caused a marked reduction in α-Btx binding to the peptide (Conti-Tronconi et al., 1991). Substitution of this residue in a *Torpedo* 188–197 synthetic peptide caused a partial reduction in binding (Tzartos & Remoundos, 1990). The present results confirm the importance of Pro-194 in toxin binding. The role of this residue most likely is in maintaining a conformation conducive to binding. The toxin-binding region of the α subunit is considered to be a β-barrel structure (Finer-Moore & Stroud, 1984; McCarthy & Stroud, 1989; Stroud et al., 1990). Proline residues at positions 194 and 197 may produce a loop with Cys-192 and Cys-193 near the tip. Disruption of this structure may interfere with or prevent formation of complementary interactions between the toxin and this region of the α subunit.

Conversion of three *Torpedo* α1-subunit residues to neuronal α3-subunit residues, W187E, Y189K, and T191N, markedly reduced α-Btx binding to the fusion protein. Two of the residues, Trp-187 and Thr-191, are substituted in both the snake and neuronal fusion proteins, although with different residues. In the case of the snake, it was concluded that substitution of Trp-187 and Thr-191 in the fusion protein had little influence on the affinity of toxin binding. Thus, substitution of Tyr-189 appears to be primarily responsible for the decreased affinity of α-Btx for the mutated fusion protein. This conclusion is confirmed by the absence of toxin binding to the mutant in which Y189 was converted to Asn. Position 189 is occupied by Tyr or Phe in muscle α1 subunits except for human (Thr) and snake (Asn). This substitution in the human sequence could be responsible for the lower affinity binding of human peptides (Gotti et al., 1988; Tzartos & Remoundos, 1990). Sequence 183–204 containing Tyr-189 binds toxin with higher affinity than sequences with Phe-189 (Ohana & Gershoni, 1990). Substitution of Tyr-189 with Gly in *Torpedo* peptide 181–200 (Conti-Tronconi et al., 1991) and with Ala or Gly in *Torpedo* and human peptides 188–197 (Tzartos & Remoundos, 1990) significantly reduced α-Btx binding. In the neuronal α3 subunit, the polar, aromatic tyrosine is replaced with a positively charged lysine. This substitution could result in an unfavorable electrostatic repulsion of toxin.

^{125}I-κ-Btx binding could not be detected to any of the fusion proteins including a construct in which three *Torpedo* residues were converted to neuronal α3 residues. It might have been expected that the latter mutations might restore some κ-Btx binding. However, McLane et al. (1990) have shown that in solid-phase assays κ-Btx does not bind to neuronal α3 residues 180–199 but does bind to peptides comprising residues 51–70 and 1–18. Peptides α3 180–189 and α3 183–201 did partially inhibit κ-Btx binding to native receptor on PC12 cells. Thus, κ-Btx appears to bind to neuronal AChRs at a different major determinant and by a different mechanism than α-Btx binds to muscle α1 subunits.

It is concluded that two substitutions in the *Torpedo* sequence, Y189K or Y189N and P194L, each markedly reduce

α-Btx affinity for α-subunit peptides. It is surprising that single residues have such a pronounced effect on binding because the neurotoxins interact with the receptor at multiple points and modification of individual toxin residues does not abolish binding or toxicity (Martin et al., 1983; Lentz & Wilson, 1988). The most likely explanation is that the receptor modifications that significantly affect binding disrupt the initial recognition event between the toxin molecule and the receptor. If recognition can occur, many other residues undoubtedly take part in subsequent interactions with the large toxin surface, but mutation of these would have less effect because of the formation of multipoint interactions.

A large change in conformation as a result of substitution of Pro-194 could significantly disrupt recognition and binding. Alteration of Tyr-189 could disrupt the initial interaction of the toxin with the receptor. Tyr-189 is located in a cluster of residues with polar side groups (Tyr-189, Tyr-190, and Thr-191). Tyr-190 is labeled by *p*-(dimethylamino)-benzenediazonium fluoroborate, a photoaffinity label of the ACh-binding site (Dennis et al., 1988; Langenbuch-Cachat et al., 1988). These residues could contribute to the formation of an electronegative site which may interact with the quaternary ammonium group of ACh or Arg-37 of the neurotoxins. Arg-37 is the only cationic group conserved among all neurotoxins (Karlsson, 1979) and has been proposed to be the counterpart of the quaternary ammonium of ACh (Tsernoglou et al., 1978). In toxin binding, electrostatic interactions may be particularly important in recognition and the initial binding event (Lentz & Wilson, 1988). Alternatively, the quaternary ammonium group of ACh or the guanidinium group of Arg-37 could interact directly with the π electrons of the electron-rich aromatic group of tyrosine residues (Dougherty & Stauffer, 1990). Tyr-189 could also interact with a hydrophobic region on the toxin through aromatic–aromatic interactions. The substitution of Y189 and P194, however, does not appear to affect the binding of ACh because the α3 subunit, when expressed with the β2 subunit in *Xenopus* oocytes, responds to ACh (Boulter et al., 1987). The fact that certain substitutions in this region which contributes to the ACh-binding site (Dennis et al., 1986, 1988; Kao & Karlin, 1986) affect α-Btx binding but not ACh binding brings into question the presence of an ACh mimic on loop 2 of the neurotoxins (Tsernoglou et al., 1978; Lentz & Wilson, 1988) and indicates the toxins bind to the receptor by a different mechanism than ACh.

Added in Proof

It was reported that mutation of Y189 to threonine in a recombinant fusion protein containing residues 183–204 of *T. californica* α1 subunit abolishes α-bungarotoxin binding (Ohana et al., 1991), providing further evidence of the importance of Y189 in toxin binding.

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Registry No. α-Btx, 11032-79-4.

References

Aronheim, A., Eshel, Y., Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 9933–9937.

Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S. J., & Ballivet, M. (1987) *Science 235*, 77–80.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., & Patrick, J. (1986) *Nature 319*, 368–374.

Boulter, J., Connolly, J., Deneris, E., Goldman, D., Heinemann, S., & Patrick, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7763–7767.

Burden, S. J., Hartzell, H. C., & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3245–3249.

Chiappinelli, V. A. (1985) *Pharmacol. Ther. 31*, 1–32.

Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221–6230.

Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, T., Schröder, B., Reinhardt-Maelicke, S., & Maelicke, A. (1991) *Biochemistry 30*, 2575–2584.

Deneris, E. S., Connolly, J., Rogers, S. W., & Duvoisin, R. (1991) *Trends Pharmacol. Sci. 12*, 34–40.

Dennis, M., Giraudat, J., Kotzyba-Hilbert, F., Goeldner, M., Hirth, C., Chang, J.-Y., & Changeux, J.-P. (1986) *FEBS Lett. 207*, 243–249.

Dennis, M., Giraudat, J., Kotzyba-Hilbert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chrêtien, M., & Changeux, J.-P. (1988) *Biochemistry 27*, 2346–2357.

Donnelly-Roberts, D. L., & Lentz, T. L. (1991) *Biochemistry 30*, 7484–7491.

Dougherty, D. A., & Stauffer, D. A. (1990) *Science 250*, 1558–1560.

Finer-Moore, J., & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155–159.

Galzi, J.-L., Revah, F., Bessis, A., & Changeux, J.-P. (1991) *Annu. Rev. Pharmacol. 31*, 37–72.

Gershoni, J. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4318–4321.

Gershoni, J. M., Hawrot, E., & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4973–4977.

Gotti, C., Frigerio, F., Bolognesi, M., Longhi, R., Racchetti, G., & Clementi, F. (1988) *FEBS Lett. 228*, 118–122.

Griesmann, G. E., McCormick, D. J., DeAizpurua, H. J., & Lennon, V. A. (1990) *J. Neurochem. 54*, 1541–1547.

Kao, P. N., & Karlin, A. (1986) *J. Biol. Chem. 261*, 8085–8088.

Kao, P. M., Dwork, A. J., Kaldany, R.-R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.

Karlin, A., Kao, P. N., & DiPaola, M. (1986) *Trends Pharmacol. Sci. 7*, 304–308.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 158–212.

Koerner, T. J., Hill, J. E., Myers, A. M., & Tzagoloff, A. (1991) *Methods Enzymol. 194*, 477–490.

Langenbuch-Cachat, J., Bon, C., Mulle, C., Goeldner, M., Hirth, C., & Changeux, J.-P. (1988) *Biochemistry 27*, 2337–2345.

Lentz, T. L., & Wilson, P. T. (1988) *Int. Rev. Neurobiol. 29*, 117–160.

Martin, B. M., Chibber, B. A., & Maelicke, A. (1983) *J. Biol. Chem. 258*, 8714–8722.

McCarthy, M. P., & Stroud, R. M. (1989) *Biochemistry 28*, 40–48.

McCarthy, M. P., Earnest, J. P., Young, E. F., Choe, S., & Stroud, R. M. (1986) *Annu. Rev. Neurosci. 9*, 383–413.

McLane, K. E., Tang, F., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 1537–1544.

Mulac-Jericevic, B., & Atassi, M. Z. (1986) *FEBS Lett. 199*, 68–74.
Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008–3011.
Neumann, D., Barchan, D., Fridkin, M., & Fuchs, S. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9250–9253.
Neumann, D., Barchan, D., Horowitz, M., Kochva, E., & Fuchs, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7255–7259.
Noda, M., Takahashi, H., Tanabe, T., Toyasato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature 299*, 793–797.
Ohana, B., & Gershoni, J. M. (1990) *Biochemistry 29*, 6409–6415.
Ohana, B., Fraenkel, Y., Navon, G., & Gershoni, J. M. (1991) *Biochem. Biophys. Res. Commun. 179*, 648–654.
Pearce, S. F., & Hawrot, E. (1990) *Biochemistry 29*, 10649–10659.
Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, J. L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261–3266.
Rodbard, D., & Frazier, G. R. (1975) *Methods Enzymol. 37*, 3–22.
Sarkar, G., & Sommer, S. S. (1990) *BioTechniques 8*, 404–409.
Stroud, R. M., McCarthy, M. P., & Shuster, M. (1990) *Biochemistry 29*, 11009–11023.
Takamori, M., Okumura, S., Nagata, M., & Yoshikawa, H. (1988) *J. Neurol. Sci. 85*, 121–129.
Tsernoglou, D., Petsko, G. A., & Hudson, R. A. (1978) *Mol. Pharmacol. 14*, 710–716.
Tzartos, S. J., & Remoundos, M. S. (1990) *J. Biol. Chem. 265*, 21462–21467.
Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E. S., Swanson, L. W., Heinemann, S., & Patrick, J. (1988) *Science 240*, 330–334.
Wilson, P. T., & Lentz, T. L. (1988) *Biochemistry 27*, 6667–6674.
Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790–8794.
Wilson, P. T., Hawrot, E., & Lentz, T. L. (1988) *Mol. Pharmacol. 34*, 643–650.

The Observed Inhibitory Potency of 3′-Azido-3′-deoxythymidine 5′-Triphosphate for HIV-1 Reverse Transcriptase Depends on the Length of the Poly(rA) Region of the Template†

Qi-Feng Ma,‡ Ian C. Bathurst,§ Philip J. Barr,§ and George L. Kenyon*,‡

Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Chiron Corporation, 4560 Horton Street, Emeryville, California 94608

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ABSTRACT: The inhibitory potency of 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) against HIV-1 reverse transriptase (HIV-1 RT) has been further evaluated. The results indicate that the previously reported low K_i values for AZTTP against HIV-1 RT (2–35 nM) are due neither to the direct tight binding of AZTTP to HIV-1 RT nor to the interaction of the enzyme with AZTMP moiety terminated primer-templates, but instead they are an artifact of the use of a homotemplate-primer [poly(rA)·oligo(dT)]. With a set of RNAs of defined sequence as templates, we demonstrate that the observed K_i value for AZTTP depends on the length of the poly(rA) region following the primer in the RNA template. The more adenosyl residues in the RNA template that are available for processive incorporation of TMP moieties, the lower is the observed K_i value for AZTTP. Since the potencies of new inhibitors of HIV-1 RT are usually compared with that for AZTTP, these results have important consequences for the process of discovery of new HIV inhibitors that are of potential use in AIDS therapy.

3′-Azido-3′-deoxythymidine (AZT),[1] the first drug used clinically for the treatment of human immunodeficiency virus (HIV) infection, is considered to be a prodrug that is converted into 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) by cellular kinases (Mitsuya et al., 1985; Fischl et al., 1987; Furman et al., 1986). In vitro kinetic studies show that AZTTP is a very potent competitive inhibitor of HIV-1 RT with observed K_i values ranging from 2 to 35 nM (Furman et al., 1986; Kedar et al., 1990; Reardon et al., 1990; Eriksson et al., 1989; Vrang et al., 1987, 1988; Cheng et al., 1987; St. Clair et al., 1987; Ono et al., 1986; Matthes et al., 1987; White et al., 1989; Starnes et al., 1989), and the therapeutic effect of AZT toward HIV infection has been attributed to this inhibition (Eriksson et al., 1989; Vrang et al., 1987; Cheng et al., 1987).

The inhibitory mechanism of AZTTP has been studied extensively. It was demonstrated that AZTTP is a substrate for HIV-1 RT (Kedar et al., 1990; Reardon & Miller, 1990)

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* To whom correspondence should be addressed.
‡ University of California.
§ Chiron Corporation.

[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; DE, (diethylamino)ethyl-cellulose; EDTA, ethylenediaminetetraacetic acid; HIV-1 RT, human immunodeficiency virus 1 reverse transcriptase; NMP, nucleoside 5′-monophosphate; TMP, thymidine 5′-monophosphate.

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