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# Amino Acids within Residues 181–200 of the Nicotinic Acetylcholine Receptor α1 Subunit Involved in Nicotine Binding

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**ABSTRACT.** Structural determinants of L-[$^3$H]nicotine binding to the sequence flanking Cys 192 and Cys 193 of the *Torpedo* acetylcholine receptor α1 subunit were investigated using synthetic peptides (residues 181–200) and fusion proteins (residues 166–211). Nicotine binding at a single concentration (30 nM) was compared with 71 peptides and fusion proteins in which individual amino acids at positions 181–200 were substituted. Substitution of Lys 185, Tyr 190, Cys 192, Cys 193, Thr 196, and Tyr 198 resulted in the greatest reduction in nicotine binding. Equilibrium binding of [$^3$H]nicotine to peptide 181–200 revealed a binding component with an apparent $K_D$ of 1.2 μM. Substitution of Lys 185 (with Glu), His 186, Tyr 190, Cys 192, Cys 193, and Tyr 198 resulted in a significant reduction in affinity. Affinity was not affected significantly by substitution of Arg 182, Lys 185 (with Gly or Arg), Val 188, Tyr 189, Pro 194, Asp 195, Thr 196, and Asp 200. It is concluded that Lys 185, His 186, Tyr 190, Cys 192, Cys 193, and Tyr 198 play the greatest role in nicotine binding to residues 181–200 of the α1 subunit. Previous studies have implicated Tyr 190, Cys 192, Cys 193, and Tyr 198 in agonist binding to the acetylcholine receptor. These results confirm a role for these residues and also demonstrate a function for Lys 185 and His 186 in nicotine binding. BIOCHEM PHARMACOL **55**;3:341–347, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** acetylcholine receptor; amino acids; fusion proteins; nicotine; structure–function relationships; synthetic peptides

The nicotinic AChR‖ transduces a chemical signal, the neurotransmitter 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 cations to enter and depolarize the membrane. The AChR from *Torpedo californica* electric organ and that on the postsynaptic surface of the neuromuscular junction are transmembrane glycoproteins composed of four subunits arranged in a stoichiometry of 2α1, β1, ε or γ, and δ [1, 2]. This pentameric complex contains the binding sites for agonists, e.g. ACh, carbamylcholine, and nicotine, and for competitive antagonists, e.g. α-Btx and *d*-tubocurarine. *Torpedo* and immature muscle AChRs contain two cholinergic binding sites located at the interfaces of the α and γ and the α and δ subunits [1–4]. The α subunit contains major determinants for binding of agonists and competitive antagonists. Amino acid residues on the γ and δ subunits also contribute to the cholinergic binding site [4–6].

Synthetic peptides and fusion proteins have proven useful in localizing components of the neurotoxin binding site on the α subunit and in studying structural determinants for toxin binding through multiple, systematic substitution of residues (reviewed in Ref. 7). Short peptide sequences have the potential disadvantages that they may not reflect the function of the intact receptor, may not fold into the native conformation, and may exclude discontinuous components of the binding site. Still, they have been shown repeatedly to retain the ability to bind curaremimetic neurotoxins and cholinergic ligands by virtue of their ability to compete with neurotoxin binding. Studies investigating the binding of α-Btx to synthetic peptides [8–15] and fusion proteins containing receptor sequences [16–19] located a major neurotoxin binding site in the sequence flanking Cys 192 and Cys 193 and identified residues that are critical for neurotoxin binding. Although the binding sites for neurotoxins and agonists appear to overlap, there are significant differences between the two sites [20]. A previous study investigated the binding of L-nicotine to synthetic α1 subunit peptides [20]. It was demonstrated that [$^3$H]nicotine binding to peptides could be assayed directly, that binding could be competed with cholinergic agonists

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‖ *Abbreviations:* AChR, acetylcholine receptor; ACh, acetylcholine; and α-Btx, α-bungarotoxin.

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and antagonists, and that nicotine and α-Btx interact preferentially with different determinants within residues α1 173–204.

Nicotine, an agonist of the AChR, is addictive and a drug of abuse but also has potential as a medication in disorders such as ulcerative colitis, Alzheimer's disease, and Parkinson's disease [21, 22]. Characterization of the mechanism of nicotine interaction with the AChR could identify potential targets for therapeutic intervention in addiction and in diseases in which cholinergic function is disrupted. Here, we investigated structure–function relationships of nicotine binding to the AChR by comparing the binding of L-[$^3$H]nicotine to 71 synthetic sequences and fusions proteins of the AChR α1 subunit in which individual amino acids at positions 181–200 were substituted. We found that residues Lys 185, His 186, Tyr 190, Cys 192, Cys 193, and Tyr 198 play a role in nicotine binding to this region of the α1 subunit.

## MATERIALS AND METHODS

### *Nicotine*

Nicotine, L-(-)-[*N-methyl*-$^3$H]-, was purchased from the NEN/Dupont Co. Upon receipt, [$^3$H]nicotine was diluted 1:10 in 0.02 M phosphate buffer, pH 7.2, divided into aliquots, and stored at −20°. The initial specific activity of [$^3$H]nicotine was 75–125 cpm/fmol.

### *Synthetic Peptides*

Synthetic peptides comprising residues 181–200 of the *Torpedo* α1 subunit sequence were those employed by Conti-Tronconi and coworkers [14, 15] to investigate the binding of α-Btx. The peptides and single residue substituted analogs of that sequence were synthesized by manual parallel synthesis, and their purity and amino acid composition were characterized as described previously [14]. Lyophilized peptides were resuspended in 50% acetonitrile/$H_2O$ to a concentration of 0.5 mg/mL.

Fusion proteins, also used to investigate α-Btx binding [19], were produced as described previously [18, 19]. Briefly, a fusion protein consisting of the TrpE protein fused to *Torpedo* α1 subunit residues 166–211 was produced in *Escherichia coli*. A 138 bp fragment of *Torpedo* α1 subunit cDNA was inserted into a pATH 10 expression vector and used to transform *E. coli* strain XL1 Blue. The residues between positions 184 and 200 were mutated either by means of oligonucleotide-directed mutagenesis or a polymerase chain reaction-based method. Bacterial clones containing cDNA fragments were induced and harvested as described. Partially purified bacterial lysates were resuspended in distilled $H_2O$ containing 0.01% phenylmethanesulfonyl fluoride to a concentration of 1 mg/mL and stored at −70°.

The sequence of residues 181–200 of the native *Torpedo* α1 subunit is shown in Table 1. The rationale for the amino acid substitutions has been described previously. Each residue in peptide α1 181–200 was nonconservatively substituted with a glycine residue [14]. In addition, a series of conservative substitutions were made [15]. In the fusion protein, amino acids were substituted with residues present in the snake α1 subunit, with an alanine, or with a functionally dissimilar residue [19]. Substituted peptides and fusion proteins are designated by the one-letter code of the native residue, followed by the position in the sequence and the one-letter code for the substitution.

**TABLE 1. Sequence of residues 181–200 of *Torpedo* α1 subunit***

| 181 | 185 | 190 | 195 | 200 |
|---|---|---|---|---|
| Y | R G W K H W V Y Y T C C P D T P Y L D | | | |

* Substitutions shown in Table 2 and Fig. 2.

### *Binding of [$^3$H]Nicotine to Synthetic Peptides*

[$^3$H]Nicotine binding to synthetic peptides was measured by a solid phase assay as described [20]. For coating wells, stock solutions of peptides and fusion proteins were diluted in distilled $H_2O$, and 100 μL containing 8 μg peptide or 5 μg fusion protein was placed in the wells of a microtiter plate (Immulon Removawell Strips, Dynatech Laboratories) and allowed to evaporate overnight at 45°. Wells were washed three times with 200 μL PBS. In the case of fusion proteins, wells were quenched with 300 μL of 5% bovine serum albumin in PBS. After washing, the wells were incubated with [$^3$H]nicotine in 50 μL of 0.02 M phosphate buffer (~150,000 cpm, 30 nM) for 30 min. Wells were washed rapidly two times with PBS. Then wells were placed in vials containing the fluorophore Cytoscint (ICN) and counted in a scintillation counter. Background binding to wells lacking peptide but exposed to labeled ligand was subtracted from the binding in the presence of peptide. All determinations were performed in triplicate, and all peptides and fusion proteins were assayed at least three times.

Equilibrium saturation experiments were performed for [$^3$H]nicotine binding to some peptides in the solid phase assay system described above. Wells were coated with peptides and incubated with increasing concentrations of [$^3$H]nicotine (0.16 to 20 μM). Incubation was performed for 3 hr at room temperature. Equilibrium binding data were analyzed in Scatchard plots. Because the peptides exhibited non-specific binding at high concentrations of nicotine, the data were corrected for non-specific binding as described by Chamness and McGuire [23]. Significance of differences in dissociation constants ($K_D$) between native and substituted peptides was determined with the two-tailed Student's *t*-test. Competition studies with unlabeled nicotine were performed as described [20]. The $IC_{50}$ values were determined from logit-log plots of the competition data [24].

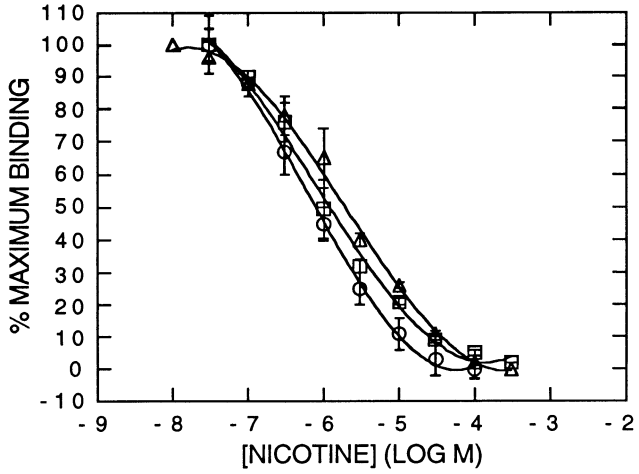


**FIG. 1. Competition of [$^3$H]nicotine binding to *Torpedo* α1 166–211 fusion protein (○), 173–204 32mer (□), and 181–200 20mer (△). Wells of microtiter plates were coated with fusion protein or peptide, washed, and incubated with [$^3$H]-nicotine (150,000 cpm) and a range of concentrations of unlabeled nicotine for 30 min. The wells were washed, and bound radioactivity was measured. Values represent the average of three replicates. Data for 173–204 32mer are from Lentz [20].**

## RESULTS

Binding of [$^3$H]nicotine to peptide α1 173–204 was described previously [20]. To compare the sequences α1 166–211 and α1 181–200 with the peptide α1 173–204, we tested the ability of unlabeled nicotine to compete with [$^3$H]nicotine. The $IC_{50}$ values for nicotine competition to the sequences α1 166–211, α1 173–204, and α1 181–200 were $8.4 \times 10^{-7}$, $1.4 \times 10^{-6}$, and $1.9 \times 10^{-6}$ M, respectively (Fig. 1). Thus, [$^3$H]nicotine binding to the three sequences was comparable.

[$^3$H]Nicotine binding to synthetic peptides and fusion proteins in which individual residues were substituted was compared in a solid-phase radioassay at a single concentration of nicotine (30 nM) (Table 2; Fig. 2). Binding to the native sequence was taken as 100%. The following substitutions resulted in at least a 50% reduction in nicotine binding: K185G, K185E, K185R, Y190G, Y190A, C192H, C192/G/C193, C193H, C193V, T196S, Y198A, and Y198F. Other substitutions resulted in a lesser reduction (40–50%) in binding: R182G, H186G, V188I, Y189F, Y190H, C192V, P194G, P194GG, and T196V. In four cases, the peptide and fusion protein had the same substitution. For the W184F and C192G substitutions, nicotine binding to the peptide and fusion protein was comparable. On the other hand, the difference in binding to the peptide and fusion protein for the V188T and T191S substitutions was statistically significant ($P < 0.01$). The differences could indicate a modulatory effect of the TrpE protein of the fusion protein on the nicotine binding site.

Comparison of binding at a constant nicotine concentration can give an estimate of relative binding. However, there can be inaccuracies if substituted peptides differ in their solubility or ability to adhere to plastic. For this reason, equilibrium binding studies were performed on selected peptides, and $K_D$ values were determined. Analysis of nicotine binding to peptide α1 181–200 yielded a binding component with an apparent $K_D$ of 1.2 μM (Fig. 3). This value is comparable to the major binding component of peptide α1 173–204 ($K_D$ = 1.6 μM) [20].

When substituted peptides were compared with the nonsubstituted sequence, the following peptides showed a significant reduction in $K_D$: K185E, H186G, Y190T, Y190H, C192H, C193H, Y198F, and Y198H (Table 2; Fig. 3). These include some peptides that did not show a drastic reduction in [$^3$H]nicotine binding in the binding assay in which a single concentration of [$^3$H]nicotine was used. The following peptides did not show a significant reduction in $K_D$: R182G, K185G, K185R, V188I, Y189H, P194G, D195G, T196V, T196S, and D200G. D195G and D200G were tested because Asp 195 and Asp 200 are candidates for an anionic subsite and P194G because of a possible role of Pro 194 in conformation, which could affect binding. These substituted peptides did not show a reduction in affinity relative to the unsubstituted peptide.

## DISCUSSION

These studies point most strongly to a role of the α1 subunit residues Lys 185, His 186, Tyr 190, Cys 192, Cys 193, and Tyr 198 in nicotine binding within residues 181–200 of the α1 subunit. Substitution of these residues resulted in reduced nicotine binding and decreased affinity for nicotine relative to the unsubstituted sequence. These residues may be involved in binding through direct interaction of functional groups with the ligand. Alternatively, substitution of these amino acids could reduce binding by altering the conformation of the peptide to one less conducive to binding or by affecting the functional properties of neighboring residues. This could be the case with residues where some amino acid substitutions reduced binding, while others had no effect (e.g. Lys 185, Tyr 198). Another possible limitation is that the peptides may not completely mimic the nicotine-binding site on the native receptor.

The ligand-binding site on the AChR should be chemically complementary to the three-dimensional arrangement of the essential groups of ligands. Nicotinic agents are characterized by a positively charged alkylammonium moiety (quaternary ammonium group of ACh and pyrrolidine nitrogen in nicotine) located 5.9 Å from a hydrogen bond acceptor (carbonyl oxygen in ACh and pyridine nitrogen in nicotine) [25]. Thus, the binding site on the AChR should contain at least two subsites: an anionic center and a hydrogen-bonding site that is likely to also contain hydrophobic residues [1].

Studies that used affinity-labeling reagents and native AChR have identified residues on the α1 subunit that likely contribute to the cholinergic-binding site [26–30]. They are Tyr 93, Trp 149, Tyr 190, Cys 192, Cys 193, and Tyr 198. These residues are expected to be in close proximity in the native AChR molecule [31]. [$^3$H]Nicotine

**TABLE 2. Effects of amino acid substitutions on [$^3$H]nicotine binding to AChR α1 subunit peptides and fusion proteins**

| Peptide | % Binding to unsubstituted peptide | $K_D$ (μM) | *P** | Peptide | % Binding to unsubstituted peptide | $K_D$ (μM) | *P** |
|---|---|---|---|---|---|---|---|
| Native | 100 | 1.2 ± 0.1 | | | | | |
| Y181G | 105 ± 5 | | | C192G | 68 ± 9 | | |
| R182G | 59 ± 6 | 1.7 ± 0.6 | > 0.10 | †C192G | 63 ± 11 | | |
| G183A | 79 ± 4 | | | †C192A | 77 ± 3 | | |
| W184G | 106 ± 7 | | | C192S | 60 ± 7 | | |
| W184F | 94 ± 7 | | | C192H | 46 ± 7 | 3.1 ± 0.2 | < 0.001 |
| †W184F | 97 ± 6 | | | C192V | 54 ± 5 | | |
| K185G | 49 ± 7 | 1.3 ± 0.2 | > 0.10 | C192/G/C193 | 46 ± 4 | | |
| K185E | 38 ± 10 | 2.6 ± 0.3 | < 0.001 | C193G | 72 ± 9 | | |
| K185R | 39 ± 6 | 1.2 ± 0.3 | > 0.10 | †C193A | 69 ± 10 | | |
| †K185W | 99 ± 9 | | | C193S | 61 ± 6 | | |
| H186G | 60 ± 6 | 3.4 ± 0.4 | < 0.001 | C193H | 31 ± 7 | 3.6 ± 0.2 | < 0.001 |
| †H186A | 96 ± 7 | | | C193V | 41 ± 9 | | |
| W187G | 104 ± 5 | | | P194G | 60 ± 7 | 1.4 ± 0.3 | > 0.10 |
| W187F | 82 ± 7 | | | †P194L | 93 ± 7 | | |
| †W187S | 92 ± 7 | | | P194GG | 53 ± 6 | | |
| W187M | 107 ± 6 | | | D195G | 94 ± 8 | 1.2 ± 0.2 | > 0.10 |
| W187Y | 82 ± 5 | | | †D195A | 95 ± 6 | | |
| V188G | 90 ± 6 | | | D195E | 90 ± 7 | | |
| V188I | 51 ± 6 | 1.7 ± 0.4 | > 0.05 | D195N | 68 ± 8 | | |
| V188T | 83 ± 3 | | | †D195K | 94 ± 2 | | |
| †V188T | 95 ± 4 | | | T196G | 86 ± 9 | | |
| Y189G | 101 ± 11 | | | †T196A | 64 ± 5 | | |
| Y189F | 57 ± 5 | | | T196S | 44 ± 9 | 1.4 ± 0.2 | > 0.10 |
| Y189H | 73 ± 9 | 1.4 ± 0.3 | > 0.10 | T196V | 52 ± 5 | 1.6 ± 0.5 | > 0.10 |
| Y189T | 97 ± 5 | | | P197G | 100 ± 9 | | |
| †Y189N | 96 ± 8 | | | †P197A | 73 ± 4 | | |
| †Y190G | 46 ± 10 | | | P197GG | 85 ± 8 | | |
| †Y190A | 36 ± 10 | | | Y198G | 105 ± 8 | | |
| Y190F | 88 ± 4 | | | †Y198A | 44 ± 7 | | |
| Y190H | 59 ± 10 | 3.2 ± 0.1 | < 0.001 | Y198F | 45 ± 9 | 3.8 ± 0.3 | < 0.001 |
| Y190T | 69 ± 8 | 3.9 ± 0.8 | < 0.01 | Y198H | 72 ± 9 | 3.6 ± 0.8 | < 0.01 |
| T191G | 100 ± 7 | | | Y198T | 87 ± 6 | | |
| T191S | 61 ± 4 | | | L199G | 75 ± 9 | | |
| †T191S | 81 ± 12 | | | D200G | 66 ± 3 | 1.2 ± 0.2 | > 0.10 |
| T191V | 78 ± 6 | | | †D200A | 86 ± 7 | | |

Values are means ± SD, N = 3–6.

* Significance level for difference between $K_D$ of unsubstituted and substituted peptides.

† Fusion protein.

photolabeled primarily Tyr 198 and, to a lesser extent, Tyr 190 and Cys 192 [32]. The present study also indicates a role for Tyr 190, Cys 192, Cys 193, and Tyr 198 in nicotine binding.

There are several possibilities as to how these residues might interact with cholinergic agents. Aromatic residues could form an electronegative subsite [13, 27–29, 33]. It has been proposed that the quaternary ammonium group could interact with an aromatic hydroxyl of Tyr 93 [34, 35] and of Tyr 190 [35] and with the aromatic ring of Tyr 198 through a cation-π interaction [34–36]. On the other hand, evidence has been presented that acidic residues (aspartate and glutamate) in the γ and δ subunits contribute to the negative subsite [1, 6]. The present study indicates that negatively charged residues (Asp 195 and Asp 200) of the α1 subunit do not contribute to the anionic subsite for nicotine. It has been noted that both carboxylate and aromatic side chains could form the negative subsite [1]. In this model, other aromatic side chains, possibly including Tyr 190, may contribute to the second subsite and interact with the other end of agonists through hydrogen bonding and hydrophobic interactions [1].

Cys 192 and Cys 193 are present in all nicotinic AChR α subunits and substitution of these residues reduced nicotine binding and affinity. These residues are labeled by affinity alkylating agents and are located 9–12 Å from the negative subsite [26]. In the native receptor, the cysteines form a disulfide bond necessary for the action of agonists [37]. It was proposed that the lone pair electrons on the sulfur of cysteine could contribute to the electronegativity of the anionic subsite [27]. Alternatively, substitution of these residues could change the conformation of the peptides to one less conducive to binding. On the other hand, substitution of other residues that might affect conformation (Pro 194 and Pro 197) did not affect nicotine binding significantly.

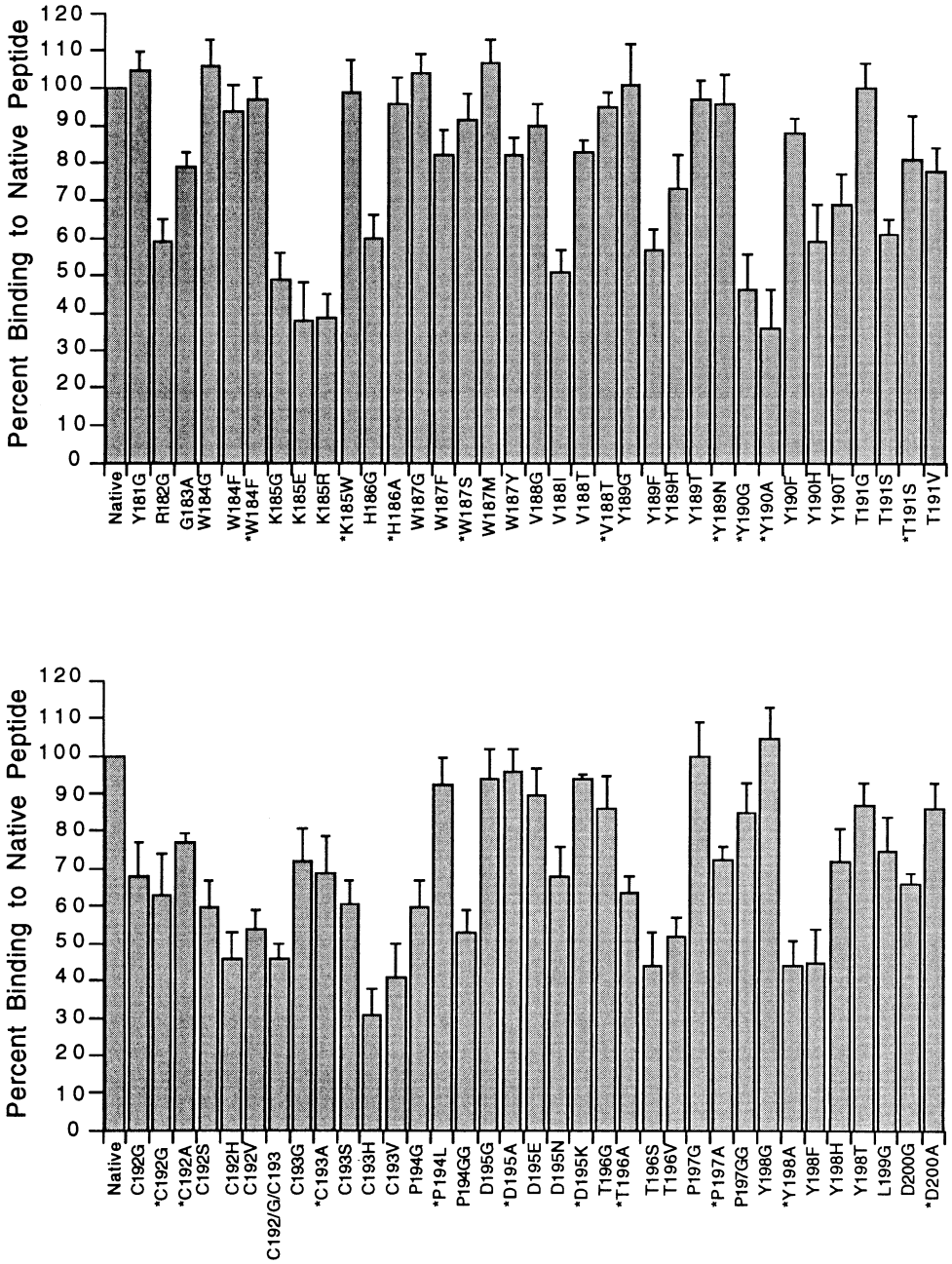


**FIG. 2. Comparison of [$^3$H]nicotine binding to native and substituted fusion proteins and peptides. Wells of microtiter plates were coated with peptides or fusion proteins, washed, and incubated with 150,000 cpm [$^3$H]nicotine (30 nM) for 90 min. The wells were washed, and bound radioactivity was measured. Binding to the native sequence is taken as 100%. Values represent the averages of at least three experiments with three replicates each. Error bars show the standard deviation. Key: * = fusion protein.**

While roles for Tyr 190, Cys 192, Cys 193, and Tyr 198 in binding are established, there is little previous evidence for functions of Lys 185 and His 186 in agonist binding to the AChR. Both residues are conserved or conservatively substituted in most α subunits. Both could participate in hydrogen bonding. It has been suggested that His 186 could act as a proton acceptor responsible for tyrosinate production [38]. However, mutation of His 186 produced only a small change in activation of the receptor by ACh [39]. While these residues could have an effect on their environment, there is evidence that nicotine can interact directly with histidine and lysine residues. Nicotine has been reported to bind to histidine residues of the 42-residue amyloid β-peptide and to inhibit amyloid formation by the peptide [40]. Binding is mediated by the N-$CH_3$ and 5′-$CH_2$ pyrrolidine moieties of nicotine. There is also evidence that nicotine interacts with lysine in the amyloid protein.*

*Zagorski MG, In molecular modeling of nicotine binding to the amyloid protein, the Lys 16 side chain is hydrogen bonded to the aromatic nitrogen of nicotine. Personal communication; cited with permission.

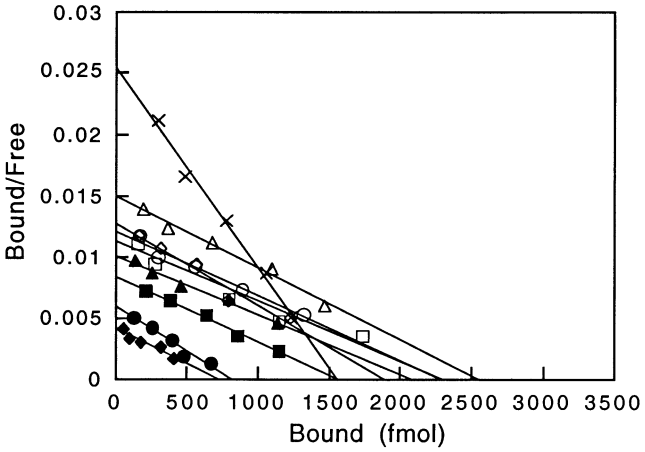


**FIG. 3. Scatchard analysis of the binding of [$^3$H]nicotine to native α1 181–200 20mer and substituted peptides. Wells of microtiter plates were coated with 8 μg of peptide and incubated with increasing amounts of [$^3$H]nicotine for 2 hr at room temperature. The wells were washed and placed in scintillation vials, and bound radioactivity was measured. Background binding in the absence of peptide was subtracted from the total binding in the presence of peptide. The data were corrected for non-specific binding by subtracting the B/F ratio at B→∞ from total bound at each point as described by Chamness and McGuire [23]. The data are presented as femtomoles of [$^3$H]nicotine bound/femtomoles free versus femtomoles bound. Specific activity of [$^3$H]nicotine was 0.05 cpm/fmol. Curves are from a single representative experiment (mean of three experiments is shown in Table 2). $K_D$ values for peptides are native (×) 1.2 × $10^{-6}$ M, K185E (●) 2.7 × $10^{-6}$ M, H186G (■) 3.7 × $10^{-6}$ M, Y190T (○) 3.7 × $10^{-6}$ M, Y190H (□) 3.1 × $10^{-6}$ M, C192H (◇) 2.9 × $10^{-6}$ M, C193H (◆) 3.4 × $10^{-6}$ M, Y198F (△) 3.4 × $10^{-6}$ M, and Y198H (▲) 4.2 × $10^{-6}$ M.**

Of the residues playing a role in nicotine binding to the *Torpedo* α1 subunit, Cys 192, Cys 193, and Tyr 198 are invariant in muscle α1 subunits of different species and in neuronal α subunits (α2–α9). Tyr 190 is present in all α subunits except α5. Lys 185 and His 186 are conserved in muscle α1 subunits but less so in neuronal α subunits. In the latter, Lys 185 is most often substituted with a Tyr or Arg residue, whereas His 186 is most often substituted with an Asn residue.

α-Btx binding to these peptides [14, 15] and fusion proteins [19] has been described. Substitution of His 186, Tyr 190, Cys 192, Cys 193, and Tyr 198 affected both α-Btx and nicotine binding, indicating that antagonists and agonists share some binding determinants. On the other hand, substitution of Val 188, Tyr 189, Pro 194, Asp 195, and Pro 197 affected only α-Btx binding, whereas substitution of Lys 185 affected only nicotine. Tyr 198 is the residue primarily photoaffinity labeled by [$^3$H]nicotine [32], while Tyr 189 is present in muscle AChRs that bind α-Btx with high affinity. Substitution of Tyr 189 did not affect nicotine binding. Thus, Tyr 189 appears to play a unique role in α-Btx binding, while Tyr 198 plays a major role in nicotine binding. The relatively large α-Btx molecule appears to interact with more residues than does nicotine. The finding that substitution of proline residues affects α-Btx binding more than nicotine binding suggests that conformation may play a greater role in the binding of the neurotoxin molecule than of nicotine.

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## References

1. Karlin A and Akabas MH, Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* **15:** 1231–1244, 1995.
2. Hucho F, Tsetlin VI and Machold J, The emerging three-dimensional structure of a receptor. The nicotinic acetylcholine receptor. *Eur J Biochem* **239:** 539–557, 1996.
3. Dunn SMJ, Conti-Tronconi BM and Raftery MA, A high-affinity site for acetylcholine occurs close to the α-γ subunit interface of *Torpedo* nicotinic acetylcholine receptor. *Biochemistry* **32:** 8616–8621, 1993.
4. Sine SM, Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of residues that determine curare selectivity. *Proc Natl Acad Sci USA* **90:** 9436–9440, 1993.
5. Czajkowski C and Karlin A, Agonist binding site of *Torpedo* electric tissue nicotinic acetylcholine receptor. A negatively charged region of the δ subunit within 0.9 nm of the α subunit binding site disulfide. *J Biol Chem* **266:** 22603–22612, 1991.
6. Czajkowski C and Karlin A, Structure of the nicotinic receptor acetylcholine-binding site. Identification of acidic residues in the δ subunit within 0.9 nm of the α subunit-binding site disulfide. *J Biol Chem* **270:** 3160–3164, 1995.
7. Conti-Tronconi BM, McLane KE, Raftery MA, Grando SA and Protti MP, The nicotinic acetylcholine receptor: Structure and autoimmune pathology. *Crit Rev Biochem Mol Biol* **29:** 69–123, 1994.
8. Wilson PT, Lentz TL and Hawrot E, Determination of the primary amino acid sequence specifying the α-bungarotoxin binding site on the α-subunit of the acetylcholine receptor from *Torpedo*. *Proc Natl Acad Sci USA* **82:** 8790–8794, 1985.
9. Wilson PT, Hawrot E and Lentz TL, Distribution of α-bungarotoxin binding sites over residues 173–204 of the α-subunit of the acetylcholine receptor. *Mol Pharmacol* **34:** 643–650, 1988.
10. Neumann D, Barchan D, Safran A, Gershoni JM and Fuchs S, Mapping of the α-bungarotoxin binding site within the α subunit of the acetylcholine receptor. *Proc Natl Acad Sci USA* **83:** 3008–3011, 1986.
11. Ralston S, Sarin V, Thanh HL, Rivier J, Fox JL and Lindstrom J, Synthetic peptides used to locate the α-bungarotoxin binding site and immunogenic regions on α subunits of the nicotinic acetylcholine receptor. *Biochemistry* **26:** 3261–3266, 1987.
12. Gotti C, Frigerio F, Bolognesi M, Longhi R, Racchetti G and Clementi F, Nicotinic acetylcholine receptor: A structural model for α-subunit peptide 188–201, the putative binding site for cholinergic agents. *FEBS Lett* **228:** 118–122, 1988.
13. Wilson PT and Lentz TL, Binding of α-bungarotoxin to synthetic peptides corresponding to residues 173–204 of the α-subunit of *Torpedo*, calf, and human acetylcholine receptor and restoration of high affinity binding by sodium dodecyl sulfate. *Biochemistry* **27:** 6667–6674, 1988.
14. Conti-Tronconi BM, Diethelm BM, Wu X, Tang F, Bertazzon T, Schröder B, Reinhardt-Maelicke S and Maelicke A,

α-Bungarotoxin and the competing antibody WF6 interact with different amino acids within the same cholinergic subsite. *Biochemistry* **30:** 2575–2584, 1991.
15. McLane KE, Wu X and Conti-Tronconi BM, An α-bungarotoxin-binding sequence on the *Torpedo* nicotinic acetylcholine receptor α-subunit: Conservative amino acid substitutions reveal side-chain specific interactions. *Biochemistry* **33:** 2576–2585, 1994.
16. Barkas T, Mauron A, Roth B, Alliod C, Tzartos SJ and Ballivet M, Mapping the main immunogenic region and toxin-binding site of the nicotinic acetylcholine receptor. *Science* **235:** 77–80, 1987.
17. Ohana B and Gershoni JM, Comparison of the toxin binding sites of the nicotinic acetylcholine receptor from *Drosophila* to human. *Biochemistry* **29:** 6409–6415, 1990.
18. Chaturvedi V, Donnelly-Roberts DL and Lentz TL, 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. *Biochemistry* **31:** 1370–1375, 1992.
19. Chaturvedi V, Donnelly-Roberts DL and Lentz TL, Effects of mutations of *Torpedo* acetylcholine receptor α1 subunit residues 184–200 on α-bungarotoxin binding in a recombinant fusion protein. *Biochemistry* **32:** 9570–9576, 1993.
20. Lentz TL, Differential binding of nicotine and α-bungarotoxin to residues 173–204 of the nicotinic acetylcholine receptor α1 subunit. *Biochemistry* **34:** 1316–1322, 1995.
21. Lukas RJ and Bencherif M, Heterogeneity and regulation of nicotinic acetylcholine receptors. *Int Rev Neurobiol* **34:** 25–131, 1992.
22. Benowitz NL, Pharmacology of nicotine: Addiction and therapeutics. *Annu Rev Pharmacol Toxicol* **36:** 597–613, 1996.
23. Chamness GC and McGuire WL, Scatchard plots: Common errors in correction and interpretation. *Steroids* **26:** 538–542, 1975.
24. Rodbard D and Frazier GR, Statistical analysis of radioligand assay data. *Methods Enzymol* **37:** 3–22, 1975.
25. Beers WH and Reich E, Structure and activity of acetylcholine. *Nature* **228:** 917–922, 1970.
26. Kao PN, Dwork AJ, Kaldany R-RJ, Silver ML, Wideman J, Stein S and Karlin A, Identification of the α subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine binding site. *J Biol Chem* **259:** 11662–11665, 1984.
27. Dennis M, Giraudat J, Kotzyba-Hibert F, Goeldner M, Hirth C, Chang J-Y, Lazur C, Chrétien M and Changeux J-P, Amino acids of the *Torpedo marmorata* acetylcholine receptor α subunit labeled by a photoaffinity ligand for the acetylcholine binding site. *Biochemistry* **27:** 2346–2357, 1988.
28. Galzi JL, Revah F, Black D, Goeldner M, Hirth C and Changeux J-P, Identification of a novel amino acid α Tyr 93 within the active site of the acetylcholine receptor by photoaffinity labeling: Additional evidence for a three-loop model of the acetylcholine binding site. *J Biol Chem* **265:** 10430–10437, 1990.
29. Abramson SN, Li Y, Culver P and Taylor P, An analog of lophotoxin reacts covalently with $Tyr^{190}$ in the α-subunit of the nicotinic acetylcholine receptor. *J Biol Chem* **264:** 12666–12672, 1989.
30. Chiara DC and Cohen JB, Identification of amino acids contributing to high and low affinity D-tubocurarine (dTC) sites on the *Torpedo* nicotinic acetylcholine (nAChR) receptor subunits. *FASEB J* **6:** A106, 1992.
31. Galzi J-L, Revah F, Bessis A and Changeux J-P, Functional architecture of the nicotinic acetylcholine receptor: From electric organ to brain. *Annu Rev Pharmacol* **31:** 37–72, 1991.
32. Middleton RE and Cohen JB, Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [$^3$H]Nicotine as an agonist photoaffinity label. *Biochemistry* **30:** 6987–6997, 1991.
33. Cohen JB, Sharp SD and Liu WS, Structure of the agonist-binding site of the nicotinic acetylcholine receptor. *J Biol Chem* **266:** 23354–23364, 1991.
34. Aylwin ML and White MM, Ligand–receptor interactions in the nicotinic acetylcholine receptor probed using multiple substitutions at conserved tyrosines on the α subunit. *FEBS Lett* **349:** 99–103, 1994.
35. Sine SM, Quiram P, Papanikolaou F, Kreienkamp H-J and Taylor P, Conserved tyrosines in the α subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonists and curariform antagonists. *J Biol Chem* **269:** 8808–8816, 1994.
36. Nowak MW, Kearney PC, Sampson JR, Saks ME, Labarca CG, Silverman SK, Zhong W, Thorson J, Abelson JN, Davidson N, Schultz PG, Dougherty DA and Lester HA, Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. *Science* **268:** 439–442, 1995.
37. Kao PN and Karlin A, Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *J Biol Chem* **261:** 8085–8088, 1986.
38. Pearce SF and Hawrot E, Intrinsic fluorescence of binding-site fragments of the nicotinic acetylcholine receptor: Perturbations produced upon binding α-bungarotoxin. *Biochemistry* **29:** 10649–10659, 1990.
39. Tomaselli GF, McLaughlin JT, Jurman ME, Hawrot E and Yellin G, Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor. *Biophys J* **60:** 721–727, 1991.
40. Salomon AR, Marcinowski KJ, Friedland RP and Zagorski MG, Nicotine inhibits amyloid formation by the β-peptide. *Biochemistry* **35:** 13568–13578, 1996.