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Profile of the α-bungarotoxin-binding regions on the extracellular part of the α-chain of *Torpedo californica* acetylcholine receptor

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The continuous α-neurotoxin-binding regions on the extracellular part (residues 1–210) of the α-chain of *Torpedo californica* acetylcholine receptor were localized by reaction of ^{125}I-labelled α-bungarotoxin with synthetic overlapping peptides spanning this entire part of the chain. The specificity of the binding was confirmed by inhibition with unlabelled toxin and, for appropriate peptides, with unlabelled anti-(acetylcholine receptor) antibodies. Five toxin-binding regions were localized within residues 1–10, 32–41, 100–115, 122–150 and 182–198. The third, fourth and fifth (and to a lesser extent the first and second) toxin-binding regions overlapped with regions recognized by anti-(acetylcholine receptor) antibodies. The five toxin-binding regions may be distinct sites or, alternatively, different 'faces' in one (or more) sites.

INTRODUCTION

Post-synaptic neuromuscular transmission effected by acetylcholine receptor (AChR) is mediated by ion flux across the cell membrane caused by binding of acetylcholine (Karlin, 1980; Conti-Tronconi & Raftery, 1982; McCarthy *et al.*, 1986; Changeux *et al.*, 1984; Hucho, 1986). The receptor is a pentamer composed of four subunits ($\alpha_2\beta\gamma\delta$). Several studies have shown that the α-subunit contains the acetylcholine-binding site(s) (Sobel *et al.*, 1977; Moore & Raftery, 1979; Tzartos & Changeux, 1983). The regulatory activity of this site(s) is inhibited by binding of a snake-venom α-neurotoxin [e.g. α-bungarotoxin (BgTX) or cobratoxin (CbTX)] to AChR (Changeux, 1981).

At least two toxin-binding regions have been reported in the α-chain of AChR (McCormick & Atassi, 1984; Mulac-Jeričevič & Atassi, 1986; Wilson *et al.*, 1985). One binding region resides within (but may not include all of) the synthetic peptides 125–147 of *Torpedo* AChR α-subunit (McCormick & Atassi, 1984) and 125–148 of the human AChR α-subunit (Mulac-Jeričevič & Atassi, 1986). Another binding region resides (Mulac-Jeričevič & Atassi, 1986) within the synthetic peptide α182–198. The present paper describes studies undertaken to localize the full profile of the continuous toxin-binding regions on the extracellular part of the α-subunit by employing synthetic overlapping peptides (Kazim & Atassi, 1980) that encompass the entire extracellular part (residues 1–210) of the α-chain of *Torpedo californica* AChR.

MATERIALS AND METHODS

Materials

α-Bungarotoxin (BgTX) was obtained from Miami Serpentorium Laboratories (Salt Lake City, UT, U.S.A.). The preparation of AChR from the electric-organ tissue of *Torpedo californica* has been described elsewhere (Froehner & Rafto, 1979; Mulac-Jeričevič *et al.*, 1987).

Peptides were prepared as described previously (Mulac-Jeričevič & Atassi, 1987) by solid-phase synthesis according to the known primary structure of AChR (Noda *et al.*, 1982). The peptides were purified by passage on columns of Sephadex G-25 followed by ion-exchange column chromatography on DEAE-Sephadex or CM-Sephadex (Koketsu & Atassi, 1973; Lee & Atassi, 1977), with a buffer selection based on the solubility, overall charge and stability of each peptide. After purification, the peptides were homogeneous by high-voltage paper electrophoresis and by h.p.l.c. and had amino acid compositions that agreed very well with those expected from their covalent structures (Mulac-Jeričevič & Atassi, 1987).

Binding of ^{125}I-labelled toxin to AChR and its peptides

The binding of toxins to AChR and its synthetic peptides was studied by quantitative solid-phase radiometric titrations. Preparation of protein and peptide adsorbents, labelling of proteins with ^{125}I and solid-phase radiometric titrations were carried out as previously described (Twining & Atassi, 1979). At least six different preparations of each adsorbent were studied. Protein and peptide adsorbents contained 0.9 ± 0.1 mg/ml and 0.45 ± 0.05 mg/ml of packed volume respectively. Binding studies were carried out in 0.1% bovine serum albumin in phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2) with fixed amounts of ^{125}I-labelled BgTX and various amounts of protein or peptide adsorbents. Also, studies were performed in which fixed amounts (25 μl of 1:1, v/v, suspension) of each adsorbent were titrated with increasing amounts of ^{125}I-labelled toxins. Binding studies to each peptide were performed six or more times, each in triplicate. In all these experiments, non-specific binding was determined by titrating, under identical conditions, equivalent volumes of uncoupled Sepharose CL-4B and Sepharose adsorbents of unrelated proteins (bovine serum albumin, hen's-egg lysozyme, sperm-

Abbreviations used: AChR, acetylcholine receptor; BgTX, α-bungarotoxin; CbTX, cobratoxin.

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whale myoglobin, human adult haemoglobin) and peptides [human haemoglobin synthetic peptides α1–15 (Kazim & Atassi, 1982) and α45–56 (McCormick & Atassi, 1985), myoglobin synthetic peptide 87–100 (Yoshioka *et al.*, 1983) and a nonsense peptide (Twining & Atassi, 1979)].

The specificity of the binding of ^{125}I-labelled BgTX to a given peptide was confirmed by inhibition experiments with unlabelled toxin as inhibitor. Portions (5 μl) of 1:1 (v/v) suspensions of peptide adsorbents in phosphate-buffered saline containing 0.1% bovine serum albumin were incubated (room temperature for 3 h) with increasing concentrations (1 nM to 10 μM) of unlabelled toxin, after which ^{125}I-labelled toxin (2×10^5 c.p.m., 1.2 nM) was added (total reaction volume 30 μl). The adsorbents were agitated gently for 14 h and were then washed with phosphate-buffered saline and their radioactivities counted. The amount of radiolabel bound in the presence of inhibitor was compared with that in uninhibited controls. The binding of toxin to certain peptides that are known (Mulac-Jeričevič *et al.*, 1987) to bind anti-AchR antibodies was also inhibited by these antibodies. For inhibition by anti-AChR antibodies, constant amounts of adsorbent (5 μl packed volume) were incubated at room temperature for 3 h with different dilutions (1:10 to 1:1000, v/v, in 0.1% bovine serum albumin in phosphate-buffered saline) of mouse anti-AChR sera. Labelled toxin (5×10^5 c.p.m.) was then added (total reaction volume 30 μl) and the suspension was incubated, with gentle agitation, at 4 °C for 16 h. The adsorbents were washed on the centrifuge five times with phosphate-buffered saline and their radioactivities then counted. The amount of inhibition by antibody was calculated relative to that in uninhibited controls.

RESULTS

Binding of ^{125}I-labelled BgTX to the peptides

Fig. 1 illustrates radiometric titrations of a constant amount of ^{125}I-labelled BgTX with increasing volumes of peptide adsorbents, as well as titrations of constant amounts of adsorbents with increasing amounts of ^{125}I-labelled BgTX. Peptide 182–198 possessed the highest binding activity with BgTX, being 4-fold higher than the next most active peptide, namely peptide 100–115. The following peptides also possessed considerable binding activities: 23–38, 34–49, 122–138 and 1–16. Lower, but significant, activities were exhibited by peptides 67–82, 134–150, 194–210 and 56–71. The remaining peptides showed little or no activity. It should be noted that adsorbents of unrelated proteins and peptides did not have any toxin-binding activity. Fig. 2 summarizes the binding results in a convenient bar diagram.

Inhibition of the binding of ^{125}I-labelled toxin by unlabelled toxin or by anti-AChR antibodies

To confirm the specificity of toxin binding to the peptides, the binding of ^{125}I-labelled toxin was inhibited by unlabelled toxin. Fig. 3 shows inhibition curves by unlabelled toxin of the binding of ^{125}I-labelled BgTX to the most active peptides (182–198, 100–115, 23–38 and 122–138). It can be seen that binding was inhibited by 75–100% in the concentration range of unlabelled toxin used. These inhibition studies gave the following IC_{50} values for BgTX: peptide 182–198, 0.05 μM; peptide 100–115, 0.8 μM; peptide 122–138, 2 μM; peptide 23–38, 7 μM.

The binding of ^{125}I-labelled BgTX to peptide adsorbents was also inhibited (by 85–100%) by anti-AChR

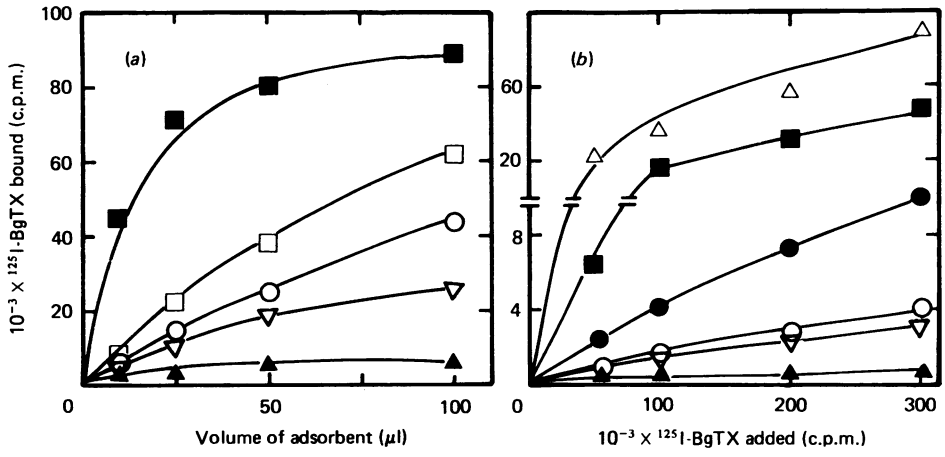

Fig. 1. Examples of radiometric titrations with ^{125}I-labelled BgTX

(*a*) illustrates titrations of various amounts of peptide adsorbents with a constant amount of ^{125}I-labelled BgTX. Increasing amounts of adsorbents were incubated, in triplicate, with a constant amount of ^{125}I-labelled BgTX (6×10^5 c.p.m.) for 16 h at 22 °C. After adsorbents had been washed five times with phosphate-buffered saline, the radioactivities of samples were counted on a γ-radiation counter. ■, Peptide 182–198; □, peptide 122–138; ○, peptide 23–38; ▽, peptide 34–49; ▲, average of the negative controls (bovine serum albumin, sperm-whale myoglobin, hen's-egg lysozyme, human adult haemoglobin, haemoglobin α-chain synthetic peptides 1–15 and 45–56, myoglobin synthetic peptide 87–100 and a nonsense peptide). The results varied ±2.8% or less. (*b*) Increasing amounts of labelled BgTX were added to a constant volume (25 μl of 1:1, v/v, suspension) of each adsorbent. After 16 h incubation at room temperature, adsorbents were washed five times with phosphate-buffered saline and their radioactivities were counted on a γ-radiation counter. △, AChR; ■, peptide 182–198; ●, peptide 100–115; ○, peptide 23–38; ▽, peptide 34–49; ▲, average of the negative controls. Note that the solvent in all these titrations was 0.1% bovine serum albumin in phosphate-buffered saline. The results varied ±3.1% or less.

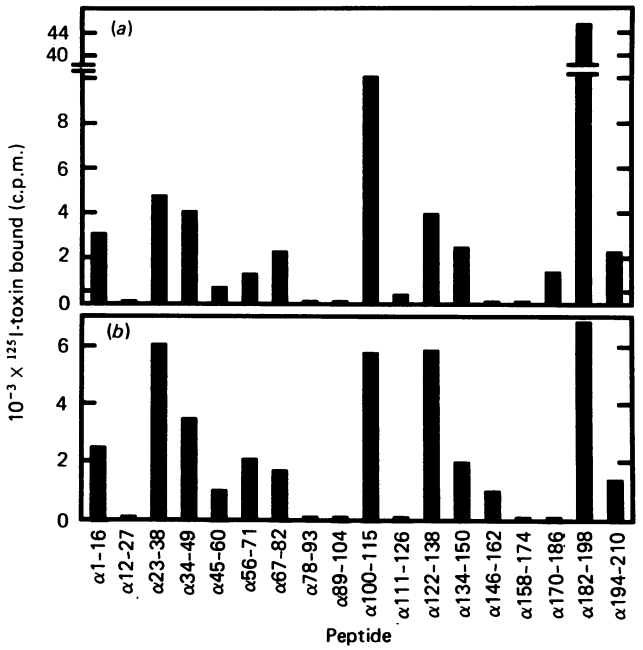

Fig. 2. Summary of the binding profiles of (*a*) BgTX and (*b*) CbTX to the synthetic overlapping peptides

The bars represent the maximum binding values to 25 μl of a 1:1 (v/v) suspension of each peptide adsorbent (see Fig. 1*b*). The results of the CbTX binding to the peptides were obtained from Mulac-Jeričevič & Atassi (1987).

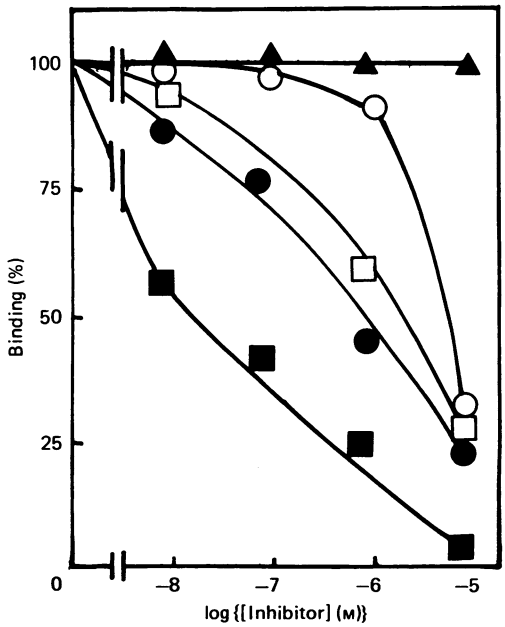

Fig. 3. Inhibition by unlabelled BgTX of the binding of ^{125}I-labelled BgTX to synthetic peptides

Peptide adsorbents (5 μl of 1:1, v/v, suspension) were incubated at room temperature for 3 h with increasing concentrations of unlabelled BgTX, followed by the addition of ^{125}I-labelled (2×10^5 c.p.m.) BgTx. After 14 h at room temperature, the adsorbents were washed five times with phosphate-buffered saline and their radioactivities were counted. ○, Peptide 23–38; ●, peptide 100–115; □, peptide 122–138; ■, peptide 182–198. The amounts of label bound in the absence of inhibitor were: peptide 23–38, 6340 c.p.m.; peptide 100–115, 5630 c.p.m.; peptide 122–138, 5140 c.p.m.; peptide 182–198, 8350 c.p.m. ▲, Average of the negative controls (see Fig. 1). Results represent the average of six replicates, which varied $\pm 1.9\%$ or less.

antibodies (Fig. 4). Non-immune sera or antisera against unrelated proteins did not exhibit any inhibitory activity, thus confirming that the inhibition by anti-AChR antibodies was specific.

DISCUSSION

Recent studies have indicated that there are at least two neurotoxin binding regions on the α-subunit of AChR (McCormick & Atassi, 1984; Wilson *et al.*, 1985; Mulac-Jeričevič & Atassi, 1986; Neumann *et al.*, 1986*a*). One toxin-binding region resides around the residues Cys-128 and Cys-142, and its binding activity has been demonstrated in the synthetic disulphide-loop peptides 125–147 of *Torpedo* AChR α-chain (McCormick & Atassi, 1984) and 125–148 of human AChR α-chain (Mulac-Jeričevič & Atassi, 1986). The other toxin-binding region resides within the synthetic peptide 182–198 of *Torpedo* AChR α-chain (Mulac-Jeričevič & Atassi, 1986). In the present work we have employed uniform overlapping synthetic peptides, comprising the extracellular part (residues 1–210) of the α-chain of *Torpedo* AChR, to screen systematically for the profile of the toxin-binding regions on this part of the α-chain. The radiometric quantitative adsorbent titration procedure (Twining & Atassi, 1979) is an effective method to study direct ligand binding to peptides (Twining & Atassi, 1978, 1979; Kazim & Atassi, 1981, 1982; Yoshioka & Atassi, 1986*a*,*b*,*c*; Atassi, 1978), even those that are slightly soluble or insoluble in aqueous solvents (Atassi & Webster, 1983; Atassi, 1985). The application and appropriate conditions for this procedure have been discussed elsewhere (Twining & Atassi, 1979; Atassi *et al.*, 1979).

The results of BgTX binding to the 18 peptides showed that the binding activity of the α-chain resides predominantly in five regions localized within peptides 1–16 (I), 23–38/34–49 overlap (II), 100–115 (III), 122–138/134–150 overlap (IV) and 182–198 including its overlap with peptide 194–210 (V). It is also likely, although difficult to determine at present, that the slight amounts of labelled toxin bound by peptides 56–71 and 67–82 represent significant binding and that a sixth region in that part of the α-chain contributes slightly (with very weak binding energy) to binding of toxin by AChR. From the homologies, within these regions of α-subunits of AChR from other species (Fig. 5), regions I and II can be further narrowed down to fall within residues 1–10 and 32–41 respectively. The activities of peptide pairs 23–38/34–49, 122–138/134–150 and 182–198/194–210 indicated that the overlap between a peptide pair contains some of the essential contact residues of the respective binding region. On the other hand, because the binding of peptide 182–198 was much higher than that of peptide 194–210, the binding region must extend to the left of the overlap (i.e. to the left of residue 194 rather than to the right of residue 198). Therefore peptide 182–198, like peptides 1–16 and 100–115, carries an intact binding region, whereas the peptides in the pairs 23–38/34–49

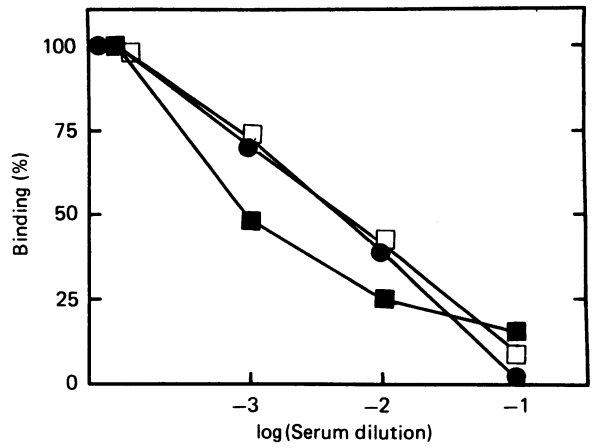

Fig. 4. Inhibition by mouse anti-AChR antibodies of the binding of ^{125}I-labelled BgTX to the synthetic peptides

Inhibition experiments were carried out as described for Fig. 3, except that various dilutions (1:10, 1:100 and 1:1000, v/v) of anti-AChR antisera were used as inhibitor. ●, Peptide 100–115; □, peptide 122–138; ■, peptide 182–198. The amounts of label bound in the absence of inhibitor were: peptide 100–115, 6500 c.p.m.; peptide 122–138; 14840 c.p.m.; peptide 182–198, 18580 c.p.m. Note that pre-immune sera and antisera against unrelated proteins and peptides did inhibit the binding of toxin to the peptides. Results represent the average of six replicates, which varied ±2.2% or less.

Species	Binding region I	
	1	16
(A)	SEHETRLVANLLEDYN	
(B)	SEHETRLVAKLFKDYS	
(C)	SEHETRLVAKLFEDYN	
(D)	SEHETRLVAKLFEDYS	
(F)	SEAEHRLFQYLFEVYN	

	Binding region II	
	23	49
(A)	EHHTHFVDITVGLQLIQLISVDEVNDI	
(B)	EDHRQVVEVTVGLQLIQLINVDEVDQI	
(C)	EDHRQAVEVTVGLQLIQLINVDEVNQI	
(D)	EDHRQIVQVTVGLQLIQLINVDEVNQI	
(F)	ANVSHPVIIQFEVSMSQLVKVDEVNQI	

	Binding region III	
	100	115
(A)	FAIVHMTKLLLDYTGK	
(B)	FAIVKFTKVLLQYTGH	
(C)	FAIVKFTKVLLDYTGH	
(D)	FAIVKFTKVLLDYTGH	
(E)	FAIVKYTKVLLEHTGK	
(F)	FQVDDKTKALLKYTGE	

	Binding region IV	
	122	150
(A)	AIFKSYCEIIVTHFPFDQQNCTMKLGTWT	
(B)	AIFKSYCEIIVTHFPFDEQNCSMKLGTWT	
(C)	AIFKSYCEIIVTHFPFDEQNC-MKLGTWT	
(D)	AIFKSYCEIIVTHFPFDEQNC-MKLGTWT	
(E)	AIFKSYCEIIVTYFPFDQQNCSMKLGTWT	
(F)	AIFKSSCKIDVTYFPFDYQNCTMKFGSWS	

	Binding region V	
	182	198
(A)	RGWKHWVYYTCCPDTPY	
(B)	RGWKHSVTYSCCPDTPY	
(C)	RGWKHWVFYACCPSTPY	
(D)	RGWKHWVFYSCCPTTPY	
(E)	RGWKHWVYYACCPDTPY	
(F)	PGYKHEIKYNCCEE-IY	

Fig. 5. Sequence comparison of the toxin-binding regions of the α-chain of *Torpedo californica* (A) with the corresponding regions of the α-chains of (B) *Homo sapiens*, (C) *Bos taurus* striated-muscle, (D) *Mus musculus*, (E) *Gallus domesticus* erythrocyte and (F) neuronal *Rattus norvegicus* neuronal ACRRs

From the homologies among the various α-subunits within sequences 1–16 and 23–49, binding regions I and II can be further narrowed down to reside within residues 1–10 and 32–41 respectively.

and 122–138/134–150 each carry only a part of a binding region. The activity of the peptide pair 122–138/134–150 confirms previous findings that peptide α125–147 contains a toxin-binding region (McCormick & Atassi, 1984; Mulac-Jeričevič & Atassi, 1986). To define the precise boundaries of the sites, the activity of a series of synthetic peptides spanning different overlaps within each of these indicated regions will have to be studied (Koketsu & Atassi, 1973, 1974; McCormick & Atassi, 1985).

The present finding that AChR has a number of binding regions is consistent with the proposal (Walkinshaw *et al.*, 1981) that neurotoxins have 'multiple binding sites' to AChR. Regions 182–198 and 125–148 possess the highest affinity for BgTX. Support for the involvement of the region around cysteine residues 192 and 193 in the binding of toxin is now accumulating (Wilson *et al.*, 1985; Mulac-Jeričevič & Atassi, 1986; Neumann *et al.*, 1986*a*). It should also be noted that site-directed mutagenesis experiments (Mishina *et al.*, 1985) have shown that *Torpedo* AChR mutants with serine replacements at positions α128 or α142 showed very little or no BgTX-binding activity and failed to respond to acetylcholine. Other AChR mutants with serine replacements at positions α192 or α193 had nearly normal BgTX-binding activity but were not responsive to acetylcholine (Mishina *et al.* 1985). In addition, affinity labelling experiments have shown that both regions can be labelled with 4-(*N*-maleimido) benzyltrimethylammonium iodide (Cahill & Schmidt, 1984; Kao *et al.*, 1984). These findings are in agreement with our results (McCormick & Atassi, 1984; Mulac-Jeričevič & Atassi, 1986) that both regions are involved in toxin binding. In contrast, other workers (Criado *et al.*, 1986; Neumann *et al.*, 1986*b*) have reported results that do not agree with these findings concerning the activity of region 125–148. In both cases, the peptides used by these workers [α127–143 (Criado *et al.*, 1986) and α126–143 (Neumann *et al.*, 1986*b*) of *T. californica* AChR] were several residues shorter relative to the *Torpedo* (α125–147; McCormick & Atassi, 1984) and human (α125–148; Mulac-Jeričevič & Atassi, 1986) peptides previously studied. The peptide was coupled (Criado *et al.*, 1986) to protein carrier by diazotization, a reaction that would be expected to cause drastic chemical modification of many residues in the peptide (Hornishi *et al.*, 1964; Takenaka *et al.*, 1969, 1970; Sokolovsky & Vallee, 1967; Andres & Atassi, 1973) and alteration of its antigenicity (Andres & Atassi, 1973). Antibodies against such an antigen would have lower reactivity with the unmodified species of the peptide, and consequently with the corresponding region on AChR. Nevertheless, three of these monoclonal antibodies showed low (18–25%) inhibition of the binding of toxin to AChR (Criado *et al.*, 1986). This is not insignificant, particularly in view of the fact that the

affinity of toxin to AChR is three to four orders of magnitude higher than the affinity of the average antibody and that AChR has other toxin-binding regions and therefore antibodies to one region may not inhibit toxin binding completely.

Since the toxin-binding activity of AChR resides in the α-chain (Moore & Raftery, 1979; Tzartos & Changeux, 1983) it is useful to compare the primary structures of α-subunits from various species to determine the extent of homology in the aforementioned five toxin-binding regions. Fig. 5 compares these regions in the α-subunits of *Torpedo californica* (Noda *et al.*, 1982), *Homo sapiens* (Noda *et al.*, 1983*b*), *Bos taurus* striated-muscle (Noda *et al.*, 1983*a*), *Mus musculus* (Boulter *et al.*, 1985), *Gallus domesticus* erythrocyte (Ballivet *et al.*, 1983) and *Rattus norvegicus* neuronal AChRs (Boulter *et al.*, 1986). It can be seen that the binding regions are very highly conserved in the α-subunits from the various species, thus preserving the capacity to bind toxin. The substitutions, most of which are conservative in nature, will most probably modulate the affinity of AChR for toxin. It is noteworthy that the neuronal AChR α-chain from *Rattus norvegicus* shows numerous substitutions in the toxin-binding regions localized here. Some small regions of homology occur in toxin-binding region IV within residues 120–126 (seven residues) and 135–139 (five residues). Significantly, α-neurotoxins do not block the function of this neuronal AChR (Boulter *et al.*, 1986; Patrick & Stallcup, 1977). Clearly, the binding of toxin to the receptor is impaired at four of the five potential toxin-binding regions. Therefore this neuronal receptor will not be expected to bind α-neurotoxin, or, if binding occurred, it will be weak and it will take place via region 120–139 of the neuronal receptor.

Sequence comparisons of the α-chain toxin-binding regions to the corresponding regions on the β-, γ- and δ-chains of *Torpedo californica* AChR (Noda *et al.*, 1983*a*) are shown in Fig. 6. It can be seen that, although these chains share considerable homology with the α-chain, very little of this homology occurs within the indicated toxin-binding regions. This would explain why the other three AChR subunits do not bind neurotoxin.

It is important to note that the toxin-binding regions III, IV and V, and to a lesser extent regions I and II, overlap regions recognized by anti-AChR [especially anti-(membrane-bound AChR)] antibodies (Lennon *et al.*, 1985; Mulac-Jeričevič & Atassi, 1986; Mulac-Jeričevič *et al.*, 1987). On the other hand, in research studies and in diagnostic tests, binding of anti-AChR antibodies is often examined with a complex of AChR with ^{125}I-labelled BgTX or CbTX. The overlap of the toxin-binding regions with antigenic sites would prevent binding and thus detection of some anti-AChR antibodies to this complex, depending on the affinity of the antibodies relative to that of the toxin. The use of this method is not recommended.

The folding of the AChR subunits has received considerable attention, and at least three models have been proposed. The first model (Noda *et al.*, 1983*a*; Claudio *et al.*, 1983) proposed four transmembrane regions. A second model (Guy, 1984; Finer-Moore & Stroud, 1984) added a fifth amphipathic transmembrane region. Both these models visualized the residues 1–210 as being extracellular, but differed in the assignment of the intracellular piece and the *C*-terminal segment (residues α427–439). A third model (Criado *et al.*, 1985)

Subunit	Binding region I																
	1																16
α	S	E	H	E	T	R	L	V	A	N	L	L	-	E	N	-	Y N
β	S	V	M	E	D	T	L	L	S	V	L	F	-	E	T	-	Y N
γ	E	M	E	E	G	R	L	I	E	K	L	L	-	G	D	-	Y D
δ	V	N	E	E	E	R	L	I	N	D	L	L	I	V	N	K	Y N
	Binding region II																
	23																49
α	E	H	H	T	H	F	V	D	I	T	V	G	L	Q	L	I	Q L I S V D E V N Q I
β	Q	T	V	G	D	K	V	T	V	R	V	G	L	T	L	T	N L L I L N E K I E E
γ	K	T	L	D	H	I	I	D	V	T	L	K	L	T	L	T	N L I S L N E K E E A
δ	K	H	N	N	E	V	V	N	I	A	L	S	L	T	L	S	N L I S L K E T D E T
	Binding region III																
	100																115
α	F	A	I	V	H	M	T	K	L	L	L	D	Y	T	G	K	
β	F	E	I	T	L	H	V	N	V	L	V	Q	H	T	G	A	
γ	F	E	V	A	Y	Y	A	N	V	L	V	Y	N	D	G	S	
δ	Y	H	V	A	Y	F	C	N	V	L	V	R	P	N	G	Y	
	Binding region IV																
	122																150
α	A	I	F	K	S	Y	C	E	I	I	V	T	H	F	P	F	D Q Q N C T M K L G I W T
β	A	I	Y	R	S	S	C	T	I	K	V	M	Y	F	P	F	D W Q N C T M V F K S Y T
γ	A	I	Y	R	S	T	C	P	I	A	V	T	Y	F	P	F	D W Q N C S L V F R S Q T
δ	A	I	F	R	S	S	C	P	I	N	V	L	Y	F	P	F	D W Q N C S L K F T A L N
	Binding region V																
	182																198
α	R	G	W	K	H	-	W	V	Y	-	Y	T	C	C	P	D	- T P Y
β	K	P	S	R	K	N	W	-	-	R	S	D	-	D	P	-	- S - Y
γ	R	P	A	K	K	N	Y	N	N	Q	L	T	K	D	-	D	- T D F
δ	K	P	A	K	K	N	-	I	Y	-	P	D	K	F	P	N	G T N Y

Fig. 6. Sequence comparisons of the α-chain toxin-binding regions with the corresponding regions on the β-, δ- and γ-chains of *Torpedo californica* AChR

proposed seven transmembrane segments with the residues 142–192 forming two more transmembrane amphipathic regions. The strongest neurotoxin-binding regions on the α-subunit, as reported in the present paper, reside within this segment, which also has regions that are recognized by anti-AChR [in particular by anti-(membrane-bound AChR)] antibodies (Mulac-Jeričevič *et al.*, 1987) and by AChR-primed T cells (Yokoi *et al.*, 1987). These findings would require that most, if not all, of the segment 142–192 be extracellular, with significant parts of it being exposed. Thus our findings would not support the model that proposes seven transmembrane regions (Criado *et al.*, 1985) and are compatible with the other two models, but do not permit the selection of one of them.

It is noteworthy that the five toxin-binding regions occur on separate exons of the genomic DNA and that none of these regions is divided between two exons. It should also be noted that there is no relationship between the five toxin-binding regions and their hydrophobicity or hydrophilicity character (plots not shown). Finally, it is perhaps necessary to stress that it remains unknown whether the five toxin-binding regions, localized here, form distinct binding sites or are 'faces' of one binding site, the binding energy of each face being sufficient to form a fruitful complex with toxin. This could be resolved by knowledge of the three-dimensional structure of AChR.

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