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How the mongoose can fight the snake: The binding site of the mongoose acetylcholine receptor

(ligand binding/α-bungarotoxin/polymerase chain reaction/immunofluorescence microscopy)

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ABSTRACT **The ligand binding site of the nicotinic acetylcholine receptor (AcChoR) is within a short peptide from the α subunit that includes the tandem cysteine residues at positions 192 and 193. To elucidate the molecular basis of the binding properties of the AcChoR, we chose to study nonclassical muscle AcChoRs from animals that are resistant to α-neurotoxins. We have previously reported that the resistance of snake AcChoR to α-bungarotoxin (α-BTX) may be accounted for by several major substitutions in the ligand binding site of the receptor. In the present study, we have analyzed the binding site of AcChoR from the mongoose, which is also resistant to α-neurotoxins. It was shown that mongoose AcChoR does not bind α-BTX *in vivo* or *in vitro*. cDNA fragments of the α subunit of mongoose AcChoR corresponding to codons 122–205 and including the presumed ligand binding site were cloned, sequenced, and expressed in *Escherichia coli*. The expressed protein fragments of the mongoose, as well as of snake receptors, do not bind α-BTX. The mongoose fragment is highly homologous (>90%) to the respective mouse fragment. Out of the seven amino acid differences between the mongoose and mouse in this region, five cluster in the presumed ligand binding site, close to cysteines 192 and 193. These changes are at positions 187 (Trp → Asn), 189 (Phe → Thr), 191 (Ser → Ala), 194 (Pro → Leu), and 197 (Pro → His). The mongoose like the snake AcChoR has a potential glycosylation site in the binding site domain. Sequence comparison between species suggests that substitutions at positions 187, 189, and 194 are important in determining the resistance of mongoose and snake AcChoR to α-BTX. In addition, it was shown that amino acid residues that had been reported to be necessary for acetylcholine binding are conserved in the toxin-resistant animals as well.**

The nicotinic acetylcholine receptor (AcChoR) is an integral membrane glycoprotein composed of four types of subunits present in a stoichiometry of $\alpha_2\beta\gamma\delta$ (for review, see refs. 1 and 2). The cholinergic binding site of the receptor is within the α subunit (1, 3, 4) in close proximity to a sulfhydryl group (1). A number of experimental approaches have been employed to identify the ligand binding site in AcChoR and the amino acids that participate in it. Studies based on proteolytic fragmentation of the α subunit (5–7), affinity-labeling experiments (8, 9), synthetic peptides (6, 7, 10), genetic constructs (11, 12), and site-directed mutagenesis (13) indicated that the ligand binding site of AcChoR is within a region of the α subunit that contains the two tandem cysteine residues at positions 192 and 193. We demonstrated that a synthetic dodecapeptide corresponding to amino acid residues 185–196 of the *Torpedo* AcChoR α subunit contains the essential elements of the ligand binding site (7, 10).

To analyze the detailed structure of the cholinergic binding site of AcChoR and to elucidate the structural requirements for agonist vs. α-bungarotoxin (α-BTX) binding, we studied nonconventional muscle AcChoRs of animals that are resistant to α-BTX. AcChoR of elapid snakes is unique in its pharmacological properties; it binds cholinergic ligands but, unlike other muscle AcChoRs, it does not bind α-BTX (14). We have cloned and sequenced (15) cDNA fragments that contain the presumed ligand binding site in the AcChoR α subunit from two different snakes. We demonstrated that in the binding site region, in the vicinity of cysteines 192 and 193, several major substitutions occur in the snake sequence at positions 184 (Trp → Phe), 185 (Lys → Trp), 187 (Trp → Ser), and 194 (Pro → Leu). In addition, Asn-189 is a putative N-glycosylation site, present only in the snake (15). These changes or part of them may explain the lack of α-BTX binding to snake AcChoR.

Some of the sequence differences observed in the snake AcChoR could be specific to the group. Therefore, we have extended our study to a mammal (mongoose) that is resistant to neurotoxins (16) and includes snakes in its diet. We have cloned and sequenced the region of the AcChoR α subunit (residues 122–205) that contains the binding site domain from the mongoose and from an additional primitive snake, the sand boa (*Eryx jaculus*).§ This region in the mongoose AcChoR is highly homologous (>90%) to the corresponding region in other mammalian AcChoRs. Nevertheless, there are five amino acid differences in the mongoose sequence that cluster in a very limited segment in the presumed binding site area. Sequence comparison of the binding site domains of the mongoose and snake AcChoR with those of other AcChoRs led us to propose that substitutions at positions 187, 189, and 194 of the receptor α subunit are important in conferring toxin resistance in these animals.

MATERIALS AND METHODS

Animals. Mongooses (*Herpestes ichneumon*) and snakes (the sand boa *Eryx jaculus* and the cobra *Naja naja atra*) were obtained from The Canadian Center of Ecological Zoology (Tel-Aviv University). Mice and rabbits were from the Center of Animal Breeding (The Weizmann Institute).

RNA Preparation and Northern Blot Analysis. RNA preparation and Northern blot analysis were performed as described (17).

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Abbreviations: AcCho, acetylcholine; AcChoE, acetylcholinesterase; AcChoR, acetylcholine receptor; α-BTX, α-bungarotoxin; FITC, fluorescein isothiocyanate; TMR, tetramethylrhodamine.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M93639).

Preparation and Amplification of cDNA. The preparation of cDNA and the polymerase chain reaction (PCR) were performed as described (15). Fragments were purified and subcloned into M13 bacteriophage vectors mp18 and mp19 or pBluescript KS− for sequencing and then into the pET8C vector for expression. The primer at the 5′ end (GGCCATG-GCCATCTTRAAAAGC, where R = C or T) corresponded to a highly conserved region of the α subunit (amino acid residues 122–126) and was designed in a way that enabled cloning into a pET8C-derived expression vector by adding a restriction site for *Nco* I (underlined) and an initiation codon (marked by asterisks). The primer at the 3′ end (CCGGATCCTCAAAAGTGRTAGGTGATRTC, where R = A or G) corresponded to the complementary sequence of another conserved region (amino acid residues 200–205), and contained a restriction site for *Bam*HI (underlined) and a stop codon (marked by asterisks).

Expression and Analyses of Cloned cDNA Fragments. The cloned cDNA fragments of the mongoose, snake, and mouse were subcloned into *Nco* I and *Bam*HI sites of the expression vector pET8C (18). Cloning sites were confirmed by DNA sequencing, and induction of protein expression was performed (18). After expression, the *Escherichia coli* suspension (400 ml) was centrifuged, cells were lysed by freezing and thawing the pellet and resuspended in phosphate-buffered saline (PBS, 20 ml). The resuspended material was sonicated for five 15-sec periods and kept frozen in aliquots until use. After centrifugation, the expressed protein was localized in the precipitate, probably in inclusion bodies. The proteins were analyzed by electrophoresis in SDS/polyacrylamide gel (15%), followed by blotting and toxin or antibody overlays as described (7).

Preparation of Antibodies. Antibodies to proteins expressed by the cloned cDNA fragments were elicited in rabbits by three immunizations with the homogenized gel band containing the 8-kDa protein fragment and originating from 0.5 ml of concentrated (20 times) cell suspension emulsified in complete Freund's adjuvant.

Immunofluorescence Microscopy. Diaphragms were dissected from Wistar rats and mongooses. Areas containing endplates were quickly frozen and 20-μm sections were incubated for 1 hr with purified anti-AcChoR antibodies in PBS containing 0.25% gelatin and 0.5% bovine serum albumin (PBS-GB). Sections were washed and incubated for 1 hr with a mixture containing 50 nM tetramethylrhodamine-conjugated α-BTX (TMR-α-BTX; ref. 19) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Hyland, Costa Mesa, CA) at 10 μg/ml. Slides were transferred to 70% ethanol at −20°C and mounted in glycerol/PBS Citifluor (Citifluor, London). Fluorescence photomicrographs were taken at exposures of 15–30 sec on Kodak T-Max 400 ASA film processed to ASA 800.

Acetylcholinesterase (AcChoE) was stained by the method of Karnovsky and Roots (20) or by the immunofluorescent technique using rabbit anti-*Torpedo* AcChoE serum (80b; kindly provided by Palmer Taylor, University of California, San Diego; ref. 21), followed by FITC-conjugated anti-rabbit antibody, as described above.

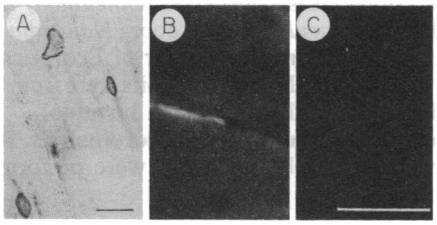

FIG. 1. Staining of AcChoE in mongoose diaphragm sections. (*A*) Karnovsky staining (20). (*B* and *C*) Immunofluorescent staining. (*B*) FITC fluorescence showing staining of AcChoE in the endplate. (*C*) TMR fluorescence of the same field showing that TMR-α-BTX did not stain the endplate. (Bar = 50 μm.)

RESULTS

Resistance of Mongoose to α-BTX. The toxic effect of α-BTX in the mongoose as compared with rabbit and mouse was examined. Intramuscular administration of α-BTX into mongoose in amounts of 0.3–2 μg/g of body weight did not kill the mongoose, whereas 0.1 and 0.3 μg/g of body weight were lethal in mice and rabbits, respectively. The resistance of mongoose to α-BTX was not due to neutralizing factors in their blood serum, since preincubation of mongoose serum with α-BTX did not abolish its toxic effect upon subsequent injection into mice, as has been observed also for snake serum (15).

Mongoose AcChoR Does Not Bind α-BTX. Extracts of either mongoose or snake (cobra or sand boa) muscle did not bind ^{125}I-labeled α-BTX, whereas extracts of mouse muscle

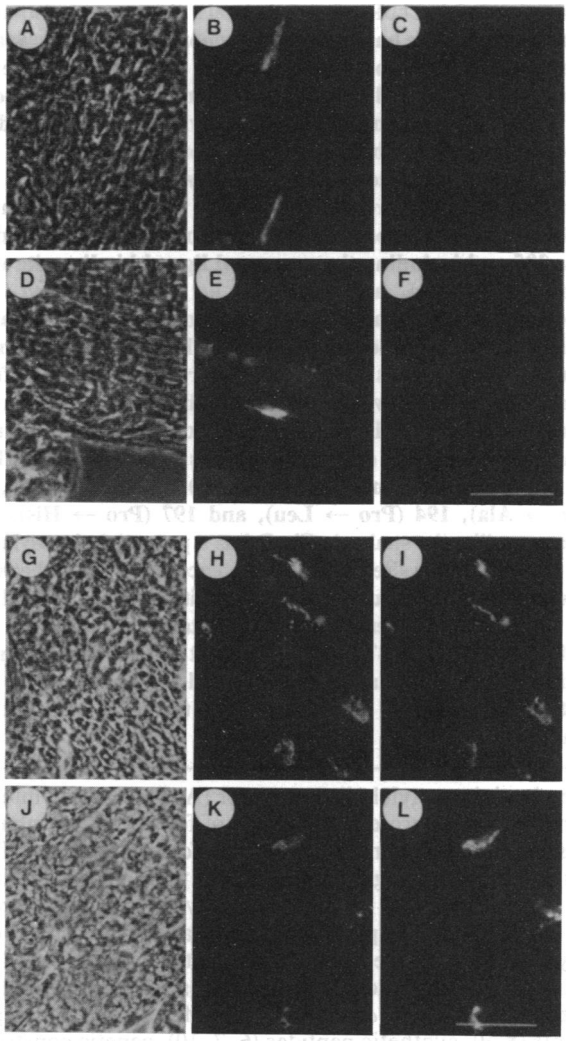

FIG. 2. Staining of AcChoR in mongoose (*A–F*) and rat (*G–L*) diaphragm sections. Sections were incubated with rabbit anti-denatured *Torpedo* AcChoR antibody (*A–C* and *G–I*) or rabbit anti-peptide 351–368 (*D–F* and *J–L*) and then with a mixture of TMR-α-BTX and FITC-conjugated goat anti-rabbit IgG. (*A*, *D*, *G*, and *J*) Phase-contrast images. (*B*, *E*, *H*, and *K*) FITC fluorescence demonstrating AcChoR staining with both types of antibodies. (*C*, *F*, *I*, and *L*) TMR fluorescence of the same fields demonstrating that TMR-α-BTX did not stain the mongoose endplate (*A–F*) and stained the rat endplate (*G–L*). (Bar = 50 μm.)

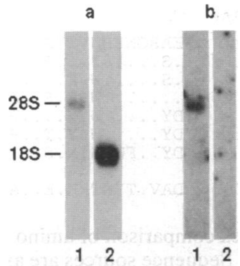

FIG. 3. Northern blot analysis of mongoose RNA. Poly(A)$^+$ RNA from mongoose (lane 1) or mouse (lane 2) was gel-electrophoresed, blotted, and probed with mouse AcChoR α-subunit cDNA (*a*) or with a mongoose-specific oligonucleotide (33-mer) corresponding to amino acid residues 187–197 (*b*) (see Fig. 4).

exhibited a specific binding that could be displaced by unlabeled α-BTX or by *d*-tubocurarine (data not shown).

As shown in Fig. 1, the endplates in mongoose diaphragm could be easily visualized by the Karnovsky method (20), which is based on the activity of the enzyme, or by fluorescent microscopy employing anti-AcChoE antibodies. Rhodamine-conjugated α-BTX did not stain the mongoose endplates. AcChoR in the mongoose endplate was visualized by rabbit antibody against denatured *Torpedo* AcChoR (22) or against a synthetic peptide corresponding to residues 351–368 of the α subunit of human AcChoR (23). This latter antibody is specific for mammalian AcChoR (23). Sections from mongoose and for comparison, from rat diaphragm, were incubated with either of the two antibodies, followed by FITC-labeled goat anti-rabbit immunoglobulins. The same sections were also incubated with TMR-conjugated α-BTX. As can be seen in Fig. 2 (*A–F*), both antibodies stained the mongoose endplate whereas no staining was observed with α-BTX. In contrast, both the anti-AcChoR antibodies and α-BTX stained the endplate regions in sections of the rat diaphragm (Fig. 2 *G–L*).

Cloning the Binding Site Domain of Mongoose AcChoR. We have cloned and sequenced a mongoose cDNA fragment that includes the binding site domain (i.e., the segment from the α subunit containing the tandem cysteines 192 and 193). We first verified by Northern blot analysis that mongoose poly(A)-containing RNA hybridizes with the mouse AcChoR α-subunit cDNA probe. As shown in Fig. 3*a*, a 4-kilobase transcript hybridized specifically with the mouse probe. The mongoose transcript for the α subunit is larger than the homologous mouse transcript (2.3 kilobases).

The PCR was used to amplify the cDNA fragment encompassing the binding site region, from mongoose single-stranded cDNA. The resulting 250-base-pair fragment hybridized to the mouse AcChoR α-subunit cDNA. Sequence analysis of this amplified mongoose fragment (Fig. 4) revealed high homology with the respective mouse fragment, corresponding to amino acid residues 122–205 of the α subunit (homology of 89% in nucleotides and 92% in amino acids). The mongoose segment contains the four cysteines at positions 128, 142, 192, and 193, thus verifying that it corresponds to the AcChoR α subunit. Interestingly, 5 of the 7 amino acid differences between the mouse and the mongoose fragments concentrate in the vicinity of the tandem cysteines in a stretch of 11 amino acid residues (residues 187–197). Three of these 5 differences are at positions 187, 189, and 194 where major substitutions take place also in the snake AcChoR (15), and one of them at position 187 creates a potential N-glycosylation site in the mongoose AcChoR. A synthetic oligonucleotide corresponding to amino acids 187–197 of the mongoose sequence hybridized to the mongoose and not to the mouse poly(A)-containing RNA (Fig. 3*b*).

Cloning the Binding Site Domain of the Sand Boa AcChoR. Binding experiments with muscle Triton extracts from the sand boa did not reveal any significant binding to ^{125}I-labeled α-BTX (data not shown). We have then PCR-amplified the 250-base-pair fragment from single-stranded cDNA from the sand boa, by employing the primers used to clone the mongoose fragment. Sequence analysis of the boa fragment revealed a very high similarity to the cobra and water snake respective fragments (15). In this fragment there is only one amino acid difference between the boa and cobra (residue 170 is histidine in the boa and tyrosine in cobra and water snake) and another difference between the boa and water snake (residue 149 is tryptophan in the boa and arginine in the water snake). All three snakes are completely identical in the putative binding site area.

Expression and Binding Properties of the Mongoose and Snake Fragments. The cloned fragments corresponding to amino acid residues 122–205 of the mongoose, cobra, and, for comparison, the mouse AcChoR were expressed employing a pET8C-derived expression vector. The expressed protein fragments were localized in the insoluble pellet, probably in inclusion bodies. These expressed fragments have the expected molecular mass of 8 kDa in SDS/polyacrylamide gel and constitute the major protein in the pellet (Fig. 5*a*). Antibodies against a synthetic peptide corresponding to residues 143–158 of the *Torpedo* AcChoR α subunit stained all three fragments (Fig. 5*b*), indicating that the expressed fragments are indeed from the AcChoR α subunit. Overlay of the blotted proteins with ^{125}I-labeled α-BTX showed that the toxin binds only to the mouse fragment and not to snake or mongoose fragments (Fig. 5*c*). In some cases a very long exposure of the blots gave a faint signal with the mongoose fragment.

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         122                     *                                               140     *
         A   I   F   K   S   Y   C   E   I   I   V   T   H   F   P   F   D   E   Q   N   C
Mongoose GCC ATC TTC AAA AGC TAC TGT GAG ATC ATC GTC ACC CAC TTT CCC TTT GAT GAA CAG AAC TGC
Mouse    ... ... ..T ... ... ... ... ... ... ..T ... ..T ... ... ... ..C ... ..G ... ... ...
         .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

                                                                             160
         S   M   K   L   G   T   W   T   Y   D   S   S   V   V   V   I   N   P   E   S   D
Mongoose AGC ATG AAG CTG GGT ACC TGG ACC TAT GAC AGC TCT GTG GTT GTC ATC AAC CCG GAA AGC GAC
Mouse    ... ... ... ... ..C ... ... ... ... ... G.. ... ... ..G .C. ..T ... ... ... ..T ...
         .   .   .   .   .   .   .   .   .   .   G   .   .   .   A   .   .   .   .   .   .

                                                                         180
         Q   P   D   L   S   N   F   M   E   S   G   E   W   V   I   K   E   A   R   G   W
Mongoose CAA CCT GAC CTA AGC AAC TTC ATG GAA AGC GGA GAG TGG GTG ATC AAG GAG GCC CGG GGC TGG
Mouse    ..G ..C ... ..G ..T ... ... ... ..G ... ..G ... ... ... ... ... ..A ..T ... ... ...
         .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

                                     *   *                           200
         K   H   N   V   T   Y   A   C   C   L   T   T   H   Y   L   D   I   T   Y   H   F
Mongoose AAG CAC AAT GTG ACC TAC GCC TGC TGC CTC ACC ACC CAC TAC CTG GAC ATC ACC TAC CAC TTC
Mouse    ... ... TGG ... TT. ... T.. ... ... .C. ... ..T .C. ... ... ... ... ... ... ... ...
         .   .   W   .   F   .   S   .   .   P   .   .   P   .   .   .   .   .   .   .   .
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FIG. 4. Alignment of nucleotide and deduced amino acid sequences for the mongoose PCR fragment of the AcChoR α subunit and the corresponding mouse fragment. Amino acid residues are numbered from 122 to 205, corresponding to their position in the mouse AcChoR α subunit. Cysteine residues are marked with an asterisk. Nucleotides or amino acids identical to the mongoose sequence are designated by dots.

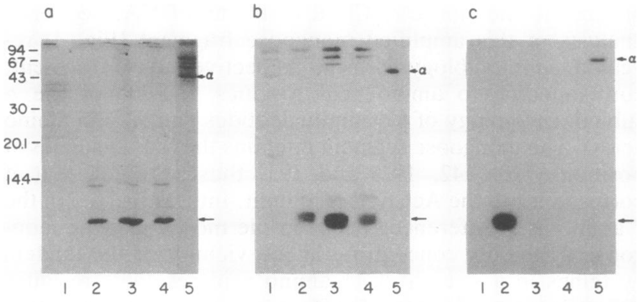

FIG. 5. Analysis of expressed protein fragments. The expressed protein fragments (20 μg of protein) or purified *Torpedo* AcChoR (10 μg) were resolved by polyacrylamide gel electrophoresis (15% gel). The gels were stained for proteins by Coomassie brilliant blue (*a*) or blotted (*b* and *c*) and overlaid with rabbit anti-peptide 143–156, followed by ^{125}I-labeled protein A (*b*) or with ^{125}I-labeled α-BTX (*c*). Lanes: 1, pET8C proteins without inserted DNA; 2–4, expressed mouse, mongoose, and cobra fragment, respectively; 5, *Torpedo* AcChoR.

Antiserum elicited against the expressed mongoose fragment stained specifically the endplates of both mongoose and rat diaphragms in immunofluorescence microscopy (Fig. 6). To allow for efficient staining of the receptor with the anti-fragment antibodies, the sections had to be pretreated with 0.2% SDS in PBS for 1 hr to partially denature the extracellular portion of the receptor. Similar results were obtained with the antibodies against the expressed mouse fragment.

DISCUSSION

Most mammals are highly sensitive to snake neurotoxins. The mongoose, however, is resistant to neurotoxins and can overcome various snakes and feed on them. As we have shown in this study, the highly curarimetric toxin α-BTX does not bind to mongoose AcChoR *in vivo* or *in vitro* and thus is not toxic in this animal. To understand the molecular basis for the resistance of mongoose AcChoR to α-BTX, we cloned, sequenced, and expressed a cDNA fragment corresponding to residues 122–205 of the mongoose AcChoR α subunit. We have also cloned and sequenced the same cDNA fragment from a primitive snake *Eryx jaculus* (sand boa), which like the other snakes studied (Natrix and cobra) (15), was shown to be resistant to α-BTX.

Comparison of the AcChoR binding site domains of animals that are susceptible or resistant to α-neurotoxins is an

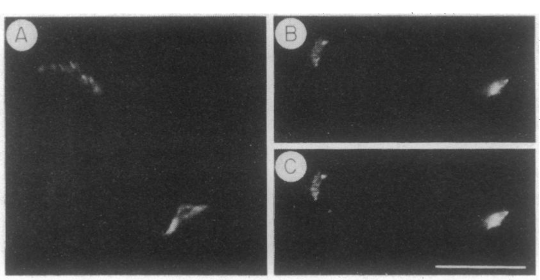

FIG. 6. Antibody to the expressed mongoose AcChoR fragment binds to mongoose and rat endplate. Sections were first incubated for 1 hr with TMR-α-BTX. These sections were then fixed for 2 hr in 2.5% (wt/vol) paraformaldehyde in PBS, washed, treated for 1 hr with 0.2% SDS in PBS, and washed with 0.1 M glycine, followed by fluorescent staining with the immunoglobulin fraction of rabbit anti-mongoose AcChoR fragment (residues 122–205). (*A* and *B*) FITC fluorescence shows the staining with anti-mongoose AcChoR fragment of mongoose and rat diaphragm, respectively. (*C*) TMR fluorescence of the field in *B*, demonstrating the colocalization of α-BTX and anti-mongoose antibody staining in rat endplate.

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              180                 200
                          **
Mongoose      EARGWKHNVTYACCLTTHYLD
Human         .S.....S...S..PD.P...
Calf          .S.....W.F....PS.P...
Mouse         .......W.F.S..P..P...
Chick         DY.....W.Y....PD.P...
Torpedo       DY.....W.Y.T..PD.P...
Snake         DY..FW.S.N.S...D.P...

Rat(PC12)     DAV.TYNTRK.E..AEI-.P.
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FIG. 7. Interspecies comparison of amino acid residues 180–200 of AcChoR α subunit. Sequence sources are as referred to in ref. 15. All sequences were compared to mongoose.

appropriate approach to distinguish between the essential elements required for binding of the natural agonist acetylcholine (AcCho) and those required for interacting with the antagonistic polypeptide toxin α-BTX. The mongoose is particularly suitable since it is very homologous to other mammals and differs from them only in its resistance to α-BTX. It was thus reasonable to predict that the sequence differences between the mongoose and other mammals will be found in the binding site domain of the AcChoR. Indeed, 5 out of the 7 amino acid differences between the mouse and mongoose α-subunit fragment (residues 122–205) cluster in a short stretch of 11 amino acids (residues 187–197) encompassing the two tandem cysteines 192 and 193 at the binding site (Fig. 4). The substitutions are at positions 187 (Trp → Asn), 189 (Phe → Thr), 191 (Ser → Ala), 194 (Pro → Leu), and 197 (Pro → His). Alignment of the mongoose-derived sequence at the ligand binding region with those of other species, including the snake and one neuronal receptor, is depicted in Fig. 7. Three of the mongoose substitutions are at the same positions (187, 189, and 194) at which major substitutions take place also in the snake. Position 194, adjacent to the tandem cysteines is proline in the toxin binders, whereas in the mongoose and snakes it is leucine. This substitution may result in a major conformational change, as proline can form a β-bend or turn. Such a conformational change presumably will not affect the interaction between the receptor and its natural ligand, AcCho, but can interfere with the binding of the much larger polypeptide antagonist α-BTX. It should be noted that, in the neuronal AcChoR, which does not bind α-BTX (24) and in the α-BTX-binding subunit from chicken brain (25), there is no proline residue adjacent to the tandem cysteines (corresponding to Pro-194 in the muscle α subunit). Nevertheless, the brain α-BTX-binding protein has a proline residue three residues after the tandem cysteines, which may play a similar role to that played by Pro-194 in the muscle toxin-binding AcChoRs.

The other two major substitutions are from aromatic residues, at positions 187 and 189 in toxin binders, to nonaromatic residues in the mongoose and snake. Position 189, which is tyrosine or phenylalanine in the toxin binders (Fig. 7) is threonine in the mongoose, asparagine in the snakes, and lysine in all neuronal AcChoRs. Replacement of Tyr-189 in the *Torpedo* binding site peptide by alanine or glycine (26) abolished its toxin-binding activity. At position 187, tryptophan in the toxin-binding AcChoRs is asparagine in the mongoose and serine in the snake. Low and Corfield (27) have proposed that Trp-187 interacts with the "Trp cleft" of the toxin reactive site and that, in the anomalous human AcChoR, the binding to toxins might be alternatively mediated by Trp-184. In the neuronal α subunits, there is no tryptophan residue in either position 187 or 184 (Fig. 7 and refs. 28 and 29). That Trp-187 is important for toxin binding is supported by the observation that its chemical modification in the synthetic *Torpedo* peptide containing residues 185–196 eliminated the binding activity to α-BTX and that the homologous human synthetic dodecapeptide with Ser-187 and Thr-189 did not bind α-BTX either (10). A longer synthetic

peptide of the human sequence (32-mer; residues 173–204) was reported to bind α-BTX 150 times weaker than the homologous *Torpedo* peptide (30). Nevertheless, intact human AcChoR binds α-BTX and it is possible that conformational factors not present in the synthetic peptides or that other domains in the receptor or the toxin molecule contribute to the toxin-binding activity of the human receptor. This point deserves further clarification.

Asn-187 in the mongoose and Asn-189 in the snake AcChoR α subunits are both putative N-glycosylation sites. It is not known yet whether these asparagines are glycosylated in the intact mongoose and snake receptors, and if they are, whether such a glycosylation contributes to toxin resistance. It should be noted that the nonglycosylated mongoose and snake protein fragments expressed in *E. coli* (Fig. 5), as well as the respective nonglycosylated synthetic peptides (residues 185–196), do not bind α-BTX. It is possible, however, that glycosylation, which adds a bulky group in the binding site domain, may provide additional protection toward the toxin without affecting AcCho binding. Thus, it seems that proline at position 194, an aromatic residue (tyrosine or phenylalanine) at 189, and tryptophan at 187 are strong requirements for toxin binding as mutations at these three positions in the mongoose and snake AcChoR abolish their binding to α-BTX. It is still not clear whether all three changes are required and what is the contribution of each in conferring toxin resistance.

Finally, although mongoose and snake AcChoRs do not bind α-BTX, they still retain their cholinergic properties and, therefore, amino acid residues that are essential for binding of AcCho should be conserved also in these receptors. Indeed, both the mongoose and snake binding site domains contain the amino acids that were shown to be labeled by dimethylaminobenzene diazonium fluoroborate (DDF; refs. 29 and 31), maleimido benzyltrimetylammonium (8), and lophotoxin (28) at the same positions as other muscle and neuronal receptors α subunits. These include Tyr-190, Cys-192 and -193, and Tyr-198 (Fig. 7). The aromatic residues Trp-149 and Tyr-151, which were reported to be labeled by DDF (29, 31), are also conserved in the mongoose and snake (Fig. 4). It should be noted that additional aromatic residues that are not contained within the fragment studied by us, such as Tyr-93 (32, 33) and probably Trp-86 (32), were also shown to contribute to cholinergic ligand binding. The participation of aromatic residues in the binding site of AcChoR might be of special interest in view of the recent crystalization of AcChoE and elucidation of its binding site as an "aromatic gorge" (34). In conclusion, our study indicates that the requirements for AcCho binding are not sufficient for α-BTX binding. Though both bind primarily to the same site in AcChoR, additional structural elements are necessary for α-BTX binding and those can be manipulated by genetic pressure without the loss of the major physiological function, which is AcCho binding.

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1. Karlin, A. (1980) in *The Cell Surface and Neuronal Functions*, eds. Cotman, C. W., Poste, G. & Nicolson, G. L. (Elsevier, Amsterdam), pp. 191–260.
2. Changeux, J. P., Devillers-Thiery, A. & Chemouilli, P. (1984) *Science* **225**, 1335–1345.
3. Haggerty, J. G. & Frohner, S. C. (1981) *J. Biol. Chem.* **256**, 8294–8297.
4. Gershoni, J. M., Hawrot, E. & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4973–4977.
5. Neumann, D., Gershoni, J. M., Fridkin, M. & Fuchs, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3490–3493.
6. Wilson, P. T., Lenz, T. L. & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8790–8794.
7. Neumann, D., Barchan, D., Safran, A., Gershoni, J. M. & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3008–3011.
8. Kao, P., Dwork, A., Kaldany, R., Silver, M., Weideman, J., Stein, S. & Karlin, A. (1984) *J. Biol. Chem.* **259**, 11662–11665.
9. Dennis, M., Giraudet, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J. Y. & Changeux, J. P. (1986) *FEBS Lett.* **207**, 243–249.
10. Neumann, D., Barchan, D., Fridkin, M. & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9250–9253.
11. Barkas, T., Mauron, A., Roth, B., Alliod, C., Tratos, S. J. & Ballivet, M. (1987) *Science* **235**, 77–80.
12. Gershoni, J. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4318–4321.
13. Mishina, M., Tobimatsu, T., Imoto, K., Tona-Ka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M. & Numa, S. (1985) *Nature (London)* **313**, 364–369.
14. Burden, S. Y., Hartzell, M. C. & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3245–3249.
15. Neumann, D., Barchan, D., Horowitz, M., Kochva, E. & Fuchs, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7255–7259.
16. Ovadia, M. & Kochva, E. (1977) *Toxicon* **15**, 541–548.
17. Asher, O., Neumann, D. & Fuchs, S. (1988) *FEBS Lett.* **233**, 277–281.
18. Studier, F. W. & Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–123.
19. Ravdin, P. & Axelrod, D. (1977) *Anal. Biochem.* **80**, 585–592.
20. Karnovsky, M. J. & Roots, L. (1964) *J. Histochem. Cytochem.* **12**, 219–222.
21. Abramson, S. N., Ellisman, M. H., Deerinck, T. J., Moulet, Y., Gentry, M. K., Doctor, B. P. & Taylor, P. (1989) *J. Cell Biol.* **108**, 2301–2311.
22. Bartfeld, D. & Fuchs, S. (1977) *FEBS Lett.* **77**, 214–218.
23. Fuchs, S., Neumann, D., Safran, A., Pizzighella, S., Montegazza, R., Daniels, M. P. & Vogel, Z. (1987) *Biochemistry* **26**, 4611–4616.
24. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. & Patrick, J. (1986) *Nature (London)* **319**, 368–374.
25. Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M. & Lindstrom, J. (1990) *Neuron* **5**, 35–48.
26. Tzartos, S. J. & Remoundos, M. S. (1990) *J. Biol. Chem.* **256**, 21462–21467.
27. Low, B. W. & Corfield, P. W. R. (1986) *Eur. J. Biochem.* **161**, 579–587.
28. Abramson, S. N., Li, Y., Culver, P. & Taylor, P. (1989) *J. Biol. Chem.* **264**, 12666–12672.
29. Galzi, J. L., Revah, F., Bessis, A. & Changeux, J. P. (1991) *Annu. Rev. Pharmacol.* **31**, 37–72.
30. Wilson, P. T. & Lentz, T. L. (1988) *Biochemistry* **27**, 6667–6674.
31. Dennis, M., Giraudet, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J. Y., Lazure, C., Chretien, M. & Changeux, J. P. (1988) *Biochemistry* **27**, 2346–2357.
32. Galzi, J. L., Revah, F., Black, D., Goeldner, M., Hirth, C. & Changeux, J. P. (1990) *J. Biol. Chem.* **265**, 10430–10437.
33. Cohen, J., Sharp, S. D. & Liu, W. S. (1991) *J. Biol. Chem.* **266**, 23354–23364.
34. Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. & Silman, I. (1991) *Science* **253**, 872–879.