the specificities of the enzymes from the two sources has allowed quantitation of these similarities through the correlation coefficient and slope obtained. The successful application of this approach here suggests that this may be a useful general method for comparing active sites of two enzymes isolated from different organisms.

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Comparison of the Toxin Binding Sites of the Nicotinic Acetylcholine Receptor from *Drosophila* to Human†

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ABSTRACT: Recombinant toxin binding proteins have been previously found to provide a convenient experimental system for the study of receptor–ligand recognition (Aronheim et al., 1988). Here, this system has been used to produce the binding sites of the cholinergic receptor derived from seven organisms, *Torpedo californica*, *Xenopus*, chick, mouse, calf, human, and *Drosophila*. These have been compared with respect to their toxin binding capacity. Scatchard analyses show that the K_D values of α-bungarotoxin binding to the above sites are 63, 536, 150, 3200, 6200, 6470, and 1700 nM, respectively. These results reiterate the importance of α183–204 as a ligand binding site. In order to increase the repertoire of sites available for study, chimeric structures were constructed. Through the analysis of such chimeras, some themes of the gross anatomy of the binding site can be learned. A positive subsite followed by a hydrophobic patch preceding a nucleophilic domain appears to be required for efficient toxin binding.

Over the years, numerous receptors have been purified, cloned, and sequenced (Numa et al., 1983; Dohlman et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987; Bunzow et al., 1988; Greve et al., 1989; Mendelsohn et al., 1989; Staunton et al., 1989). However, the nature of the basic recognition process is still unclear. What is the mechanism responsible for ligand binding to its receptor?

In order to address this question, the ligand binding domain of a given receptor must be identified and then analyzed systematically. A case in point is the toxin binding site of the nicotinic acetylcholine receptor (nAChR).[1] Numerous reports have shown that the area of residues 170–210 of the α-subunit

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; BTX, α-bungarotoxin; CTX, α-cobratoxin.

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Table I: Recombinant Binding Sites

SOURCE	AMINO ACID SEQUENCE OF INSERT [a] (183 … 204)	HOMOLOGY (%)	K_D (nm) [b]		RELATIVE BINDING (%) [c]
TORPEDO [d]	G W K H W V Y Y T C C P D T P Y L D I T Y H	100	63 ± 9.5	(5)	100.0
XENOPUS [e]	K	95	536 ± 81	(5)	11.7
CHICK [f]	A	95	150 ± 82	(5)	42.0
MOUSE [g]	F S T	86	3200 ± 660	(6)	1.9
CALF [h]	F A S	86	6200 ± 1900	(6)	1.0
HUMAN [h]	S T S	86	6470 ± 1550	(6)	0.9
DROSOPHILA [i]	A V R N E K F S - E E V F N	36	1700 ± 1060	(8)	3.7

[a] A series of binding sites corresponding to the sequence α183–204 derived from different organisms. Only the residues which differ from *T. californica* are shown. [b] Numbers in parentheses represent the number of experiments performed. [c] Taking the binding of *T. californica* as 100%, the relative binding of each was calculated. Moreover, it should be noted that the affinity of the intact AChR for BTX is 2.3×10^{-10} M (Lukas et al., 1981). [d] Noda et al. (1982). [e] Baldwin et al. (1988). [f] Ballivet et al. (1983). [g] Boulter et al. (1985). [h] Noda et al. (1983). [i] Bossey et al. (1988).

Table II: Chimeric Binding Sites

SOURCE	AMINO ACID SEQUENCE OF INSERT [a] (183 … 204)	HOMOLOGY (%)	K_D (nm) [b]		RELATIVE BINDING (%)
TORPEDO	G W K H W V Y Y T C C P D T P Y L D I T Y H	100	63 ± 9.5	(5)	100.0
M/T	F S	91	1820 ± 740	(6)	3.4
T/M	T	95	687 ± 90	(6)	9.2
D/T	A V R N E K F S	64	1200 ± 900	(7)	5.2
T/D	- E E V F N	73	580 ± 44	(5)	10.8
Ca/T	F A	91	1538 ± 407	(6)	4.0
T/Ca	S	95	1300 ± 560	(8)	4.7

[a] Chimeric binding sites are compared with the binding of BTX to α183–204 of *T. californica*. Each chimera is composed of NH_2-terminal and COOH-terminal halves. The chimera between the mouse NH_2 half and the *Torpedo* COOH terminus is designated M/T, for example. [b] Numbers in parentheses represent the number of experiments performed. M, mouse; D, *Drosophila*; Ca, calf; T, *Torpedo*.

of the nAChR can be affinity labeled (Kao et al., 1984; Dennis et al., 1986) or can independently bind α-neurotoxins such as α-bungarotoxin (BTX) (Neumann et al., 1985, 1986a,b; Wilson et al., 1985; Mulac-Jericevic & Atassi, 1986; Pedersen et al., 1986; Barkas et al., 1987; Gershoni, 1987) with reasonable affinity, $K_D = (0.7–350) \times 10^{-8}$ M (Neumann et al., 1986a,b; Gotti et al., 1987; Lentz et al., 1987; Ralston et al., 1987; Arohneim et al., 1988; Wilson & Lentz, 1988). These observations can lead to two very different lines of investigation: (i) What other components of the intact nAChR participate in toxin binding [see, for example, McCormick and Atassi (1984), Mulac-Jericevic and Atassi (1986), and Oblas et al. (1986)], thus generating high affinity ($K_D < 10^{-10}$ M)? Alternatively, (ii) what molecular mechanisms enable a remarkably short sequence, e.g., α184–200, to recognize cholinergic ligands with such specificity? In this paper, we concentrate on the latter.

In trying to elucidate rules that govern the binding process, a number of questions have been formulated. Which amino acids are actually involved in forming contact points with toxins? Which amino acids are responsible for generating and maintaining a functional three-dimensional configuration which places the contact residues in their proper orientation in space?

In dealing with these questions, we have employed an experimental system which allows easy manipulation of the toxin binding site (Aronheim et al., 1988). Toxin binding sequences are expressed as fusion proteins in bacteria which are then biochemically analyzed. In the past using this approach, we have demonstrated that α184–200 of *Torpedo californica* binds BTX and other cholinergic ligands, as well as does the α-subunit itself (Aronheim et al., 1988).

In this paper, we have continued our analysis of the bacterially expressed cholinergic binding sites and characterized the binding of toxin to the corresponding domain in the nAChR derived from assorted animal species.

Materials and Methods

Constructs. In principle, all the constructs in this study were produced as previously described (Aronheim et al., 1988). Synthetic oligonucleotides were prepared on an Applied Biosystem 380 B DNA automated synthesizer. They were designed by using bacterially preferred codons to code for the amino acid sequences described in Tables I and II. The constructs were ligated into the expression vector pATH2 (Dieckmann & Tzagoloff, 1985). The new plasmids, containing the different inserts, were used to transform *Escherichia coli* HB101 cells. The transformed bacterial clones, which contained the insert in proper orientation, were selected by ^{125}I-labeled BTX overlay of colony blots as described (Gershoni, 1987), except for deleting the sodium dodecyl sulfate lysis and electrophoretic removal of the detergent.

Thus, the filters were put directly into the quench solution and processed accordingly.

Polyacrylamide Gel Electrophoresis and Protein Blotting. Bacterial cells were solubilized in sample buffer (125 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 3% β-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue) and sonicated in an E/MC ultrasonic cleaner. In each lane, a sample of 0.5 OD_{600} bacterial homogenates was loaded. The proteins were resolved on 10% polyacrylamide gels by using the Laemmli buffer system. After electrophoresis, the gels were either stained in Coomassie brilliant blue and destained or used for protein blotting. Gels were blotted onto nitrocellulose membrane filters at 50 V for 2 h, in a gradient electric field (Gershoni & Palade, 1982; Gershoni et al., 1985). Blots were then quenched for 5 h at room temperature with 1% hemoglobin in 10 mM phosphate buffer, pH 7.4, and incubated in the same solution containing (2–5) × 10^6 cpm of ^{125}I-labeled BTX [(2–5) × 10^{-9} M] for 12 h. The filters were washed 3 × 15 min in ice-cold 10 mM phosphate buffer, pH 7.4, and autoradiographed with Kodak XAR-5 film. Filters which were overlayed with ^{125}I-labeled α-cobratoxin (CTX) were first quenched 5 h with 25% low-fat milk in 10 mM phosphate buffer, pH 7.4. Then 5 × 10^6 cpm of radiolabeled CTX [(2–5) × 10^{-9} M] were added to the filters which were then incubated at room temperature for 12 h. The filters were washed 3 × 15 min with ice-cold 10 mM phosphate buffer, pH 7.4, containing 0.05% Triton X-100 and autoradiographed with Kodak XAR-5 film.

Preparation of trpE Fusion Protein. Positive clones were grown on M-9 medium supplemented with 0.2% glucose, 30 μg/mL ampicillin, 0.5% casamino acids, 5 μg/mL thiamin, and 5 μg/mL 3-indoleacrylic acid (33 °C, 12–24 h). The culture was pelleted and resuspended to 0.1 of its original volume with high-salt buffer (10 mM phosphate buffer, pH 7.4, containing 500 mM NaCl, 1 mM EDTA, 1 mM 1,4-dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride). The suspension was sonicated (1–2 min on ice) and then centrifuged at 3000 rpm for 10 min. The pellet was resuspended with water and resonicated. The supernatant was highly enriched for the fusion protein and was used for the toxin binding assays.

Toxin Binding Assay. Toxin binding was measured essentially as described by Schmidt and Raftery (1973) and modified according to Aronheim et al. (1988). In principle, aliquots of enriched fusion protein preparations (50–100 μL) were incubated in 10 mM phosphate buffer, pH 7.4, containing 2 × 10^{-8} M ^{125}I-labeled BTX (2 × 10^4 cpm) and nonlabeled BTX as required. All the incubations were carried out at 4 °C for 15–18 h to ensure equilibrium even for the low-affinity binders. Afterward, the reaction was terminated by adding 2–3 mL of the same buffer plus 0.1% Triton X-100 and filtering the mixture through a positively charged nylon membrane filter (Zetabind, CUNO Inc., Meriden, CT). The efficiency of separation of bound from free ligand was improved by using a Geneco Hyvac 14 vacuum pump and further washing the filters with 5 mL of 10 mM phosphate buffer, pH 7.4, containing 0.1% Triton X-100 at 4 °C. Background was determined by adding 500–1000× excess of nonradioactive toxin.

Results

We have previously demonstrated that the sequence α184–200 in the nAChR of *T. californica* could be produced using the strategy depicted in Figure 1. In principle, the sequence to be produced is split into two oligonucleotides, sense and antisense, connected by a short overlapping junction, "filled in" enzymatically and cloned into the pATH2 expression vector. Such an arrangement is especially convenient as it allows simple modification of the domain being studied. For the series of experiments described here, the junction has been kept constant in all the constructs produced, thus providing the option to mix oligonucleotide pairs at will, allowing the formation of chimeric binding sites as well (see below).

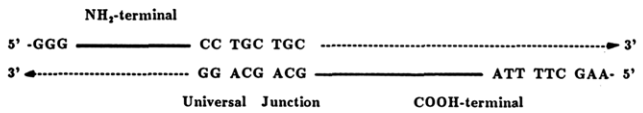

FIGURE 1: Formation of synthetic binding sites. Two oligonucleotides, a "sense", corresponding to the NH_2-terminal half of the site, and an "antisense", corresponding to the COOH-terminal half, were joined via an eight base pair universal junction. The junction corresponds to the last two bases of the codons for Ser, Ala, or Thr and the complete codons for Cys-192 and Cys-193. The 5′-GGG has been added to maintain a functional *Sma*I site, and the 3′ end of the construct was designed to generate a termination site and a *Hin*dIII site. The construct was "filled in" and cloned into the pATH2 vector.

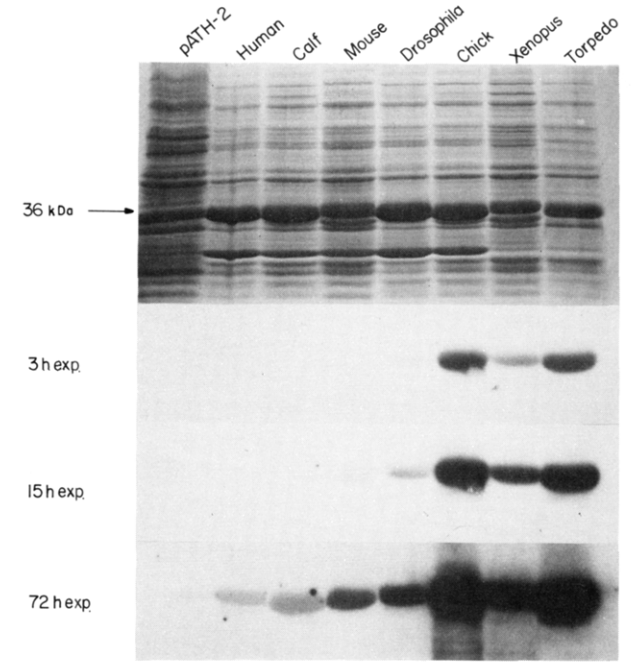

FIGURE 2: Protein blot analysis of the binding sites. Total cell homogenates were prepared from eight clones as indicated. The samples were separated by polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue (top) or transferred onto nitrocellulose filters and overlayed with ^{125}I-labeled BTX and autoradiographed for different exposure times as indicated (bottom three panels). The position of the 36-kDa peptide is marked.

First, the sequence α183–204 derived from the nAChR of chordates *T. californica*, *Xenopus*, chick, mouse, calf, and human and the invertebrate *Drosophila* was prepared (Table I). As can be seen in Figure 2, all the bacterial clones efficiently produced an inducible fusion protein of similar size (36 kDa). However, it is immediately obvious from the ligand overlay protein blot analysis that there is a considerable degree of variation in ligand binding (compare the three exposure times, BTX binds *T. californica* > chick > *Xenopus* > *Drosophila* > mouse > calf > human). These differences were quantified by Scatchard analyses (Figure 3). The K_D values measured for the seven constructs were 63, 536, 150, 3200, 6200, 6470, and 1700 nM, respectively.

Compared with our previous report (Aronheim et al., 1988), more accurate measurements were achieved by increasing the vacuum intensity during filtration. Thus, for *T. californica*, α183–204 bound BTX with a K_D = 6 × 10^{-8} M, which is 3.3-fold lower than the value previously reported for equilib-

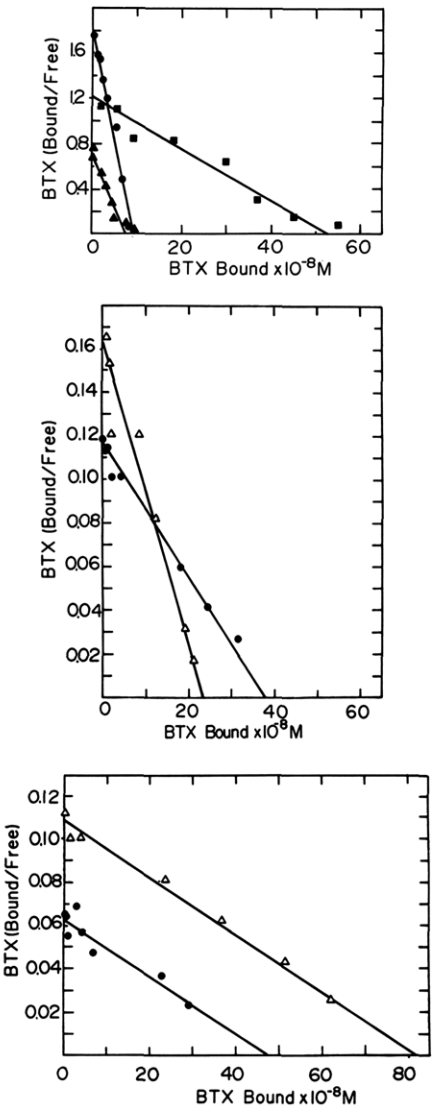

FIGURE 3: Scatchard analysis of α-bungarotoxin binding to the sites. The seven clones containing α183–204 of the different organisms were grown under inductive conditions (200 mL of each culture). From the induced cells, the corresponding trpE fusion proteins were partially purified and used for Scatchard analysis as depicted in the figure. The nonspecific binding was determined by adding a 1000× excess of nonlabeled BTX to the reactions. This value was subtracted accordingly. In addition, binding assays for protein derived from the pATH2 vector alone were performed as negative controls. In all cases, random and low values were obtained, and no reproducible low affinity could be demonstrated. (Upper panel) K_D(*Torpedo*) = 5.3 × 10^{-8} M (●), K_D(chick) = 1.07 × 10^{-7} M (▲), and K_D(*Xenopus*) = 4.5 × 10^{-7} M (■). (Middle panel) K_D(mouse) = 3.27 × 10^{-6} M (●), K_D(*Drosophila*) = 1.55 × 10^{-6} M (△). (Bottom panel) K_D(human) = 7.53 × 10^{-6} M (●), K_D(calf) = 7.36 × 10^{-6} M (△).

rium binding yet 4.6 times higher than the constant calculated from the kinetic study (Aronheim et al., 1988). The major significance, however, of the more precise binding assay is that it enables the analysis of the low-affinity binders. For example, despite the relatively high dissociation constants of human and calf for BTX, the method provides consistent results within a given experiment (as the affinity drops, however, the deviation between experiments does increase). All the sites tested were found to be sensitive to *d*-tubocurarine competition (data not shown).

Most intriguing is the fact that CTX binding does not precisely reflect BTX binding (see Figure 4). The *T. californica* site binds CTX 10 times less than BTX (the dissociation constant for CTX was 5 × 10^{-7} M, not shown). In

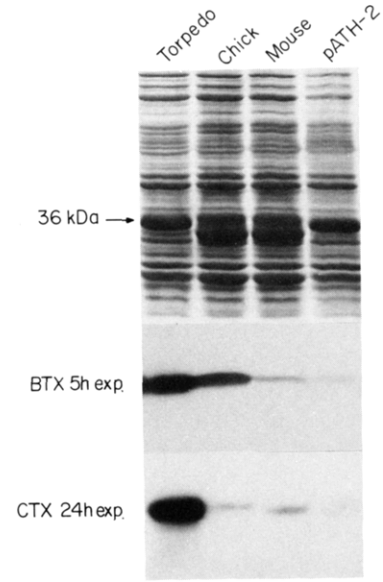

FIGURE 4: Comparison of α-neurotoxin binding to various sites. Total cell homogenates were prepared, resolved, and analyzed by toxin overlay of protein blots as described in Figure 2 and under Materials and Methods: top, gel stained with Coomassie brilliant blue; middle, gel blotted 2 h at 50 V, overlayed with ^{125}I-labeled BTX, washed, and autoradiographed for 5 h; bottom, overlayed with ^{125}I-labeled CTX, washed, and autoradiographed for 24 h.

marked contrast to this, the chick-derived site binds BTX almost as well as *T. californica* yet is considerably less efficient in CTX binding. Such a selective drop in CTX binding is not observed for the mouse site.

The midpoint of each binding site is the area of the tandem cysteines, Cys-192 and Cys-193. Thus, the sites can be considered to be comprised of NH_2-terminal and COOH-terminal halves. In human and chick, the variations are confined only to the NH_2-terminal half, while in *Xenopus* only the COOH-terminal half is modified. However, with mouse, calf, and *Drosophila*, there are changes in both halves. Therefore, chimeric structures were prepared enabling further evaluation of the effect of individual changes. In these experiments, a *T. californica* NH_2-terminal half was matched with the complementary COOH-terminal counterparts derived from *Drosophila*, mouse, or calf (referred to as T/D, T/M, and T/Ca, respectively). The reciprocal chimeras D/T, M/T, and Ca/T were also prepared (Table II). Chimeras which contain the NH_2 part of *T. californica*, such as T/D and T/M, bind BTX much better than chimeras which contain the NH_2 part of the mouse, M/T, or *Drosophila*, D/T. Figure 5 exemplifies the BTX binding to *Torpedo*/*Drosophila* or mouse chimeras. The K_D values for T/D, T/M, T/Ca, D/T, M/T, and Ca/T were 550, 750, 1300, 1100, 1830, and 1538, respectively.

Discussion

The 22 amino acid sequence α183–204 of the *T. californica* nAChR binds BTX with an affinity of K_D = 6 × 10^{-8} M, a value similar to that reported for the 32-mer α173–204 and the 17-mer α184–200 (Lentz et al., 1987; Aronheim et al., 1988; Wilson & Lentz, 1988). Yet it is not obvious why and how these synthetic binding sites work. Which residues are essential and provide contact points with the toxin and which confer the structural framework of the binding site? One strategy which comes to mind for the study of such questions could be to systematically prepare representative sequences of the possible 440 single-exchange permutations and test their binding capacity. Then one could continue to examine selected multiple variations. Indeed, saturation mutagenesis has pro-

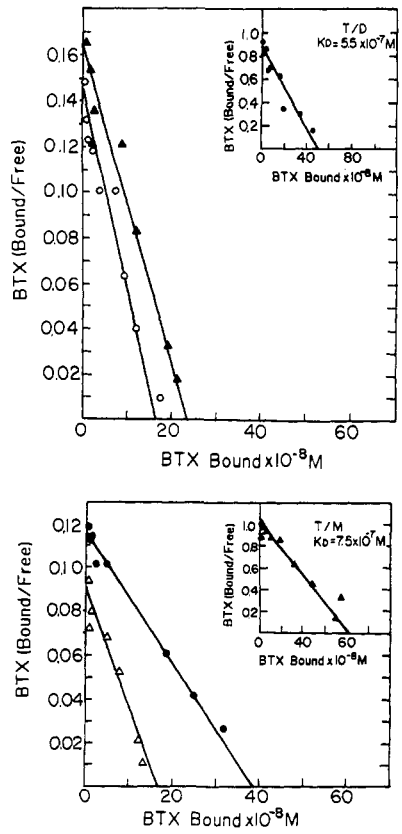

FIGURE 5: Scatchard analysis of chimeric sites. Total cell homogenates of chimeric binding sites and nonchimeric sites were prepared and analyzed as described in Figure 3. (Upper panel) K_D(*Drosophila*) = 1.55×10^{-6} M (▲), K_D(D/T) = 1.1×10^{-6} M (○), K_D(T/D) = 5.5×10^{-7} M (insert). (Lower panel) K_D(mouse) = 33.27×10^{-6} M (●), K_D(M/T) = 1.83×10^{-6} M (△), K_D(T/M) = 7.5×10^{-7} M (insert).

vided insight to the folding and activity of DNA binding proteins (Bowie & Sauer, 1989). However, until an efficient means for production of multiple peptides becomes available, the mechanics and cost make such a study both tedious and extremely expensive.

Therefore, in the present investigation, we focused on seven variations of the toxin binding domain which have been chosen for us through natural selection. These have been derived from organisms which are sensitive to BTX and other curarimimetic toxins (Vogel et al., 1972; Burden et al., 1975; Dreyer et al., 1978; Dudai, 1978; Lukas et al., 1981; Sine & Taylor, 1981; Stephenson et al., 1981; Behling et al., 1988a; Schloss et al., 1988). The demonstration that the seven sequences bind toxin, as one would hope, emphasizes the fact that the area α183–204 is a genuine component of the cholinergic binding site. Surprisingly, the *T. californica* site continues to remain the "all time champion" for BTX binding. The evolutionary significance of this, however, is still not understood. The explanation for this pecularity might be that in the course of evolution, fish encountered terrestrial venomous snakes less often than did humans and other mammals, leading to less effective selection pressure.

Examination of the sequences indicates that nine residues are conserved in all seven variants and among the vertebrates an additional seven residues are conserved. However, this type of comparison can only point out the commonality or differences between the sites which have been selected. In order to appreciate the effect of these changes, the sites had to be synthesized and tested for BTX binding. Indeed, the use of synthetic peptides can, and has been found to, provide binding data for this region [e.g., see Neumann et al. (1985, 1986a,b), Wilson et al. (1985), and Mulac-Jericevic and Atassi (1986)]. In such instances, each peptide must be prepared individually and cannot be reused to create chimeras or novel combinations as demonstrated in this study. Moreover, synthetic peptides of this region are rather insoluble which imposes the need to employ solid-phase assays (Neumann et al., 1986a,b; Mulac-Jericevic & Atassi, 1986; Lentz et al., 1987; Wilson & Lentz, 1988). Another approach could be to produce a series of site mutations in the α-subunit, to be used in reconstitution experiments. Whereas such experiments have been done in the past (Mishina et al., 1985), the intepretation of the results obtained is often complicated by allosteric effects. The recombinant approach used here represents an intermediate strategy which has been found to be extremely efficient, versatile, and amenable to biochemical scrutiny. As more information is learned about the fragment of the receptor α183–204, new mutations can be designed to specifically analyze the binding process. In addition, one must consider the possible effect of the flanking residues that may not necessarily be directly involved in the ligand binding per se but could otherwise have a profound effect on the stabilization of the functional conformation.

Our data and those reported by Wilson and Lentz (Lentz et al., 1987; Wilson & Lentz, 1988) show that the areas α173–204 and α183–204 from human bind BTX 50–100 times less efficiently than the same region in *T. californica*. Neumann et al. (1986b) have found that the human sequence α185–196 has virtually no toxin binding ability. In contrast to this, the intact nAChR of human binds toxins with an affinity ($K_D = 5 \times 10^{-10}$ M) similar to that found for *T. californica* (Lukas et al., 1981; Stephenson et al., 1981). This discrepancy between the intact receptors and the isolated sites might indicate that there could be other domains in the human receptor that compensate for α183–204 and contribute significantly to toxin binding [see, for example, Mulac-Jericevic and Atassi (1986)]. Alternatively, human α183–204 is markedly less hydrophobic and therefore may be unable to maintain the necessary configuration when in the isolated form and can only assume the functional conformation when present in its natural milieu, i.e., as part of the intact nAChR and opposing the lipid bilayer.

Another finding of this study is the observed differential affinity of the chick site for BTX as compared to CTX. Interestingly, only a single exchange (Thr-191 → Ala) exists. Recently, a similar situation for substrate differential affinity has been reported. In the case of β-lactamase, exchanging Leu-76 for Ile greatly modified the enzyme's ability to neutralize nitrocephin yet had no effect on ampicillin neutralization (Oliphant & Struhl, 1989).

No simple correlation between high affinity and homology with *T. californica* can be identified. For example, *Drosophila* binds BTX with higher affinity compared with that observed for the human or the calf site. The relative drop in the affinity of mouse as compared with both chick and *T. californica* corresponds well with previous observations (Hawrot et al., 1986). From our study, it becomes clear that the NH_2 and COOH parts of α183–204 are both essential, although the NH_2 terminal appears to be more critical than the other.

A number of possible common themes for the gross anatomy of a toxin binding site can be identified. The site may form a loop (or protrusion) turning around the conserved prolines-194 and -198, bringing two hydrophobic patches opposing each other possibly forming aromatic–aromatic interactions [Tyr-189, Tyr-190 with Tyr-198, Tyr-203; see Burley and Petsko (1985)]. There appears to be a requirement for a

positive charge preceding the first hydrophobic domain in the NH_2-terminal domain; this is then followed by a nucleophilic patch in the COOH-terminal region. All the toxin binding vertebrates studied thus far have preserved both prolines-194 and -198 and also cysteines-192 and -193 in the peripheral nervous system. The total conservation of residues α197–200 reiterates their significance as has been previously indicated (Aronheim et al., 1988). The significant drop in affinity found in the case for human α183–204 as compared to *T. californica* might be due to the marked increase in hydrophilicity of the required hydrophobic patch. On the other hand, both calf and mouse preserve a hydrophobic nature for the area α187–190, and yet the chimeras M/T or Ca/T are still markedly perturbed for BTX binding. This could indicate a required participation of Tyr-189 in hydrogen binding which cannot be satisfied by phenylalanine. Tyr-190 is conserved in all the sites and therefore may also have a special role [see also Dennis (1986), Aronheim et al. (1988), and Wilson and Lentz (1988)]. The reciprocal chimeras T/M and T/Ca are also less efficient for toxin binding and thus focus our attention on the possible involvement of Asp-195 in hydrogen binding. Hydrogen binding to charged donors is at least twice as strong as bonds formed with uncharged donors (3 kcal/mol^{-1} vs 0.5–1.5 kcal/mol^{-1}; Fersht et al., 1985). The exchange of Asp-195 for Ser or Thr (in T/Ca and T/M, respectively) should therefore be expected to drop in affinity for BTX. Moreover, serine appears to be more detrimental relative to threonine which may reflect the size and steric similarity between threonine and aspartic acid. The possible requirement for a negative subsite provided by Asp-195 might explain the drop in affinity seen in the *Xenopus* site where the exchange of the Thr-196 for a lysine introduces a positive charge in the middle of the postulated nucleophilic site.

The study of seven different binding sites and six chimeras has provided demonstration of the universal importance of the domain α183–204 as a BTX binding site. The multiple residue exchanges examined here indicate the extent of the flexibility of this site. The general requirements for efficient binding have become more clear. The toxin binding site requires an electrophilic subsite followed by a hydrophobic patch that can complement the carbonyl group of acetylcholine and its hydrophobic cluster (Behling et al., 1988b) or Asp-31 of BTX followed by residues Ala-32 and the conserved Phe-33. The significance of a negative subsite has also been pointed out. The latter might interact electrostatically with the guanidinium moiety of the toxin's Arg-37. Arg-37 could also hydrogen bond with Thr-195 or Ser-195 of the mouse or calf, respectively. On the basis of these postulates, a series of selected mutations are being studied so as to determine the individual contributions of specific residues. Moreover, the NMR analysis of the agonist binding to these sites (Fraenkel et al., 1990) is being conducted.

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Cross-Linking of Endothelin 1 and Endothelin 3 to Rat Brain Membranes: Identification of the Putative Receptor(s)

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ABSTRACT: Affinity-labeling experiments with ^{125}I-endothelin derivatives using bifunctional cross-linking reagents were carried out in an attempt to identify the polypeptide component(s) of the endothelin/sarafotoxin receptors in rat brain tissues. In rat cerebellum, cortex, and caudate putamen, endothelin 1 specifically labeled a major component with a molecular mass of around 53 000. In the same tissues endothelin 3 specifically labeled, in addition to the 53 000 band, a band of molecular mass of 38 000. This result clearly indicates that in the brain the endothelin binding site resides within a polypeptide of apparent M_r = 53 000. The possible presence of receptor subtypes is discussed with reference also to the reported identification of endothelin receptors in chick cardiac membrane and in rat mesangial cells.

In brain and in other tissues, endothelins (ET)[1] and sarafotoxins (SRTX) have been shown to bind specifically to a class of receptors that are associated with the hydrolysis of phosphoinositides (PI) (Ambar et al., 1988, 1989; Kloog et al., 1988; Bousso-Mittler et al., 1989; Jones et al., 1989) and with the mobilization of intracellular Ca^{2+} (Miasiro et al., 1988; Van Renterghen et al., 1988). Mammalian endothelin (ET-1) (Yanagisawa et al., 1988a) and sarafotoxin *b* (SRTX-*b*) (Takasaki et al., 1988) are highly potent vasoconstrictive peptides. Other homologues of this family of peptides, all of which possess 21 amino acid residues, are the mammalian ET-2 and ET-3 (Yanagisawa et al., 1988a,b; Inoue et al., 1989) and vasoactive intestinal contractor (Saida et al., 1989) and the snake toxins SRTX-*a*, SRTX-*c*, and SRTX-*d* (Kochva et al., 1982; Takasaki et al., 1988; Bdolah et al., 1989). All of these peptides bind to the ET/SRTX receptors in rat brain and atrium and induce contractions of smooth muscles in various tissues.

Affinity labeling by means of bifunctional cross-linking reagents has proved to be an extremely useful procedure in the biochemical and pharmacological analysis of many receptors, especially when the questions to be addressed concern multiple receptor subtypes [for review, see Pilch and Czech (1984)]. Recent studies point to the possible existence of receptor subtypes for the endothelin/sarafotoxin family of peptides [Kloog & Sokolovsky, 1989; Kloog et al. (1989) and references cited therein]. This possibility was now examined by the cross-linking technique in three rat tissues: cerebellum, cortex, and caudate putamen. The ^{125}I derivatives of endothelin 1 (ET-1) and endothelin 3 (ET-3) were employed. Cross-linking was achieved by the use of two reagents, di-

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[1] Abbreviations: ET, endothelin; SRTX, sarafotoxin; DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidyl propionate).

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