The Binding Site of the Nicotinic Acetylcholine Receptor in Animal Species Resistant to α-Bungarotoxin[†,‡]

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ABSTRACT: The ligand binding site of the nicotinic acetylcholine receptor (AChR) is located in the α-subunit, within a small fragment containing the tandem cysteines at positions 192 and 193. We have been analyzing the binding site domain of AChRs from several animal species exhibiting various degrees of resistance to α-bungarotoxin (α-BTX). Our earlier work on the snake and mongoose AChR, both of which do not bind α-BTX, suggested that amino acid substitutions at positions 187, 189, and 194 of the AChR α-subunit are important in determining the resistance of these AChRs to α-BTX. In the present study, we have examined the correlation between α-BTX binding and the structure of the binding site domain of AChR from the hedgehog, shrew, cat, and human. Fragments of the AChR α-subunit corresponding to residues 122−205 from these species were cloned, sequenced, and expressed in *Escherichia coli*. The hedgehog fragment does not bind α-BTX, in common with the snake and mongoose AChR, and the human fragment is a partial binder. The shrew and cat fragments bind α-BTX to a similar extent as the mouse fragment. The hedgehog and human AChRs have nonaromatic amino acid residues at positions 187 and 189 of the α-subunit, as is seen with the "toxin resistant" snake and mongoose, and in contrast with the "toxin binders", which have aromatic residues at these two positions. Thus, it appears that aromatic amino acid residues at positions 187 and 189 of the AChR α-subunit are required for proper α-BTX binding, and that changes at these positions to nonaromatic residues are important in determining resistance to α-BTX.

The nicotinic acetylcholine receptor (AChR)[1] is a multisubunit glycoprotein composed of four types of subunits in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ (Karlin, 1980; Changeux, 1990). The structure of the binding site of AChR for cholinergic ligands and for α-neurotoxins, and its localization within the molecule, has been an attractive question for a number of years. The cholinergic binding site of the receptor was shown to be located in the α-subunit, in a portion of its extracellular domain, within a region containing the tandem cysteines at residues 192 and 193 (Haggerty & Froehner, 1981; Kao et al., 1984; Neumann et al., 1985, 1986a,b). Several experimental approaches have been employed to identify the ligand binding site in AChR and the amino acid residues that participate in it. These included proteolytic fragmentation of the α-subunit, affinity labeling experiments, synthetic peptides, genetic constructs, and site-directed mutagenesis [reviewed in Barchan et al. (1992)]. All these studies have localized the ligand binding site in close proximity or contiguous to the two tandem cysteine residues 192 and 193, which are unique to the α-subunit and are present at this position in all α-subunits of muscle and neuronal AChRs thus far sequenced. Some of these studies have also demonstrated that other regions in the extracellular domains of the α-subunit, and possibly in other subunits as well, may be in close contact with the ligand. We have demonstrated previously that a synthetic dodecapeptide corresponding to residues 185−196 of the *Torpedo* and mouse AChR α-subunit contains the essential elements for α-bungarotoxin (α-BTX) binding (Neumann et al., 1986a,b).

To further analyze the binding site of AChR, we have chosen to study nonconventional muscle AChRs from animal species which are resistant to α-neurotoxins. From our studies on the binding site domains of AChR from the snake (Neumann et al., 1989) and the mongoose (Barchan et al., 1992), both of which were shown to be resistant to α-BTX, we have concluded that this resistance is governed mainly by amino acid substitutions within the binding site region, in the vicinity of cysteines 192 and 193. Amino acid substitutions at three positions are common to snake and mongoose AChR; residues 187 and 189, which are aromatic in animal species that are sensitive to α-BTX, are nonaromatic in both snakes and mongoose (as well as in the human AChR), and residue 194, which is a proline in α-BTX binders, is a leucine in snakes and in the mongoose. In addition, both snake and mongoose AChRs have a putative glycosylation site in the ligand binding site (Asn 189 and 187 in snake and mongoose, respectively).

We have now extended these studies to additional animal species: the hedgehog (*Erinaceus europeus*) and shrew (*Crocidura russula*), which belong to the Order Insectivora (a group of primitive mammals) and were suspected to be

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[‡] The nucleotide sequences for shrew, cat, and hedgehog have been submitted to GenBank under Accession Numbers U17006, U17007, and U17008, respectively.

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[1] Abbreviations: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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Table 1: Lethality of α-BTX in Various Animals Species

species	dose (μg of α-BTX/ g of body weight)	death (min after injection)
snake	100	survived (Neumann et al., 1989)
	10	survived
mongoose	2	survived (Barchan et al., 1992)
hedgehog	0.2	survived
	0.4	survived
	1.2	30
shrew	0.1	survived
	0.2	survived
	0.4	120
	0.8	60
cat	0.2	90
mouse	0.1	10

resistant or partially resistant to α-BTX. The hedgehog was previously reported to exhibit some resistance to snake venoms (Domont et al., 1991). We have also tested the cat (*Felis domestica*) which, like the mongoose, belongs to the Order Carnivora. The domains of the AChR α-subunit (corresponding to amino acid residues 122−205) of these species, as well as of the human AChR, were cloned, sequenced, and expressed in bacteria. We report here that the hedgehog fragment does not bind α-BTX and the human fragment is a partial binder. Both species have substitutions at positions 187 and 189 of their AChR α-subunit, from aromatic to nonaromatic residues, as previously shown by us for the snake and mongoose, confirming the role of these positions in determining resistance to α-BTX.

EXPERIMENTAL PROCEDURES

Animals. Hedgehogs (*Erinaceus europeus*) and shrews (*Crocidura russula*) were obtained from The I. Meyer Segals Gardens for Zoological Research (Tel Aviv University). Cats and mice were from the Center of Animal Breeding (The Weizmann Institute of Science).

Muscle Extracts and α-BTX Binding. Muscle AChR preparations were made essentially by extraction in Triton X-100 as described (Souroujon et al., 1985), and ^{125}I-α-BTX binding to muscle Triton extracts was performed according to Aharonov et al. (1977).

Cloning, Expression, and Analysis of cDNA Fragments. RNA preparations were made as described (Asher et al., 1988); hedgehog, shrew, and cat poly(A+) RNA was prepared from muscle tissue; total human RNA was prepared from the TE671 cell line, which expresses muscle type nicotinic AChR (Schoepfer et al., 1988). The preparation of cDNA and the polymerase chain reaction (PCR) were performed as described (Barchan et al., 1992). The primers employed to amplify cDNA fragments corresponding to residues 122−205 of the AChR α-subunit from mouse, hedgehog, shrew, cat, and human were described previously (Barchan et al., 1992). The obtained PCR fragments were subcloned into *Nco*I and *Bam*HI sites of the expression vector pET8C (Studier & Moffatt, 1986) for sequence analysis and expression. The expressed proteins were analyzed by electrophoresis in a SDS/polyacrylamide gel, followed by blotting and ^{125}I-α-BTX overlay, as described (Neumann et al., 1986).

RESULTS

α-BTX Resistance and Binding to Muscle AChR. We have tested the resistance to α-BTX by examining the toxic effect of intramuscular administration of α-BTX in hedgehog, shrew, and cat, in comparison with the effect in mouse, snake, and mongoose (Table 1). A resistance to α-BTX was observed in the hedgehog, in comparison with the mouse. It survived 0.4 μg of α-BTX/g of body weight, which is 4 times the dose (0.1 μg) that killed the mice within 10 min of α-BTX administration. Some marginal resistance was observed in the shrew, which survived at least twice the amount of α-BTX that was lethal in the mouse. It should be noted, however, that these resistances are much lower than those observed in the snake (Neumann et al., 1989) and mongoose (Barchan et al., 1992). In the cat, α-BTX appeared to be toxic to an extent similar to that in the mouse. The increased resistance to α-BTX of the hedgehog and shrew was not due to neutralizing factors in their blood serum, since preincubation of their sera with α-BTX did not neutralize its toxic effect upon subsequent injection into mice.

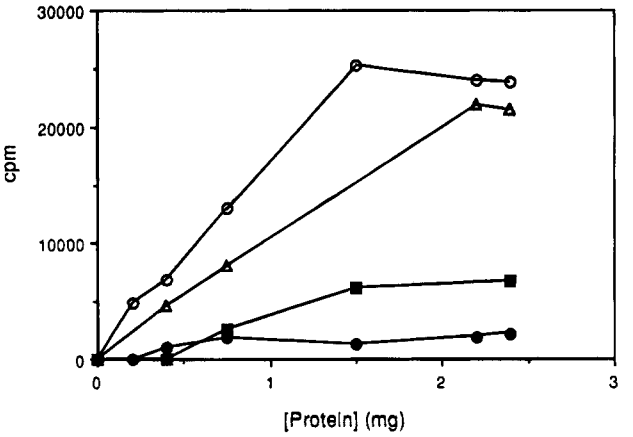

FIGURE 1: ^{125}I-α-BTX binding to muscle Triton extracts from mouse (○), shrew (△), hedgehog (■), and mongoose (●).

Analysis of the binding of ^{125}I-α-BTX to muscle Triton extracts from mouse, cat, shrew, and hedgehog showed just marginal levels of binding of the hedgehog AChR to α-BTX (Figure 1), whereas muscle extracts of the shrew and cat (data not shown) exhibited specific binding in a concentration-dependent manner.

Sequence Analysis of the Binding Site Domain of the Hedgehog, Shrew, and Cat AChR. We have cloned and sequenced the cat, shrew, and hedgehog cDNA fragments corresponding to residues 122−205 of the AChR α-subunit using PCR. These sequences have been deposited to GenBank. Sequence analysis of these fragments (Figure 2A) revealed a high homology with the respective mouse fragment (92%, 89%, and 91% for hedgehog, shrew, and cat, respectively). Most differences observed are at the third position of the codons, thus not affecting the deduced amino acids. The amino acid sequences, deduced from the nucleotide sequences (Figure 2B), revealed a homology of 94%, 94%, and 98% for the hedgehog, shrew, and cat fragments, respectively, with that of the mouse fragment. The hedgehog fragment has two nonconservative amino acid substitutions at positions 187 and 189, from aromatic (tryptophan and tyrosine) to nonaromatic residues (arginine and isoleucine). These are the positions that are also substituted in the snake, mongoose, and human AChR. In the shrew fragment, there are nonconservative changes, from the mouse sequence, at positions 155, 160, and 164 (Figure 2B).

α-BTX Binding of the Hedgehog, Shrew, Cat, and Human AChR Expressed Fragments. The cloned hedgehog, shrew,

A

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          364                                                                             426
Mouse     GCC ATC TTT AAA AGC TAC TGT GAG ATC ATT GTC ACT CAC TTT CCC TTC GAT GAG CAG AAC TGC
Cat       ... ... ..C ... ... ... ... ... ... ..C ... ..C ... ... ... ... ... ..A ... ... ...
Shrew     ... ... ... ... ... ... ... ... ... ... ... ..C ..T ... ... ..T ..C ..A ... ... ...
Hedgehog  ... ... ..C ... ... ... ... ... ... ..C ... ..C ... ... ..G ..T ... ..A ... ... ...

                                                                                          489
Mouse     AGC ATG AAG CTG GGC ACC TGG ACC TAT GAC GGC TCT GTG GTG GCC ATT AAC CCG GAA AGT GAC
Cat       ... ... ... ... ..T ... ... ... ..C ..T ... ..C ... ... ... ..C ... ... ... ..C ...
Shrew     ... ... ... ... ... ... ... ... ... ..T ... ..G AA. ... ... ..C ..T G.G ..G ... ..G
Hedgehog  ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C ... ... ... .A. ...

                                                                                          552
Mouse     CAG CCC GAC CTG AGT AAC TTC ATG GAG AGC GGG GAG TGG GTG ATC AAG GAA GCT CGG GGC TGG
Cat       ..A ..A ... ..A ..C ... ... ... ..A ... ..A ..A ... ... ... ... ..G T.. ..A ... ...
Shrew     ..C ..G ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ..G ..C ... ..T ...
Hedgehog  ... ..G ... ... ..C ... ... ... ... ..T ... ... ... ..C ... ... ..G ..C ... ... ...

                                                                                          615
Mouse     AAG CAC TGG GTG TTC TAC TCC TGC TGC CCC ACC ACT CCC TAC CTG GAC ATC ACC TAC CAC TTC
Cat       ... ... ... ... ... ... G.. ... ... ... ... ..C ... ... ... ..T ... ... ... ... ...
Shrew     ... ... ... ... ... ..T G.. ... ... ... ... ..C ... ... T.. ... ... ... ..T ... ..T
Hedgehog  ... ... C.. ... A.. ..T G.. ... ... ... T.. ... ... ... ... ... ... ... ... ... ...
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B

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           122               140                 160                 180                 200
           .     *            . *                  .                   .          **       .
Mouse      AIFKSYCEIIVTHFPFDEQNCSMKLGTWTYDGSVVAINPESDQPDLSNFMESGEWVIKEARGWKHWVFYSCCPTTPYLDITYHF
Cat        ..........................................................S..........A..............
Shrew      .................................K....A..EH..........................A..............
Hedgehog   ........................................N........................R.I.A...S..........
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FIGURE 2: (A) Alignment of nucleotide sequences for the cat, shrew, and hedgehog PCR fragment of the AChR α-subunit, and the corresponding mouse fragment. Nucleotides are numbered from 364 to 615. Nucleotides identical to the mouse sequence are designated by dots. (B) Deduced amino acid sequences for the cat, shrew, and hedgehog fragments of the AChR α-subunit, in comparison to the mouse fragment (residues 122−205). Cysteine residues are marked with an asterisk. Amino acid residues identical to the mouse sequence are designated by dots.

and cat PCR fragments, corresponding to amino acid residues 122−205 of the α-subunit, were expressed in *E. coli.* The expressed protein fragments were localized in the insoluble pellet, in inclusion bodies. These expressed fragments have the expected molecular mass of 8 kDa in an SDS/polyacrylamide gel and constitute the major protein in the pellet (Figure 3A). Overlay of the blotted proteins with ^{125}I-α-BTX showed that, as with the mongoose fragment, the hedgehog fragment hardly bound α-BTX. On the other hand, the shrew and cat fragments both bound α-BTX to the same extent as the mouse fragment (Figure 3B).

We and others have previously shown that synthetic peptides from the binding site domain of the human AChR α-subunit bind α-BTX to a lower extent than the respective peptides from the mouse or *Torpedo* AChR (Neumann et al., 1986b; Wilson & Lentz, 1988). This may correlate with the fact that human AChR also has two nonaromatic residues at positions 187 and 189, in common with the snake, mongoose, and hedgehog AChR. It was therefore interesting to analyze, in addition to the protein fragments of the abovementioned species, the homologous fragment from human AChR. This fragment was amplified by RT-PCR from cDNA which has been prepared from RNA isolated from the human TE671 cell line. Overlay of three different amounts of the blotted human AChR fragment with ^{125}I-α-BTX indicated that it is an intermediate binder and binds less than the mouse and more than the mongoose and hedgehog respective fragments (Figure 4).

DISCUSSION

In an attempt to analyze in detail the structure of the ligand binding site of AChR and to elucidate the structural requirements for agonist *versus* α-BTX binding, we chose to study the AChR binding site of animals that are resistant to α-neurotoxins. For the various tested animals, we have examined (i) resistance to α-BTX in the intact animal, (ii) binding of α-BTX to muscle AChR, and (iii) amino acid sequence in the binding site domain. These studies indicate that there is a correlation between resistance to α-BTX and amino acid sequence at the binding site of AChR. Our earlier work on the snake and mongoose AChRs, both of which do not bind α-BTX (Neumann et al., 1989; Barchan et al., 1992), suggested that amino acid substitutions at positions 187, 189, and 194 of the AChR α-subunit are important in determining the resistance of these AChRs to α-BTX. In the present study, we have examined the correlation between α-BTX binding and the structure of the binding site domain of AChR, from additional animal species. We have demonstrated that an AChR α-subunit fragment (corresponding to amino acid residues 122−205) from the hedgehog, which is phylogenetically among the most primitive mammals (insectivores), does not bind α-BTX, in common with the snake

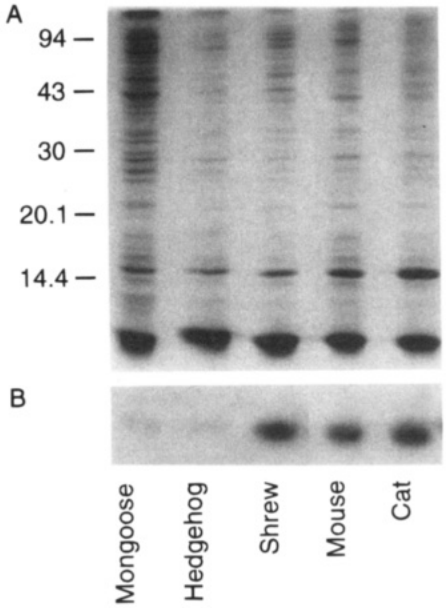

FIGURE 3: α-BTX binding to expressed fragments. The expressed protein fragments (20 μg of protein) of mongoose, hedgehog, shrew, mouse, and cat AChR α-subunit were resolved by polyacrylamide gel electrophoresis (15% gel). The gel was stained for proteins by Coomassie brilliant blue (A) or blotted and overlaid with ^{125}I-α-BTX (B).

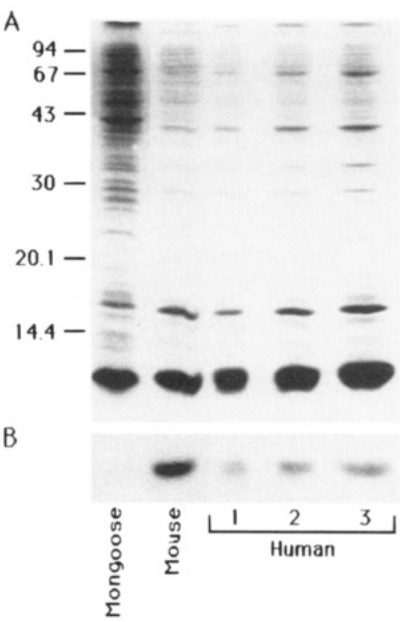

FIGURE 4: α-BTX binding to expressed human fragments. Samples of 10 μg, 20 μg, and 40 μg of human protein fragment in lanes 1, 2, and 3, respectively, were resolved by polyacrylamide gel electrophoresis. The gel was stained for proteins (A) or blotted and overlaid with ^{125}I-α-BTX (B).

and mongoose AChR, and that the human fragment is a partial binder. Comparison of the deduced amino acid sequences of the mouse α-subunit fragment (residues 122–205) with those of the hedgehog and human indicates that differences occur mainly in the proximity of cysteines 192 and 193 and that both species have nonaromatic amino acid residues at positions 187 and 189, in contrast with the "toxin-binders", which have aromatic residues at these two positions. Taken together with our earlier studies, it now appears that positions 187 and 189 represent a hot spot or target for evolutionary modifications, resulting in a functional change, namely, sensitivity to α-BTX. It should be noted that the

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          180      187 189   194 197  200
          .         | |   **|  |    .
Mouse     EARGWKHWVFYSCCPTTPYLD
Cat       .S.........A.........
Calf      ...........A...S.....
Shrew     ...........A.........
Chick     DY.......Y.A...D.....
Torpedo   DY.......Y.T...D.....
Human     .S.....S.T.S...D.....
Hedgehog  .......R.I.A...S.....
Mongoose  .......N.T.A..L..H...
Snake     DY..FW.S.N.S..LD.....
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FIGURE 5: Interspecies comparison of amino acid residues 180–200 of AChR α-subunit.

α-subunit fragment of the shrew, another member of the Order Insectivora, binds α-BTX like the other toxin-binders. There are no amino acid differences in the shrew fragment, in proximity of cysteines 192 and 193, but there are four changes in the region of residues 155–164 (Figure 2B). It is possible that changes in this latter region might contribute to the somewhat lower level of α-BTX binding to Triton extracted and to the partial resistance to α-BTX in the whole animal.

Alignment of amino acid residues 180–200 of the AChR α-subunit from several species is depicted in Figure 5. Muscle AChRs from species which are sensitive to α-BTX all have a tryptophan at position 187 and an aromatic residue (phenylalanine or tyrosine) at position 189. These two positions are replaced together by nonaromatic amino acid residues in the snake, mongoose, hedgehog, and human AChR, although in each of these species the changes are different. We have not found any case of a muscle AChR in which only one position (187 or 189) has been changed. It should be noted that in the neuronal α-BTX binding protein there is an aromatic residue at the position corresponding to 189 in the muscle AChR α-subunit, but a glutamic acid at position 187 (Schoepfer et al., 1990; Couturier et al., 1990). It is possible that this difference may contribute to the lower affinity of this neuronal AChR to α-BTX, than that of muscle AChR (Keyser et al., 1993).

There appear to be several means by which different degrees of resistance to α-BTX have developed during evolution, in taxonomically different groups of animals. Experiments of α-BTX administration have demonstrated that the resistance of the snake and the mongoose to α-BTX is much higher than that of the hedgehog. The nature of the amino acid substituted at positions 187 and 189 is different in the various resistant animals. The additional changes at prolines 194 (snake and mongoose) and 197 (mongoose), and the putative glycosylation in both the snake (Asn 189) and mongoose (Asn 187) AChR, probably add to the resistance of these species to α-neurotoxins. On the level of the expressed α-subunit fragments (122–205 residues), the snake, mongoose, and hedgehog fragments do not bind α-BTX, and the human fragment binds less than the mouse fragment. It should be noted that the binding experiments of ^{125}I-α-BTX to the fragments are performed on protein blots and it is quite likely that under these conditions the fragments do not fully assume their conformation as in the intact receptor. This may explain some of the differences in binding observed between the fragments, and intact AChR (as measured in Triton extracts or α-BTX administration). In addition, changes in other regions of the α-subunit or in other subunits may also contribute to the overall structure of the binding site.

The investigation of AChRs from animals that are resistant to α-neurotoxins, for the elucidation of structural elements which determine toxin sensitivity, should be preferred as a first approach over studies dealing with random mutagenesis. Our strategy made it possible to pinpoint the critical positions of functional importance, created by "nature" during the evolutionary development of toxin resistance. With this information in hand, one can now carry out logically plausible point mutations at a limited number of specific positions, in order to elucidate the relative contribution of each of them to biological function.

In conclusion, it appears that aromatic residues at positions 187 and 189 of the muscle AChR α-subunit are essential for proper α-BTX binding. Changes at these positions to nonaromatic residues are most important in determining resistance to α-BTX, though the nature of these changes, as well as additional structural elements, probably contributes to the degree of this resistance.

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