Differential Binding of Nicotine and α-Bungarotoxin to Residues 173−204 of the Nicotinic Acetylcholine Receptor α1 Subunit†

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ABSTRACT: The binding of the agonist L-[^{3}H]nicotine and the competitive antagonist α-[^{125}I]bungarotoxin to synthetic peptides comprising residues 173−227 of the *Torpedo* nicotinic acetylcholine receptor α subunit were compared using a solid phase-assay. Equilibrium saturation binding of [^{3}H]nicotine to peptide 173−204 revealed a minor binding component with an apparent K_D of 1.9 nM and a major component with a K_D of 1.6 μM. Nicotine bound to α subunit peptides 173−204, 181−198, and 194−204 and less well to 179−192 and 186−196, and it did not bind to 173−180 and 205−227. α-Bungarotoxin bound to peptides 173−204 and 186−196 and less well to 179−192 and 181−198, and it did not bind to 173−180, 194−204, and 205−227. Agonists (nicotine, suberyldicholine, carbamylcholine, and cytisine) effectively competed [^{3}H]nicotine binding to the 173−204 peptide but competed α-[^{125}I]bungarotoxin binding at millimolar concentration and with loss of rank order of potency. The competitive antagonists α-bungarotoxin, α-cobratoxin, and *d*-tubocurarine effectively blocked α-[^{125}I]bungarotoxin binding but competed [^{3}H]nicotine binding only at millimolar concentration. These results indicate that nicotine and α-bungarotoxin preferentially bind to different determinants within residues 173−204. Alternatively, nicotine and α-bungarotoxin could bind to different conformations of the peptide. Both agents appear to interact with common residues, most likely Tyr 190 and Cys 192, in the region of Cys 192 so that there is overlap of binding sites. However, a determinant to the N-terminal side of this region, most likely Tyr 189, plays a greater role in α-bungarotoxin binding, and a determinant to the C-terminal side, most likely Tyr 198, plays a more important role in nicotine binding.

The nicotinic acetylcholine receptor (AChR)[1] of muscle is composed of four subunit types, α1, β1, γ or ϵ, and δ, arranged in a stoichiometry of $\alpha1_2\beta1\gamma\delta$ or $\alpha1_2\beta1\epsilon\delta$. The α subunit contains major determinants for binding of both agonists and competitive antagonists, although other subunits contribute to the binding sites. Cholinergic affinity alkylating agents label two consecutive cysteine residues at positions 192 and 193 (numbering for *Torpedo* α subunit) (Kao et al; 1984; Kao & Karlin, 1986). On the basis of the dimensions of the alkylating agents, the acetylcholine binding site is located within 10 Å of the disulfide bond between Cys 192 and Cys 193 (Karlin, 1980). The snake venom neurotoxin α-bungarotoxin (α-Btx) is a competitive antagonist of the AChR and has been widely employed as a probe for the receptor because it binds specifically and with high affinity. The binding of affinity alkylating agents such as [4-(*N*-maleimido)benzyl]trimethylammonium iodide and bromoacetylcholine, which act as either cholinergic agonists or competitive antagonists, are inhibited by the neurotoxins (Karlin, 1980). Conversely, the affinity labels inhibit the binding of neurotoxins. Thus, the neurotoxin binding site on the receptor appears to include the acetylcholine binding site.

Other evidence, however, indicates that the neurotoxin and acetylcholine binding sites are not identical, although they may overlap. First, the cholinergic agonist carbamylcholine and the competitive antagonist *d*-tubocurarine, which are competitive with α-Btx, differ greatly in their ability to displace bisquaternary analogs of the cholinergic ligand decamethonium (Bode et al., 1979) and the antagonist propidium (Sator et al., 1977). These findings suggest the presence of distinct cholinergic binding subsites. Small cholinergic ligands in high concentration cause accelerated dissociation of α-cobratoxin−receptor complexes, indicating the presence of different binding sites for toxins and small ligands (Kang & Maelicke, 1980). From competition studies between anti-AChR monoclonal antibodies and cholinergic ligands, it was concluded that the cholinergic binding region contains three subsites that interact respectively with monoquaternary agonists (acetylcholine, carbamylcholine, and succinyldicholine), bismethonium compounds (decamethonium, a depolarizing competitive blocking agent, and hexamethonium), and *d*-tubocurarine (Watters & Maelicke, 1983; Fels et al., 1986). The effects of anti-AChR monoclonal antibodies that are inhibited by α-Btx on cholinergic ligand binding properties were studied (Mihovilovic & Richman, 1984, 1987). It was concluded that the agonist carbamylcholine and the antagonist *d*-tubocurarine bind to different but overlapping sites within the α-Btx binding region of the receptor. Similarly, cholinergic agonists and snake neurotoxins but not hexamethonium and *d*-tubocurarine compete with an anti-AChR monoclonal antibody (Chinchetru et al., 1989). One explanation for these findings on differential binding of cholinergic ligands is that different

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

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Table 1: Sequences of Acetylcholine Receptor and Other Peptides[a]

Peptide	Sequence
	175 180 185 190 195 200 205 210 215 220 225
Torpedo AChR α1 173-204 32mer	SGEWVMKDYRGWKHWVYYTCCPDTPYLDITYH
Torpedo AChR α1 171-180 8mer	SGEWVMKD
Torpedo AChR α1 179-192 14mer	KDYRGWKHWVYYTC
Torpedo AChR α1 181-198 18mer	YRGWKHWVYYTCCPDTPY
Torpedo AChR α1 186-196 11mer	HWVYYTCCPDT
Torpedo AChR α1 194-204 11mer	PDTPYLDITYH
Torpedo AChR α1 205-227 23mer	FIMQRIPLYFVVNVIIPCLLFSF
Human $GABA_AR$ β1 188-201 14mer	IVDYKMVSKKVEFT
Human GlyR α1 189-209 21mer	LKEEKD-LRYCTKHYN-TGKFTC
Human Glucagon 1-29 29mer	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

[a] Peptides are designated by the residues they comprise and the number of residues. Glucagon is not aligned.

classes of ligands interact preferentially with different amino acids within a common cholinergic binding determinant.

Synthetic peptides and fusion proteins have proven useful in localizing and investigating the neurotoxin binding site on the AChR α subunit. Although the affinity of α-Btx for the peptides is reduced by 2−4 orders of magnitude relative to the native receptor, it is comparable to the affinity for denatured, intact α subunit, indicating that the peptides contain the major toxin binding determinant on the α subunit. Studies investigating the binding of α-Btx to synthetic peptides (Wilson et al., 1985, 1988; Neumann et al., 1986; Ralston et al., 1987; Gotti et al., 1988; Wilson & Lentz, 1988; McLane et al., 1991) and fusion proteins containing receptor sequences (Barkas et al., 1987; Ohana & Gershoni, 1990; Chaturvedi et al., 1992, 1993) indicate that a major neurotoxin binding site is located between residues 173 and 204. Although the affinity of α-Btx for the peptides is reduced, it is sufficiently high so that binding characteristics and structure−function relationships can be investigated. Comparable studies on the binding of agonists to synthetic peptides have not been reported. The ability to investigate agonist binding would allow direct comparison of agonist and antagonist binding. In this study, the binding of L-nicotine to synthetic α1 subunit peptides is described and compared with that of α-Btx. The results of studies on the binding of these agents to overlapping peptides within residues 173 and 227 and the effects of competitors on binding to a 173−204 peptide indicate that nicotine and α-Btx interact preferentially with different determinants within this region.

EXPERIMENTAL PROCEDURES

L-(−)-[*N-methyl*-^{3}H]Nicotine was purchased from NEN/Dupont Co. (Boston, MA). Upon receipt, [^{3}H]nicotine was diluted 1:10 in 0.02 M phosphate buffer, pH 7.2, aliquoted, and stored at −20 °C. The specific activity of [^{3}H]nicotine was 75−125 cpm/fmol. α-Btx was obtained from the Miami Serpentarium (Salt Lake City, UT) and iodinated with ^{125}I by the chloramine T method (Wang & Schmidt, 1980). The monoiodinated α-Btx used in these experiments was separated from diiodinated α-Btx using a CM-25 ion-exchange column. The initial specific activity of the labeled toxin was greater than 500 cpm/fmol. Labeled toxin was aliquoted and stored at −20 °C.

Synthetic Peptides. All AChR and other peptides used in these studies were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory, Boyer Center for Molecular Medicine, Yale University. The peptides correspond to portions of the *Torpedo* α1 subunit and are designated by the numbering of the sequence and the total number of residues (Table 1). All peptides were synthesized with their amino- and carboxy-termini blocked. The integrity of the peptide sequences was determined by amino acid composition analysis and by reverse-phase high-performance liquid chromatography. The molecular weights of the peptides were confirmed by mass spectroscopy. Peptides were purified by reverse-phase high-performance liquid chromatography using a Vydac C_4 column and a gradient of 0 to 80% acetonitrile/0.05% trifluoroacetic acid. The eluted peptide was lyophilized and resuspended in 50% acetonitrile/H_2O to a concentration of 0.5 mg/mL. One peptide, α1 194−204, was iodinated by incubating the peptide with 50 μg of IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, Pierce Chemical Co., Rockford, IL) plated onto glass tubes in the presence of a 10-fold molar excess of KI for 20 min. The solution was removed and incubated with AG 1-X8 anion-exchange resin (Bio-Rad Laboratories, Richmond, CA) for 30 min to remove free iodide.

Binding of [^{3}H]Nicotine and α-[^{125}I]Btx to Synthetic Peptides. [^{3}H]Nicotine and α-[^{125}I]Btx binding to synthetic peptides was measured by a solid-phase assay. For [^{3}H]-

nicotine binding, the peptide preparation, the solvent used for solubilization, the degree of solubilization of the peptide, and the nicotine preparation were critical. Although not all of these conditions have been thoroughly characterized, the conditions which yielded consistent results are described here. The most consistent results were obtained using freshly prepared [^{3}H]nicotine. It is necessary that the AChR peptides be completely solubilized. Peptides not completely solubilized in 50% acetonitrile, as evidenced by turbidity of the solution, were further diluted in acetonitrile until the solution was clear. Distilled H_2O was added to make a final concentration of 50 μg/mL. Peptide solution (100 μL, 5 μg) was placed in the wells of a microtiter plate (Immulon Removawell Strips, Dynatech Labs, Chantilly, VA) and allowed to evaporate overnight at 45 °C. Wells were washed three times with 200 μL of phosphate-buffered saline (PBS). Wells were then quenched with 300 μL of 2.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Quenching was not necessary with [^{3}H]nicotine because background binding to uncoated wells was negligible. After being washed, the wells were aspirated and incubated with [^{3}H]nicotine in 50 μL of 0.02 M phosphate buffer (~150 000 cpm, 30 nM) or α-[^{125}I]Btx in 100 μL of 0.02 M phosphate buffer/0.2% BSA (~150 000 cpm, 5 nM) for 30 min. Wells were washed rapidly two times with PBS for nicotine and three times for α-Btx. Wells exposed to [^{3}H]nicotine were placed in vials containing the fluorophore Cytoscint (ICN) and counted in a scintillation counter. Wells exposed to α-[^{125}I]Btx were placed in plastic tubes, and radioactivity was counted in a γ 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. The average standard deviation for each data point with the solid-phase assay was ±5%. Competitive binding assays with unlabeled nicotine, suberyldicholine, carbamylcholine, cytisine, α-Btx, α-cobratoxin, *d*-tubocurarine, and NaCl at increasing concentrations were performed as described previously (Wilson et al., 1988). From these competition experiments, the binding affinities were approximated by measuring the concentration of unlabeled ligand that resulted in a 50% reduction in the binding of [^{3}H]nicotine or α-[^{125}I]Btx (IC_{50} value). IC_{50} values were determined from logit−log plots of the competition data (Rodbard & Frazier, 1975). Competition curves were fitted by polynomial least squares analysis as performed by the computer program KaleidaGraph (Synergy Software, Reading, PA).

Equilibrium saturation experiments were performed for [^{3}H]nicotine in the solid-phase assay system described above. Incubation was performed for 90 min at room temperature. Equilibrium binding data were analyzed in Scatchard plots. Nonlinear curves were fitted to two linear binding curves by applying the limiting slope technique of Hunston (1975) to the data. Measurement of the rate of binding of [^{3}H]-nicotine to the 173−204 32mer revealed that half-maximal binding occurred at 10 min and maximal binding occurred at 1 h (data not shown).

RESULTS

[^{3}H]Nicotine Binding. [^{3}H]Nicotine binding to the *Torpedo* AChR α1-subunit 173−204 32mer and other peptides was demonstrated using a solid-phase radioassay (Figure 1; Table 2). [^{3}H]Nicotine bound to the 173−204 32mer but not significantly to $GABA_A$ receptor β1 subunit and glycine receptor α1 subunit peptides and glucagon. A study of nicotine binding to overlapping AChR α1 subunit peptides showed that nicotine bound to the α1 181−198 18mer almost as well as to the 32mer. Nicotine also bound to the α1 194−204 11mer and to a lesser extent to peptides 179−192 and 186−196. Residues 173−180 and 205−227 did not bind nicotine. Iodination of tyrosine residues in peptide α1 194−204 greatly reduced [^{3}H]nicotine binding. Peptide incubated with IODO-GEN and anion-exchange resin but not KI bound 1805 ± 186 cpm. After iodination in the presence of KI, 571 ± 59 cpm was bound.

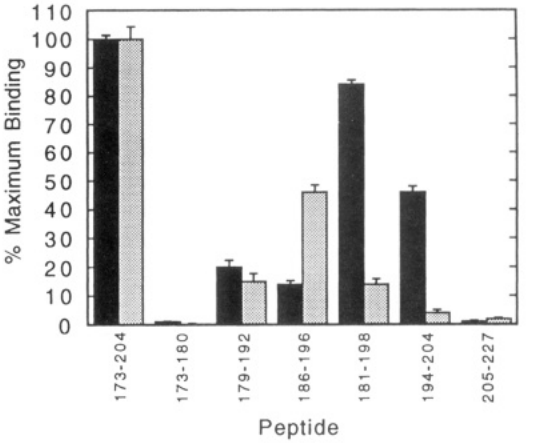

FIGURE 1: Wells of microtiter plates were coated with 1×10^{-9} mol of peptide by evaporation. Wells were incubated with 150 000 cpm [^{3}H]nicotine (30 nM) or 150 000 cpm α-[^{125}I]Btx (5 nM) for 30 min. The wells were washed, and bound radioactivity was measured (solid bars, nicotine; stippled bars, α-Btx). Binding to the α1 173−204 32mer is taken as 100%. Values represent the average of at least two experiments with three replicates each.

Table 2: [^{3}H]Nicotine and α-[^{125}I]Btx Binding to AChR α-Subunit Peptides[a]

	[^{3}H]nicotine		α-[^{125}I]Btx	
peptide	cpm bound	% max	cpm bound	% max
AChR α1 173−204 32mer	6745 ± 100	100	6521 ± 289	100
AChR α1 173−180 8mer	80 ± 12	1	27 ± 33	0
AChR α1 179−192 14mer	1319 ± 162	20	965 ± 181	15
AChR α1 186−196 11mer	944 ± 85	14	3000 ± 160	46
AChR α1 181−198 18mer	5665 ± 110	84	900 ± 119	14
AChR α1 194−204 11mer	3133 ± 137	46	229 ± 72	4
AChR α1 205−227 23mer	58 ± 27	1	138 ± 26	2
$GABA_AR$ β 188−201 14mer	0 ± 12	0	66 ± 70	1
GlyR α 189−209 21mer	4 ± 9	0	80 ± 86	1
glucagon 1−29 29mer	0 ± 10	0	61 ± 28	1

[a] Binding studies were performed as described in Figure 1. Values ± SD represent the average of at least two experiments with three replicates each.

The ability of cholinergic ligands to inhibit binding of [^{3}H]-nicotine to the α1 32mer was tested (Figure 2; Table 3). In order of decreasing affinity, the agonists nicotine, suberyldicholine, carbamylcholine, and cytisine inhibited binding of labeled nicotine. The ability of cholinergic agonists to compete binding of [^{3}H]nicotine to the 32mer indicates that binding is specific. The competitive antagonists α-Btx, α-cobratoxin, and *d*-tubocurarine inhibited nicotine binding at high concentration. NaCl inhibited binding with an IC_{50} value of 2.0×10^{-1} M.

Equilibrium saturation binding studies were performed for the binding of [^{3}H]nicotine to the 173−204 32mer using the

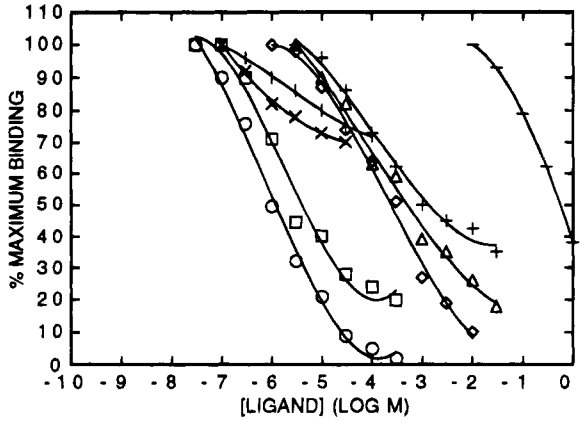

FIGURE 2: Competition of [^{3}H]nicotine binding to *Torpedo* AChR α1 173−204 32mer with cholinergic agonists and antagonists. Wells of microtiter plates were coated with 5 μg of the peptide and washed. The peptide was incubated with [^{3}H]nicotine (150 000 cpm), and a range of concentrations of unlabeled nicotine (○), suberyldicholine (□), carbamylcholine (◇), cytisine (△), *d*-tubocurarine (+), α-Btx (×), α-cobratoxin (|), and NaCl (−) were added and incubated for 30 min. The wells were washed, and bound radioactivity was determined. Values represent the average of at least two experiments with three replicates each. One hundred percent binding represents approximately 3000 cpm for most experiments.

Table 3: Competition of [^{3}H]Nicotine and α-[^{125}I]Btx Binding to Tα1 173−204 AChR Peptide by Agonists and Competitive Antagonists[a]

	IC_{50} (M)	
competitor	[^{3}H]nicotine	α-[^{125}I]Btx
nicotine	1.4×10^{-6}	5.9×10^{-3}
suberyldicholine	4.9×10^{-6}	2.9×10^{-3}
cytisine	5.6×10^{-4}	6.8×10^{-3}
carbamylcholine	2.3×10^{-4}	3.6×10^{-2}
NaCl	2.0×10^{-1}	1.6×10^{-2}
α-bungarotoxin	1.8×10^{-3}	4.2×10^{-8}
α-cobratoxin	2.0×10^{-3}	4.4×10^{-7}
d-tubocurarine	1.3×10^{-3}	8.6×10^{-5}

[a] Competition experiments were performed as described in Figures 2 and 4. IC_{50} values were determined from logit−log plots of the data shown in Figures 2 and 4. The data for competition of α-[^{125}I]Btx by α-bungarotoxin, α-cobratoxin, *d*-tubocurarine, suberyldicholine, and NaCl are from Wilson et al. (1988).

solid-phase assay. Saturable binding of [^{3}H]nicotine to the peptide adsorbed onto plastic wells was obtained (Figure 3). Scatchard analysis of nicotine binding to the peptide yielded a nonlinear plot indicating the presence of two binding components. A minor component with an apparent dissociation constant (K_D) of 1.9 nM comprised 2% of the total binding sites, and a major component with an apparent K_D of 1.6 μM comprised 98% of the sites. However, unlike α-Btx binding (Wilson & Lentz, 1988), low concentrations of sodium dodecyl sulfate (SDS) did not convert the low-affinity [^{3}H]nicotine binding component to high affinity (data not shown).

α-[^{125}I]Bungarotoxin Binding. The binding of α-[^{125}I]Btx to the AChR α1 subunit peptides was measured to compare with nicotine binding. Residues 173−204 have been shown previously to represent a major determinant of α-Btx binding (Wilson et al., 1988). Measurement of α-[^{125}I]Btx binding to peptides with the solid-phase assay revealed little or no binding to AChR α1 173−180 8mer, α1 194−204 11mer, α1 205−227 23mer, $GABA_A$ β1 subunit peptide, glycine α1 subunit peptide, and glucagon (Table 2). The greatest

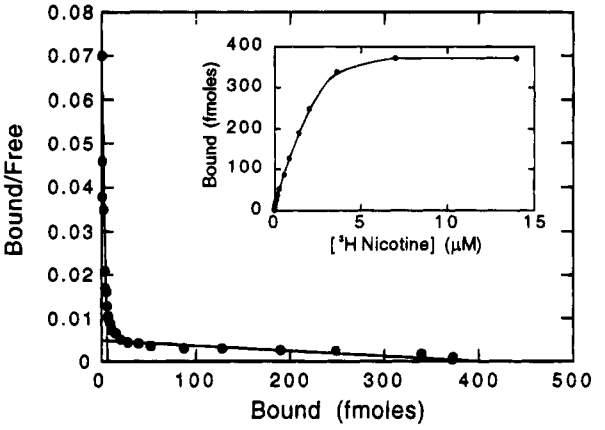

FIGURE 3: Scatchard analysis of the binding of [^{3}H]nicotine to α1 173−204 32mer. Wells of microtiter plates were coated with 5 μg of peptide and incubated with increasing amounts of [^{3}H]nicotine for 90 min at room temperature. An aliquot was removed to determine the free [^{3}H]nicotine concentration. The wells were washed and placed in scintillation vials, and bound radioactivity was measured. Background binding in the absence of peptide was subtracted from total binding in the presence of peptide. The data are presented as (femtomoles of [^{3}H]nicotine bound/femtomoles free) versus femtomoles bound. The high affinity binding component had a K_D of 1.9 nm and a B_{max} of 6.5 fmol, while the low affinity binding component had a K_D of 1.6 μM and a B_{max} of 404 fmol. The inset shows femtomoles bound versus the concentration of [^{3}H]nicotine. Points are each the average of three replicates.

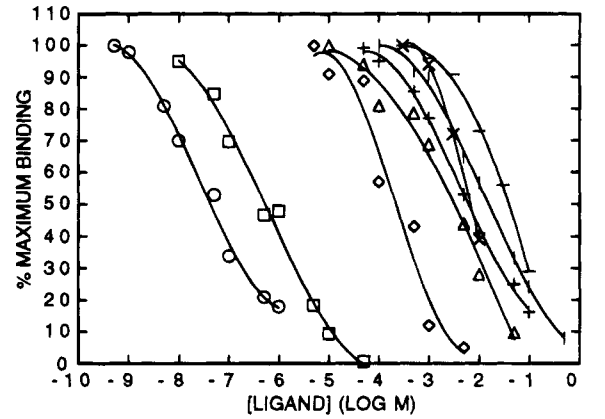

FIGURE 4: Competition of α-[^{125}I]Btx binding to α1 173−204 32mer with cholinergic agonists and antagonists. Wells of microtiter plates were coated with 5 μg of the peptide, quenched with 2.5% BSA, and washed. The peptide was incubated with α-[^{125}I]Btx (150 000 cpm) and a range of concentrations of unlabeled α-Btx (○), α-cobratoxin (□), *d*-tubocurarine (◇), suberyldicholine (△), nicotine (+), cytisine (×), NaCl (|), and carbamylcholine (−). Data for α-Btx, α-cobratoxin, *d*-tubocurarine, suberyldicholine, and NaCl are from Wilson et al. (1988). Each point represents the average of triplicate determinations.

amount of binding was that to the AChR α1 186−196 11mer, with less to the 179−192 14mer and the α1 181−198 18mer.

As with [^{3}H]nicotine, cholinergic ligands were tested for their ability to compete α-[^{125}I]Btx binding to the α1 173−204 32mer (Figure 4; Table 3). The effects of α-Btx, α-cobratoxin, *d*-tubocurarine, suberyldicholine, and NaCl were described in a previous study (Wilson et al., 1988). The competitive antagonists α-Btx, α-cobratoxin, and *d*-tubocurarine effectively inhibited binding. The agonists nicotine, suberyldicholine, cytisine, and carbamylcholine competed α-Btx binding, but only at millimolar concentrations. NaCl was as effective in inhibiting α-Btx binding as were the agonists.

Equilibrium saturation binding of α-[^{125}I]Btx to the AChR 173−204 32mer revealed a minor component with an apparent K_D of 4.2 nM and a major component with a K_D of 63 nM (Wilson & Lentz, 1988). In the presence of 0.01% SDS, a single binding component with a K_D of 7.8 nM was observed.

DISCUSSION

In this study, the binding of [^{3}H]nicotine and α-[^{125}I]Btx to synthetic peptides of the AChR α1 subunit were compared. Possible drawbacks to the use of short receptor sequences are that they may not reflect the function of the intact receptor, they do not contain binding determinants separated on the primary sequence or located on other subunits but brought into proximity in the folded protein, and they may not maintain native conformation. In the case of the AChR, studies have indicated that relatively short synthetic peptides can retain some of the binding properties of the native receptor and that the peptides interact specifically with cholinergic ligands. Although the use of peptides may exclude some components of the binding site, if they comprise a major portion of the site, detailed investigation of structure−function relationships of that region becomes possible through multiple systematic substitutions. Finally, it is possible that peptides can adopt and maintain nativelike conformations in solution, especially if they comprise structural domains in the native protein (Leszczynski & Rose, 1986). Circular dichroism spectroscopy shows that AChR peptides in solution have considerable secondary structure. Circular dichroism analysis of an α1 181−200 peptide yielded 50−60% β-pleated sheet structure and 25−30% random coil (Conti-Tronconi et al., 1991), and circular dichroism analysis of the α1 173−204 peptide yielded 0.8% α-helix, 80.3% β-sheet, and 18.9% random coil (Donnelly-Roberts & Lentz, 1993). Thus, the AChR peptides can assume secondary structure, although it is not known in what form they are adhered to the plastic. Although the three-dimensional structure of the receptor is not known, this region of the receptor is predicted to be a β-barrel structure (Finer-Moore & Stroud, 1984). Another possibility is that binding of ligands changes the conformation of the peptide. It has been shown that carbamylcholine produces a small but significant change in the circular dichroism spectrum of the α1 173−204 peptide (Donnelly-Roberts & Lentz, 1993).

Direct binding of [^{3}H]nicotine to a synthetic peptide comprising residues 173−204 of the *Torpedo* AChR α1 subunit was demonstrated in a solid-phase radioassay. A low-affinity component (K_D = 1.6 μM) comprising the majority of sites and a high-affinity component (K_D = 1.9 nM) were observed by Scatchard analysis of equilibrium binding data. Reported affinities of nicotine for native *Torpedo* AChR are K_I = 0.5 μM (Gibson, 1976), K'_L = 0.24 μM (Weiland & Taylor, 1979), and K_{eq} = 0.6 μM (Middleton & Cohen, 1991). High- and low-affinity binding components have been observed with rat brain membranes; K_D = 28 nM and 0.46 μM (Romano & Goldstein, 1980), 8.9 nM and 0.0449 μM (Yamada et al., 1985), and 2−3 nM and 0.109 μM (Lippiello & Fernandez, 1986). The affinity of nicotine for the peptide is about an order of magnitude lower than for binding to membranes. Maximum [^{3}H]nicotine binding achieved was 404 fmol. This compares with a B_{max} of 75 fmol for α-[^{125}I]Btx binding to the 32mer in the absence of SDS and a B_{max} of 609 fmol in the presence of 0.01% SDS (Wilson & Lentz, 1988).

The findings of this study indicate that nicotine and α-Btx probably bind preferentially to different determinants within residues 173−204 of the nicotinic AChR α subunit. An alternative explanation is that nicotine and α-Btx bind to different conformations of the peptide. α-Btx binds to peptides comprising residues 179−192, 181−198, and 186−196. α-Btx binding to each of these peptides was less than to peptide 173−204, suggesting that α-Btx binding determinants are scattered throughout residues 179−198. α-Btx bound only at low levels to peptide 194−204. The highest degree of binding was to the 186−196 peptide, indicating that the sequence flanking Cys 192 and Cys 193 is the most important in binding α-Btx.

It might be expected that peptide 181−198 would bind more α-Btx than peptide 186−196, since the latter is included within the former. In a previous study, it was found that, in the presence of 0.01% SDS, peptide 181−198 bound more α-Btx than peptide 186−196 (Wilson & Lentz, 1988). It was suggested that SDS enhances a conformation of peptide 181−198 more conducive to binding. Because SDS has not been found to enhance nicotine binding, the present studies comparing nicotine and α-Btx binding were performed under the same conditions using buffer lacking SDS. However, because assay conditions and possibly conformation affect binding, small differences in relative binding of ligands are probably not significant, while large differences or absence of binding are more relevant.

Nicotine bound to peptide 181−198 almost as well as to 173−204, indicating that the major nicotine determinants are located between residues 181 and 198. Unlike α-Btx, substantial nicotine binding took place to residues 194−204. Nicotine also bound to a lesser extent to residues 179−192 and 186−196. These findings indicate that nicotine shares some binding regions with α-Btx, but that a nicotine determinant which does not play a crucial role in α-Btx binding lies between residues 194 and 204. Binding of nicotine to peptide 179−192, although at lower levels, indicates the presence of some determinants to the N-terminal side of Cys 192 and Cys 193.

Competition studies further support a difference between nicotine and α-Btx binding determinants. α-Btx binding to the α1 173−204 32mer was effectively competed by competitive antagonists. Binding was also competed by agonists but only at high concentration and with loss of rank order of potency. NaCl was as effective as agonists in competing α-Btx binding but inhibited nicotine binding only at a higher concentration, possibly indicating a greater role of an anionic receptor group in α-Btx binding. In contrast, agonists competed nicotine binding to the 173−204 peptide with IC_{50} values 1−3 orders of magnitude higher than those for competition of α-Btx binding. The competitive antagonists α-Btx, α-cobratoxin, and *d*-tubocurarine inhibited nicotine binding only at high concentration. The rank order of potency of cholinergic agents for competition of α-Btx and nicotine in the intact receptor differs from that observed with the peptides. For α-Btx binding to intact AChR, the rank order of potency of competitors was reported to be toxins > suberyldicholine > nicotine > carbamylcholine > *d*-tubocurarine (Lukas, 1986). For nicotine binding, the rank order of potency was toxins > suberyldicholine > *d*-tubocurarine, cytisine, carbamylcholine > nicotine (Lukas,

1990). In the intact receptor, competitors, in addition to binding to functional residues, can also inhibit ligands by blocking access to critical residues through steric hindrance, possibly accounting for the differences between receptor and peptides. The loss of agonist-specific competition of α-Btx binding in peptides could also be due to the absence of components of the agonist binding site or to altered conformation of the binding site (Wilson et al., 1988). However, the demonstration of nicotine binding to the peptide indicates that agonist binding determinants are present. Similarly, antagonist binding sites are clearly present even though antagonists do not effectively inhibit nicotine binding. The inability of agonists to effectively compete α-Btx binding to the peptide and the inability of competitive antagonists to effectively compete nicotine binding could be explained by the preferential interaction of each class of agents with different regions within residues 173−204. Different types of interactions between functional groups of ligands and receptor amino acids could also explain differences in binding. In some cases, these agents may interact with the same residue, but the nature of the interaction and the resultant contribution to overall affinity could be quite different.

Studies in which amino acids are substituted in synthetic peptides and fusion proteins have provided information on residues important for neurotoxin binding (Wilson & Lentz, 1988; Ohana & Gershoni, 1990; Tzartos & Remoundos, 1990; Conti-Tronconi et al., 1991; McLane et al., 1991; Ohana et al., 1991; Chaturvedi et al., 1992, 1993). These studies point most strongly to a major function of Tyr 189, Tyr 190, Cys 192, Cys 193, Pro 194, and Asp 195. A role for His 186, Val 188, and Tyr 198 is also suggested, although there is not complete agreement regarding these residues. Mutation of Tyr 198 was reported to partially reduce (Chaturvedi et al., 1993) or to have no effect on α-Btx binding (Conti-Tronconi et al., 1991). In studies using antagonist affinity labels, *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate (DDF) photoaffinity 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), and a lophotoxin analog labeled Tyr 190 of the α subunit (Abramson et al., 1989). These studies indicate that within residues 173−204, Tyr 190, Cys 192, and Cys 193 are reactive to the affinity label and are in close proximity to the binding site for antagonists. Tyr 189, present in muscle AChRs which bind α-Btx with high affinity, and Asp 195, conserved or conservatively substituted in α subunits, additionally appear to play a role in neurotoxin binding. In species where one or both of these residues are substituted (human, bovine, mouse, *Drosophila*, and cobra), α-Btx binding to synthetic peptides or fusion proteins corresponding to the sequences of these species is considerably reduced (Wilson & Lentz, 1988; Ohana & Gershoni, 1990; McLane et al., 1991). After photoaffinity labeling of the *Torpedo* AChR, [^{3}H]nicotine was found to label primarily Tyr 198 and to a smaller extent Tyr 190 and Cys 192 (Middleton & Cohen, 1991), indicating that these residues are located near the nicotine binding site. On the basis of nicotine labeling, Tyr 198, which is invariant in α subunits, plays a greater role in agonist binding than in antagonist binding. Together, these studies indicate that the major α-Btx binding determinants are located between residues 189 and 195 and that the nicotine determinants are between residues 190 and 198. Thus, while there is overlap of binding sites with both agents interacting with Tyr 190 and Cys 192, Tyr 189 plays a unique role in α-Btx binding and Tyr 198 plays a greater role in nicotine binding.

Further evidence for a role of Tyr 198 in nicotine binding is provided by the observation that nicotine binding to peptide 194−204, which binds nicotine but not α-Btx, is reduced by iodination of tyrosine residues. Although this peptide contains two tyrosine residues, Tyr 198 and Tyr 203, there is little evidence from other studies that Tyr 203 plays a role in binding, so that the reduction in binding is most likely due to modification of Tyr 198.

McLane et al. (1990), using synthetic peptides, suggested that residues 1−18, 51−70, 180−199, and 183−201 contribute to the neuronal bungarotoxin (κ-bungarotoxin, toxin F) binding site on the α3 subunit. Luetje et al. (1993), on the other hand, found that residues 195−215 but not residues 1−84 are of importance for neuronal bungarotoxin sensitivity. The latter results suggest different mechanisms of binding for α-Btx and neuronal bungarotoxin, since major α-Btx determinants are located between residues 189 and 195.

The present findings on nicotine binding are in agreement with those of Luetje et al. (1993) who showed, on the basis of a study of chimeric neuronal nicotinic AChR α subunits, that amino acids determining agonist sensitivity of neuronal nicotinic AChR α subunits are located between positions 195 and 215. In addition, amino acids within this region are responsible for the different pharmacological properties of different α subunits. Residues within sequence segment 1−84 also contributed to agonist sensitivity. It will be of interest to extend the present studies to the investigation of neuronal nicotinic AChR α subunits which should bind nicotine with higher affinity than the muscle type subunit. In addition, it should be possible to investigate the role of individual amino acids in determining the differential sensitivities of neuronal AChRs to nicotine.

REFERENCES

Abramson, S. N., Li, Y., Culver, P., & Taylor, P. (1989) *J. Biol. Chem. 264*, 12666−12672.

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

Bode, J., Moody, T., Schimerlik, M., & Raftery, M. (1979) *Biochemistry 18*, 1855−1861.

Chaturvedi, V., Donnelly-Roberts, D. L., & Lentz, T. L. (1992) *Biochemistry 31*, 1370−1375.

Chaturvedi, V., Donnelly-Roberts, D. L., & Lentz, T. L. (1993) *Biochemistry 32*, 9570−9576.

Chinchetru, M. A., Marquez, J., Garcia-Borron, J. C., Richman, D. P., & Martinez-Carrion, M. (1989) *Biochemistry 28*, 4222−4229.

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.

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

Donnelly-Roberts, D. L., & Lentz, T. L. (1993) *Mol. Brain Res. 19*, 55−61.

Fels, G., Plümer-Wilk, R., Schreiber, M., & Maelicke, A. (1986) *J. Biol. Chem. 261*, 15746−15754.

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

Galzi, J. L., Revah, F., Black, D., Goeldner, M., Hirth, C., & Changeux, J.-P. (1990) *J. Biol. Chem. 265*, 10430−10437.

Gibson, R. E. (1976) *Biochemistry 15*, 3890−3901.

Gotti, C., Frigerio, F., Bolognesi, M., Longhi, R., Racchetti, G., & Clementi, F. (1988) *FEBS Lett. 228*, 118−122.
Hunston, D. L. (1975) *Anal. Biochem. 63*, 99−109.
Kang, S., & Maelicke, A. (1980) *J. Biol. Chem. 255*, 7326−7332.
Kao, P. N., & Karlin, A. (1986) *J. Biol. Chem. 261*, 8085−8088.
Kao, P. N., 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. (1980) in *The Cell Surface and Neuronal Function* (Cotman, C. W., Poste, G., & Nicholson, G. L., Eds.) pp 191−260, Elsevier, New York.
Leszczynski, J. F., & Rose, G. D. (1986) *Science 234*, 849−855.
Lippiello, P. M., & Fernandez, K. G. (1986) *Mol. Pharmacol. 29*, 448−454.
Luetje, C. W., Piattoni, M., & Patrick, J. (1993) *Mol. Pharmacol. 44*, 657−666.
Lukas, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5741−5745.
Lukas, R. J. (1990) *J. Pharmacol. Exp. Ther. 253*, 51−57.
McLane, K. E., Tang, F., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 1537−1544.
McLane, K. E., Wu, X., Diethelm, B., & Conti-Tronconi, B. M. (1991) *Biochemistry 30*, 4925−4934.
Middleton, R. E., & Cohen, J. B. (1991) *Biochemistry 30*, 6987−6997.
Mihovilovic, M., & Richman, D. P. (1984) *J. Biol. Chem. 259*, 15051−15059.
Mihovilovic, M., & Richman, D. P. (1987) *J. Biol. Chem. 262*, 4978−4986.
Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008−3011.
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.
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.
Romano, C., & Goldstein, A. (1980) *Science 210*, 647−650.
Sator, V., Raftery, M. A., & Martinez-Carrion, M. (1977) *Arch. Biochem. Biophys. 184*, 95−102.
Tzartos, S. J., & Remoundos, M. S. (1990) *J. Biol. Chem. 265*, 21462−21467.
Wang, G.-K., & Schmidt, J. (1980) *J. Biol. Chem. 255*, 11156−11162.
Watters, D., & Maelicke, A. (1983) *Biochemistry 22*, 1811−1819.
Weiland, G., & Taylor, P. (1979) *Mol. Pharmacol. 15*, 197−212.
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.
Yamada, S., Isogai, M., Kagawa, Y., Takayanagi, N., Hayashi, E., Tsuji, K., & Kosuge, T. (1985) *Mol. Pharmacol. 28*, 120−127.

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