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# MOLECULAR DISSECTION OF CHOLINERGIC BINDING SITES: HOW DO SNAKES ESCAPE THE EFFECT OF THEIR OWN TOXINS?

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Snakes have evolved a novel binding site demonstrating selective biorecognition. The snake nicotinic acetylcholine receptor is sensitive to acetylcholine while resistant to the effect of the lethal neurotoxins secreted in their own venom. By subjecting recombinant binding sites to point mutagenesis, biochemical analyses and NMR spectroscopy the binding characteristics of three cholinergic ligands have been measured. The amino acid residue at position 189 has been found to be of particular importance to toxin binding. 

Cobras, and related snakes, produce α-neurotoxins that specifically bind to the nicotinic acetylcholine receptor of their prey, thereby blocking the neuromuscular junction, causing paralysis leading to eventual death (1-4). Snakes and other reptiles have, however, acquired an impressive resistance to these α-neurotoxins (5) through means that are only now becoming understood. The cobratoxin binding site of the electric organ of *Torpedo californica* has been isolated, cloned and expressed in *Escherichia coli* (6). This recombinant toxin binding site has been systematically analyzed biochemically (7) and by NMR (8). These studies have shown that this site (α184-200) not only binds α-bungarotoxin and cobratoxin but also other cholinergic ligands such as d-tubocurarine, gallamine, nicotine and acetylcholine itself. Recently, the homologous sequences derived from various other vertebrates (including human) were also found to bind cholinergic ligands (9). It was of interest, therefore, to test whether or not the sequence α183-204, derived from the cobra (*Naja naja atra*) cholinergic receptor (10), also binds neurotoxins. In this paper

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we demonstrate that this snake sequence binds both acetylcholine and d-tubocurarine, yet has no affinity for α-bungarotoxin, a fact which might explain how snakes have escaped the effect of their own toxins.

## Materials and Methods

Production of Recombinant Binding Sites: Recombinant binding sites were prepared as previously described (7, 9). In principle, synthetic oligonucleotides were designed to correspond to the amino acid sequence α183-204 with various modifications as indicated. The duplex DNA was cloned into the SmaI site of the polylinker of the bacterial expression vector pATH2 (11). Transformed *E.coli* strain HB101 were cultivated on M9 medium as described (7) and the induction of expression was elicited by addition of 3β–indoleacrylic acid (10μg/ml) at 33°C. The 36kDA fusion protein was enriched from lysates of sonicated cultures by differential centrifugation (7). These preparations were then used for the binding assays, the NMR measurements and ligand overlays of protein blots as previously described (8, 9).

Binding Assay: The binding of $^{125}$I-labelled α-bungarotoxin to the recombinant proteins was assayed using a filtration assay based on that of Schmidt and Raftery (12) as modified by Ohana (9).

NMR Measurements: All NMR work was done on a Bruker AM-360-WB spectrometer equipped with an ASPECT 3000 computer. Selective $T_1$'s were measured by the inversion recovery pulse sequence, where the desired resonance for the selective $T_1$ was inverted with a long pulse from the decoupler. $K_D$'s were determined by the following equation:

$$[R]_0 T_{1p} = ([L]_0 + K_D)(T_{1b} + \tau_b)$$

$[R]_0$ is the total receptor binding site concentration. $[L]_0$ is the total ligand concentration. $T_{1p} = (1/T_1 - 1/T_{1free})^{-1}$. $T_{1b}$ and $\tau_b$ are the relaxation time and the lifetime of the bound state respectively. The $T_{1free}$ for the $K_D$ of acetylcholine was determined by titrating the bound acetylcholine with d-tubocurarine, for further details see references 8,13 and 14.

## Results and Discussion

The sequence α183-204 of the snake acetylcholine receptor (see figure 1) was expressed in *E. coli* using the pATH2 expression system (11). In this system a fusion protein is produced which contains the first 323 residues of the bacterial *trpE* protein followed by the foreign peptide, generating a 36 kDa polypeptide. Such a fusion protein, containing the sequence derived from *T. californica* binds α-bungarotoxin with an affinity of $K_D$=6x10$^{-8}$M (9). The

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          183                                                 204
Torpedo: G [W K] H [W] V [Y] Y [T] C C [P] D T P Y L D I T Y H
Snake  : G [F W] H [S] V [N] Y [S] C C [L] D T P Y L D I T Y H
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Figure 1.

Comparison between the cholinergic binding sites of the snake and the *Torpedo* acetylcholine receptors. The sequence α183-204 differs in the six positions indicated in the boxes.

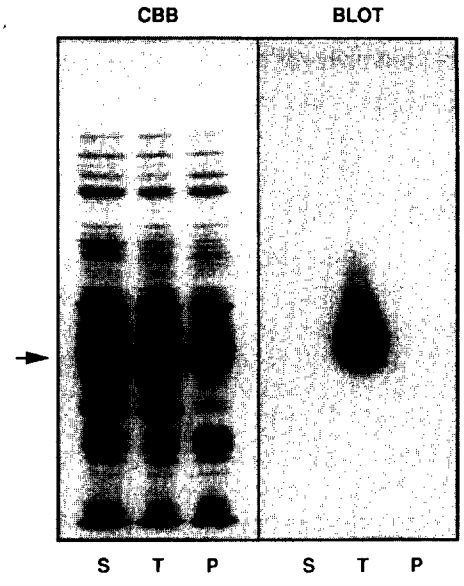


Figure 2.
Bacterial expression of the snake (S) and the *Torpedo* (T) cholinergic binding sites. The position of the fusion protein (36kDa) is indicated by the arrow next to the Coomassie brilliant blue stained gel (CBB). Such a gel was blotted and overlayed with $^{125}$I-labelled α-bungarotoxin. Over exposure (40 h) of such a blot (above) illustrates that only the *Torpedo* sequence binds toxin. Bacteria transformed with the pATH2 vector alone provided a negative control (P). Methods for the preparation and analysis of these recombinant binding sites are as described (7, 9).

comparable construct derived from snake, however, while expressed efficiently has no affinity for toxin (figure 2). Whereas this observation is intriguing and agrees well with the original report of Burden (5) and a most recent study using synthetic peptides (15), it is imperative to test whether or not this region of the α-subunit of the snake continues to bind the agonist, acetylcholine.

Although we are able to employ the biochemical binding assays to evaluate the capacity for toxin binding, the question of acetylcholine binding has only been approachable through NMR measurements. As we have shown previously, selective $T_1$ relaxation has been found to be extremely useful for such studies (8,13,14). The snake binding site was analyzed for its capacity to increase the relaxation rate of acetylcholine in solution. Indeed this is the case, thus illustrating its ability to bind acetylcholine. In order to further determine the specificity and affinity of this binding, it is necessary to titer the bound ligand with an effective displacing antagonist. In our previous studies this displacement was easily accomplished using α-bungarotoxin. However, as can be seen in figure 3, α-bungarotoxin is absolutely ineffective for the snake site. Displacement could be achieved, however, using the alkaloid d-tubocurarine (figure 3). The affinity constants measured for the different sites are given in Table 1.

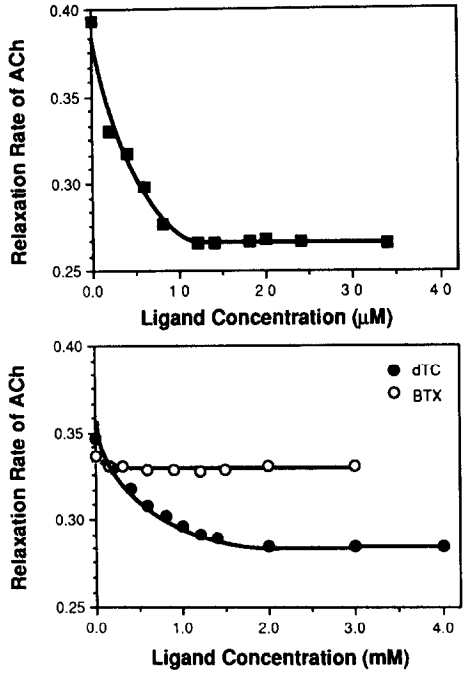


Figure 3.

Titration curves of acetylcholine (ACh) bound to *Torpedo* (upper graph) and to snake (lower graph) cholinergic binding sites. The $T_1$ NMR relaxation rate of ACh was measured as previously described (8). As can be seen, α-bungarotoxin was able to effectively displace ACh from the *Torpedo* sequence (top) and had absolutely no effect on that of the snake (bottom, **O**) [note the difference in the scale for the ligand concentrations]. On the other hand, d-tubocurarine (dTC) was able to titrate the ACh in the snake (●) illustrating that this site is resistant to α-bungarotoxin while sensitive to both dTC and ACh.

These NMR measurements illustrate that, whereas the snake binding site has escaped the deleterious effects of toxin binding, it has retained the necessary capacity to bind the native neurotransmitter, acetylcholine. Moreover, during the course of evolution the snake has not found an answer to

TABLE 1. Ligand binding to recombinant sites

| | ACh (mM) | dTC (mM) | BTX (nM) |
|---|---|---|---|
| *Torpedo* | 1.9 | 0.2 | 63.0 |
| Snake | 2.6 | 0.1 | - |
| pATH2 | 13.0 | 14.0 | - |

The $K_D$ values for acetylcholine (ACh) and d-tubocurarine (dTC) were determined using $T_1$ relaxation and the $K_D$ value for α-bungarotoxin (BTX) to the *Torpedo* site was determined by Scatchard analysis. No binding of BTX could be demonstrated for the snake nor for the negative control protein derived from bacteria transformed with the pATH2 vector alone.

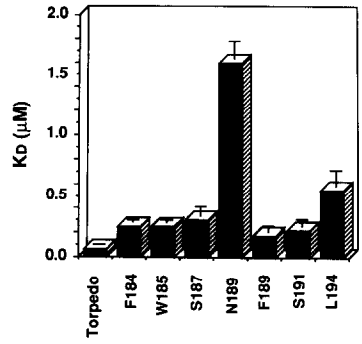


Figure 4.

Quantitative analysis of α-bungarotoxin binding to *Torpedo* point mutations. The *Torpedo* sequence α183-204 was modified *via* a series of point mutations as indicated above. Each column of the histogram is derived from 4-6 independent measurements and the deviations are given. The mutation Y189 to threonine was also expressed but no toxin binding could be demonstrated (not shown). The conditions and method for the Scatchard analyses were as previously described (9).

the plant-derived poison d-tubocurarine. It was therefore of interest to systematically dissect the site and learn which residues are responsible for this unique behavior.

There are six residue differences in the site of snake as compared to that of *Torpedo* (figure 1). Therefore, six independent point mutations were prepared in which the *Torpedo* sequence was modified, one residue at a time, so as to correspond with the snake sequence. Each one of these mutations were cloned and expressed and were tested for toxin binding using Scatchard analysis. The summary of these experiments is given in figure 4. It is clear that all the mutant sites have at least some affinity for α-bungarotoxin illustrating that the unique characteristic of the snake site is the combinatory effect of a number of residues.

The most striking effect is the change of tyrosine to asparagine at position 189. This tyrosine has been altered in other receptors as well and so the point mutations exchanging tyrosine for phenylalanine (corresponding to the mouse (16) and calf sites (17)) and threonine (to correspond to the human binding site (17)) were prepared and tested (figure 4). Recently, using synthetic polypeptides, additional support showing the particular importance of this tyrosine has been reported (18). It appears therefore, that a hydrophobic or aromatic residue at position 189 is necessary in order to maintain efficient toxin binding.

Asn189 of snake could be compatible with *N*-glycosylation and one could obviously imagine that such glycosylation in the midst of the ligand

binding site would cause considerable steric hindrance. The recombinant sites examined in this study, however, are bacterial and thus no glycosylation is expected. It is also noteworthy that the effect of the exchange of Pro194 for Leu may directly compromise a loop structure and thus cause the detected drop in toxin binding.

The nicotinic acetylcholine receptor must be viewed as a micro-molecular machine, constructed of numerous components that in harmony carry out its function. These components are comprised of "functional domains" with in the polypeptide chains of the receptor. Indeed, some of these domains might be dependent on the coordinated action of residues derived from dispersed regions of one or more subunits, "conformational domains". However, this notion does not exclude the possibility that "linear domains" can also exist. The cholinergic binding site, at least in part, is an example of such a linear functional domain. In the present study we illustrate that this relatively short sequence has the capacity to harbor *two* functional domains. The first domain (residues 183-192) is required to interface with aspects of the snake neurotoxins. The five modifications observed in this region of the snake site, and especially the alterations of the Tyr189 render resistance to the effects of snake toxins. The second domain (residues 193-204) is almost completely conserved in snake and very highly conserved in the other organisms studied thus far. This domain is postulated to be particularly important for agonist recognition and as has been previously suggested (7, 9) to provide the nucleophilic subsite that should interact with the quaternary ammonium of acetylcholine or the analogous moiety of the antagonist neurotoxins. Through the maintenance of this second domain and selective modification of the first, the snakes have evolved an ingenious example of selective biorecognition.

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