

DTIC FILE COPY

①

AD-A230 342

CONTRACT NO.: DAMD17-89-C-9061

TITLE: MOLECULAR RECOGNITION OF ALPHA-NEUROTOXINS

PRINCIPAL INVESTIGATOR: ZOUHAIR M. ATASSI

PI ADDRESS: Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

REPORT DATE: November 30, 1990

TYPE OF REPORT: Midterm

DTIC
SELECTED
JAN 03 1991
S E D

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited		
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION Baylor College of Medicine		6b. OFFICE SYMBOL (if applicable)		7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) One Baylor Plaza Houston, Texas 77030			7b. ADDRESS (City, State, and ZIP Code)		
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (if applicable)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-89-C-9061	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21702-5012			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO. 61102A	PROJECT NO. 3M1- 61102BS12	TASK NO. AA
11. TITLE (Include Security Classification) (U) Molecular Recognition of Alpha-Neurotoxins					
12. PERSONAL AUTHOR(S) Zouhair M. Atassi					
13a. TYPE OF REPORT Midterm		13b. TIME COVERED FROM 3/15/89 TO 9/14/90		14. DATE OF REPORT (Year, Month, Day) 1990 November 30	
15. PAGE COUNT 27					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) Synthetic peptides; RA 1; Alpha-neurotoxins		
FIELD	GROUP	SUB-GROUP			
06	03				
06	01				
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian			22b. TELEPHONE (Include Area Code) 301-663-7325		22c. OFFICE SYMBOL SGRD-RMI-S

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

all 29 MZA Where copyrighted material is quoted, permission has been obtained to use such material.

all 29 NA Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

all 29 MZA Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

all 29 MZA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

all 29 Clay 12-05-90
PI - Signature DATE

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By _____	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

TABLE OF CONTENTS

	Page
FRONT COVER	1
DD Form 1473 Report Documentation page	2
FOREWORD	3
TABLE OF CONTENTS	4
A. DETERMINATION OF THE REGION-TO-REGION CONTACTS IN ACETYLCHOLINE RECEPTOR-BUNGAROTOXIN INTERACTIONS AND MODELING OF THE RECEPTOR CAVITY	5
A1. Introduction	5
A2. Body	5
A2.1. Experimental Procedure	5
A2.2. Results	6
A2.3. Discussion	6
A3. Conclusions	8
A4. References	8
A5. Appendix	10
B. THE SHORT NEUROTOXIN BINDING REGIONS ON THE α -CHAIN OF HUMAN AND <i>TORPEDO CALIFORNICA</i> ACETYLCHOLINE RECEPTORS	17
B1. Introduction	17
B2. Body	17
B2.1. Experimental Procedure	17
B2.2. Results	18
B3.3. Discussion	18
B3. Conclusions	20
B4. References	21
B5. Appendix	23

SCIENTIFIC REPORT

The work under this contract is being carried out on different fronts of α -neurotoxin function and immunology, with the ultimate goal being the achievement of protection against toxin by synthetic toxin peptides. In order to facilitate the organization of the data, the latter are subdivided into appropriate sections.

A. DETERMINATION OF THE REGION-TO-REGION CONTACTS IN ACETYLCHOLINE RECEPTOR-BUNGAROTOXIN INTERACTIONS AND MODELING OF THE RECEPTOR CAVITY.

A1. Introduction

The nicotinic acetylcholine receptor (AcChoR) effects postsynaptic neuromuscular transmission by permitting ion flux across the cell membrane in response to binding of acetylcholine (1,2). The α -chain of AcChoR contains the acetylcholine binding site(s) (3-5). The regulatory effect of acetylcholine is inhibited by the binding of an α -neurotoxin to AcChoR. The toxin-binding site(s) also resides in the α -subunit of AcChoR (6). Recent studies from this laboratory using synthetic uniform-sized overlapping peptides encompassing the entire extracellular parts of the α -chain of human AcChoR and Torpedo AcChoR enabled the localization of the full profile of the toxin binding regions on the Torpedo (7-9) and human (10) receptors. Conversely, the binding sites for AcChoR on α -bungarotoxin (Bgt) were mapped by synthetic peptides representing each of the Bgt loops (11,12).

In these investigations, we have developed a new approach for studying the details of protein-protein recognition. Each of the active peptides of one protein is allowed to interact with each of the active peptides of the other protein. Based on relative binding affinities of peptide-peptide interactions the position of AcChoR peptides relative to the Bgt molecule was assessed. The receptor peptides were docked onto the appropriate regions of Bgt, whose 3-D structure is known (13), by computer graphics and energy minimization, thus allowing a three dimensional model to be constructed of the binding site cavity for the toxin on human AcChoR.

A2. Body

A2.1. Experimental Procedure

Synthesis, purification and characterization of the human AcChoR α -chain peptides have been reported (10). The α -neurotoxin binding regions on human AcChoR reside (10) in the peptides shown in Fig. 1. These peptides were employed in the present work. The receptor-binding regions on Bgt are present in the three loop peptides shown in Fig. 1 (12). The synthesis, purification and characterization of the monomeric forms of the three cyclic peptides (Fig. 1) have been described (12). Bgt and its synthetic peptides were labeled with iodine-125 using the chloramine-T method (14). Radioiodinated materials were used immediately after labeling. The specific activities of the labeled peptides were: LIN, 3.1×10^5 cpm/p mole; L2, 2.4×10^5 cpm/p mole; L3E, 2.1×10^5 cpm/p mole.

The coupling of proteins and peptides to CNBr-activated Sepharose CL-4B was carried out under optimum conditions as described previously (15). The binding of 125 I-labeled Bgt or Bgt peptides to adsorbents of the human AcChoR peptides was determined by a quantitative solid-phase radiometric binding assay (12,15,16). All titrations were carried out in PBS containing 0.1% bovine serum albumin. Non-specific binding was determined by titrating equivalent volumes of uncoupled Sepharose and Sepharose coupled to unrelated proteins (bovine serum albumin, hen lysozyme) and peptides [synthetic peptides of myoglobin and a nonsense peptide (ESSGTGISSGTGI) (15)] under identical conditions. Dissociation constants of the binding of 125 I-labeled Bgt or its peptides to each of the receptor peptides were calculated by Scatchard analysis (17) from titrations employing a fixed amount (10 μ l) of adsorbent suspension (1:1, vol/vol) in PBS/0.1% bovine serum albumin and increasing amounts of labeled ligand (Fig. 2). The specificity of the binding of the Bgt peptides was confirmed by their inhibitory activity towards the binding of 125 I-labeled Bgt to adsorbents of the receptor peptides.

For structural prediction algorithms, the computer program ALB (18) was employed. Standard conditions (pH 7.0, ionic strength 0.15 M, dielectric constant 78.5 and temperature 300 K) were used to predict the globular structure of the entire human AcChoR α chain from sequence information. The secondary structures for the peptides were then chosen based on this calculation. Model building was done by the graphics program PSFRODO (19) on an Evans-Sutherland PS300 Graphics System connected to a VAX 8550 computer. The conformations of the predicted peptide secondary structures were constructed by replacing residues in similar peptides obtained from refined structures to match perfectly with the composition of the target peptides. The replaced residues were then regularized by using the REFINE option in PSFRODO. The resulting structures of the receptor peptides were docked with the appropriate loop(s) in the refined Bgt structure (13). Initial docking was done to agree with the results of receptor peptide-Bgt loop binding studies. Hydrogen bond and positive Van der Waal's interactions were then maximized. Structural refinement of the receptor peptide segments was then accomplished by energy minimization, using the computer program Yeti (20), while maintaining the X-ray coordinates for Bgt.

A2.2. Results

The results of titrations of fixed amounts of human AcChoR peptide adsorbents with varying amounts of ^{125}I -labeled Bgt or its active (i.e. receptor-binding) loop peptides are summarized in Fig. 2. It was found that each of the receptor peptides 34-49, 100-115 and 122-138 bound more than one Bgt peptide. Conversely, a given Bgt loop peptide bound to more than one receptor peptide. Thus, the receptor peptides exhibited the following binding activities: peptide 34-49 bound Bgt and its peptide L2 and LIN; peptide 100-115 bound Bgt and its peptides L2, L3E and LIN; peptide 122-138 bound Bgt and peptides L2 and LIN; peptide 194-210 bound Bgt and effectively only peptide LIN. However, the affinities of the toxin peptides that bound to a given AcChoR peptide differed. The dissociation constants (K_d) of the binding of ^{125}I -labeled Bgt or its synthetic peptides to the AcChoR peptides are summarized in Table 1. In the binding to the AcChoR peptide 34-49, the K_d value for the Bgt peptide L2 was about 7.3 times smaller than that of peptide LIN and the K_d of Bgt was 6.5 times smaller than that of L2. In binding to the receptor peptide 100-115, the K_d values were: Bgt < L2 = L3E < LIN. For binding to AcChoR peptide 122-138 the K_d values increased in the following order: Bgt < L2 < LIN. Finally, only toxin peptide LIN (and of course Bgt itself) bound to the AcChoR peptide 194-210.

The specificity of binding of the synthetic Bgt peptides to the AcChoR peptides was confirmed by inhibition experiments. The binding of ^{125}I -labeled Bgt to an adsorbent of a given AcChoR peptide was inhibited by the appropriate Bgt loop peptide, or by an equimolar mixture of the relevant Bgt peptides (Fig. 3). The experiments showed that incubation of inhibitor peptides with receptor peptides 34-49 and 100-115 for 4 hrs gave IC_{50} values of 8.0×10^{-7} and 1.5×10^{-6} M, respectively. When the incubation time was extended to 14 hrs, the IC_{50} values were improved to 5×10^{-7} M for peptide 34-49 and 2×10^{-6} M for peptide 100-115. The IC_{50} values of the peptides 122-138 and 194-210 were not improved by increasing the incubation time from 4 hrs to 14 hrs. The appropriate optimum interaction time was, therefore, used for each of the AcChoR peptides. Under these optimum conditions, the following inhibition results of Bgt binding were obtained: binding to receptor peptide 34-49 was inhibited completely by an equimolar mixture of Bgt peptides LIN and L2 (IC_{50} , 5×10^{-7} M); to peptide 100-115 by an equimolar mixture of Bgt peptides L2 and L3E (IC_{50} , 2×10^{-6} M) and to the peptide 122-138 by Bgt peptide L2 only (IC_{50} , 2×10^{-6} M). Finally, Bgt binding to receptor peptide 194-210 was inhibited only by toxin peptide L3 (IC_{50} , 5×10^{-6} M). It should be noted that an unrelated nonsense peptide, bovine serum albumin and hen lysozyme had no inhibitory effect (Fig. 3) on this binding, even when employed at concentrations of 10^{-4} M, thus further confirming the specificity of the interactions of the toxin peptides with the AcChoR peptides.

A2.3. Discussion

The recent mapping of the full profiles of the α -neurotoxin binding regions on the α -chains of *Torpedo californica* (7-9) and human (10) AcChoR by synthetic overlapping peptides encompassing the entire extracellular parts of each of these subunits revealed a complex toxin-binding site on each receptor.

The *Torpedo* receptor has five toxin-binding regions. In the human receptor, four of these regions retain the ability to bind Bgt and cobratoxin while the binding activity of the fifth region (residing within residues 1-16) is lost because of adverse amino acid replacements (10). The four toxin-binding regions of human

AcChoR reside within, but do not necessarily include all of, the α -chain peptides 34-49, 100-115, 122-138 and 194-210. Previously, adsorbents of peptide 100-115 were found (10) to have low capacity for Bgt. Exhaustive analysis carried out in the present work with several different ^{125}I -labeled Bgt preparations confirmed both the previously-reported (10) low binding activity of AcChoR peptide 100-115 and the profile of the α -neurotoxin binding regions on the α -chain of human AcChoR. However, in spite of the low capacity for Bgt of peptide 100-115 adsorbents, Bgt binds to peptide 100-115 with high affinity (Table 1). The binding activity of peptide 100-115 was highly dependent on the labeled Bgt preparation. Perhaps, radioiodinations may have resulted in varying degrees of modification on Bgt of residues that are essential for binding to the receptor. Modification reactions that can occur during radioiodination of proteins by the chloramine-T method have been reviewed (21). By sequence comparison of the α -chains of AcChoR from different species, the α -neurotoxin binding regions were further narrowed down (10,22) to the following residues: 32-41, 100-110, 125-136, and 194-208. These assigned regions were used in the present structural prediction and model building studies.

The AcChoR binding regions on Bgt have been recently mapped using synthetic peptides corresponding to the various loops and most of the surface areas of the toxin (12). It was found that Bgt has three main AcChoR-binding regions within the loop peptides (Fig. 1): LIN (residues 1-16), L2 (residues 26-41, with an artificial disulfide bond between the two ends of the peptide, see ref. 12) and L3E (residues 45-59).

The binding studies reported here between Bgt peptides and receptor peptides revealed that there are extensive contacts between the two molecules. Each of the AcChoR peptides was bound to the intact Bgt molecule with greater affinity than to any of its loop segments. One interpretation of this would imply that the AcChoR segments have interactions with more than one loop. The major interaction would be with the loop which displayed the higher affinity. Indeed, except for the Bgt loop L3E, each of the other Bgt loops that participates in the binding makes contact with more than one peptide region on the receptor. Conversely, each active AcChoR peptide, except for peptide 194-210, makes contact with more than one Bgt loop. The relative importance of these contacts to complex formation has been ordered arbitrarily on the basis of the binding affinities displayed by a given peptide towards the peptides of the other molecule (Table 1). It is assumed that the greater the affinity (i.e., the smaller the K_d), the better the fit. Thus, from Table 1, receptor peptide 34-49 would make better contact with Bgt peptide L2 than with LIN. Similarly, Bgt peptide L2 interacts better with both receptor peptides 34-49 and 100-115 than with peptide 122-138.

Knowledge of the regions involved in the interactions between two protein molecules and cross-binding studies between the correlate synthetic peptides should provide binding affinity data indicative of specific interactions between the native molecules. In addition, if the three-dimensional structure of one molecule is known, then the binding areas formed by the other molecule can be appropriately fitted to yield a tentative three-dimensional description of its binding site. The 3-D structure of the Bgt molecule as determined by X-ray crystallography (13) displays surface features that can be utilized in combination with peptide binding studies to construct a possible active site model for AcChoR.

Before applying this approach to modeling the AcChoR binding cavity for Bgt, it was necessary to test the approach on two interacting polypeptides both having known three-dimensional structures, i.e., the α and β subunits of human adult Hb. Previously, using synthetic uniform-sized overlapping peptides encompassing the entire Hb subunits, we have determined the regions involved in the α - β subunit interacting surfaces in solution (23) and compared them with those expected from the X-ray structure of Hb (24). Four regions of the β chain of Hb (β 10-18, β 25-32, β 74-86 and β 100-118), which bind to the Hb α -chain in solution (23), were fitted by the approach described in the Methods section onto the appropriate regions of the α -chain, using the known three-dimensional structure of the α -chain within the Hb molecule (24). The calculated structures of the α -carbon backbone of the three β -chain regions (β 10-18, β 25-32 and β 100-118) were in good agreement with those expected for these regions from the known three-dimensional structure of Hb (Figure 4). However, for the region β 74-86, the calculated structure for the free peptide in solution showed reversed polarity and very poor resemblance to the shape expected for this region within the X-ray derived structure of Hb (Figure 4). This divergence could be due to the freedom of the peptide in solution and might indeed reflect its true conformation when the free peptide is allowed to bind to the α -chain of Hb. Also, it should be noted that the orientation of the side chains could not be reliably determined by this method.

With this limitation in mind, the approach was employed to derive a tentative model for the three-dimensional backbone structure of the Bgt-binding cavity on AcChoR. Based on affinity of binding considerations, and with the knowledge of the X-ray coordinates of Bgt at 2.5Å resolution (13), the AcChoR binding peptides were fitted onto the appropriate regions of Bgt. While the modeled AcChoR structure is not a unique solution, it represents an energetically favorable model that satisfies both the binding data and structural constraints. The main interactions are summarized region by region in Fig. 5. The binding site is shown in a stereo drawing in Fig. 6, with and without the Bgt backbone. The site comprises a deep conical cavity (30.5Å in depth), the dimensions of which are indicated in Fig. 6. The binding between Bgt and AcChoR involves several areas of contact on both molecules. It is important to point out that one of these areas of contact on the receptor (region 125-136) resides in the acetylcholine binding site (25). Since the affinity of α -neurotoxin to the receptor is several orders of magnitude higher than that of acetylcholine, the binding of toxin will be expected to prevent that of acetylcholine (and thus disrupt receptor function) completely, even in the presence of a large excess of the latter.

A3. Conclusions

In previous studies from this laboratory, the binding regions of α -neurotoxins on human and torpedo acetylcholine (AcCho) receptors and the binding regions for the receptor on the toxin were characterized with synthetic peptides of the respective molecules. In the present work, peptides representing the active regions of one molecule are each allowed to bind to each of the active region peptides of the other molecule. Thus, the interaction of three α -bungarotoxin (Bgt) synthetic loop peptides with four synthetic peptides representing the toxin-binding regions on human acetylcholine receptor (AcChoR) permitted the determination of the region-region interactions between α -bungarotoxin and the human receptor. Based on the known three-dimensional structure of the toxin, the active peptides of the receptor were then assembled to their appropriate toxin-contact regions by computer model building and energy minimization. This allowed the three-dimensional construction of the toxin-binding cavity on human acetylcholine receptor. The cavity appears to be conical, 30.5Å in depth, involving several receptor regions which make contact with the Bgt loop regions. One AcChoR region (within residues 125-136) involved in the binding to Bgt also resides in a known AcCho binding site, thus demonstrating in three dimensions a critical site involved in both AcCho activation and Bgt blocking. The validity of this approach was first established for 3 of 4 peptides corresponding to regions on the β -chain of human hemoglobin involved in binding to the α -chain. Thus, studying the interaction between peptides representing the binding regions of two protein molecules may provide a novel approach in molecular recognition by which the binding site on one protein can be described if the three-dimensional structure of the other protein is known.

A4. References

1. McCarthy, M.P., Earnest, J.P., Young, E.T., Choe, S. & Stroud, R.M. (1986) *Annu. Rev. Neuroscience* **9**, 383-413.
2. Changeux, J.-P., Devillers-Thiery, A. & Chemouilli, P. (1984) *Science* **225**, 1335-1345.
3. Sobel, A., Weber, M. & Changeux, J.-P. (1977) *Eur. J. Biochem.* **80**, 215-224.
4. Moore, H.P. & Raftery, M.A. (1979) *Biochemistry* **18**, 1862-1867.
5. Tzartos, S.J. & Changeux, J.-P. (1983) *EMBO J.* **2**, 381-387.
6. Lee, C.Y. (1979) *Adv. Cytopharmacol.* **3**, 1-16.
7. Mulac-Jericevic, B. & Atassi, M.Z. (1986) *FEBS Letters* **199**, 68-74.
8. Mulac-Jericevic, B. & Atassi, M.Z. (1987) *J. Protein Chemistry* **6**, 365-373.

9. Mulac-Jericevic, B. & Atassi, M.Z. (1987) *Biochem. J.* **248**, 847-852.
10. Mulac-Jericevic, B. & Atassi, M.Z. (1988) *J. Protein Chemistry* **7**, 173-177.
11. McDaniel, C.S., Manshour, T. & Atassi, M.Z. (1987) *J. Protein Chemistry* **6**, 455-461.
12. Atassi, M.Z., McDaniel, C.S. & Manshour, T. (1988) *J. Protein Chemistry* **7**, 655-666.
13. Love, R.A. & Stroud, R.M. (1986) *Protein Engineering* **1**, 37-46.
14. Hunter, W. & Greenwood, F. (1962) *Nature* **194**, 495-496.
15. Atassi, M.Z., Yoshioka, M., Bean, M. & Bixler, G.S. (1987) *Biochem. J.* **246**, 307-312.
16. Kazim, A.L. & Atassi, M.Z. (1980) *Biochem. J.* **185**, 285-287.
17. Scatchard, G. (1949), *Ann. New York Academy Sciences* **51**, 660-672.
18. Ptitsyn, O.B. & Finkelstein, A.V. (1983) *Biopolymers* **22**, 15-25.
19. Pflugrath, J.W., Saper, M.A. & Quiocho, F.A. (1984) in *Methods and Applications in Crystallographic Computing*, eds. Hall, S.R. and Ashida, T., (Clarendon Press, Oxford), pp. 404-410.
20. Vedani, A. (1988) *J. Comput. Chem.* **9**, 269-280.
21. Atassi, M.Z. (1977) in *Immunochemistry of Proteins*, ed. Atassi, M.Z., (Plenum Press, New York and London), Vol. 1, pp. 1-161.
22. Atassi, M.Z., Mulac-Jericevic, B., Yokoi, T. & Manshour, T. (1987) *Fed. Proc.* **46**, 2538-2547.
23. Yoshioka, N. & Atassi, M.Z. (1986) *Biochem. J.* **234**, 457-461.
24. Fermi, G., Perutz, M.F., Shaanan, B. & Fourme, R. (1984) *J. Molecular Biology* **175**, 159-174.
25. McCormick, D.J. & Atassi, M.Z. (1984) *Biochem. J.* **224**, 995-1000.

A5. Appendix

Table 1. Dissociation constants of the binding of ^{125}I -labeled Bgt and its peptides to the human AcChoR peptides.

Labeled ligand	Kd (M) of binding to human AcChoR peptides*			
	Peptide <u>34-49</u>	Peptide <u>100-115</u>	Peptide <u>122-138</u>	Peptide <u>194-210</u>
Bgt	8.6×10^{-9}	4.7×10^{-9}	2.2×10^{-8}	4.0×10^{-8}
Peptides				
LIN	4.1×10^{-7}	2.3×10^{-7}	1.0×10^{-6}	2.0×10^{-7}
L2	5.6×10^{-8}	6.2×10^{-8}	2.9×10^{-7}	+
L3E	+	6.9×10^{-8}	+	+

*Dissociation constants of the binding of ^{125}I -labeled Bgt or its active synthetic loop peptides to adsorbents of each of the four active human AcChoR peptides were determined in the text.

+ No binding is obtained between these two peptides.

A5. Appendix

A. SYNTHETIC PEPTIDES OF ACHR α -CHAIN WHICH ARE INVOLVED IN BINDING TO BGT

Peptide 34-49	34 G-L-Q-L-I-Q-L-I-N-V-D-E-V-D-Q-I(G) 49
Peptide 100-115	100 F-A-I-V-K-E-T-K-V-L-L-Q-Y-T-G-H(G) 115
Peptide 122-138	122 A-I-F-K-S-Y-C-E-I-I-V-T-H-F-P-F-D(G) 138
Peptide 194-210	194 P-D-T-P-Y-L-D-I-T-Y-H-F-V-M-Q-R-L(G) 210

B. SYNTHETIC LOOP PEPTIDES OF BGT WHICH ARE INVOLVED IN BINDING TO ACHR

L1N	3 I-V-C-H-T-T-A-T-I-P-S-S-A-V-T-C-(G) 16 S
L2	26 C-K-M-W-A-D-A-F-T-S-S-R-G-K-V-V-E-C-G(G) 41 S
L3E	45 A-A-T-C-P-S-K-K-P-Y-E-E-V-T-C-(G) 59 S

FIG. 1. Covalent structures of the synthetic peptides employed in this work. (A) The peptides of the AcChoR α -chain which are involved in binding to α -neurotoxins (10). (B) The peptides of the Bgt molecule, which are involved in binding to AcChoR (11,12). Note that the disulfide bonds in L1N and L2 are artificial (for details, see ref. 12). The binding areas on the AcChoR peptides have been assigned (10) to the underlined regions 32-41, 100-110, 125-136 and 198-208. Note that the glycine residues in parentheses are not part of the Bgt or the AcChoR sequences but the peptides were synthesized on a Gly-resin for convenience.

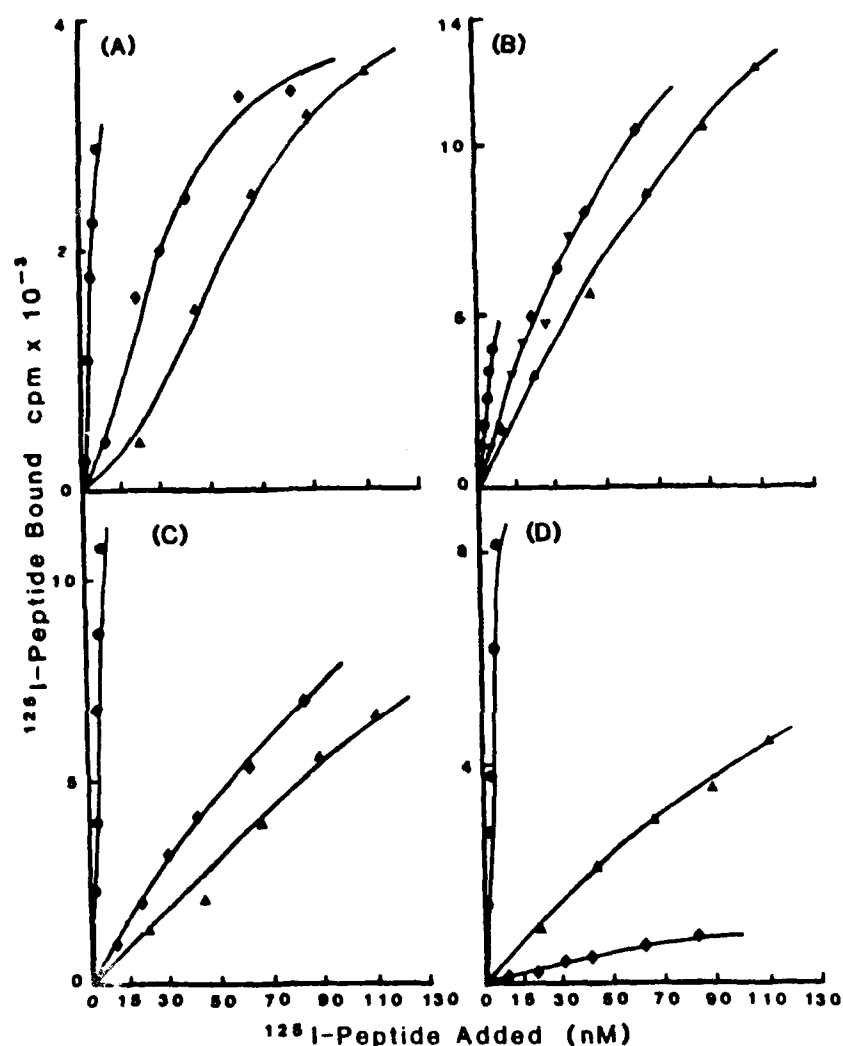


FIG. 2. Concentration-dependent binding of ^{125}I -labeled Bgt and its receptor-binding synthetic peptides to each of the Bgt-binding peptides of human AcChoR. Fixed amounts (10 μl) of suspension (1:1, vol/vol) of each AcChoR peptide adsorbent, were incubated with increasing amounts of ^{125}I -labeled Bgt or its peptides LIN, L2 and L3E at room temperature for 14 hours. The binding was done in a reaction volume of 40 μl in 0.01 M sodium phosphate buffer which was 0.15 M with respect to NaCl (PBS) and 0.1% with respect to bovine serum albumin. After reaction, the adsorbents were washed four times with PBS, transferred to clean tubes and their radioactivity counted on a Beckman 4000 gamma counter. (A) Binding to peptide 34-49; (B) binding to peptide 100-115; (C) binding to peptide 122-138; (D) binding to peptide 194-210. ▲, LIN; ◆, L2; ▼, L3E; ●, Bgt.

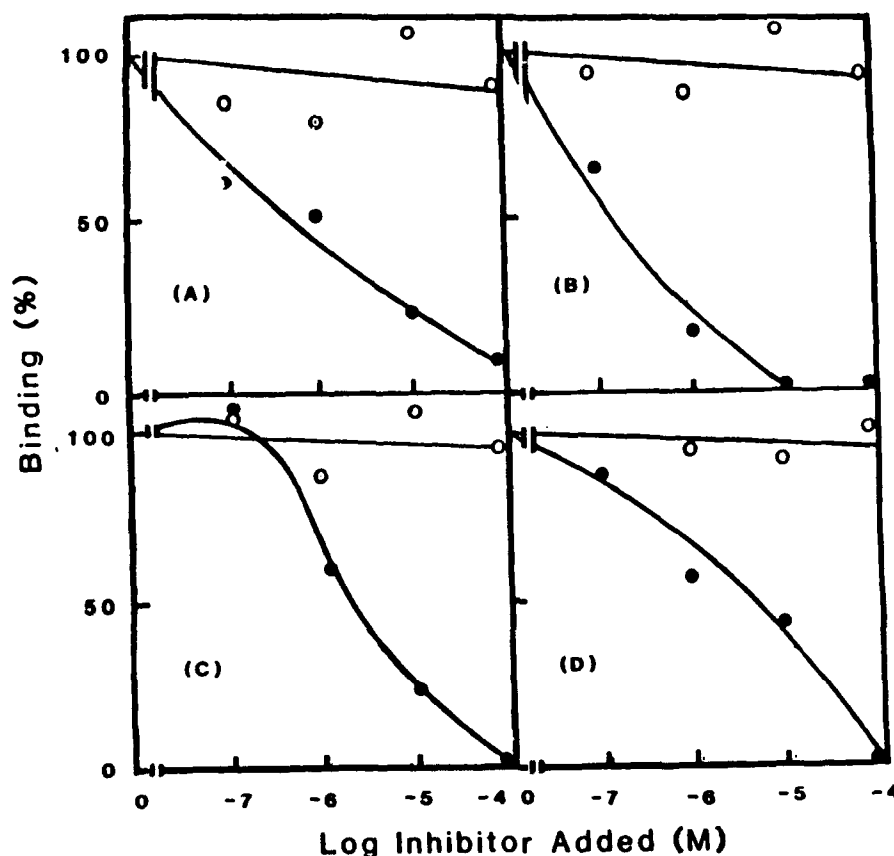


FIG. 3. Inhibition of the binding of ^{125}I -labeled Bgt to AcChoR peptides by the synthetic Bgt peptides. Fixed amounts ($10\ \mu\text{l}$) of suspension (1:1 vol/vol) of each AcChoR peptide adsorbent were mixed with $10\ \mu\text{l}$ 0.2% bovine serum albumin in PBS (4°C , overnight). Aliquots ($10\ \mu\text{l}$) containing increasing amounts of the appropriate unlabeled Bgt peptide (or controls) in PBS-0.1% bovine serum albumin were added and the mixture incubated with gentle agitation at room temperature for four hours. ^{125}I -labeled Bgt (2.5×10^5 cpm) was then added and the binding reaction allowed to take place with gentle agitation at room temperature (4 hours for peptides 122-138 and 194-210, or 14 hours for peptide 34-49 and 100-115). The binding was done in PBS-0.1% bovine serum albumin in a final reaction volume of $40\ \mu\text{l}$. The adsorbents were then washed and counted as described in Figure 2. (A) Inhibition of the binding of ^{125}I -labeled Bgt to peptide 34-49 by an equimolar mixture of Bgt peptides LIN and L2; (B) inhibition of Bgt binding to peptide 100-115 by an equimolar mixture L2 and L3E; (C) inhibition of the binding to peptide 122-138 by peptide L2; (D) inhibition of the binding to peptide 194-210 by peptide LIN. ●, Inhibition by the Bgt peptides, ○, control inhibitors which included hen egg lysozyme and an unrelated nonsense peptide with amino acid sequence, ESSGTGIESSGTGI.

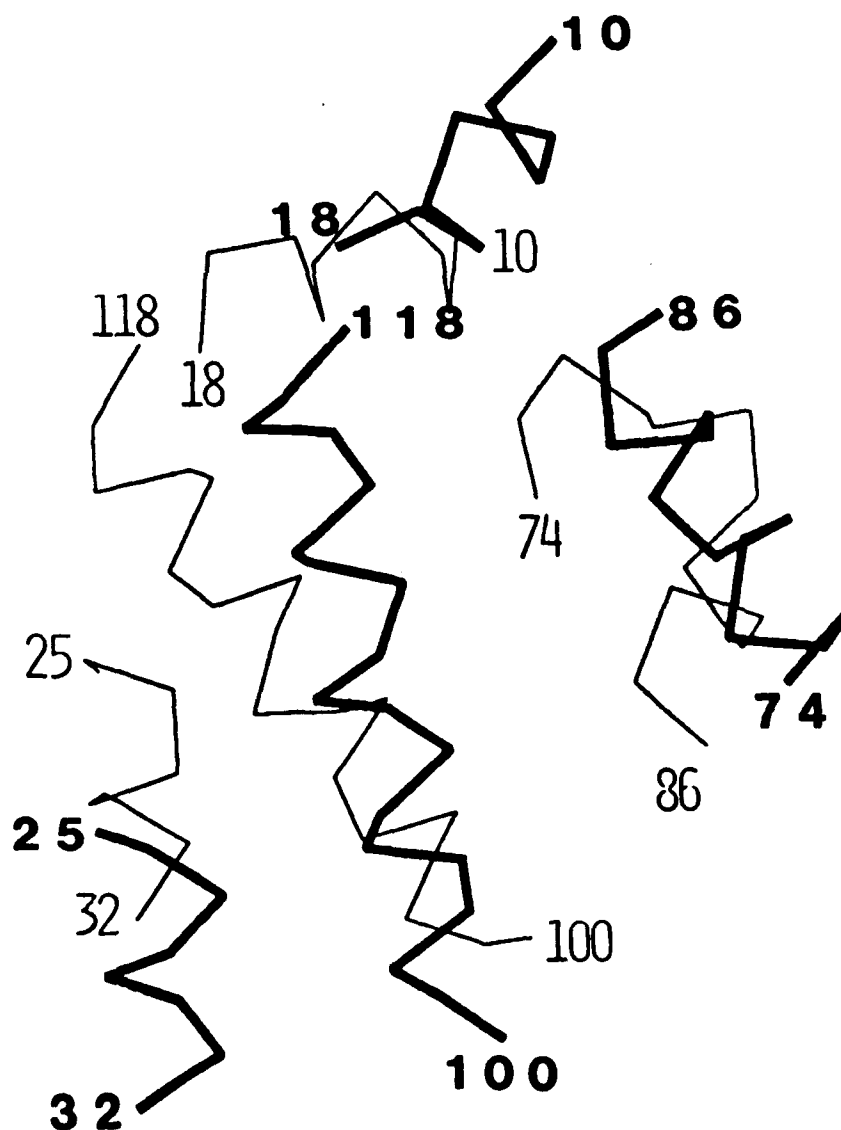


FIG. 4. Computer-graphic drawings of the calculated (heavy lines) α -carbon backbones of human hemoglobin β -chain peptide regions β 10-18, β 25-32, β 74-86 and β 100-118 [which have been shown to bind in solution to intact α -chain of Hb (23)]. The β -chain regions were fitted by the approach described in the Methods section onto the appropriate regions of the α chain using its known 3-D structure within Hb. The calculated conformations are compared with those expected (thin lines) for these β -chain regions within Hb, as obtained from the X-ray structure of the crystalline Hb tetramer.

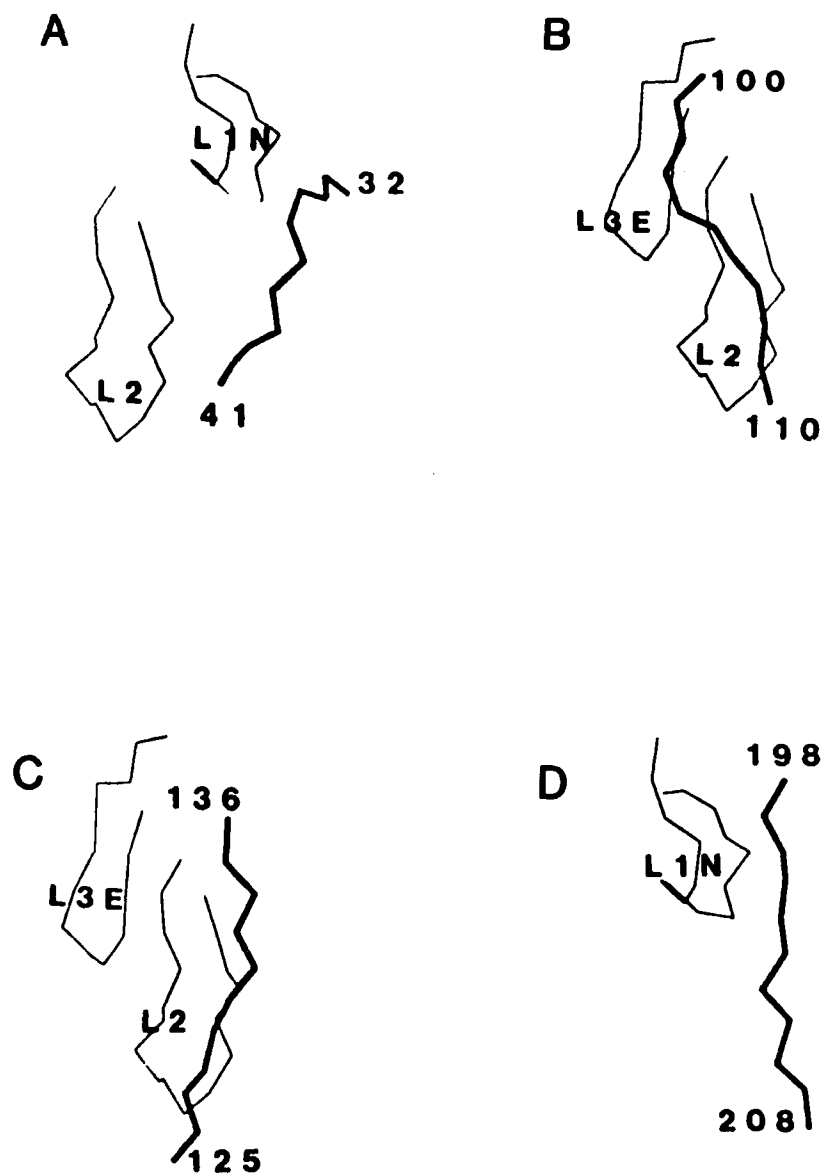


FIG. 5. A computer-graphics drawing of the α -carbon backbones showing the association of the various AcChoR regions (heavy lines) with the appropriate part(s) of the three-dimensional structure of Bgt. (A) Receptor region 32-41 bound to Bgt loops LIN and L2; (B) receptor region 100-110 bound to Bgt loops L2 and L3E; (C) receptor region 125-136 bound to loop L2; (D) receptor region 198-208 bound to loop LIN.

A5. Appendix

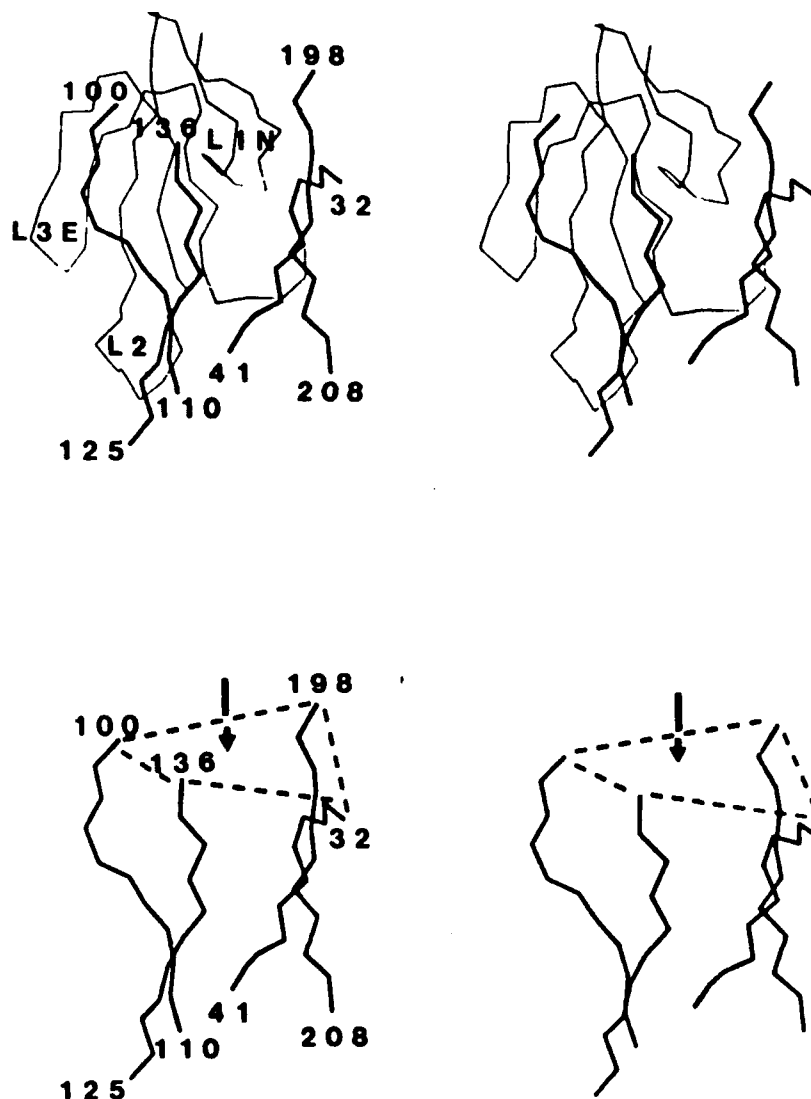


FIG. 6. A stereo drawing of a three-dimensional construction of the toxin-binding cavity in AcChoR, with the Bgt molecule (backbone only) bound in the cavity (upper diagram), and without the Bgt molecule (lower diagram). The somewhat conical cavity has the following dimensions: residues 100-to-136, 21.32Å; residues 136-to-32, 35.01Å; residues 32-to-198, 16.06Å; residues 198-to-100, 22.13Å. The depth of the cavity is 30.48Å.

B. THE SHORT NEUROTOXIN BINDING REGIONS ON THE α -CHAIN OF HUMAN AND *TORPEDO CALIFORNICA* ACETYLCHOLINE RECEPTORS

B1. Introduction

The nicotinic acetylcholine receptor (AChR) plays a central role in postsynaptic neuromuscular transmission by mediating ion flux across the cell membrane in response to binding of acetylcholine (1-4). The binding of an α -neurotoxin to AChR blocks postsynaptic neuromuscular transmission by inhibiting the channel-opening activity of acetylcholine (5-7). Snake venom postsynaptic neurotoxins form a large family of homologous proteins, of which two subgroups, the long and short neurotoxins, are major constituents (see Discussion). Both long and short neurotoxins are known to bind specifically to the α chain of AChR in a competitive manner with cholinergic ligands (8-10), but display differences in their association and dissociation kinetics. Identification of the binding sites on the toxins and the receptor should provide a molecular explanation for the observed differences between the two toxin groups in their actions on AChR.

By application of a comprehensive synthetic peptide strategy (11), we have recently reported the localization of the full profile of the continuous binding regions for long α -neurotoxin on the extracellular part (residues α 1-210) of the α -chains of *Torpedo* (12,13) and human AChRs (14,15). In *Torpedo* AChR, the binding-regions reside within (but may not include all of) residues α 1-10, α 32-49, α 100-115, α 122-138 and α 182-198. In human AChR, long neurotoxins bind to regions: α 32-49, α 100-115, α 122-138 and α 194-210. In the present work, the two panels of *Torpedo* and human AChR synthetic overlapping peptides were employed to localize short neurotoxin-binding regions on the extracellular part of α chains of both two respective species of AChR. Comparison between the short and long neurotoxin-binding regions on AChR revealed important differences in the AChR-toxin contacts, particularly with *Torpedo* AChR.

B2. Body

B2.1. Experimental Procedure

Cobrotoxin and erabutoxin b were prepared by Dr. Bruce Meade (Fort Detrick, Frederick, MD). Cobrotoxin (Cot) was isolated from the venom of Formosan Cobra (*Naja naja atra*) as described (16,17). Erabutoxin b (Eb) was prepared from the venom of *Laticauda semifasciata* by the procedure of Tamiya and his co-workers (18). Crude venoms were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The preparations were monitored by polyacrylamide (15%) gel electrophoresis in SDS. The authenticity of the pure toxin preparations was confirmed by their amino acid compositions and partial N-terminal sequence analysis. The amino acid composition of each neurotoxin and the sequence of its first 15 amino acid residues were in excellent agreement with those expected from its respective reported covalent structure (17,19). The LD₅₀ values (3 μ g/28-30g mouse) were very similar to the reported values (18). α -Bungarotoxin (Bgt) and cobratoxin (Cbt) were obtained from Miami Serpentarium Laboratories (Salt Lake City, UT, USA).

The preparation of AChR from the electric organ tissue of *Torpedo californica* (Pacific Bio-Marine Laboratories, Venice, CA, USA) was carried out as described elsewhere (20). The four-subunit composition ($\alpha_2\beta_2$) of pure AChR and the binding activity of its α -chain were confirmed by SDS-PAGE and Western blotting (21). Freshly prepared AChR had a Bgt-binding activity of 8.7-9.1 nmoles/mg AChR. The peptides (Fig. 1), which corresponded to the extracellular part (residues 1-210) of the α -chains of *Torpedo* and human AChR (22,23) were synthesized, purified and characterized as previously described (12,14).

The toxins were labeled with iodine-125 by using the chloramine-T method (24). Radioiodinated materials were used immediately after labeling. The specific activities of the labeled toxins were: Cot, 2.5×10^5 cpm/p mol; and Eb, 3.3×10^5 cpm/p mol. The coupling of proteins and peptides to CNBr-activated Sepharose CL-4B was carried out under optimum conditions as described (25). At least three preparations of each adsorbent were studied. Protein and peptide contents of the adsorbents were determined by duplicate amino acid analysis of acid hydrolysates. The adsorbents contained 0.8 ± 0.1 mg/ml and 0.43 ± 0.04 mg/ml of packed volume, respectively.

Quantitative adsorbent titrations were performed in phosphate-buffered saline (0.15M-NaCl in 0.01 M-sodium phosphate buffer, pH 7.2) containing 0.1% BSA with fixed amounts of ^{125}I -labelled toxin and various amounts of protein or peptide adsorbents. Titrations were also performed using fixed amounts (25 μl of 1:1 v/v, suspension) of each adsorbent with increasing amounts of ^{125}I -labelled toxin. Binding studies were done at room temperature for 16 hrs. with gentle rocking, after which the tubes were washed four times on the centrifuge with PBS and then counted on a gamma counter. The studies on each panel of peptides were done three times, each in triplicate. Non-specific binding was determined by titrating, under identical conditions, equivalent volumes of uncoupled Sepharose CL-4B and Sepharose adsorbents of unrelated proteins (BSA, horse myoglobin) and synthetic peptides of similar size [sperm whale myoglobin synthetic peptides 1-17, 25-41 and 121-137 (26)]. The specificity of binding ^{125}I -labeled toxin to fixed amounts (5 μl , packed volume) of peptide adsorbents was confirmed by inhibition studies using various amounts of unlabeled toxin (13) as inhibitor. Unrelated proteins [BSA, myoglobin and the aforementioned myoglobin synthetic peptides (Bixler and Atassi, 1983)] were used as control inhibitors.

B2.2. Results

The binding profiles of ^{125}I -labeled short neurotoxins to *Torpedo* AChR peptides are summarized in Fig. 2. The results showed that the main binding activity for both neurotoxins, Cot and Eb, resided within region α 122-138. A lower binding activity was exhibited by the peptides α 23-38/ α 34-49 overlap and α 100-115. Peptides α 1-16 and α 194-210 had low binding activity only with Cot and little or no activity with Eb. On the other hand, peptide α 45-60 showed low, but significant binding activity to Eb, whereas its binding to Cot was considerably lower. Finally, ^{125}I -short neurotoxins bound, as expected, to *Torpedo* AChR (positive control), but not to any unrelated proteins and peptides (negative controls).

The finding here that the region α 182-198 of *Torpedo* AChR did not show any significant binding to short neurotoxins was unexpected in view of the fact that this constitutes a major binding region to long neurotoxins (12,13,27). To further confirm this major difference in the binding site for long and short neurotoxins on *Torpedo* AChR, quantitative radiometric titrations were carried out using a constant amount of peptide adsorbents (25 μl , 1:1 v/v suspension in PBS) and increasing amounts of ^{125}I -labeled short (Cot and Eb) or long (Bgt) neurotoxins. The peptide α 122-138, which binds both long and short neurotoxins equally well, was used as a positive control. The results (Fig. 3) showed that the long neurotoxin, Bgt, bound to both peptides α 122-138 and α 182-198, clearly confirming that the binding activity of the peptide α 182-198 had not been destroyed in the present adsorbent preparation. This same preparation did not bind the short neurotoxins Cot and Eb (Fig. 3), while peptide α 122-138 was fully capable of binding these two toxins. Finally, adsorbents of unrelated proteins and peptides did not bind any of these toxins, thus confirming the specificity of the aforementioned binding results.

The binding results of the short neurotoxins to human AChR peptides are summarized in Fig. 4. The main binding activity for both toxins resided within peptide α 122-138. A lower binding activity was present in the overlap α 23-38/ α 34-49/ α 45-60 and in peptide α 194-210. Peptides α 100-115 and α 56-71 showed strong and medium binding activities, respectively, to Eb, but low activity to Cot. Peptide α 1-16 had a low binding activity only with Cot and showed negligible binding to Eb. Finally, these two toxins did not bind to unrelated proteins and peptide controls.

B2.3. Discussion

Venoms of snakes from the *Elapidae* and *Hydrophiidae* families possess proteins having very pronounced pharmacological activities (28,29). Some members of this family are potent cytotoxins while others are presynaptic or postsynaptic neurotoxins. The postsynaptic neurotoxins are divided into short and long neurotoxins. Both classes of toxins are known to bind specifically (8-10,30) and tightly (31) to the nicotinic acetylcholine receptor. This binding is, in a competitive manner, linked to the binding of cholinergic ligands (one of which is the physiological native channel-opening molecule, acetylcholine). However, unlike the cholinergic ligands, binding of neurotoxins to AChR does not lead to opening but rather to relatively permanent closure of the channel. The extremely tight, non-covalent association between receptor and neurotoxins (dissociation constant range, 10^{-9}M to 10^{-11}M) in comparison to that of acetylcholine (10^{-6}M) makes them useful tools with which to investigate the function of the neuromuscular synapse and its

receptors.

Short and long neurotoxins have very similar dissociation constants with AChR (32,33) (10^{-10} to 10^{-11} M) and LD₅₀ values (typically for mice, between 50 and 150 μ g/kg). They differ chiefly in their rates of association and dissociation from the receptor. Long neurotoxins generally associate and dissociate much more slowly (31,32). These differing rates are reflections of major sequence differences between the two types of toxins.

The primary structure of short neurotoxins is composed of 60, 61 or 62 amino acid residues all of which are intramolecularly cross-linked by four disulfide bridges. One short neurotoxin, erabutoxin b, has been crystallized and its X-ray structure has been determined (34-38). The disulfide bonds of this short neurotoxin are localized at one end of the molecule and accordingly, produce a knotted structure with a globular head and three protruding loops. The predominant secondary structural characteristic is β -sheet with β -turns located at the chain reversals. Most of the invariant residues are either localized in the immediate vicinity of the disulfide bridge in the globular head or are found toward the distal ends of the three major loops. In contrast, the least conserved residues tend to be grouped across the top of the globular head.

Long neurotoxins also have the four disulfide bridges of short neurotoxins but possess an additional disulfide bond in the central loop of the molecule. In addition, apart from insertions and deletions within the main chain itself, long neurotoxins have a longer polypeptide chain (between 65 and 74 residues) giving rise to a characteristic C-terminal tail. The 3-D structures of two long neurotoxins (α -cobratoxin and α -bungarotoxin) have been determined and the overall structure is highly similar to that of erabutoxin b (39). Apparently, where there are differences in sequence or chain length, these alterations do not disrupt the clustering of the disulfide bridges or the major loops. There are proportionately fewer conserved or invariant residues in the long neurotoxins. However, there are marked similarities in and around the disulfide bridges and in the loops. In long neurotoxins, the least conserved regions tend to be found in the C-terminal tail and the first loop.

The application of a comprehensive synthetic approach, previously introduced in this laboratory (11,40), enabled the mapping of the full profile of binding regions for long neurotoxins on the extracellular part of the α chains of *Torpedo californica* (12,13) and human (14) AChR. Determination of the binding regions for short neurotoxins on *Torpedo* and human AChR should, therefore, permit the comparison of the binding regions for the two classes of toxin on a given AChR and provide a rationale for the differences in their binding kinetics.

AChR of *T. californica* has five regions on its α subunit which are involved in the binding to long neurotoxins (α -bungarotoxin and cobra toxin) (12,13,27). These regions reside within, but may not include all of, residues α 1-16, the overlap α 23-38/ α 34-49, α 100-115, the overlap α 122-138/ α 134-150, α 182-198. In the human receptor the affinity to long neurotoxins is decreased, relative to *Torpedo* AChR. The binding activities of peptides α 1-16 and α 182-198 are lost because of adverse amino acid replacements (Fig. 1) (14). A low binding activity is retained by the human peptide α 194-210. The main difference, however, in the binding of long neurotoxins to the overlapping peptides of *T. californica* and human AChR, is the great decrease in the contribution of peptide α 182-198 to the binding of the human receptor. It has been found (41) that Bgt binds to human and *T. californica* AChR with the same forward rate constant (1.8×10^5 M⁻¹ Sec⁻¹). But there were remarkable differences in the dissociation of the toxin from human and *T. californica* receptors. The dissociation time constant was 6 hrs for the human receptor from intact TE671 human medulloblastoma cells and 24 hrs for membrane-associated *Torpedo* receptor. The differences in the reversibility of long neurotoxins binding to *Torpedo* and human AChR must be due to the contribution of region α 182-198 to binding in *Torpedo* AChR and the absence of this contribution in human AChR.

Binding studies with whole human AChR and short neurotoxins have not been performed. Reversibility studies of neuromuscular blockade by long and short neurotoxins were done with species other than human and *Torpedo* (33). With rat phrenic nerve preparations, the short neurotoxins Eb and Cot were slowly reversible, while Bgt was not. Binding of Cot to the sciatic nerve sartorius muscle preparation of the frog (*Rana tigrina*) was reversible, while that of Bgt was irreversible. This clearly indicates that the sequence differences between short and long neurotoxins are reflected in their binding properties to AChR. In the present work, the main difference in the binding of long and short neurotoxins to the overlapping peptides

of *T. californica* AChR, lies in the behavior of peptide α 182-198. This peptide possessed the highest binding activity of all the *T. californica* peptides for long neurotoxins, but showed little or no binding to short neurotoxins. The inability of the region α 182-198 in both human and *Torpedo* AChR to bind short neurotoxins and in human AChR to bind long neurotoxins confirms the previous conclusions (14) that the region α 182-198 may not play a significant role in neuromuscular blockage. It may be concluded that the differences in reversibility between long and short neurotoxins are due to the inability of short neurotoxins to bind to the contact region within residues α 182-198 of AChR. Thus, the participation, or otherwise, of region α 182-198 in the neurotoxin binding may explain the differences in the association and dissociation rates between long and short neurotoxins.

The AChR binding regions on Bgt were recently mapped using synthetic peptides corresponding to the various loops and most of the surface areas of the toxin (42). It was found that Bgt has three AChR binding regions within the loop peptides LIN, L2 and L3E (Fig. 5). Comparison of the loop sequences in the short neurotoxins, Cot and Eb, with the corresponding regions in the long neurotoxins, Bgt and Cbt (Fig. 5), will help to explain some of the differences in their binding behaviors towards the region α 182-198 of *Torpedo* AChR. The consensus sequences exhibited several amino acid replacements within the three loops, but loop LIN exhibited a much higher number of amino acid replacements than loops L2 and L3E. This could indicate that loop LIN of the long neurotoxins is their main contact with region α 182-198 of the *Torpedo* AChR. Short neurotoxins are unable to bind to peptide α 182-198 of *Torpedo* AChR probably because of several adverse amino acid replacements in loop LIN (Fig. 5).

With the peptide panel of a given AChR, there were differences in the binding profiles of Cot and Eb (Figures 2 and 4). The main difference was that peptide α 1-16 in both receptors bound to Cot but not to Eb. Sequence comparison of Cot and Eb showed several amino acid differences within the AChR-binding loops (Fig. 5). These replacements may explain the slight differences in the binding profiles of the two short neurotoxins. It should also be noted that some quantitative differences were observed between human and *Torpedo* AChR peptides in the binding to a given short neurotoxin. For example, Cot showed considerably lower binding to *Torpedo* peptide α 45-60 than to the corresponding human peptide (Figures 2 and 4). This region has two amino acid replacements (Thr-51 $\rightarrow$ Glu and Lys-57 $\rightarrow$ Arg) in *Torpedo* AChR relative to human receptor (Fig. 1). The decrease in binding is most likely due to the adverse effect resulting from the creation of a negatively charged side chain at position 51. On the other hand, Eb showed lower binding to *Torpedo* peptides to α 34-49, α 56-71, α 100-115 and α 194-210 than to the corresponding human peptides (Figures 2 and 4). These differences are caused by the amino acid substitutions in these regions (Fig. 1). The quantitative differences in the effects of these substitutions on the binding of the two short neurotoxins should also be influenced by sequence differences between Eb and Cot (Fig. 5).

The Bgt-binding cavity on human AChR was recently derived from peptide-to-peptide binding studies of the human receptor peptides and the aforementioned Bgt synthetic loops, followed by modeling (15). The region α 122-138, which is a main universal long neurotoxin binding region on AChR α -chain from various species (14), forms a face (subsite) within the toxin-binding cavity. This region also carries essential contact residues of the acetylcholine binding site (43). The present studies have shown that this region possessed the highest binding activity to the short neurotoxins. Clearly, this region is the main universal binding site for both long and short neurotoxins. It is important to note that the affinity of neurotoxins to the receptor is several orders of magnitude higher than that of acetylcholine. Therefore, the binding of toxin will be expected to prevent that of acetylcholine (and thus disrupt receptor function) completely, even in the presence of a large excess of the latter.

B3. Conclusions

The continuous regions for short neurotoxin-binding on the α -chains of *Torpedo californica* and human acetylcholine receptors (AChR) were localized by reaction of 125 I-labeled cobrotoxin (Cot) and erabutoxin b (Eb) with synthetic overlapping peptides spanning the entire extracellular part of the respective α chains. On *Torpedo* AChR, five Cot-binding regions were found to reside with peptides α 1-16, α 23-38/ α 34-49 overlap, α 100-115, α 122-138 and α 194-210. The Eb-binding regions were localized within peptides α 23-38/ α 34-49/ α 45-60 overlap, α 100-115 and α 122-138. The main binding activity for both toxins resided

within region α 122-138. In previous studies, we had shown that the binding of long α -neurotoxins [α -bungarotoxin (Bgt) and cobratoxin (Cbt)] involved the same regions on *Torpedo* AChR as well as an additional region within residues α 182-198. Thus region α 182-198, which is the strongest binding region for long neurotoxins on *Torpedo* AChR, was not a binding region for short neurotoxins. On human AChR, peptide α 122-138 possessed the highest activity with both toxins and lower activity was found in the overlap α 23-38/ α 34-49/ α 45-60 and in peptide α 194-210. In addition, peptides α 100-115 and α 56-71 showed strong and medium binding activities to Eb, but low activity to Cot, while peptide α 1-16 exhibited low binding to Cot and no binding to Eb. Comparison with previous studies indicated that, for human AChR, the binding regions of short and long neurotoxins were essentially the same. The finding that the region within residues α 122-138 of both human and *Torpedo* AChR possessed the highest binding site for long and short neurotoxins on AChR from various species.

B4. References

1. Changeux, J.P., Devillers-Thiery, A. and Chemouilli, P. (1984) *Science* **225**, 1335-1345.
2. Conti-Tronconi, B.M. and Raftery, M.A. (1982) *Annu. Rev. Biochem.* **51**, 491-530.
3. Karlin, A. (1980) Poste, G., Nicolson, G.L. and Colman, C.W., Editors. Cell surface and neuronal function. Elsevier/North-Holland: New York, 191-260.
4. McCarthy, M.P., Earnest, J.P., Young, E.T., Choe, S. and Stroud, R.M. (1986) *Ann. Rev. Neurosci.* **9**, 383-413.
5. Popot, J.L. and Changeux, J.P. (1984) *Physiol. Rev.* **64**, 1162-1239.
6. Stroud, R. and Finer-Moore, J. (1985) *Annu. Rev. Cell. Biol.* **1**, 317-351.
7. Hucho, F. (1986) *Eur. J. Biochem.* **158**, 211-226.
8. Meunier, J.C., Sealock, R., Olsen, R. and Changeux, J.P. (1974) *Eur. J. Biochem.* **45**, 371-394.
9. Maelicke, A., Fulpius, B.W., Klett, R.P. and Reich, E. (1977) *J. Biol. Chem.* **252**, 4811-4830.
10. Haggerty, J.G. and Froehner, S.C. (1981) *J. Biol. Chem.* **256**, 8294-8297.
11. Kazim, A.L. and Atassi, M.Z. (1980) *Biochem. J.* **191**, 261-264.
12. Mulac-Jericevic, B. and Atassi, M.Z. (1987a) *Biochem. J.* **248**, 847-852.
13. Mulac-Jericevic, B. and Atassi, M.Z. (1987b) *J. Prot. Chem.* **6**, 365-373.
14. Mulac-Jericevic, B., Manshour, T., Yokoi, T. and Atassi, M.Z. (1988) *J. Prot. Chem.* **7**, 173-177.
15. Ruan, K.H., Spurlino, J., Quirocho, F.A. and Atassi, M.Z. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6156-6160.
16. Lee, C.Y., Chang, C.C. Chiu, T.H., Chi, P.J.S., Tseng, T.C. and Lee, S.Y. (1968) *Naunyn Schmiedeberg's Arch. Pharmacol.* **259**, 360-365.
17. Yang, C.C., Yang, H.J. and Huang, J.S. (1969) *Biochim. Biophys. Acta* **188**, 65-70.
18. Nishida, S., Kokubun, Y. and Tamiya, N. (1985) *Biochem. J.* **226**, 879-880.
19. Tamiya, N. and Arai, H. (1966) *Biochem. J.* **99**, 624-630.
20. Mulac-Jericevic, B., Kurisaki, J. and Atassi, M.Z. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3633-3637.

21. Towbin, H. Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
22. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) *Nature (London)* **299**, 793-797.
23. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikuyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) *Nature (London)* **302**, 528-532.
24. Hunter, W. and Greenwood, F. (1962) *Nature (London)* **194**, 495-496.
25. Twining, S.S. and Atassi, M.Z. (1979) *J. Immunol. Methods* **30**, 139-151.
26. Bixler, G.S. and Atassi, M.Z. (1983) *Immunol. Commun.* **12**, 593-603.
27. Mulac-Jericevic, B. and Atassi, M.Z. (1986) *FEBS Lett.* **199**, 68-74.
28. Dufton, M.J. and Hider, R.C. (1983) *Crit. Rev. Biochem.* **14**, 113-171.
29. Endo, T. and Tamiya, N. (1987) *Pharmacol. Ther.* **34**, 403-451.
30. Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, T., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M., and Numa, S. (1984) *Nature* **307**, 604-608.
31. Weber, M. and Changeux, J. (1974) *Mol. Pharmacol.* **10**, 1-4.
32. Chicheportiche, R., Vincent, J.P., Kopeyan, C., Schweitz, H. and Lazdunski, M (1975) *Biochemistry* **14**, 2081-2091.
33. Lee, C.Y., Chang, C.C. and Chen, Y.M. (1972) *J. Formosan Med. Assoc.* **71**, 344-349.
34. Low, B.W., Bourne, P.E. and Corfield, R. (1984) in *Proceedings of the 6th European Symposium on Animal, Plant and Microbial Toxins* (Meyer, J., Stocker, K. and Freyvogel, T.A., Editors.) p. 109, International Society on Toxinology European Section, Basle.
35. Low, B.W., Preston, H.S., Sato, A., Rosen, L.S., Searl, J.E., Rudko, A.D. and Richardson, J.S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2991-2994.
36. Tsernoglou, D. and Petsko, G.A. (1976) *FEBS Lett.* **68**, 1-4.
37. Tsernoglou, D. and Petsko, G.A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 971-974.
38. Kimball, M.R., Sato, A., Richardson, J.S., Rosen, L.S. and Low, B.W. (1979) *Biochem. Biophys. Res. Commun.* **88**, 950-959.
39. Walkinshaw, M.D., Saenger, W. and Maelicke, A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2400-2404.
40. Kazim, A.L. and Atassi, M.Z. (1982) *Biochem. J.* **203**, 201-208.
42. Atassi, M.Z., McDaniel, C.S. and Manshour, T. (1988) *J. Prot. Chem.* **7**, 655-666.
43. McCormick, D.J. and Atassi, M.Z. (1984) *Biochem. J.* **224**, 9950-10000.
41. Sine, S.M. (1988) *J. Biol. Chem.* **263**, 18052-18062.

B5. Appendix

Peptide Position	Species	Structure
α 1-16	Human Torpedo	S E H E T R L V A K L F K D Y S - - - - - N - L E N - N
α 12-27	Human Torpedo	F K D Y S S V V R P V E D H R Q L E N - N K - I - - - - H - T H
α 23-38	Human Torpedo	E D H R Q V V E V T V G L Q L I - H - T H F - D I - - - - -
α 34-49	Human Torpedo	G L Q L I Q L I N V D E V N Q I - - - - - S - - - - -
α 45-60	Human Torpedo	E V N Q I V T T N V R L K Q Q W - - - - - E - - - - - R - - -
α 56-71	Human Torpedo	L K Q Q W V D Y N L K W N P D D - R - - - I - V R - R - - - A -
α 67-82	Human Torpedo	W N P D D Y G G V K K I H I P S - - - A - - - - I - - - - R L - -
α 78-93	Human Torpedo	I H I P S E K I W R P D L V L Y - R L - - D D V - L - - - - -
α 89-104	Human Torpedo	D L V L Y N N A D G D F A I V K - - - - - - - - - - - - - H
α 100-115	Human Torpedo	F A I V K F T K V L L Q Y T G H - - - - H M - - L - - D - - - K
α 111-126	Human Torpedo	Q Y T G H I T W T P P A I F K S D - - - K - M - - - - - - - -
α 122-138	Human Torpedo	A I F K S Y G E I I V T H F P F D - - - - - - - - - - - - - -
α 134-150	Human Torpedo	H F P F D E Q N G S M K L G T W T - - - - - Q - - - - T - - - - I - -
α 146-162	Human Torpedo	L G T W T Y D G S V V A I N P E S - - I - - - - - T K - S - S - - -
α 158-174	Human Torpedo	I N P E S D Q P D L S N F M E S G - S - - - - R - - - - T - - - -
α 170-186	Human Torpedo	F M E S G E W V I K E S R G W K H - - - - - - - M - D Y - - - - -
α 182-198	Human Torpedo	R G W K H S V T Y S G G P D T P Y - - - - - W - Y - T - - - - - -
α 194-210	Human Torpedo	P D T P Y L D I T Y H F V M Q R L - - - - - - - - - - - I - - - I
α 262-276	Human Torpedo	E L I P S T S S A V P L I G K - - - - - - - - - - - - -

Fig. 1. Covalent structures of the synthetic overlapping peptides representing the extracellular part of each of the α chains of human and *Torpedo californica* acetylcholine receptors. The upper sequences of each pair of peptides give the full primary structures of the human AChR peptides and, under these, only the residues that are different in the corresponding Torpedo peptides are given. Segments in bold type represent the 5-residue overlaps between consecutive peptides.

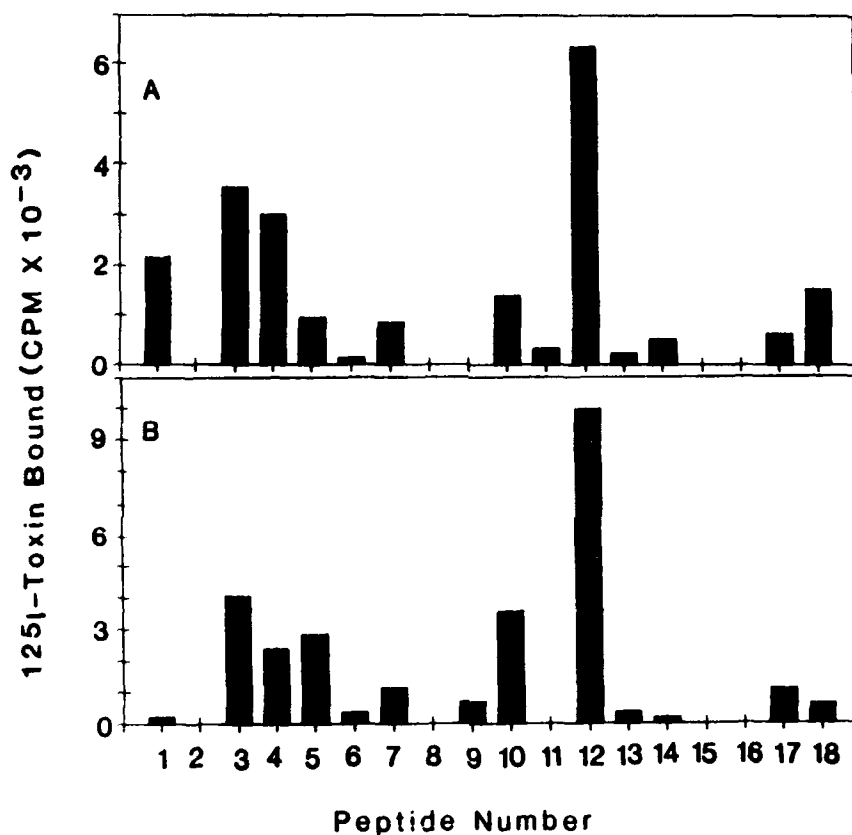


Fig. 2. Summary of the binding profiles of (upper panel) Cot and (lower panel) Eb to the synthetic overlapping peptides of the extracellular part of the α chain of *Torpedo* AChR. The bars represent the binding values to 25 μl of a 1:1 suspension (v/v) of each peptide adsorbent. Titrations were carried out in triplicates in PBS containing 0.1% BSA. The reaction volume was 60 μl and the amount of ^{125}I -labeled toxin added was 350,000 cpm/tube. After the reaction, the adsorbents were washed on the centrifuge four times with PBS and their radioactivity was counted. *Torpedo* AChR was used as a positive control. The results, which represent the average of three experiments, each in triplicate, have been corrected for nonspecific binding to unrelated proteins and peptides. The peptides were: 1, α 1-16; 2, α 12-27; 3, α 23-38; 4, α 34-49; 5, α 45-60; 6, α 56-71; 7, α 67-82; 8, α 78-93; 9, α 89-104; 10, α 100-115; 11, α 111-126; 12, α 122-138; 13, α 134-150; 14, α 146-162; 15, α 158-174; 16, α 170-186; 17, α 182-198; 18, α 194-210 (12,14). The binding values of ^{125}I -labeled Cot and Eb to *T. californica* AChR were $55,140 \pm 1,350$ and $68,550 \pm 1,520$ cpm, respectively. Binding to unrelated proteins (BSA, horse myoglobin) and peptides [sperm whale myoglobin synthetic peptides 1-17, 25-41 and 121-137 (26)] (negative controls) was 650 ± 220 cpm.

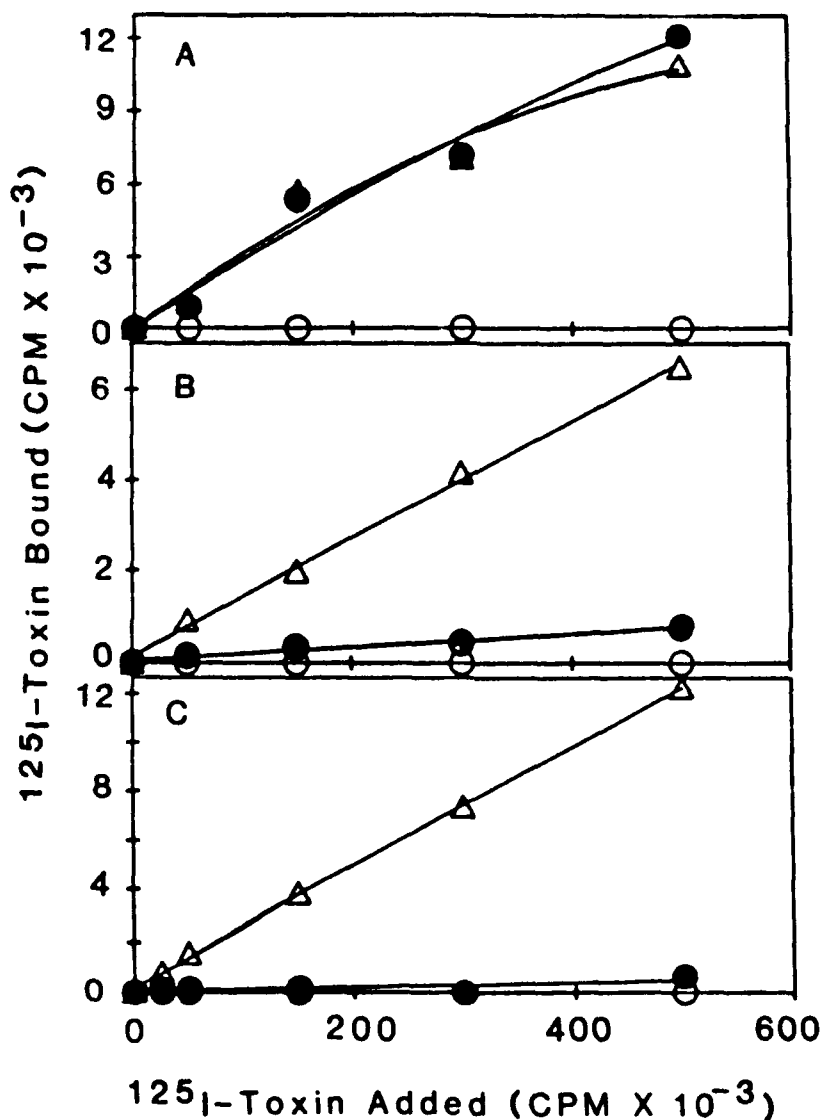


Fig. 3. Comparison of the binding activities of ^{125}I -labeled (A) Bgt, (B) Cot and (C) Eb to peptides (Δ) $\alpha 122-138$ and ($\bullet$) α chain of *Torpedo* AChR and to ($\circ$) the unrelated synthetic peptides given in Fig. 2. Increasing amounts of the ^{125}I -labeled toxins were added to a fixed volume ($25\ \mu$, 1:1 v/v suspension in PBS/0.1% BSA) of each peptide adsorbent. The experiments were carried out as described in Fig. 2 and the text. Each experiment point represents the average of six replicate analyses which varied $\pm 2.4\%$ or less.

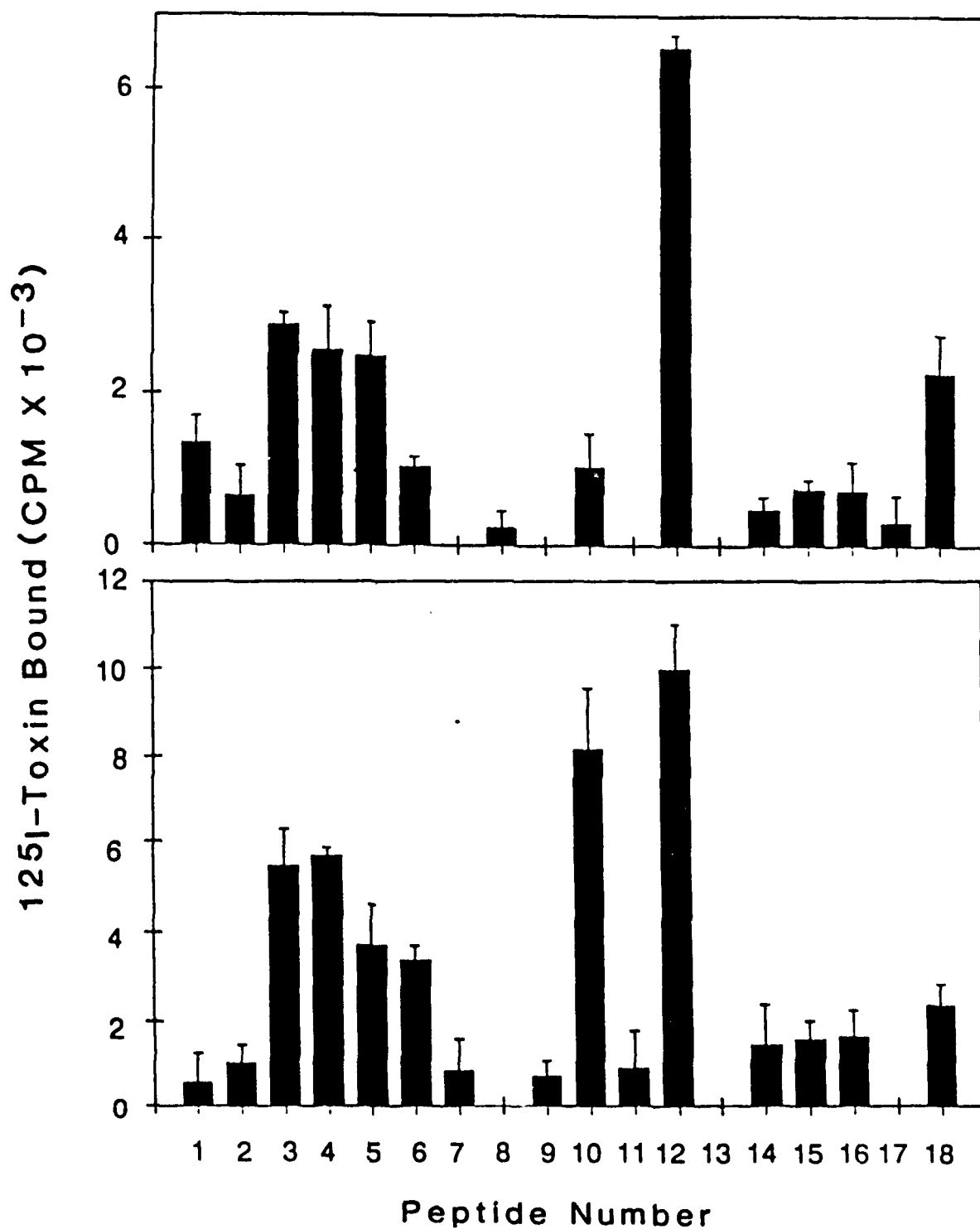


Fig. 4. Summary of the binding profiles of (upper panel) Cot and (lower panel) Eb to the synthetic overlapping peptides of the extracellular part of the α chain of human AChR. The sequence positions of the eighteen synthetic overlapping peptides of the α chain of human AChR and the assay conditions were as described in Fig. 1.

B5. Appendix

	1	L1N																							23
Bgt	I V C H T T A T I P S S A V T C P P G E N L																								
Cbt	I R C F I T P D I T S K D C P N G H V																								
Eb	R I C F N H Q S S Q P Q T T K T C S P G E S S																								
Cot	L E C H N Q Q S S Q T P T T T G C S G G E T N																								
	24	L2																							45
Bgt	C Y R K M W C D A F C S S R G K V V E L G C																								
Cbt	C Y T K T W C D A F C S I R G K R V D L G C																								
Eb	C Y H K Q W S D F R G T I I E R G C																								
Cot	C Y K K R W R D H R G Y R T E R G C																								
	46	L3E																							68
Bgt	A A T C P S K K P Y E E V T C C S T D K C N H																								
Cbt	A A T C P T V K T G V D I Q C C S T D N C N P																								
Eb	G C P T V K P G I K L S C C E S E V C N N																								
Cot	G C P S V K N G I E I N C C T T D R C N N																								
	69	75																							
Bgt	P P K R Q P G																								
Cbt	F P T R K R P																								

Fig. 5. Comparison of the consensus sequences of the short neurotoxins, Cot and Eb, and the long neurotoxins, Bgt and Cbt. Letters in bold denote differences between consensus sequences of long and short neurotoxins. The boxed regions in Cot and Eb denote sequence differences between the two short neurotoxins. The shaded boxed parts in the Bgt structure indicate the regions of the AChR-binding loops which were localized and confirmed by synthetic peptides (42).