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 polytope 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 α2βγδ (Karlin 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.
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 σγ and αδ 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 pharmaceutical 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 Asher 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 Asher 1976).

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-ATTGCCCTTTGATGAGCA-3' (sense) and 5'-AT-GATGACGTTGACAAATGAAGTAGAGTA-3' (antisense; PA), followed by a second amplification with primers PA and 5'-TGAAGCAAAAATCTGCAGTGAGCCTGG-3' (sense). The final product was digested with HincII and PsI 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, αN1, 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 αN2–αN10

Segments or point mutations were introduced into wild-type α (to construct αN2, αN4, αN6, and αN8), αN1 (to construct αN3 and αN7), or αN3 (to construct αN5, αN9, and αN10) 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 DraiIII-561, were used together with primers upstream of the Eagl-295 site, 5′-CTTGGAAAAATGGAATCCAGATGAC-3' (sense; for αN2, αN3), or downstream of the BstXI-1215 site, 5′-AAACACACAGCCAGGTCCCGATGAG-3' (antisense; for all other mutants), to PCR amplify the mutated segment from templates α (to construct αN3, αN8, and αN9), αN1 (to construct αN2 and αN7), αN3 (to construct αN4, αN5, and αN6), or αN8 (to construct αN10). The PCR products were double-digested with DraiIII and EaglI or with DraiIII and BstXI restriction enzymes, gel-purified, and ligated into α, αN1, or αN3 at the homologous position. All mutations were confirmed by sequencing both strands.

Expression of nAChR in Xenopus Oocytes

nAChR subunit α (BamHI), αN1–αN10 (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

Snake α-Neurotoxin Binding Site Is Conserved in Cobra 1801

Materials and Methods

Cloning the N. haje Ligand Binding Domain and Construction of αN1

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.
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 2α : β : γ : δ (wild-type M. musculus) or 2αN1–αN10 : β : γ : δ (chimeric Naja/Mus αN1 and mutant αN2–αN10 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 MΩ 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 mM ATP, 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 × 10⁻⁵ M of ACh for 10 s, and peak current amplitude (Iₘₐₓ) 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 × 10⁻⁷ M to 2 × 10⁻⁵ M, for 10 s, and peak current amplitude (Iₘₐₓ) was recorded as the maximum change of holding current. At the end of each protocol, the application of 4 × 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 = Iₘₐₓ/[1 + (Kₘᵢₚ/[ACh])ⁿ]. Each data point displayed along the curves is the mean of Iₜₑˢᵗ/Iₘₐₓ ± 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 (Iₘₐₓ). 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 × 10⁻¹⁰ M to 3.60 × 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 (Iₜₑˢᵗ). Iₜₑˢᵗ/Iₘₐₓ 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 Iₜₑˢᵗ/Iₘₐₓ 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 Iₜₑˢᵗ/Iₘₐₓ ± 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 αN8 nAChR (single residue introduced into wild type, M. musculus α-BTX-sensitive nAChR) at the highest α-BTX concentration (3.6 × 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 μl/oocyte) in ice-cold 50 mM Na₂HPO₄/NaH₂PO₄ (pH 7.5), 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 5 μg/ml soybean trypsin inhibitor, then centrifuged at 15,000 × g for 20 min at 4°C. The pellet was resuspended in Laemmli sample buffer (15 μl/oocyte), incubated at 60°C for 20 min, and centrifuged at 15,000 × g at 4°C for 20 min. Electrophoresis was performed after the supernatant was loaded onto a 12% SDS-polyacrylamide gel. Kaleidascoppe 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),
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 αN1−αN10 (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 (αN1−αN10) 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

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**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).
Fig. 2.—Sequences of the mutated α subunits that were constructed to test the α-BTX binding site of *Naja haje* nAChR. A, Schematic representation of a chimeric nAChR α subunit (αN1) indicating the introduction of the *N. haje* 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. haje* (upper panel) and *M. musculus* (lower panel) ligand binding domains. A dot indicates residues identical to the top sequence of each panel, αN1 or α. Shaded residues are from *N. haje*, 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 IC50 of 3.50 × 10−9 M (fig. 4A and F). However, nAChR containing the full-length *N. haje* ligand binding domain T154-L208 (αN1) 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 (αN2 and αN3) demonstrated that the α-BTX resistance-mediating elements were inherent in *N. haje* segment S187-L208 (fig. 4B). Introduction of T154-H186 alone (αN2) did not alter the α-BTX sensitivity.

In contrast to most α-BTX-sensitive species, positions 187 and 189 of the *N. haje* 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 (αN4, αN5, and αN6) in which aromatic amino acid residues present only in α-BTX-sensitive species at positions 187 and 189 were introduced into *N. haje* segment S187-L208. The ACh-induced response of αN4 was resistant, while αN5 and αN6 were sensitive to the inhibitory action of α-BTX, with IC50 = 0.67 × 10−9 M and IC50 = 0.81 × 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-glycosylation 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. haje* ligand binding domain T154-L208 was mutated to alanine (αN7), 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 αN7 when compared with αN1 that had the intact *N. haje* ligand binding domain (fig. 5). This single point mutation of the *N. haje* ligand binding domain eliminated the resistance to α-BTX, yielding an IC50 of 9.11 × 10−9 M, comparable with the wild-type *M. musculus* IC50 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 αN7, including S187, N189, L194, and the absence of the parallel P194 and P197 pattern.

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

Single-residue mutations presented above were introduced into the *N. haje* sequence where they had no
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 IC₅₀ 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 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 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

Table 1

<table>
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<tr>
<th>nAChR</th>
<th>ACh, Mean Iₘ₅₀ ± SD (µA)</th>
<th>ACh, E₅₀ (×10⁻⁶ M)</th>
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<td>αN1</td>
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<td>αN3</td>
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<td>αN4</td>
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</tr>
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<td>αN10</td>
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Note.—Resistant = <10% inhibition by 3.6 × 10⁻⁷ M 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 (Mc-Cormick et al. 1993), adjacent aromatic residues at po-
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), T^{191} (Mulac-Jericevic and Atassi 1986; Neumann et al. 1986a; Gotti et al. 1988; Mulac-Jericevic et al. 1988), and P^{192} 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 P^{197} (Ohana and Gershoni 1990; McLane, Wu, and Conti-Tronconi 1994; Kachalsky et al. 1994) 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 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 1993; Fuchs et al. 1993; McCormick et al. 1993; Barchan et al. 1995; Kachalsky et al. 1995; Balass, Katchalski-Katzir, and Fuchs 1997). Rather, Y^{188}, Y^{190}, P^{197}, and D^{200} were pointed out as binding determinants for another α-neurotoxin, Naja mossambica Nnml, 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 evolution at the molecular level (fig. 6). The diet of the mongoose (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 (N^{187, X^{188}, T^{189}}) 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 Hsua 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-neurotoxins. The N. haje nAChR α subunit lacks residues W187, T189, Y189, other aromatic residues at positions 187 and 189, and 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).

Acknowledgments

We thank Arthur Karlin (Columbia University), Robert S. Wilkinson (Washington University), Jon M. Lindstrom, and Rene Anand (University of Pennsylvania) for their suggestions and technical comments during the study, and we thank Arthur Karlin for his remarks on the manuscript.

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WILLIAM TAYLOR, reviewing editor

Accepted June 1, 2001