

Snake α -Neurotoxin Binding Site on the Egyptian Cobra (*Naja haje*) Nicotinic Acetylcholine Receptor Is Conserved

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Evolutionary success requires that animal venoms are targeted against phylogenetically conserved molecular structures of fundamental physiological processes. Species producing venoms must be resistant to their action. Venoms of Elapidae snakes (e.g., cobras, kraits) contain α -neurotoxins, represented by α -bungarotoxin (α -BTX) targeted against the nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction. The model which presumes that cobras (*Naja* spp., Elapidae) have lost their binding site for conspecific α -neurotoxins because of the unique amino acid substitutions in their nAChR polypeptide backbone per se is incompatible with the evolutionary theory that (1) the molecular motifs forming the α -neurotoxin target site on the nAChR are fundamental for receptor structure and/or function, and (2) the α -neurotoxin target site is conserved among Chordata lineages. To test the hypothesis that the α -neurotoxin binding site is conserved in Elapidae snakes and to identify the mechanism of resistance against conspecific α -neurotoxins, we cloned the ligand binding domain of the Egyptian cobra (*Naja haje*) nAChR α subunit. When expressed as part of a functional *Naja*/mouse chimeric nAChR in *Xenopus* oocytes, this domain confers resistance against α -BTX but does not alter responses induced by the natural ligand acetylcholine. Further mutational analysis of the *Naja*/mouse nAChR demonstrated that an *N*-glycosylation signal in the ligand binding domain that is unique to *N. haje* is responsible for α -BTX resistance. However, when the *N*-glycosylation signal is eliminated, the nAChR containing the *N. haje* sequence is inhibited by α -BTX with a potency that is comparable to that in mammals. We conclude that the binding site for conspecific α -neurotoxin in Elapidae snakes is conserved in the nAChR ligand binding domain polypeptide backbone per se. This conclusion supports the hypothesis that animal toxins are targeted against evolutionarily conserved molecular motifs. Such conservation also calls for a revision of the present model of the α -BTX binding site. The approach described here can be used to identify the mechanism of resistance against conspecific venoms in other species and to characterize toxin-receptor coevolution.

Introduction

The biological function of snake venom neurotoxins is to immobilize potential prey and predator species. For evolutionary success, the molecular structure of the neurotoxin target site must be conserved in a phylogenetically wide spectrum of taxa among Chordata lineages. In addition, the same target site must be associated with fundamental physiological mechanisms, such as release or binding or degrading of the neurotransmitter at the neuromuscular junction, in order to provide a basis for the immediate and potentially lethal pharmacological effect of the neurotoxins.

Venoms of the Elapidae snakes (e.g., cobras, kraits) contain several postsynaptic polypeptide α -neurotoxins, such as α -bungarotoxin (α -BTX) isolated from the venom of the banded krait (*Bungarus multicinctus*) (Mebs et al. 1972). The common target of α -neurotoxins is the muscle-type nicotinic acetylcholine receptor (nAChR), a ligand-gated ion channel on the postsynaptic fold of the

neuromuscular junction with the subunit stoichiometry of $\alpha_2\beta\gamma\delta$ (Karlín 1993). Upon binding to the nAChR, α -neurotoxins prevent the binding of the natural ligand acetylcholine (ACh) and the subsequent ACh-induced ion flow, resulting in a neuromuscular inhibition of the envenomated species. The primary binding site of α -BTX on Chordata nAChR has been localized within segment E¹⁷²-F²⁰⁵ of the α subunits (Wilson, Lentz, and Hawrot 1985; Neumann et al. 1986a, 1986b; Barkas et al. 1987; Gotti et al. 1988; Wilson and Lentz 1988; Ohana and Gershoni 1990; Pearce and Hawrot 1990; Conti-Tronconi et al. 1991; McLane et al. 1991; Chaturvedi, Donnelly-Roberts, and Lentz 1992, 1993; McLane, Wu, and Conti-Tronconi 1994; Lentz 1995; Arias 2000). Specifically, α subunit residues H¹⁸⁶, W¹⁸⁷, V¹⁸⁸, Y, T, or F at position 189, Y¹⁹⁰, T¹⁹¹, C¹⁹², C¹⁹³, P¹⁹⁴, D¹⁹⁵, P¹⁹⁷, and D²⁰⁰ have been postulated to be the principal elements forming the α -BTX binding site in torpedo (*Torpedo* spp.), mouse (*Mus musculus*), and human (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988; Ohana and Gershoni 1990; Conti-Tronconi et al. 1991; McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992; Chaturvedi, Donnelly-Roberts, and Lentz 1992; Chaturvedi, Donnelly-Roberts, and Lentz 1993; Fuchs et al. 1993; McCormick et al. 1993; McLane, Wu, and Conti-Tronconi 1994; Kachalsky et al. 1995; Lentz 1995; Ackermann and Taylor 1997; Spura et al. 1999, 2000). In addition, a two-subsite model was proposed that requires aromatic amino acid residues at positions 187 and 189, plus the parallel presence of P¹⁹⁴ and P¹⁹⁷

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Abbreviations: ACh, acetylcholine; α -BTX, α -bungarotoxin; *N. haje*, Egyptian cobra (*Naja haje*, Elapidae, Reptilia); nAChR, muscle-type nicotinic acetylcholine receptor.

Key words: evolution, snake neurotoxin, resistance, acetylcholine receptor, *Naja*, Elapidae.

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in order to bind α -BTX (Barchan et al. 1995; Kachalsky et al. 1995).

The primary binding site of α -BTX, the α subunit segment E¹⁷²-F²⁰⁵, is in the vicinity of $\alpha\gamma$ and $\alpha\delta$ subunit interfaces (Blount and Merlie 1989; Sine and Claudio 1991; Czajkowski and Karlin 1995). As predicted by the theory that the target sites of neurotoxins are associated with fundamental physiological mechanisms, this segment also includes major determinants for ACh binding, namely, residues Y¹⁹⁰, C¹⁹², C¹⁹³, and Y¹⁹⁸ (Karlin 1993). All of these residues are conserved among Chordata lineages.

Venomous snakes exhibit a natural resistance to components of conspecific venoms. Considering the phylogenetic conservation and physiological relevance of the target site for venom components, the evolutionary and pharmacological basis for such resistance is rather interesting. To date, only one resistance strategy has been demonstrated, where components of pit viper (Crotalinae) venoms are neutralized by humoral factors present in conspecific blood plasma (Straight, Glenn, and Snyder 1976). Such a neutralization mechanism has not been established in Elapidae snakes (Ovadia and Kochva 1977), and serum-free neuromuscular preparations of snakes generally are insensitive to α -neurotoxins (Burden, Hartzell, and Yoshikami 1975; Liu, Xu, and Hsu 1990; Endo and Tamiya 1991). Earlier studies suggested that α -BTX resistance in cobras (*Naja* spp., Elapidae) was (1) due to the absence of the binding site because of the inherent amino acid substitutions in the polypeptide backbone of the nAChR α subunit per se (Neumann et al. 1989; McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992, 1995; Chaturvedi, Donnelly-Roberts, and Lentz 1992; Fuchs et al. 1993; Kachalsky et al. 1993, 1995) or (2) due to the glycosylation of the nAChR ligand binding domain that is absent in α -BTX-sensitive species (Kreienkamp et al. 1994; Keller et al. 1995). These proposals, however, (1) are not consistent with or cannot address the hypothesis that the α -BTX binding site is conserved across the species of Chordata, including the Elapidae snakes; (2) provide contradictory results regarding the role of amino acid residues forming the α -BTX binding site; and (3) have never been demonstrated using the ligand binding domain of the cobra (*Naja* spp.) or any other α -neurotoxin-resistant species as part of a functional nAChR.

In the present study, we tested the hypothesis that the α -neurotoxin binding site of the cobra (*Naja* spp.) nAChR is conserved per se and addressed the unsettled question of the molecular mechanism of resistance to conspecific α -neurotoxin. We cloned the Egyptian cobra (*N. haje*) nAChR ligand binding domain and expressed it as part of a functional nAChR in *Xenopus* oocytes. Using a two-microelectrode voltage clamp to monitor the ACh-induced currents, we tested the pharmacological action of α -BTX on nAChR containing the *N. haje* ligand binding domain and several subchimeric and point-mutated derivatives.

Materials and Methods

Cloning the *N. haje* Ligand Binding Domain and Construction of α N1

Total RNA isolated from the trunk skeletal muscle of the Egyptian cobra (*N. haje*, Elapidae, Reptilia) was reverse-transcribed with M-MLV reverse transcriptase using oligo (dT)-18 primer. The first-strand cDNA was PCR-amplified using gene-specific primers 5'-CACCT-ATTCCCCTTTGATGAGCA-3' (sense) and 5'-ATGATGACGTTGACAATGAAGTAGAGA-3' (anti-sense; PA), followed by a second amplification with primers PA and 5'-TGAGCAAACACTGCAGTATGAAGCTGG-3' (sense). The final product was digested with *HincII* and *PstI* restriction enzymes (recognition sites are underlined) and ligated into the homologous position of the mouse (*M. musculus*) nAChR α subunit that was subcloned into a pSP64T vector (provided together with nAChR subunits β , γ , and δ by Arthur Karlin, Columbia University). The resulting chimeric subunit, α N1, was sequenced in both strands and was also used as a template to construct subsequent α subunit derivatives. All nucleotide and amino acid sequences are numbered according to the *M. musculus* nAChR α subunit sequence (GenBank accession number X03986).

Construction of Mutant α Subunits α N2– α N10

Segments or point mutations were introduced into wild-type α (to construct α N2, α N4, α N6, and α N8), α N1 (to construct α N3 and α N7), or α N3 (to construct α N5, α N9, and α N10) by specially designed PCR primers (within nucleotide positions 545–578) encoding the respective mutations. These special mutated primers, which also overlapped the unique restriction site *DraIII*-561, were used together with primers upstream of the *EagI*-295 site, 5'-CTTGAAATGGAATCCAGATGAC-TA-3' (sense; for α N2, α N3), or downstream of the *BstXI*-1215 site, 5'-AAACACAGCCAGCGTCCCGATGAG-3' (antisense; for all other mutants), to PCR amplify the mutated segment from templates α (to construct α N3, α N8, and α N9), α N1 (to construct α N2 and α N7), α N3 (to construct α N4, α N5, and α N6), or α N8 (to construct α N10). The PCR products were double-digested with *DraIII* and *EagI* or with *DraIII* and *BstXI* restriction enzymes, gel-purified, and ligated into α , α N1, or α N3 at the homologous position. All mutations were confirmed by sequencing both strands.

Expression of nAChR in *Xenopus* Oocytes

nAChR subunit α (*BamHI*), α N1– α N10 (*BamHI*), β (*XbaI*), γ (*XbaI*), and δ (*BamHI*) cDNAs were linearized with the restriction enzymes indicated. cRNAs were transcribed in vitro using the mMessage mMachine kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. The resulting cRNAs were quantified by electrophoresis. *Xenopus laevis* were anesthetized with 0.15 % (w/v) 3-aminobenzoic acid ethyl ester, and ovarian lobes were removed and incubated in Ca²⁺-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES [pH 7.6]) containing 50 mg/ml collagenase B (Boehringer Mannheim, Indianapolis, Ind.) for 45 min at room temperature. Follicular layers were removed by forceps, and oocytes were transferred into ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM

CaCl₂, 1 mM MgCl₂, 5 mM HEPES [pH 7.4]), supplemented with 2.5 mM Na-pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 % (v/v) fetal bovine serum, and incubated for 12 h before injection. cRNAs were mixed at a molar ratio of 2 α : β : γ : δ (wild-type *M. musculus*) or 2 α N1– α N10 : β : γ : δ (chimeric *Naja/Mus* α N1 and mutant α N2– α N10 subunits). Then 50 nl (200 pg/nl) of one of these mixtures was injected into the vegetal pole of the oocytes. Oocytes were incubated at 16°C and used for electrophysiological studies 24–72 h postinjection.

Acetylcholine (ACh) Dose-Response Recording

ACh-induced currents of the injected *Xenopus* oocytes were assayed with a two-microelectrode voltage clamp at a holding potential of –40 mV, using electrodes with <1.5 M Ω resistance when filled with 3.3 M KCl. ACh (Sigma, St. Louis, Mo.) was applied in the bath solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 1 μ M atropine, and 10 mM HEPES (pH 7.5) at a flow rate of 6 ml/min at room temperature. Five to ten minutes after impalement with the electrodes, oocytes were exposed to 4 \times 10^{–5} M of ACh for 10 s, and peak current amplitude (I_{\max}) was recorded as the maximum change of holding current. Thereafter, at 5-min intervals, oocytes were exposed to various concentrations of ACh, ranging from 1 \times 10^{–7} M to 2 \times 10^{–5} M, for 10 s, and peak current amplitude (I_{test}) was recorded as the maximum change of holding current. At the end of each protocol, the application of 4 \times 10^{–5} M of ACh for 10 s was repeated to ensure reproducibility. Representative normalized ACh dose-response relationships were calculated by fitting the peak currents to the Hill equation, $I = I_{\max} / \{1 + (K_{\text{app}}/[ACh])^n\}$. Each data point displayed along the curves is the mean of $I_{\text{test}}/I_{\max} \pm$ SE recorded from two to five individual oocytes.

α -BTX Dose-Response Recording

Five to ten minutes after impalement with the electrodes, oocytes were exposed twice (5 min apart) to 10^{–5} M of ACh for 10 s, and the peak current amplitudes were averaged (I_{\max}). Following these control recordings, the same oocytes were superfused with bath solution containing various concentrations of α -BTX (isolated from the banded krait, *B. multicinctus*, Elapidae, Reptilia; Mebs et al. 1972; Calbiochem-Novabiochem, San Diego, Calif.), ranging from 3.60 \times 10^{–10} M to 3.60 \times 10^{–7} M for 10 min, then exposed again twice (5 min apart) to 10^{–5} M of ACh for 10 s in the continuous presence of the α -BTX. The peak current amplitudes from the last two ACh exposures were averaged (I_{test}). I_{test}/I_{\max} values were plotted as a function of α -BTX concentration. For nAChRs that were inhibited by α -BTX, representative normalized dose-response curves were calculated by fitting the peak currents to the Hill equation. For nAChRs that were resistant to α -BTX, mean I_{test}/I_{\max} data points were fitted by a linear regression. Each data point displayed for both α -BTX-sensitive and α -BTX-resistant nAChRs along the curves is the mean of $I_{\text{test}}/I_{\max} \pm$ SE recorded from 2–13 individual oocytes.

Because of the slow onset of action of α -BTX, the time of incubation in this protocol does not allow measurements at equilibrium conditions. While not reaching equilibrium limits the precision of our measurements, it does not alter the conclusions from our experiments. Incubation of α N8 nAChR (single residue introduced into wild type, *M. musculus* α -BTX-sensitive nAChR) at the highest α -BTX concentration (3.6 \times 10^{–7} M) tested for 30 min resulted in no current reduction in response to ACh.

Western Blot Analysis

Twenty-three to forty-eight *Xenopus* oocytes per cRNA samples were injected and incubated as described in the *Expression of nAChR in Xenopus Oocytes* section. After 2 days, oocytes were homogenized (20 μ l/oocyte) in ice-cold 50 mM Na₂HPO₄/NaH₂PO₄ (pH 7.5), 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamide, 15 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, and 5 μ g/ml soybean trypsin inhibitor, then centrifuged at 15,000 \times g for 20 min at 4°C. The pellet was resuspended in Laemmli sample buffer (15 μ l/oocyte), incubated at 60°C for 20 min, and centrifuged at 15,000 \times g at 4°C for 20 min. Electrophoresis was performed after the supernatant was loaded onto a 12% SDS-polyacrylamide gel. Kaleidoscope prestained standards (Bio-Rad, Hercules, Calif.) were used to monitor protein migration. After electrophoresis, gels were equilibrated in the transfer buffer (48 mM Tris base [pH 8.5], 192 mM glycine, 0.02% SDS, 20% methanol) for 30 min, then blotted for 3 h at 4°C onto NitroBind nitrocellulose membranes (Micron Separations, Westborough, Mass.) in a Bio-Rad transblot apparatus at 50 V in transfer buffer. Nonspecific binding sites were blocked by incubating the membrane in 50 mM Tris base (pH 7.5), 100 mM NaCl, 0.05 % (v/v) Tween 20 (TBS buffer) containing 5% (w/v) filtered nonfat dry milk (TBS-milk buffer) for 2 h at room temperature. Primary antibody, mAb210 (stock 1/94, 5 mg/ml IgG, provided by Jon Lindstrom, University of Pennsylvania), was diluted in TBS-milk buffer at 1:1,000 and incubated with the membrane for 2 h at room temperature, followed by washing for 30 min in TBS-milk buffer. Peroxidase-labeled affinity purified antibody to rat IgG produced in goat (Kirkegaard and Perry, Gaithersburg, Md.) was used as a secondary antibody. The anti-rat IgG antibody was diluted in TBS-milk buffer at 1:1,000 and incubated with the membrane for 1 h at room temperature. Membranes were then washed for 10 min in TBS-milk buffer, followed by 30 min in TBS buffer. The signal was detected by ECL Western blotting detection reagents (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions and recorded on Fuji medical RX film.

Results

Cloning and Expression of the *N. haje* nAChR Ligand Binding Domain

To test the hypothesis that the α -neurotoxin binding site is conserved in Elapidae snakes (Elapidae, Reptilia),

	.440			.450			.460			
Naja	GA	ACA	TGG	ACT	TAC	GAT	GGC	ACC	GTG	GTT
Naja		T	W	T	Y	D	G	T	V	V
Mus		-	-	-	-	-	-	S	-	-
				.150						
	.470			.480			.490			.500
GCC	ATC	TAC	CCG	GAA	GAT	CCC	CGT	CCA	GAT	TTG
A	I	Y	P	E	D	P	R	P	D	L
-	-	N	-	-	S	D	Q	-	-	-
				.160						
	.510			.520			.530			
AGT	AAC	TAC	ATG	CAG	AGT	GGA	GAA	TGG	ACA	TTG
S	N	Y	M	Q	S	G	E	W	T	L
-	-	F	-	E	-	-	-	-	V	I
				.170						
	.540			.550			.560			
AAA	GAT	TAC	CGA	GGT	TTT	TGG	CAC	TCG	GTG	AAC
K	D	Y	R	G	F	W	H	S	V	N
-	E	A	-	-	W	K	-	W	-	F
				.180						
	.570			.580			.590			.600
TAT	TCT	TGT	TGC	CTT	GAT	ACG	CCG	TAT	CTT	GAT
Y	S	C	C	L	D	T	P	Y	L	D
-	-	-	-	P	T	-	-	-	-	-
				.190						.200
	.610			.620			.630			
ATC	ACC	TAC	CAC	TTT	ATA	TTG	CTG	CGT	TTG	CC
I	T	Y	H	F	I	L	L	R	L	P
-	-	-	-	-	V	M	Q	-	-	-
									.210	

FIG. 1.—cDNA nucleotide (*Naja*, upper row) and deduced amino acid (*Naja*, middle row) sequence of the Egyptian cobra (*Naja haje*) nAChR α subunit ligand binding domain. For comparison, the homologous portion of the mouse (*Mus musculus*) amino acid (*Mus*, bottom row) sequence is also shown. Residues in *M. musculus* identical to *N. haje* are indicated with dashes. Nucleotide and amino acid sequences are numbered according to the *M. musculus* sequence (GenBank accession number X03986).

we cloned segment T¹⁴⁸-P²¹¹ of the muscle-type nAChR α subunit from the Egyptian cobra (*N. haje*, Elapidae) (fig. 1). This segment contains several major residues implicated in the binding of the physiological ligand ACh and also contains the binding site for α -BTX in numerous other Chordata species representing various classes from Chondrichthyes to Mammalia (Karlin 1993). Specific residues of the muscle-type nAChR that are conserved across species and have been implicated in agonist binding by affinity-labeling include W¹⁴⁹, Y¹⁵¹, Y¹⁹⁰, C¹⁹², C¹⁹³, and Y¹⁹⁸ (Karlin 1993). These residues are conserved in the *N. haje* sequence.

In order to pharmacologically test for the presence of the α -BTX binding site in the *N. haje* ligand binding domain, we constructed several chimeric and point-mutated nAChRs, termed α N1- α N10 (fig. 2). In these constructs, segments and/or single residues from *N. haje* or the chicken (*Gallus gallus*) were introduced into the homologous position of the mouse (*M. musculus*) nAChR

α subunit, then coexpressed with the wild-type *M. musculus* β , γ , and δ subunits in *Xenopus* oocytes. All constructs were assayed for ACh-induced currents with two-microelectrode voltage clamp to monitor receptor function. The peak current amplitudes, individual time courses, and dose-response characteristics of ACh-induced responses were comparable with the wild-type *M. musculus* (α) in all chimeric and point-mutated (α N1- α N10) nAChRs (fig. 3 and table 1). These measurements indicated that any structural alterations in the nAChRs as a result of chimera construction or point mutation were minimal enough to maintain basic receptor functions.

Mutational Analysis of the *N. haje* nAChR Ligand Binding Domain

Upon exposure to α -BTX, the wild-type (α) nAChR was inhibited in a dose-dependent manner with

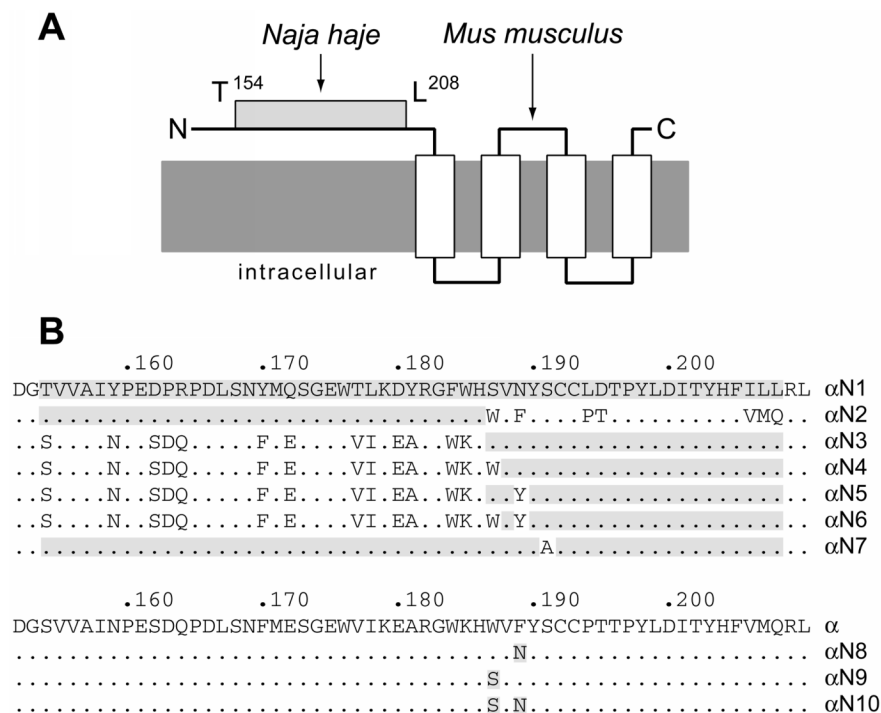


FIG. 2.—Sequences of the mutated α subunits that were constructed to test the α -BTX binding site of *Naja haja* nAChR. **A**, Schematic representation of a chimeric nAChR α subunit (α N1) indicating the introduction of the *N. haja* ligand binding domain (T¹⁵⁴-L²⁰⁸) into the homologous position of the *Mus musculus* α subunit. **B**, Mutations of the α subunits introduced into the *N. haja* (upper panel) and *M. musculus* (lower panel) ligand binding domains. A dot indicates residues identical to the top sequence of each panel, α N1 or α . Shaded residues are from *N. haja*, and others are from *M. musculus*, except Y¹⁸⁹ and A¹⁹¹, which are from the chicken (*Gallus gallus*). For simplicity, all nAChRs are referred to only by their respective α subunits in the text, figures, and table.

an IC_{50} of 3.50×10^{-9} M (fig. 4A and F). However, nAChR containing the full-length *N. haja* ligand binding domain T¹⁵⁴-L²⁰⁸ (α N1) was resistant (<10% inhibition) to the inhibitory action of 3.60×10^{-7} M α -BTX (fig. 4A and F). Construction of two subchimeric nAChRs (α N2 and α N3) demonstrated that the α -BTX resistance-mediating elements were inherent in *N. haja* segment S¹⁸⁷-L²⁰⁸ (fig. 4B). Introduction of T¹⁵⁴-H¹⁸⁶ alone (α N2) did not alter the α -BTX sensitivity.

In contrast to most α -BTX-sensitive species, positions 187 and 189 of the *N. haja* ligand binding domain contain nonaromatic residues. Lack of aromatic residues at these positions in *Naja* was claimed to eliminate the α -BTX binding site from the nAChR (McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992, 1995; Fuchs et al. 1993; Kachalsky et al. 1995). To address the role of these residues, we constructed three additional mutations (α N4, α N5, and α N6) in which aromatic amino acid residues present only in α -BTX sensitive species at positions 187 and 189 were introduced into *N. haja* segment S¹⁸⁷-L²⁰⁸. The ACh-induced response of α N4 was resistant, while α N5 and α N6 were sensitive to the inhibitory action of α -BTX, with $IC_{50} = 0.67 \times 10^{-9}$ M and $IC_{50} = 0.81 \times 10^{-9}$ M, respectively (fig. 4C). These results demonstrated that either N¹⁸⁹ alone or the intact *N*-glycosylation signal (N¹⁸⁹-X¹⁹⁰-S¹⁹¹) was responsible for mediating resistance to α -BTX.

To differentiate between these two alternatives, a point mutation was designed that eliminated the *N*-gly-

cosylation consensus sequence while leaving N¹⁸⁹ intact. In addition to N¹⁸⁹, this mutation also left all other residues (S¹⁸⁷, L¹⁹⁴, absence of parallel P¹⁹⁴ and P¹⁹⁷ pattern) that have been claimed to confer α -BTX resistance per se (McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992, 1995; Fuchs et al. 1993; Kachalsky et al. 1995) unaltered. S¹⁹¹ in the full *N. haja* ligand binding domain T¹⁵⁴-L²⁰⁸ was mutated to alanine (α N7), a residue that is present in both α -BTX-sensitive (e.g., genus *Xenopus*, *Gallus*, *Crocidura*) α -BTX-resistant (e.g., *Herpestes ichneumon*) taxa. Western blot analysis was consistent with the lack of glycosylation in α N7 when compared with α N1 that had the intact *N. haja* ligand binding domain (fig. 5). This single point mutation of the *N. haja* ligand binding domain eliminated the resistance to α -BTX, yielding an IC_{50} of 9.11×10^{-9} M, comparable with the wild-type *M. musculus* IC_{50} of 3.50×10^{-9} M (fig. 4D and F). A major consideration in this case is that all residues and motifs that have been claimed to confer α -BTX resistance per se (McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992, 1995; Fuchs et al. 1993; Kachalsky et al. 1995; Arias 2000) are present in α N7, including S¹⁸⁷, N¹⁸⁹, L¹⁹⁴, and the absence of the parallel P¹⁹⁴ and P¹⁹⁷ pattern.

Transferring the *N. haja* α -BTX Resistance to *M. musculus* nAChR

Single-residue mutations presented above were introduced into the *N. haja* sequence where they had no

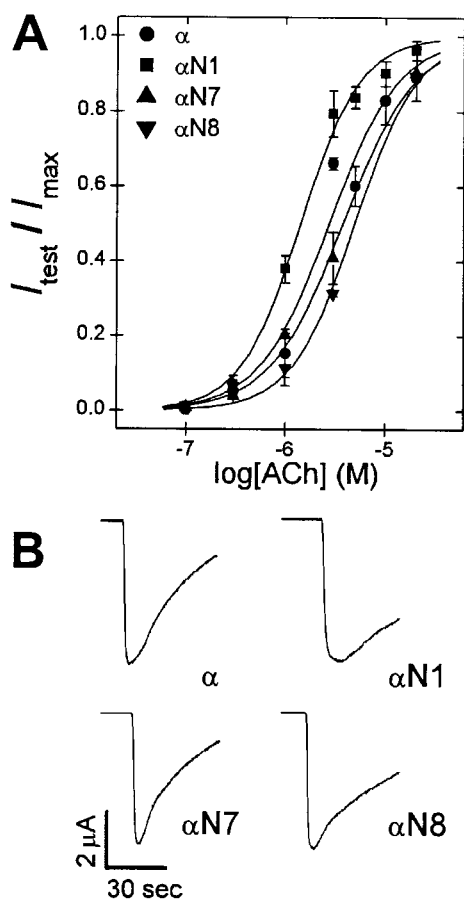


FIG. 3.—ACh-induced responses of wild-type and representative mutated nAChRs. A, Normalized ACh dose-response relationships of α , αN1 , αN7 , and αN8 . B, Representative whole-cell currents induced by the application of 10^{-5} M ACh in α , αN1 , αN7 , and αN8 .

effect on α -BTX resistance or converted the *N. haje* ligand binding domain to α -BTX-sensitive. Can the *N*-glycosylation signal that is responsible for α -BTX resistance be transferred to a different species where it will confer its associated pharmacological effect observed in *N. haje*? We tested this transferability of α -BTX resistance and introduced the *N. haje* *N*-glycosylation signal into the wild-type *M. musculus* nAChR as a single point mutation, F189N (αN8), and as a double mutant, W187S/F189N (αN10). The W187S mutation was also introduced alone (αN9). Western blot analysis was consistent with the presence of glycosylation in αN8 , with the *M. musculus* nAChR containing only one residue from *N. haje* (fig. 5). In agreement with the above findings, nAChRs αN8 and αN10 were resistant to 3.60×10^{-7} M α -BTX, while αN9 , which lacked the consensus sequence for *N*-glycosylation, was inhibited with an IC_{50} of 7.51×10^{-9} M (fig. 4E).

Discussion

The present study demonstrated that the binding site for conspecific α -neurotoxin in Elapidae snakes is conserved in the nAChR per se and supports the hypothesis that animal toxins are targeted against evolutionarily conserved molecular motifs. In addition, we

Table 1
Physiological and Pharmacological Characteristics of the Mutated nAChRs

nAChR	ACh, Mean $I_{\text{max}} \pm \text{SD}$ (μA)	ACh, EC_{50} ($\times 10^{-6}$ M)	α -BTX, IC_{50} ($\times 10^{-9}$ M)
α	4.19 ± 1.06	2.73	3.50
αN1	4.96 ± 0.83	1.41	Resistant
αN2	2.60 ± 0.87	13.27	3.58
αN3	4.74 ± 0.56	1.73	Resistant
αN4	4.51 ± 0.87	0.37	Resistant
αN5	3.86 ± 0.94	2.40	0.67
αN6	3.28 ± 0.82	2.79	0.81
αN7	3.64 ± 0.41	3.74	9.11
αN8	4.94 ± 0.34	4.93	Resistant
αN9	4.06 ± 0.56	0.99	7.51
αN10	3.38 ± 1.20	0.39	Resistant

NOTE.—Resistant = $<10\%$ inhibition by 3.6×10^{-7} M α -BTX.

presented direct evidence that an animal species can be resistant to a component of conspecific venom by structural modification of the target molecule. These results also call for a revision of the binding site for α -BTX, one of the most widely utilized animal toxins.

Expression of the Egyptian cobra (*N. haje*) ligand binding domain as part of a functional nAChR showed that the presence of the unique *N*-glycosylation signal in the middle of the ligand binding domain prevents the inhibitory action of α -BTX. The *N*-glycosylation signal, however, does not interfere with currents induced by the substantially smaller natural ligand ACh. Furthermore, the introduction of the *N*-glycosylation signal to the homologous position of mouse (*M. musculus*) nAChR also transfers the α -BTX resistance but has no effect on currents induced by ACh. Consistent with these observations, *M. musculus* nAChR carrying such *N*-glycosylation signal expressed in mammalian tissue culture exhibits greatly reduced affinity for α -BTX (Kreienkamp et al. 1994; Keller et al. 1995).

We demonstrated that the *N*-glycosylation is masking a genuine binding site for α -BTX on the nAChR. Several earlier studies based on synthetic peptides and bacterial fusion proteins suggested that the cobra (*Naja* spp.) has no binding site for α -BTX on the nAChR because of distinct amino acid substitutions in the polypeptide backbone per se, namely, S¹⁸⁷ (McLane et al. 1991; Barchan et al. 1992, 1995; Fuchs et al. 1993; Kachalsky et al. 1995), N¹⁸⁹ (McLane et al. 1991; Ohana et al. 1991; Barchan et al. 1992, 1995; Fuchs et al. 1993; Kachalsky et al. 1995), L¹⁹⁴ (McLane et al. 1991; Barchan et al. 1992; Kachalsky et al. 1995), and/or lack of parallel P¹⁹⁴ and P¹⁹⁷ (Fuchs et al. 1993; Kachalsky et al. 1995). This proposal is in conflict with the hypothesis that the α -BTX binding site is conserved in Elapidae and inconsistent with results of the present experiments. When the *N*-glycosylation signal is removed by a single point mutation or multiple point mutations from the *N. haje* ligand binding domain, the nAChR retains its normal response to ACh, but a sensitivity to α -BTX that is comparable with mammalian nAChR is revealed (table 1). This inhibition demonstrates that the α -BTX binding site is in fact present in the *Naja* nAChR polypeptide

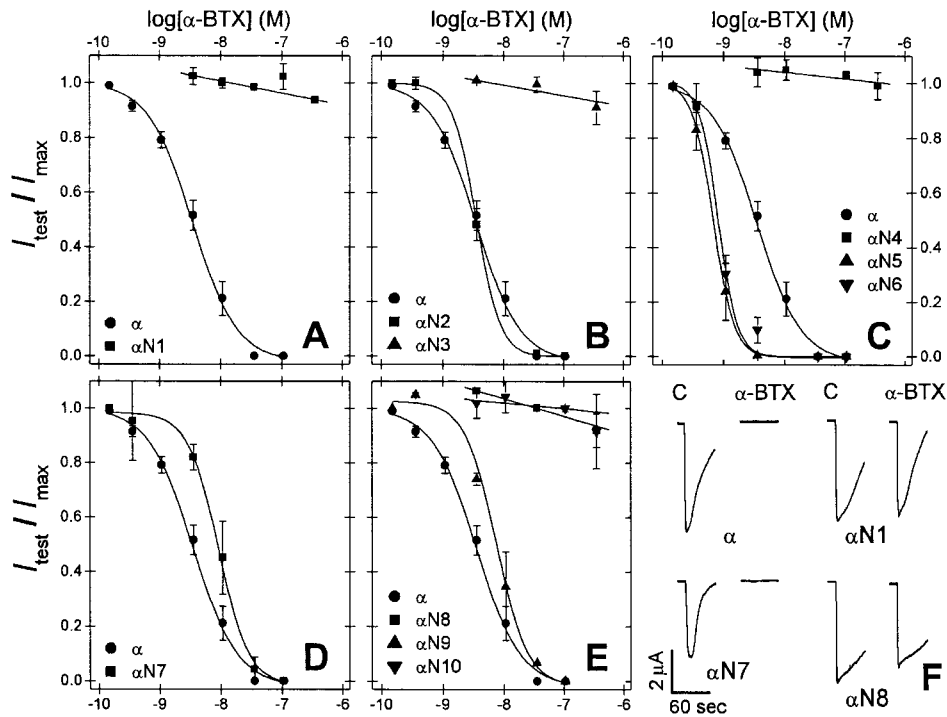


FIG. 4.—Effect of α -BTX on the ACh-induced whole-cell currents on nAChR containing the *Naja haje* ligand binding domain and its mutated derivatives. Shown are normalized α -BTX dose-response relationships of (A) αN1 ; (B) αN2 and αN3 ; (C) αN4 , αN5 , and αN6 ; (D) αN7 ; and (E) αN8 , αN9 , and αN10 . The dose-response relationship of the *Mus musculus* wild-type α is also shown for comparison (A–E). F, Representative ACh-induced whole-cell currents of the nAChRs before (C) and after (α -BTX) superfusion with 3.6×10^{-8} M (α , αN7) or 3.6×10^{-7} M (αN1 , αN8) α -BTX.

backbone per se. While such conservation of the α -BTX binding site in Elapidae snakes that produce lethal α -neurotoxins is remarkable, it is not unexpected. The function of α -neurotoxins is to inhibit the nAChR in a phylogenetically wide spectrum of species. Therefore, residues forming the target site for α -BTX on the nAChR must be highly conserved in most lineages of Chordata and, consequently, likely to be essential for receptor structure and/or function in those species, including the Elapidae snakes. The requirement of the α -neurotoxin target site for nAChR physiology is probably explained by its overlap with or proximity to the binding

site for the natural transmitter ACh (and possibly other domains critical for receptor physiology). Within the α -BTX binding segment E¹⁷²-F²⁰⁵, there are several residues that are major determinants for ACh binding (Karlin 1993) and all of those residues are conserved among different taxa of Chordata. Evolutionary pressure to conserve the ACh binding site (and possibly other domains critical for receptor physiology) in Elapidae snakes, however, did not permit significant enough structural alteration in this segment of the nAChR polypeptide backbone, likely a major factor contributing to the conservation of the neurotoxin binding site.

The fact that the *N. haje* ligand binding domain is inhibited by α -BTX when the *N*-glycosylation signal is eliminated also calls for a revision of the current model of the α -BTX binding site. On the Chordata nAChR α subunit, residues W¹⁸⁷ (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988; Fuchs et al. 1993; Spura et al. 1999, 2000), Y¹⁸⁹ (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988; Conti-Tronconi et al. 1991; McLane et al. 1991; Ohana et al. 1991; Chaturvedi, Donnelly-Roberts, and Lentz 1992, 1993; McCormick et al. 1993; McLane, Wu, and Conti-Tronconi 1994; Levandoski et al. 1999) and other aromatic residues at positions 187 and/or 189 (Neumann et al. 1986a; Fuchs et al. 1993; Barchan et al. 1995; Kachalsky et al. 1995; Balass, Katchalski-Katzir, and Fuchs 1997; Spura et al. 1999, 2000), T¹⁸⁹ (McCormick et al. 1993), adjacent aromatic residues at po-

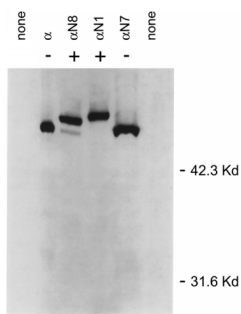


FIG. 5.—nAChR α subunits carrying the consensus sequence for *N*-glycosylation exhibit slower electrophoretic mobility compared with α subunits lacking this signal. Shown is Western blot analysis of membrane proteins expressed in *Xenopus* oocytes after injection with water (none), or with cRNAs encoding the α , αN8 , αN1 , and αN7 nAChRs. The presence of the *N*-glycosylation consensus sequence is indicated by a plus sign, and its absence by a minus sign.

sitions 189 and 190 (Conti-Tronconi et al. 1991; Chaturvedi, Donnelly-Roberts, and Lentz 1993; McLane, Wu, and Conti-Tronconi 1994; Balass, Katchalski-Katzir, and Fuchs 1997), T¹⁹¹ (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988), and P¹⁹⁴ alone (Conti-Tronconi et al. 1991; Ohana et al. 1991; Chaturvedi, Donnelly-Roberts, and Lentz 1992, 1993; Fuchs et al. 1993; McCormick et al. 1993; Spura et al. 1999) or in parallel with P¹⁹⁷ (Ohana and Gershoni 1990; McLane, Wu, and Conti-Tronconi 1994; Kachalsky et al. 1995) have been identified by others as being required for α -BTX binding based on synthetic peptides, bacterial fusion proteins, screening of phage-epitope libraries, and binding assays with cysteine-substituted mutants. However, we show here that the ligand binding domain of the *N. haje* nAChR lacks all of these residues or residue patterns but is still inhibited by α -BTX once the *N*-glycosylation signal is removed, indicating that these elements are not specifically required for α -BTX action. The discrepancies between these earlier findings by others and the present study are likely due to the lack of conformational forces (e.g., disulfide bridges, intersubunit contacts, effects of plasma membrane environment) in small peptides that are needed for proper protein folding in vivo, the presence of exogenous protein sequences, the lack of posttranslational modification, and the inability of in vitro binding assays to represent physiological receptor-ligand interaction. Furthermore, point mutations introduced into a short peptide sequence may have a more drastic effect than would be seen in the native protein that is subject to a large number of conformational constraints. In contrast, nAChRs functionally expressed in *Xenopus* oocytes exhibiting normal responses to ACh are not subject to such conformational limitations and should model native receptor characteristics with much higher fidelity. Consistent with our results, studies on α -BTX binding to nAChRs expressed in mammalian tissue cultures (Kreienkamp et al. 1994; Keller et al. 1995; Ackermann and Taylor 1997) or in *Xenopus* oocytes (Tomaselli et al. 1991) provided no support for the requirements of some of the residues identified by the various in vitro peptide or protein studies (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988; Ohana and Gershoni 1990; Conti-Tronconi et al. 1991; McLane et al. 1991; Ohana et al. 1991; Chaturvedi, Donnelly-Roberts, and Lentz 1992, 1993; Fuchs et al. 1993; McCormick et al. 1993; McLane, Wu, and Conti-Tronconi 1994; Barchan et al. 1995; Kachalsky et al. 1995; Balass, Katchalski-Katzir, and Fuchs 1997). Rather, V¹⁸⁸, Y¹⁹⁰, P¹⁹⁷, and D²⁰⁰ were pointed out as binding determinants for another α -neurotoxin, *Naja mossambica* Nmml, to *M. musculus* nAChR (Ackermann and Taylor 1997). Since these residues are also present in the *N. haje* ligand binding domain, they further support the conservation of the α -neurotoxin binding site in Elapidae snakes.

The pharmacological data presented here, along with an analysis of Chordata nAChR protein sequences, provide evidence for a striking example of convergent

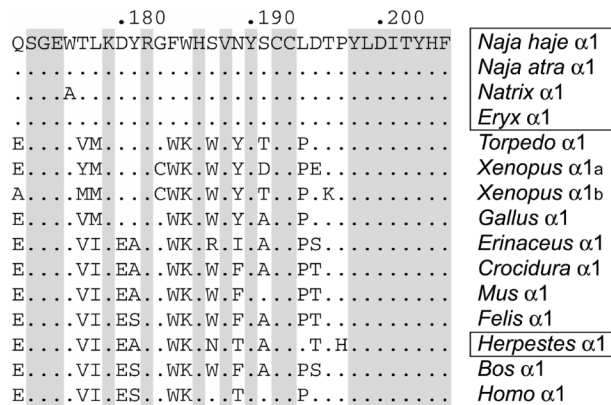


FIG. 6.—Maximum-homology alignment of the protein sequences of nAChR ligand binding domains from various Chordata species. Boxed species are resistant to α -neurotoxins and contain an *N*-glycosylation signal in the ligand binding domain. Sequences are aligned in comparison with *Naja haje*. Dots indicate residues identical to those of *N. haje*; shaded residues are conserved in Chordata muscle-type nAChR α subunits.

evolution at the molecular level (fig. 6). The diet of the mongooses (*Herpestes* spp., Viverridae, Carnivora, Mammalia) includes cobras (*Naja* spp.), and they are the only nonreptilian Chordata species that are known to be naturally resistant to Elapidae venoms (Ovadia and Kochva 1997). The nAChR ligand binding domain of *Herpestes ichneumon* does in fact contain an *N*-glycosylation signal (Barchan et al. 1992), only two residues N-terminal (N¹⁸⁷-X¹⁸⁸-T¹⁸⁹) from the position in which it is present in *N. haje*. The taxonomically closest species examined, the domestic cat (*Felis catus*, Felidae), another member of Carnivora (Barchan et al. 1995), as well as all other Chordata except advanced Squamata (Anguimorpha lizards and snakes, Reptilia), are α -BTX-sensitive (Burden, Hartzell, and Yoshikami 1975; Liu, Xu, and Hsu 1990; Endo and Tamiya 1991) and they all lack such a glycosylation signal in the ligand binding domain of the nAChR.

Based on the present work and theoretical considerations, we propose that the modified receptor structure resistance mechanism has a widespread occurrence among poisonous and venomous animals. For example, neurotoxins ATX II from the sea anemone *Anemonia sulcata* and BmK I from the scorpion *Mesobuthus martensi* share a common binding site on the voltage-gated Na⁺ channel (Catterall et al. 1980). Both of these toxins remain without effect in serum-free preparations of *M. martensi* abdominal nerve fibers (Terakawa et al. 1989). Similarly, tetrodotoxin (TTX) is another blocker of certain voltage-gated Na⁺ channels (Narahashi, Moore, and Scott 1964) that occurs in numerous species of animals, including the puffer fish, Tetraodontidae spp., and newts, *Taricha* spp. and *Cynops* spp. TTX is distributed in various tissues of the host species (Yotsu, Iorizzi, and Yasumoto 1990), and conspecific serum-free nerve fibers (Kao and Fuhrman 1967), retinal neurons (Kaneko, Matsumoto, and Hanyu 1997), and muscles (Kidokoro, Grinnell, and Eaton 1974) are insensitive to its action. Both of these examples suggest a modified receptor site

for the toxins on the Na⁺ channel. The characters of the suggested structural modifications in these examples are unknown, but they can obviously occur by means other than *N*-glycosylation.

In summary, we demonstrated that the α -neurotoxin binding site on the nAChR of the Elapidae snakes is conserved. This genuine binding site is masked by an *N*-glycosylation signal that confers resistance against conspecific α -neurotoxin. The *N. haje* nAChR α subunit lacks residues W¹⁸⁷, T¹⁸⁹, Y¹⁸⁹, other aromatic residues at positions 187 and 189, adjacent aromatic residues at positions 189 and 190, T¹⁹¹, and P¹⁹⁴ alone or in parallel with P¹⁹⁷; therefore, these structural motifs are not specifically required for α -BTX binding. The approach described here can be used to identify the mechanism of resistance against conspecific venoms in other species and to characterize toxin-receptor coevolution.

Supplementary Material

The sequence reported in this paper has been deposited in the GenBank database (accession number AF077763).

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