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TITLE: STRUCTURE-FUNCTION RELATIONSHIP OF HYDROPHIIDAE
POSTSYNAPTIC NEUROTOXINS

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Introduction

Lapemis toxin, a sea snake neurotoxin from Lapemis hardwickii venom, has been under intense investigation in this laboratory. The many studies performed in this laboratory on this toxin have demonstrated that this neurotoxin binds specifically to and inhibits the acetylcholine receptor (AChR) and its function in neurotransmission.

In order to understand this toxin's action, the current study has been designed to elucidate the binding region(s) of the neurotoxin on the AChR. In other words, the study asks the questions:

1. Which part or parts of the neurotoxin bind(s) to the receptor?
2. What are the relative binding abilities of the selected synthetic regions of the toxin to the receptor?
3. Why are the five subunit assembly essential for a neurotoxin binding instead of just one subunit?
4. Which amino acids of the toxin play an important role in binding to the AChR?

Answers to these questions will allow further effective design of experiments aimed at finding which regions and amino acids of the toxin are important in the direct binding to the receptor. The answers will also help determine the structure-function relations of the toxin and explain the tight specific binding to this receptor. From this data, neurotoxin analogs may be designed to

antagonize the neurotoxin and its effects. These antagonists may also, with appropriate studies, prove to be useful drugs in various aspects of neuromedical science studies and treatments.

Sea snakes (family Hydrophiidae) are the only truly marine members of the diverse suborder Serpentes. They are abundant in the tropical and subtropical coastal waters of the Pacific and Indian oceans and are particularly common in the Gulf of Thailand. Though most sea snakes are not aggressive, their venoms are extremely toxic, largely due to the presence of highly specific neurotoxins. Envenomation by sea snakes is characterized by muscle paralysis and severe respiratory dysfunction, and occasionally results in death.

The acetylcholine receptor is perhaps the most studied membrane receptor. The recent reviews by McCarthy et al. (1986), Conti-Tronconi and Raftery (1982), and Changeux (1981) give excellent up-to-date accounting of what is now known about the neuromuscular cholinergic receptor.

The sequences of the subunits of the neuromuscular nicotinic acetylcholine receptor have been deduced from the cDNA sequences of the four subunits known to make up the pentamer receptor. These subunits are designated as $\alpha, \beta, \gamma, \delta$ for several species including the Torpedo californica receptor (Noda et al., 1983, Claudio et al., 1983).

The receptor binding of acetylcholine, specific antibodies, and α -neurotoxins have been extensively studied. Neurotoxins which exhibit high-affinity binding to the nicotinic AChR are prominent

components of nearly all elapid and hydrophiid venoms (Lee, 1979; Tu, 1990). Typically these are small basic proteins with molecular weights of approximately 6000-8000 Daltons; all of the α -toxins have considerable sequence homology (Chiapinnelli, 1985). Computer-assisted modeling of α -toxin binding to AChR suggests that a "tryptophan cleft" on the toxin may entrap trp187 on the AChR subunit (Low and Cornfield, 1987). These toxins bind to the synaptic surface of each α -subunit of the AChR (Kubalek et al., 1987). Interestingly, a thyroid-derived protein, thymopoietin, has been shown to be a potent inhibitor of α -bungarotoxin binding to neuronal nicotinic AChR; the authors suggest that thymopoietin may be an endogenous ligand for α -bungarotoxin receptors in neuronal tissue (Quik et al., 1989). Several viruses have been shown to utilize receptors for endogenous ligands to target and to infect host cells (e.g., Epstein et al., 1985). Rabies virus glycoprotein has considerable amino acid sequence similarity to several snake venom neurotoxins (Lentz et al., 1984). The finding that the rabies virus glycoprotein binds to the α -subunit of the nicotinic AChR and inhibits binding of α -bungarotoxin further supports the probability of this site being "intended for" a native ligand (Bracci et al., 1988). These data raise the possibility that α -toxins (such as Lapemis neurotoxin) may recognize binding site(s) on muscle AChR that are also actually designed for endogenous peptides/proteins (perhaps a modulator binding site) in muscle endplate tissue.

It has been suggested that the AChR α -subunits each contain

five α -toxin binding regions (Mulac-Jericevic and Atassi, 1987). The α -neurotoxins α -bungarotoxin (from Bungarus multicinctus; Formosan Krait) and cobratoxin (from Naja naja; Indian cobra) have been localized to five main regions on the α -subunit of the receptor of the Torpedo receptor but only three regions of the human receptor. At this time it is not known if these regions bind different neurotoxin molecules or are different faces interacting with the toxin (Mulac-Jericevic and Atassi, 1987a, 1987b; Mulac-Jericevic et al., 1988).

Recent studies investigating binding of α -toxins to AChR have utilized synthetic peptide fragments of the α -subunit of the AChR and snake venom neurotoxins, most notably α -bungarotoxin. A peptide consisting of residues 183-200 of the AChR α -subunit (containing the disulfide bridge of cys192 and cys193) has been shown to bind α -bungarotoxin, indicating that this sequence may also participate in cholinergic binding (Takamori et al., 1988). When the cys192-193 disulfide is reduced, binding of α -bungarotoxin decreases by 85% (Gotti et al., 1987). Antibodies against the AChR α -subunit region 173-204 inhibited α -bungarotoxin binding, and subsequent epitope mapping indicated that residues 183-194 are major determinants of toxin binding (Donnelly-Roberts and Lentz, 1989). The toxin binding sites are quite distinct from the main immunogenic region (MIR) targeted by an autoimmune response in the disease state of myasthenia gravis, which appears to be limited to the residues 67-76 on the α -subunit (Das and Lindstrom, 1989; Tzartos et al., 1988). These and other studies have helped to

define specific binding regions on the AChR α -subunits.

Another less frequently utilized approach to determine the mechanisms of AChR-ligand binding is to use intact AChR and modify specific aspects of the ligand. This approach provides information about the functionally important portions of a ligand's structure. For example, Lapemis neurotoxin has three major structural domains defined by disulfides (loops A, B and C). When the sole tryptophan residue was chemically modified, the protein lost toxicity (Tu and Hong, 1971) and was unable to bind to the AChR (Allen and Tu, 1985). Tryptophan resides on loop B, indicating that this loop is essential to binding. Loss of toxicity may have resulted from the inability of the modified toxin to bind to receptor. By synthesizing each of the domain loops and assaying toxicity and binding, one should be able to define sequence regions necessary for receptor binding or for toxicity.

Low and Corfield (1987) presented a working theoretical model attempting to map the neurotoxin binding site on the acetylcholine receptor. The study pointed out that the differences in human receptor sequences and that of the Torpedo receptor may account for clinical differences in toxicity of sea snake and short chain α -neurotoxins with that of venoms containing long chain α -neurotoxins. However, this model did not consider or explain the other reported binding regions of the receptor known to participate in binding to α -neurotoxins.

The phosphorylation sites of the AChR subunits have been reported. Huganir and Miles (1989) discuss the sites of

phosphorylation on the AChR subunits which are not at sites involved in acetylcholine or neurotoxin binding. They implied that an associated 43 kDa protein(s) are phosphorylating protein kinase(s), perhaps regulatory protein(s). LaRoche et al. (1989) demonstrated that the 43 kDa protein(s) are absent from genetic variants of C2 muscle cells and that acetylcholine receptor expression is reduced.

The minimum number of lipids required for a functional AChR was determined and reported by Jones et al. (1988). The structures of the carbohydrate moieties linked to each AChR protein subunits were elegantly determined by Nomoto et al. (1986).

The chemical modification study by Mori and Tu (1988) demonstrated that the chemical linking of the free amino groups of the receptor subunits does not affect the binding of Lapemis toxin; however, Lin and Tu (1988) demonstrated that chemical linking of free sulfhydryl groups of the receptor subunits does reduce Lapemis toxin binding.

Lapemis toxin was first isolated from the venom of the sea snake Lapemis hardwickii by Tu and Hong (1971). Lapemis toxin was studied and the primary sequence structure determined by Fox et al. (1977). Chemical modification, toxicity and binding studies of the Lapemis toxin showed that the toxin belongs to the snake venom toxin class of Type I short chain postsynaptic α -neurotoxins. Type I α -neurotoxins consist of 60-62 amino acids and Type II being the long chain postsynaptic neurotoxins consisting of 71-74 amino acids (Tu, 1990; Lee, 1979).

Juillerat et al. (1982) published results of peptide synthesis of the Naja naja philippines neurotoxin fragment (residues 16-48) that bound receptor although weaker than the intact neurotoxin. They hypothesized the existence of a lethal "active center" of a cobra (Naja naja philippinensis) neurotoxin by synthesizing the matching residues 16-48 and showed the peptide to be highly active in binding to the AChR protein. However, the toxicity of the peptide was not indicated. The strength of binding of the peptide was several-fold weaker than the intact neurotoxin yet slightly stronger than the normal ligand, acetylcholine. These results do suggest the existence of the active center but also suggest that other portions of the neurotoxin play either a direct binding role or structural role for the optimization of the neurotoxin's binding.

In order to localize the binding domains of Lapemis toxin toward the acetylcholine receptor three main strategies have been useful. The use of the combined strategies will enable the direct amino acid interactions of the toxin with the receptor to be determined. The main strategies are:

1. Enzymatic/Chemical cleavage of the toxin and evaluation of the relative binding of the purified fragments.
2. Chemical modification of selected neurotoxin amino acids for which a known specific modification method is available.
3. The use of peptide synthesis to produce peptides of the same or modified sequence of Lapemis toxin to deduce each

region and each amino acid's involvement in the binding of the receptor.

All of these methods are useful to attain ultimate goal of determining (at the molecular level) the nature of the neurotoxin-AChR binding and related structure-function information.

The major neurotoxin from Lapemis hardwickii venom (Lapemis toxin) inhibits binding of I-125 labeled α -bungarotoxin to the acetylcholine receptor by binding to the toxin sites on the α -subunit of the receptor (Allen and Tu, 1985). The three dimensional structure of Lapemis toxin is thought to be similar to other known three dimensional structures of sea snake neurotoxins (Allen and Tu, 1985) and will be useful for determining the mechanism of toxin binding to the AChR.

The most recent work has concentrated on using peptide synthesis of three primary loop domains of Lapemis toxin to determine relative importance of each portion to binding. This sea snake neurotoxin (Lapemis toxin) is a protein molecule made up of 60 covalently linked amino acids which fold into three structural loops termed loops A, B, and C named from the N-terminal to the C-terminal, respectively.

From this data, neurotoxin analogs may be designed to antagonize the neurotoxin and its effects and may serve as a nontoxic antigen to produce antibodies that recognize and neutralize the neurotoxin effects *in-vivo* thus serving as a toxoid to produce a vaccine. The designed antagonists may also, with

appropriate studies, prove to be useful drugs in various aspects of neuromedical science studies and treatments. Envenomation by sea snakes is characterized by muscle paralysis and severe respiratory dysfunction, and occasionally results in death.

The study of the synthetic peptide fragments of Lapemis toxin has reached the point of being able to study the relative binding ability of these fragment to the acetylcholine receptor.

The question: Will these synthetic peptides be able to bind the isolated acetylcholine receptor? If the answer is yes then the conclusion can be drawn that this portion of the neurotoxin (Lapemis toxin) plays a role in the binding to the receptor. These results will suggest which amino acids play a major role in the toxin's ability to bind this physiologically important neuromuscular receptor.

When the results are compared with other studies more clues to the structure-function of not only the neurotoxins but the receptor itself will be determined. Future experiments can then be designed to probe even deeper the exact structure-function of the toxin with its known receptor. The more that is known of this interaction, the more aspects of the basic research and medical research of the acetylcholine receptor and the neurotoxins can be studied.

In this report the evidence of the binding of peptide B₁ (18 mer) to the receptor, while evidence that the peptide B₂ (16 mer) does not appear to bind the receptor is submitted.

In order to study this binding the DE-81 filter paper assay

method reported by Schmidt and Raftery (1973) with modifications by Allen and Tu (1985) have been used. The pI of the receptor and receptor-toxin complex (4.9-5.5) is such that the radio-labelled ligand-receptor complex will bind the filter paper while radio-labelled free toxin or peptide will wash out. The amount of radioactivity detected on the filter paper is directly proportional to the amount of bound toxin or peptide to the receptor. The details of this method appears in the experimental methods.

The extremely high toxicity of Hydrophiidae (sea snakes) venom is due to the presence of potent postsynaptic toxins. Hydrophiidae neurotoxins have not been studied as much as neurotoxins of Elapidae; however, a number of neurotoxins have been isolated from sea snake venoms, and the amino acid sequences of some toxins have been determined. Lapemis binds to the AChR (Ishizaki et al. 1984; Allen and Tu 1985).

The chemical structures of many snake postsynaptic neurotoxins have been identified, but the mechanism of binding to the nicotinic AChR is still not clear. In the past, most chemical modification was done on free toxins; however, the conclusions from such an approach are less reliable than methods using receptor-bound toxin. Comparing the results of chemical modification of AChR-bound neurotoxin with that of free neurotoxin will yield more precise information as to the role of a particular amino acid on toxin binding to AChR.

Recently Garcia-Borron et al. (1987) chemically modified lysine residues of the receptor-bound α -bungarotoxin instead of

modifying unbound (free) neurotoxin. Reactivity of Lys-26, Lys-38, and other lysine residues to methylation is different when the toxin is bound to the receptor. It was concluded that Lys-26 binds to the nicotinic AChR.

By using the same approach, one can determine which of the three arginine residues in Lapemis toxin is most likely to bind to the nicotinic AChR. The modification of a number of arginine residues of the receptor-bound Lapemis toxin was investigated and compared with the modification of arginine residues of unbound (free) toxin. The positions of modified and unmodified arginine residues in the receptor-bound toxin and free toxins were identified from the amino acid sequence of fragments of these toxins.

Materials and Methods

Materials. Sephadex G-50 and G-10, Cm-cellulose, CNBr-activated Sepharose 4b, and molecular weight standards were purchased from Sigma Chemical Co. Torpedo californica electroplax, excised and then quick frozen in liquid nitrogen, was obtained from Pacific Bio-Marine Laboratories (Venice, CA) and stored at -70°C until needed. ¹²⁵I- α -bungarotoxin and Econofluor were purchased from New England Nuclear (Boston, MA). Phenylglyoxal monohydrate was

purchased from Aldrich Chemical Co. (Milwaukee, WI). Phenyl [2-¹⁴C]glyoxal was obtained from Research Products International Corp. (Mount Prospect, IL). Endoproteinase Glu-C (protease V8) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Extracti-Gel D and Phenylisothiocyanate (PITC) were purchased from Pierce Chemical Co. (Rockford, IL). All sequence and peptide synthesis reagents were purchased from MilliGen/Bioscience (Burlington, MA). All other chemicals were of analytical or reagent grade.

Venom. Sea snakes, L. hardwickii, were captured in the Gulf of Thailand in 1986 and 1989, and the venom was extracted as previously described (Tu and Hong, 1971).

Venom Extraction from 1989 Sea Snake Collection. Sea snakes (Lapemis hardwickii) were obtained from local fishermen in Songkhla, Thailand, during June and July, 1989. Glands were dissected out and dried in a cool room. Dried glands were then pulverized to a coarse powder using a Wiley mill. A total of 470 g powdered glands was obtained for venom extraction.

Cold glass-distilled water was added to approximately 20 g dried gland powder, and the resulting paste was ground (using a mortar and pestle) for 40 min at 4 °C. The paste was then suspended in approximately 600 ml cold distilled water, stirred vigorously and allowed to sit for 10 min at 0 °C. The suspension was then stirred, divided into two 400 ml centrifuge bottles and was centrifuged for 20 min at 4000 rpm (4 °C). The supernatant was collected, shell-frozen and lyophilized, and the remaining solids

were ground and extracted again (as above). Approximately 46.5 g of gland extract (hereafter referred to as crude venom) were obtained from 470 g dried glands.

Isolation and Comparison of 1989 and 1986 Lapemis Venom Samples. All isolations closely followed the previously published methods of Tu and Hong (1971), Tu et al. (1975) and Mori and Tu (1988). To compare venoms collected in 1986 and 1989, 240 mg samples of each were subjected to gel filtration on Sephadex G-50-50 using 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. Samples were dissolved in 3.5 ml buffer, briefly centrifuged to remove insolubles and applied to a 2.5 cm X 100 cm Sephadex column at 4 °C. Flow rate was 12 ml/hr, and fractions were collected at 15 min intervals. Absorbance at 280 nm was used to estimate protein and peptide concentration.

Toxicity of crude venom was evaluated using female Swiss/Webster mice. Crude venom was dissolved in 0.9% saline at various concentrations and 100 µl was injected IV (tail vein). All doses were adjusted to individual body weights. LD50 values (24 hr) were estimated from semi-log plots of survivorship curves.

Lapemis toxin (Neurotoxin). The major neurotoxin was isolated by a modification of the method of Tu and Hong (1971) using a two-step gel chromatography procedure at 4°C. First, 1 g of crude venom was loaded onto a Sephadex G-50-50 column (2.5 x 100 cm) that had been previously equilibrated with a 0.01 M potassium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The toxin was eluted with the same

buffer at a flow rate of 14 mL/h, and the eluate was collected in 3 mL aliquots.

The tubes that included protein peak III were pooled and lyophilized. The samples were desalted by passage through a Sephadex G-10 column (2.5 x 50 cm).

The lyophilized toxic fraction III was applied to a CM-cellulose column (1.5 x 45 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.8). The toxin was eluted with a linear gradient of NaCl from 0.0 to 0.4 M phosphate buffer. The tubes containing the toxic protein were pooled, lyophilized, and desalted by passage through a Sephadex G-10 column (2.5 x 50 cm), and the eluted toxin was lyophilized.

Toxicity was checked after each step of the isolation procedure. After G-50 chromatography, three to five Swiss Webster mice were injected with protein from peak III dissolved in 0.9% NaCl at a concentration that was twice the LD50 of crude venom. The number of mice that died in 24 h was recorded.

The homogeneity of the toxin was checked using polyacrylamide gel electrophoresis (PAGE) with the β -alanine acetate system previously described (Tu et al., 1975).

Aliquots of Lapemis toxin were injected in a Beckman ultrasphere ODS column (4.6 mm x 25 cm) operated at a flow rate of 1.0 mL/min. Gradient conditions were as follows: from solvent A (0.1% TFA) to 50% solvent B (0.1% TFA in acetonitrile) in 30 min.

Fmoc-peptide synthesis. The 9-fluorenylmethoxycarbonyl (Fmoc) protected peptides were used to synthesize the desired peptides using the MilliGen 9050 peptide synthesizer and manufacture protocols. The method is discussed in detail by Paivianan et al. (1987). The main peptide reagents and protected amino acids were purchased from MilliGen. The progress of the deprotection and acylation (chain elongation) steps were monitored at 365 nm.

Cleavage of Fmoc Peptides from Polyamide Resins. Fmoc synthetic peptides were cleaved from the solid phase resin using 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol, and 2% anisole for 8 to 10 hours. The cleavage solution containing the released peptide was removed from the resin by vacuum filtration through a glass fritted buchner funnel. The cleavage reagent was remove using a rotoevaporator at 55° C for 20 to 30 minutes leaving a thick syrup residue containing the peptide. The next step was to precipitate the peptide with 3X wash of anhydrous diethylether and the ether layers removed. The peptides were then allowed to dry by evaporation of the residue ether. The solid peptides were then resuspended in HPLC grade water with a few drops of acetic acid to aid solubility. This solution was pipetted to a tared vial, froze, lyophilized and weighed. The peptides were then subjected to further analysis and purification by RP-HPLC.

Peptide Sequencing of the Synthetic Peptides.

Each of the five synthetic peptides were subjected to automated Edman degradation and sequencing using the Milligen Model

6600 ProSequencer and manufactures protocols. The Arylamime membrane discs provided by Milligen were used to covalently attach the peptides before sequencing.

Isolation of Cobrotoxin. The venom from Naja naja atra was subjected to Sephadex G50-50 gel filtration pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The third peak containing the neurotoxin was pooled, dialyzed and lyophilized. This lyophilized fraction was then subjected to CM-Cellulose ion exchange chromatography pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.8). After one column volume of buffer had been eluted a linear salt gradient from 0 M NaCl to 1 M NaCl (in 800 ml) was applied. The first major peak after the gradient was applied was determined to be the neurotoxin after gel electrophoresis and toxicity checks. This method is essentially that of Tu and Hong (1971).

AChR Isolation. AChR was isolated from T. californica electroplax tissue using the method of Floehner and Rafto (1979). Cobrotoxin affinity resin was prepared as previously described (Brockes and Hall, 1975) using CNBr-activated Sepharose 4B. The latest preparation of the solubilized acetylcholine receptor was done based on the method of Lindstrom et.al., 1980. Re-isolation of the *Torpedo californica* nicotinic acetylcholine receptor using an alternate procedure reported to give a more stable and active receptor. The method was used with only slight modification. The details follow:

All procedures were carried out at 4°C. *Torpedo californica* electroplax tissue (rich source of receptor) (88 g) was cut into pieces using a #11 scalpel. The tissue pieces were mixed with 200 ml of buffer A. Buffer A consisted of 10 mM sodium phosphate, pH 7.5, containing 10 mM sodium azide (inhibitor of bacterial growth), 5 mM EDTA (inhibitor of metalloproteases), 5 mM iodoacetamide (inhibitor of cysteine proteases) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (inhibitor of serine proteases). The mixture was then blended on high speed in a waring blender (8 times for 15 sec each). The mixture was then strained through a wire mesh. The mixture was then centrifuged for 10 min at 5000 rpm in Beckman Ti60 rotor (4°C). The supernatant was then centrifuged for 60 min at 19,000 rpm using the same rotor. The pellet of this spin was then resuspended with a Dounce homogenizer in buffer B. Buffer B consisted of 10 mM sodium phosphate, pH 7.5, containing 10 mM sodium azide (NaN_3), 1 mM EDTA, soybean phospholipid 5mg/ml (stabilizing lipid), and sodium cholate 2% (w/v) (detergent to solubilize the receptor). The resuspension was mixed with a virtix mixer for 30 min. The mixture was then centrifuged for 30 min at 39,000 rpm in the Beckman Ti60 rotor. Affinity chromatography of the supernatant was the next step. Cobrotoxin, isolated previously from the cobra venom, was treated with the CNBr-activated sepharose 4B resin to give the affinity resin. The supernatant was mixed gently by the virtix mixer with the toxin-linked resin for 1 hour. The columns were then poured. The columns were washed with 250 to

300 column volumes of buffer C. Buffer C consisted of 4 mM sodium phosphate, pH 7.5, containing 100 mM NaCl, 5mg/ml soybean phospholipid, and 2% (w/v) sodium cholate. The resin was then transferred to a beaker and 25 ml of receptor elution buffer D and mixed for 12 to 15 hr with the vortex mixer. Buffer D consisted of buffer C containing 1 M carbamoylcholine chloride. The resin was repoured into the columns and the eluant containing the purified acetylcholine receptor was collected. The receptor eluant was then dialyzed (dialysis tubing MWCO 6,000 - 8,000) against 500 volumes of buffer E for 24 hr. Buffer E consisted of 10 mM sodium phosphate, pH 7.5, containing 100 mM NaCl, and 0.1 % (w/v) sodium cholate. The receptor eluant was then dialyzed against 500 volumes of buffer F for 24 hr. Buffer F consisted of 10 mM sodium phosphate, pH 7.5, containing 0.02% (w/v) lauryl sulfate (SDS), and 0.04% (w/v) sodium cholate. The dialysate was then aliquoted into eppendorf tubes and froze at -70 °C until needed. An aliquot was subjected to the Pierce BCA protein concentration determination assay. Another aliquot was subjected to SDS-PAGE and stained with coomassie blue for a purity check. The purified receptors were then used in the binding studies.

AChR Assay. Toxin binding to receptor was determined using the method of Schmidt and Raftery (1973). Binding of the synthetic peptides to the nicotinic acetylcholine receptor was determined by their ability to compete with Lapemis ¹²⁵I-neurotoxin. All assays were done in Torpedo Ringer's buffer containing 250 mM NaCl, 5 mM

KCl, 4 mM CaCl₂, 5 mM sodium phosphate (pH 7.0), and 0.1% bovine serum albumin at room temperature in a total volume of 125 μ L, for 1 h. Two Whatman DE-81 filter disks were placed on a Millipore filter holder connected to a vacuum flask and were rinsed with a wash buffer containing 100 mM NaCl, 0.1% Triton X-100, and 10 mM sodium phosphate (pH 7.4). Then 50 μ L of the incubation mixture was applied to the DE-81 filter disks and allowed to soak for 10 min. The disks were then rinsed five times with 5 mL of buffer. The filters were finally dried under a heat lamp, placed in 5 mL of Econofluor, and counted in a Beckman LS 7800 liquid scintillation counter. The binding of the synthetic peptides were checked by determining the decrease in ¹²⁵I-neurotoxin binding to the receptor following preincubation of the receptor with varying amounts of synthetic peptide for 1 hr. In other words, ¹²⁵I-neurotoxin was used as a nonreversible back-titrant to measure specific binding of a ligand competing for the same receptor (Juillerat et al., 1982).

Amino Acid Analysis. The analyses were performed on a Beckman Model 344 M HPLC system using reverse-phase column after precolumn derivatization by PITC. The preparation of the PTC amino acids was as described by Heinrikson and Meredith (1984). Derivatives were resolved and applied on a Beckman ultraphsere-ODS column (4.6 mm x 25 cm) operated at a flow rate of 0.9 mL/min. PTC derivatives were detected by the absorbance at 254 nm. Samples were hydrolyzed for 24 h and 48 h at 110-115°C in 6 N HCl, sequanal grade.

SDS-PAGE. Electrophoresis was performed on SDS-polyacrylamide gel (10%) using a procedure modified from Laemmli (1970). The sample buffer contained 2% SDS and 5% β -mercaptoethanol. Samples were boiled for 5 min before electrophoresis. Bovine serum albumin (66,000), egg albumen (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and α -lactalbumin (14,200) were used as standards for molecular weight determinations (Weber and Osborn, 1969). Receptor and standards were mixed and applied to the same well for molecular weight determination. Gels were stained with Coomassie brilliant blue and destained.

Arginine Modification. Modification of arginine residues in Lapemis toxin with phenylglyoxal was performed by previously published procedures (Takahashi, 1968; Yang et al., 1974) with minor modification. The commercial 14 C-labeled phenylglyoxal employed throughout this study had specific activities of 25-35 mCi/mmol and was freed of impurities by chromatography on Silicagel 60.

To a solution of Lapemis toxin (0.6 μ mol) in 1 mL of 0.2 M N-ethylmorpholine acetate buffer (pH 8.0), a 100-fold molar excess of phenylglyoxal in 0.3 mL of the same buffer was added, and then the reaction was allowed to proceed at 27°C for 3 h. The mixture was passed through a column of Sephadex G-10 (2.5 x 50 cm) followed by CM-cellulose chromatography with a linear gradient of increasing salt concentration from 0.0 to 0.4 M NaCl in 0.01 M ammonium acetate, pH 6.8. The fractions of the main protein peak were

lyophilized and desalted through a column of Sephadex G-10 (2.5 x 50 cm) equilibrated with 1% acetic acid. The protein fractions were then pooled and lyophilized.

Identification of Arginine Residues Modified by Phenylglyoxal.

In order to determine the position of the modified arginine residues, the modified derivatives were digested with endoproteinase Glu-C (protease V8) after reduction and S-carboxymethylation. Reduction and S-carboxymethylation were performed by the method described by Crestfield et al. (1963). The S-carboxymethylated derivatives were dissolved in 0.02 M NaHCO₃ buffer (pH 7.2) to give a 1% solution, and endoproteinase Glu-C (50:1) was added. Digestion was carried out at 27°C for 16 h.

Arginine-containing peptides from endoproteinase Glu-C digests were separated by RP-HPLC (Beckman ultrasphere-ODS, 4.6 mm x 25 cm). Gradient conditions were as follows: from 95% solvent A (0.1%TFA) and 5% solvent B (0.1% TFA in acetonitrile) to 40% solvent B in 40 min. Peptides were detected by their absorbance at 214 nm. The eluting peptides were collected manually, dried, and stored at -20°C until needed for amino acid analysis.

Arginine Modification of Lapenis Toxin Part in the AChR Complex.

The AChR (5 mg) from cobrotoxin-affinity chromatography was concentrated by ultrafiltration using Centriflo membrane cone (MW cutoff 25,000 daltons, Amicon Corp.). Ultrafiltration was carried out with 750 g at 4°C, and reduced 10 mL initial volume to 2 mL. In an arginine-modification experiment, the required amount of AChR

was mixed with a twofold excess of Lapemis toxin. After 1 h incubation at room temperature, AChR solution was applied onto a Sephadex G-50-80 column (2.5 x 90 cm) previously equilibrated with 0.2 M N-ethylmorpholine acetate buffer (pH 8.0) containing 0.1% Triton X-100. The AChR-bound Lapemis toxin peaks were pooled and concentrated by ultrafiltration using Centriflo membrane cone. Chemical modification of bound neurotoxin was carried out by using [¹⁴C]phenylglyoxal was added, and then the reaction was allowed to proceed at 27°C for 3 h. The mixture was dialyzed using Spectrapor membrane tubing, MW cutoff 12,000- 14,000, for 12 h against 0.01 M ammonium acetate, pH 6.8.

The labeled Lapemis toxin was released from AChR by adding 6 M NaCl and incubating at 37°C for 6 h. Separations of Lapemis toxin from AChR and removal of 0.1% Triton X-100 from the reaction mixture were carried out by using an Extracti-Gel D, detergent-removing gel, column (0.8 x 1.5 cm). The homogeneity of the toxin was checked by SDS-PAGE with Phast Gel Gradient 8-25% using Pharmacia PhastSystem, and also by RP-HPLC. The concentration of the Lapemis toxin was determined by using a MicroBCA protein assay kit (Pierce Chemical Co.) with bovine serum albumin as a standard.

[125-I] Radiolabelling of Lapemis Toxin and Tyrosine Containing Synthetic Peptides. The lapemis toxin (10 µg) was labelled using the Chloramine-T method with 1 mCi of Na¹²⁵I under optimized conditions to favor iodination of the tyrosine residue only. This resulted in a specific activity of approximately 100 µCi/10 µg (Method

reviewed by Atassi, 1977). The synthetic peptides (containing tyrosine residues) were radiolabelled using the chloramine-T method to label the tyrosine residues with I^{125} . Each peptide (5 μ g) was sent for radiolabelling. The procedure uses a phosphate buffer (pH 7.5) to dissolve each peptide. To this solution 1 mCi of NaI^{125} is added. Chloramine-T is also added and the reaction allowed to take place for 2 min at room temperature. The reaction was stopped using sodium betametasulfite. The reaction mixture was then applied to a sephadex G-25 desalting column to separate the labelled peptide from the reagents. The amount of activity incorporated was measured. These solutions were then used in the binding studies.

The [^{125}I] labelled peptides binding studies employed the DE-81 filter paper assay of Schmidt and Raftery (1973) as modified by Allen and Tu (1985). All assays were done in Torpedo Ringer's buffer containing 250 mM NaCl, 5 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 5 mM sodium phosphate (pH 7.0), and 0.1% (w/v) bovine serum albumin at room temperature. The amount of receptor was selected to give a final concentration of 5 nM in the incubation mixture. Labelled peptides were titrated in to the incubation mixture with the final volume of each assay being 250 μ l. The solutions were allowed to incubate for 1 hr. Then, 100 μ l of the incubation mixture was applied to the Whatman DE-81 filter paper disc previously placed in a Millipore vacuum filter apparatus and allowed to soak in for 10 min. The disc was then rinsed five times with 5 ml of wash buffer. The wash buffer consisted of 10 mM sodium phosphate, pH

7.4, containing 100 mM NaCl and 0.1% (v/v) Triton X-100. Each filter disc was then placed in a scintillation vial containing 5 ml of Econofluor and counted in a Beckman LS 7800 liquid scintillation counter. Background counts due to non-specific binding were determined by doing the same assay with no receptor added.

Screening Toxicity Assay of the Synthetic Peptides. Two 25 gm female Swiss Webster mice for each crude peptide sample was injected intravenously with 0.1 cc of a 2 mg/ml solution of each sample. The mice were observed for twenty four hours for signs of toxicity and lethality.

Results

Venom Extraction from Sea Snake Collection.

Sea snakes (Lapemis hardwickii) were obtained from local fishermen in Songkhla, Thailand. Glands were dissected out and dried in a cool room. Dried glands were then pulverized to a coarse powder using a Wiley mill. A total of 470 g powdered glands was obtained for venom extraction.

Cold glass-distilled water was added to approximately 20 g dried gland powder, and the resulting paste was ground (using a mortar and pestle) for 40 min at 4 °C. The paste was then suspended in approximately 600 ml cold distilled water, stirred vigorously and allowed to sit for 10 min at 0 °C. The suspension was then stirred, divided into two 400 ml centrifuge bottles and was

centrifuged for 20 min at 4000 rpm (4 °C). The supernatant was collected, shell-frozen and lyophilized, and the remaining solids were ground and extracted again (as above). Approximately 46.5 g of gland extract (hereafter referred to as crude venom) were obtained from 470 g dried glands.

All isolations closely followed the previously published methods of Tu and Hong (1971), Tu et al. (1975) and Mori and Tu (1988). To compare venoms collected in 1986 and 1989, 240 mg samples of each were subjected to gel filtration on Sephadex G-50-50 using 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. Samples were dissolved in 3.5 ml buffer, briefly centrifuged to remove insolubles and applied to a 2.5 cm X 100 cm Sephadex column at 4 °C. Flow rate was 12 ml/hr, and fractions were collected at 15 min intervals. Absorbance at 280 nm was used to estimate protein and peptide concentration.

Toxicity of crude venom was evaluated using female Swiss/Webster mice. Crude venom was dissolved in 0.9% saline at various concentrations and 100 µl was injected IV (tail vein). All doses were adjusted to individual body weights. LD50 values (24 hr) were estimated from semi-log plots of survivorship curves.

Comparison of 1989 and 1986 Lapemis hardwickii Venom Samples

Toxicity: The estimated LD50 for 1989 crude venom is 9.0 µg/g, similar to values obtained for earlier venom extractions. Crude venom has a relatively low toxicity due to "dilution" with nontoxic intracellular proteins.

Gel Filtration: Fractionation patterns for 1986 and 1989

crude venoms are similar (not shown). Patterns for the two batches of venom were qualitatively very similar. Peak I contains most protein and enzyme components with a Mr > 20,000 daltons. Peak II contains two proteins with Mr of approximately 15,000 and 10,000 daltons; the former is likely a phospholipase A2 while the latter may be the neurotoxin precursor. Peak III contained the major neurotoxin (NT) of Lapemis venom. Peaks IV-VIII are peptide components of the venom.

The fractionation patterns were also quite similar quantitatively. Some differences exist in the amounts of Peak II, the phospholipase and the putative neurotoxin precursor, and of peptide Peak IV; both were larger in the 1989 sample. However, these differences are minor, and the two batches of venom can be considered essentially the same.

The Step II CM-Sephadex C50 Ion Exchange chromatograph pattern using the toxin (8 mg) recovered from step I peak III (Figure not shown) indicates two peak fractions were recovered termed III.1 and III.2. The III.2 is the Lapemis toxin based on toxicity screen test with mice and SDS-PAGE with 480 µg recovered.

Isolation of cobrotoxin.

The venom from Naja naja atra was subjected to Sephadex G50-50 gel filtration pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The third peak containing the neurotoxin was pooled, dialyzed and lyophilized. This lyophilized fraction was then subjected to CM-Cellulose ion exchange chromatography pre-equilibrated with 10 mM sodium

phosphate buffer (pH 7.8). After one column volume of buffer had been eluted a linear salt gradient from 0 M NaCl to 1 M NaCl (in 800 ml) was applied. The first major peak after the gradient was applied was determined to be the neurotoxin after gel electrophoresis and toxicity checks. This method is essentially that of Tu and Hong (1971).

Isolation of the Torpedo californica Nicotinic Acetylcholine Receptor.

The isolated cobrotoxin from Naja naja atra cobra venom was used to make an affinity column using CNBr-activated Sepharose 4B resin. The T. californica electroplax tissue was homogenized in Torpedo Ringers Isolation buffer which included various protease inhibitors; the homogenate was filtered through several layers of cheesecloth and then passed through the affinity column. The cobrotoxin specifically binds the AChR allowing all remaining material to be eluted first. The AChR is then eluted using the competitive inhibitor carbamylcholine. The purified solubilized receptor is then used in the AChR Binding Assay. The methods followed were that of Froehner and Rafto (1979) or Linstrom et al. 1980.

Purity of Toxin. The homogeneity of the Lapemis toxin was established by two independent methods, PAGE and analytical HPLC using a Beckman ultraphere-ODS column (4.6 mm x 25 cm). A single band was observed on acrylamide gels after applying 10 µg of Lapemis toxin. The HPLC chromatography pattern also showed that only one protein

was present. The LD₅₀ of the neurotoxin was similar to the value reported previously (Tu and Hong, 1971)

Free Lapemis Toxin. Before the chemical modification of AChR-bound neurotoxin, free toxin was used as a control. In order to find the optimum reaction time, the effect of reaction time for the degree of arginine modification as detected by cpm was first investigated. As the phenyl [2-¹⁴C]glyoxal was added, the toxin was modified, as can be seen from the increased specific activity in neurotoxin, and it reached the saturation point in 180 min (Figure 1). Because the arginine modification by phenyl [2-¹⁴C]glyoxal required 3 h, we used the reaction time of 3 h for all modifications. The modification of the arginine residues in free neurotoxin was evidenced from the large amount of ¹⁴C found in the toxin after incubation with phenyl [2-¹⁴C]glyoxal (Table I). In experiment 1, 1688 cpm was found, and in Experiment 2, 1501 cpm was found. From amino acid analysis it was evident that 2 out of 3 mol were modified (Table II).

The next important step was to identify which of the three arginine residues in the neurotoxin were modified. In order to do this, the modified toxin was incubated with endoproteinase glutamine C. The proteolytically digested fragments were separated into four components by HPLC using a C₁₈ reverse-phase column (Figure 2). The amino acid composition of each fragment is summarized in Table III.

Since the amino acid sequence of Lapemis toxin is known, the cleavage sites can be determined (Figure 3). Fragment 3 showed 1

mol of arginine, but fragment 4 contained no arginine (Table III). Thus Arg-31 and Arg-34 were modified, while Arg-37 was not modified. Apparently Arg-37 was unavailable for modification because it was situated in a more interior part of the toxin.

The modified toxin was nontoxic to mice at 0.5 $\mu\text{g/g}$ concentration, while the LD_{50} of unmodified toxin was 0.06 $\mu\text{g/g}$.

AChR-Bound Lapemis Toxin. Lapemis toxin was mixed with AChR with a molar ratio of 2:1. When the complex was formed, the neurotoxin peak that normally would appear at tube 95 on Sephadex G-50 chromatography disappeared (Figure 4). Further evidence of formation of Lapemis toxin and receptor complex is shown in Figure 5. In the SDS electrophoresis, the complex dissociated into $\alpha, \beta, \gamma,$ and δ subunits of AChR, and Lapemis toxin (LTX in Figure 5).

The receptor-bound toxin was modified with phenyl [2- ^{14}C]glyoxal at 100-fold excess. When phenyl[2- ^{14}C]glyoxal was used to modify arginine residues, a very large amount of ^{14}C was incorporated. In two separate experiments, the ^{14}C incorporated was 3756 and 3793 cpm (Table I), more than double the amount (1688 and 1501 cpm) incorporated into free neurotoxin. This suggests that many arginine residues in the AChR of the AChR-Lapemis toxin complex were modified by this reagent.

For our objective, it was most important to determine how many arginine residues of AChR-bound neurotoxin were modified. For this purpose the Lapemis toxin was detached from the AChR-Lapemis toxin complex and the radioactivity of the detached neurotoxin was

determined. The receptor was removed by precipitation after incubating the complex in NaCl at 37 °C for 6 h. After removing Triton X-100, the lapemis toxin was purified by HPLC (LTX in Figure 6). Amino acid composition analysis of the HPLC peak (Figure 6) indicated that the fraction was indeed Lapemis toxin.

The Lapemis toxin detached from the AChR-neurotoxin complex had an amino acid composition identical to that of unmodified toxin, yet only 176-185 cpm, close to background radioactivity, were found in the two experiments. This indicates that none of the arginine residues of AChR-bound Lapemis toxin were modified by phenyl[2-¹⁴C]glyoxal. Presumably the two arginine residues that are available for modification in the free toxin are no longer accessible to modification because these two residues are involved in the binding to AChR.

The results of chemical modification of neurotoxin before and after binding to AChR are summarized as follows:

	Free Lapemis toxin	AChR-bound Lapemis toxin
Arg 31	modified	unmodified
Arg 34	modified	unmodified
Arg 37	unmodified	unmodified

Synthesis, Cleavage, Purification and Sequences of the Fmoc

Synthetic Peptides.

Peptide loops A, B and C were synthesized by the Fmoc synthesis method. The composition of the synthetic peptides were based on the amino acid sequence of Lapemis neurotoxin, following the one letter amino acid designations (Table 1).

The purification of the Fmoc synthetic peptides were accomplished using the Beckman Reverse Phase ODS (C18) column with an increasing linear gradient of acetonitrile. All the Fmoc peptides showed a large predominant peak for each analysis and was easily purified by applying large amounts and collecting the major peaks.

Fmoc-peptide synthesis.

The 9-fluorenylmethoxycarbonyl (Fmoc) protected peptides were used to synthesize the desired peptides using the MilliGen 9050 peptide synthesizer and manufacture protocols. The method is discussed in detail by Paivianan et al. (1987). The main peptide reagents and protected amino acids were purchased from MilliGen. The progress of the deprotection and acylation (chain elongation) steps were monitored at 365 nm.

Cleavage of the Fmoc Peptides from the Polyamide Resins.

Fmoc synthetic peptides were cleaved from the solid phase resin using 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol, and 2% anisole for 8 to 10 hours. The cleavage solution containing the released peptide was removed from the resin by vacuum filtration through a glass fritted buchner funnel. The cleavage reagent was remove using a rotoevaporator at 55° C for 20

to 30 minutes leaving a thick syrup residue containing the peptide. The next step was to precipitate the peptide with 3X wash of anhydrous diethylether and the ether layers removed. The peptides were then allowed to dry by evaporation of the residue ether. The solid peptides were then resuspended in HPLC grade water with a few drops of acetic acid to aid solubility. This solution was pipetted to a tared vial, froze, lyophilized and weighed. The peptides were then subjected to further analysis and purification by HPLC.

Purification of the Fmoc Synthetic Peptides.

The purification of the Fmoc synthetic peptides (Table 1) were accomplished using the Beckman Reverse Phase C18 column with an increasing linear gradient of acetonitrile. All the Fmoc peptides showed a large predominant peak for each analysis and was easily purified by applying large amounts and collecting the major peaks. Figure 7 shows examples of the HPLC chromatograms of purification of the Fmoc peptides. The major peak of each chromatogram was collected. Figure 7A is a chromatogram example of Loop A. Figure 7B is a chromatogram example of the nonsense peptide. Figure 7C is a chromatogram example of Loop C. Figure 7D is a chromatogram example of Loop B₁. Figure 7E is a chromatogram example of Loop B₂. Detection was at 214 nm.

Sequences of the Fmoc Synthetic Peptides.

Peptide loops A, B and C were synthesized by Fmoc synthesis method. The composition of the synthetic peptides were based on the amino acid sequence of Lapemis neurotoxin, following the one

letter amino acid designations (Table IV).

Radiolabelling of Lapemis Toxin and Tyrosine Containing Synthetic Peptides. The lapemis toxin (10 μ g) and the tyrosine containing synthetic peptides were labelled using the Chloramine-T method with 1 mCi of Na¹²⁵I under optimized conditions to favor iodination of the tyrosine residue only. This resulted in a specific activity of approximately 100 μ Ci/10 μ g for the labelled toxin and similar specific activity for the synthetic peptides. (Method reviewed by Atassi, 1977).

Isolation of Cobrotoxin and Acetylcholine Receptor.

The isolation of cobrotoxin was done repeatedly to generate a store of this neurotoxin to be used in the isolation of the AChR. We generated approximately 40 mg toxin using the two step procedure based on the method of Tu and Hong (1971) and Mori and Tu (1988). (figures not shown). Isolations of AChR were done each giving approximately 25 ml of 0.2 to 0.3 mg/ml concentration.

The results of the acetylcholine receptor isolation gave approximately 25 ml of 0.27 mg/ml solution. The SDS-PAGE showed the expected four homologous subunit bands of the receptor with a slight contamination of two weak higher molecular weight bands. Using 270,000 g/mol as the receptor molecular weight gives a receptor solution concentration of 1×10^{-6} M. 50 μ l of this receptor solution was diluted to 1 ml and 50 μ l of this diluted receptor solution was used in each assay except for the non-specific binding where the receptor solution was replaced with incubation buffer. Therefore, in the binding study, 5 nM of

receptor was used in each experiment. The general result of the peptide binding study indicates that peptide B₁ apparently binds while peptide B₂ and the non-sense peptide do not appear to bind at all. See Table IV for the sequences of the peptides studied. Figure 8 is a plot of the bound activity of peptide B₁ to the acetylcholine receptor verses the amount of B₁ added. The bold curve indicates the activity of the peptide with receptor or total activity while the light curve indicates non-specific binding. The bold curve minus the light curve will give the specific binding and thus peptide B₁ appears to bind the receptor. Plots of the other tested peptides follow the non-specific binding plot and therefore appear not to bind at all.

Screening Toxicity Assay of the Synthetic Peptides.

All tested mice survived the intravenous injections of the peptides with no apparent ill affects. The amount of sample injected (20 µg/g) is significantly higher (approx. 114 X) than the LD₅₀ dosage of 0.06 - 0.07 µg/g of purified neurotoxin indicating the synthetic peptides are relatively non-toxic. The use of a nonsense peptide was also made and used to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation.

Discussion

It is a well-established fact that snake venom postsynaptic neurotoxin binds to the acetylcholine receptor. Torpedo californica AChR is composed of five subunits, two of which are

identical. Normally the receptor is expressed as $\alpha\beta\gamma\delta$. Acetylcholine is known to bind to the α subunits of AChR. As two molecules of acetylcholine attach to two α subunits, the AChR pore in the membrane opens up, allowing cations to pass through. This is the essence of nerve transmission from the nerve to the muscle. A snake postsynaptic neurotoxin is an antagonist to acetylcholine by competing with acetylcholine in attaching to the same site of AChR. When the neurotoxin attaches to the receptor, the AChR pore does not open and allow cations to pass through. This is the mechanism of neurotoxicity caused by postsynaptic neurotoxins.

An important question is: Which part(s) of the neurotoxin attaches to the AChR? In the past, the structure-function relationship of a neurotoxin was studied using a free neurotoxin (unbound to AChR). This type of study really does not tell which residues are involved in AChR binding. In order to solve this problem, we chemically modified the arginine residues of Lapemis neurotoxin under the condition of binding to AChR and then compared the result with that of unbound neurotoxin. One assumption is that the residues involved in the binding to AChR cannot be modified.

Lapemis toxin contains three arginine residues at positions 31, 34, and 37. In free (unbound) toxin Arg-31 and Arg-34 were modified, but Arg-37 was inaccessible to the modifying reagent presumably because it is embedded in the protein coiling. Figure 9 shows that Arg-31 and Arg-34 are relatively exposed to the outside. However, when arginine residues of the neurotoxin were modified using the Lapemis-AChR complex, neither Arg-31 nor Arg-34

were modified. The logical explanation is that both Arg-31 and Arg-34 are involved in AChR complexing.

Manual t-Boc synthesis presented problems in purification and identification of correct peptide; the syntheses appear to have many side reaction products, racemization and deletion peptides. t-Boc synthesis has several drawbacks. The repetitive acid (TFA) treatments can cause side reactions in amino acids and premature cleavage of the growing peptide chain from the support, and the final HF treatment frequently destroys peptide bonds (Paivianan et al., 1987).

Fmoc-synthesis appears to have overcome the synthesis technical problems, and the correct peptide products are the predominant peak of each HPLC chromatogram. The Fmoc peptides were made quickly by an automated synthesizer (Milligen 9050) and gave a high purity product that was confirmed by peptide sequencing.

The purchase of the Milligen Model 6600 ProSequencer by this Department has proven to facilitate sequence analysis. The use of the arylamine membrane and manufacture protocol to covalently link the carboxyl groups of the peptides serves well. The coupling efficiency however, was only 24% initial yield but due to the ample amount of peptide samples available this presented no real problem. The low yield may have been due to improper storage of the membrane disks upon arrival which were left at room temperature instead of being refrigerated as stated by the manufacture. The computerized Edman sequencing was monitored at 269 nm and 313 nm and the results of the previous cycle was dynamically subtracted to aid in the

correct assignment of the current cycle amino acid.

Since peptide B₁ differs from B₂ in the fact that the B₁ peptide contains the disulfide bond due to the terminal cysteine residues while peptide B₂ does not contain the cysteine residues and therefore does not contain the disulfide bond suggests the importance of the disulfide bond. Future work will be several related binding study experiments to further clarify the importance of the disulfide bond. The B₁ peptide will be carboxymethylated forcing an open conformation and radiolabelled. The binding study with CM-peptide should confirm or disprove the importance of the disulfide bond.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo and perhaps other related neurotoxins. It should be noted that our hydrophilicity analysis of Lapemis toxin suggests that the loop B should be the prime candidate for serving as the non-toxic antigen based on its high hydrophilicity and correlation of high hydrophilicity to antibody production (method and correlation, Hopp and Woods, 1981, 1983).

Fractionation patterns for 1989 and 1986 Lapemis venom samples were virtually identical, indicating that the extraction procedures

had yielded the same product from both batches of venom glands. The 1986 crude venom has been used for isolating Lapemis neurotoxin for subsequent modification and binding studies (e.g. Lin and Tu, 1988), and it appears that the 1989 sample will yield neurotoxin as well.

The Fmoc peptides were made very quickly by an automated synthesizer and gave a high purity product. The use of a nonsense peptide was also made to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo.

Acetylcholine Receptor Binding Assay.

Binding of the synthetic peptides to the nicotinic acetylcholine receptor will be determined by their ability to compete with Lapemis ¹²⁵I-neurotoxin.

The attempt to measure the binding of the synthetic peptides will be by determining the decrease in ¹²⁵I-neurotoxin binding to the receptor following preincubation of the receptor with varying amounts of synthetic peptides for at a selected time, perhaps 1 hr

in a competition assay. The dissociation (binding) constant of Fmoc-synthesis appears to have overcome the synthesis technical problems, and the correct peptide products are the predominant peak of each HPLC chromatogram (Figure 7). The Fmoc peptides were made very quickly by an automated synthesizer and gave a high purity product. The use of a nonsense peptide was also made to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation.

The relative binding constants of each Fmoc synthetic peptide are to be determined using the established binding assay (Schmidt and Raftery, 1979) and Scatchard analysis (Scatchard, 1949), followed by the relative toxicity determinations.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo.

Ruan et al. (1990) have shown the systematic study of the long chain post-synaptic neurotoxin synthetic fragments with those of the AChR. Boyot et al. (1990) have shown that the use of another important strategy (recombinant technology) will add greatly to the goals of this research.

Conclusion

Both Arg-31 and Arg-34 of Lapemis toxin are involved in AChR complexing. Peptide B₁ binds the AChR while peptide B₂ appears not to bind at all. Since peptide B₁ differs from B₂ in the fact that the B₁ peptide contains the disulfide bond (due to the terminal cysteine residues) while, peptide B₂ does not contain the cysteine residues and therefore does not contain the disulfide bond suggests the importance of the disulfide bond in a probable structural role. With comparisons this work and that of others, the critical amino acids and regions of the toxin and the AChR which are involved in binding and structure-function are becoming increasingly more clear.

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Figure Legend

FIGURE 1: Labeling and kinetics of free Lapemis toxin by phenyl[2-¹⁴C]glyoxal. The purpose of this experiment was to find an optimum reaction time for the modification of the arginine residues.

FIGURE 2: The RP-HPLC separation of the products resulting from endopeptidase Glu-C digestion of phenylglyoxal-treated Lapemis toxin. Aliquots of endoproteinase Glu-C treated, S-carboxymethylated Lapemis toxin were injected in a Beckman ultrasphere-ODS column (4.6mm x 25 cm) operated at a flow rate of 1.0 mL/min.

FIGURE 3: Sites of cleavage of endoproteinase Glu-C on S-carboxymethylated Lapemis toxin.

FIGURE 4: Lapemis toxin (0.25 mg) was mixed with 5 mg of AChR to form a complex, and then the mixture was applied onto a Sephadex G-50-80 (2.5 x 90 cm) column. The expected location of the Lapemis toxin peak is shown by the dotted curve. Since no absorption is observed, it can be assumed that free toxin did not exist in measurable quantities in the mixture with AChR. The one major peak observed (tube 50-69) is AChR-Lapemis toxin complex.

FIGURE 5: (A) Standard proteins (66 K, bovine albumin; 45 K, egg albumin; 36 K, glyceraldehyde-3-phosphate dehydrogenase; 29 K, carbonic anhydrase; 24 K, trypsinogen; 20 K, trypsin inhibitor; 14 K, α -lactalbumin). (B) AChR undergoes dissociation to constituent subunits α , β , γ , and δ . AChR-Lapemis toxin complex and standards were treated with sample buffer containing 4% SDS, 20% glycerol, and 10% β -mercaptoethanol for 5 min at 100°C and then run on 12% polyacrylamide gel. LTX (Lapemis toxin) is clearly seen after the dissociation of AChR-LTX complex. Two sets of each sample were run on the same gel.

FIGURE 6: Aliquots of Lapemis toxin detached from AChR were purified by injecting in a Beckman ultrasphere-ODS column (4.6 mm x 25 cm) operated at a flow rate of 1.0 mL/min. Gradient conditions: from 95% solvent A (0.1% TFA) and 5% solvent B (0.1% TFA in acetonitrile) to 50 % solvents A and B in 25 min. LTX indicates the HPLC fraction of Lapemis toxin.

FIGURE 7: RP-HPLC separations of the synthetic peptides. Each of the peptides were purified by injecting aliquots of the peptides in a Beckman ultrasphere-ODS column (4.6 mm x 25 cm with 5 μ pore size) containing a guard column of ultrasphere-ODS (4.6 mm x 4.5 cm with 5 μ pore size) operated at a flow rate of 0.8 mL/min. Gradient conditions: 90% solvent A (0.1% TFA) and 10% solvent B (0.1% TFA in acetonitrile) for 5 min. Then, a linear gradient from 10 % solvent B to 90% solvent B in 30 min. Then, holding at 90% solvent B for 10 min. followed by return to 10% solvent B in 0.1 min and holding for 10 min. Absorbance at 214 nm were monitored. Peak elution times appear in min. (A) Peptide Loop A (20 μ L of 5 mg/mL) was injected. Peak Threshold was set at 200 and attenuation at 8. Absorbance units full scale (AUFS) was set at 2.0. Loop A eluted at a retention time (RT) of 23.44 min with the applied gradient. (B) Nonsense peptide (NS peptide) (20 μ L of 3 mg/mL) was injected. Peak Threshold was set at 200 and attenuation at 8. AUFS was set at 2.0. NS peptide eluted at RT of 38.77 min. (C) Peptide Loop C (100 μ L of 6 mg/mL) was injected. Peak Threshold was set at 200 and attenuation at 16 and AUFS was set at 2.0. Peptide Loop C eluted at RT of 37.87 min. (D) Peptide Loop B₂ (20 μ L of 3 mg/mL) was injected. Peak Threshold was set at 200 and attenuation at 8. AUFS was set at 1.0. Peptide Loop B₂ eluted at 33.12 min. (E) Peptide Loop B₁ (20 μ L of 25 mg/mL) was injected. Peak Threshold was set at 200 and attenuation at 8. AUFS was set at 2.0. Peptide Loop B₁ eluted at RT of 17.71 min.

FIGURE 8: AChR Binding of Peptide Loop B₁. Aliquots of [¹²⁵I] labelled peptide loop B₁ were incubated with 5 nM of AChR for 1 hr. Aliquots of the incubation mixtures were spotted on Whatman DE-81 ion exchange paper prewetted with wash buffer (10 mM sodium phosphate, pH 7.4, containing 0.1% Triton X-100 and 100 mM NaCl), vacuum filtered and washed 3X with 5ml of wash buffer. The vacuum dried ion exchange paper containing the bound receptor-peptide complex were place in 5 mL of Econofluor and counted in a Beckman LS 7800 liquid scintillation counter set for ¹²⁵I gamma counting. The nonspecific binding of the labelled peptide was determined by using the same concentrations of labelled peptide with out the receptor. Note: this is really correcting for nonspecific binding to the ion exchange paper.

FIGURE 9: The amino acid sequence and structure of Lapemis toxin. The structure is based on the crystal structure of a similar neurotoxin, toxin b (Tsernoglou and Petsko, 1976).

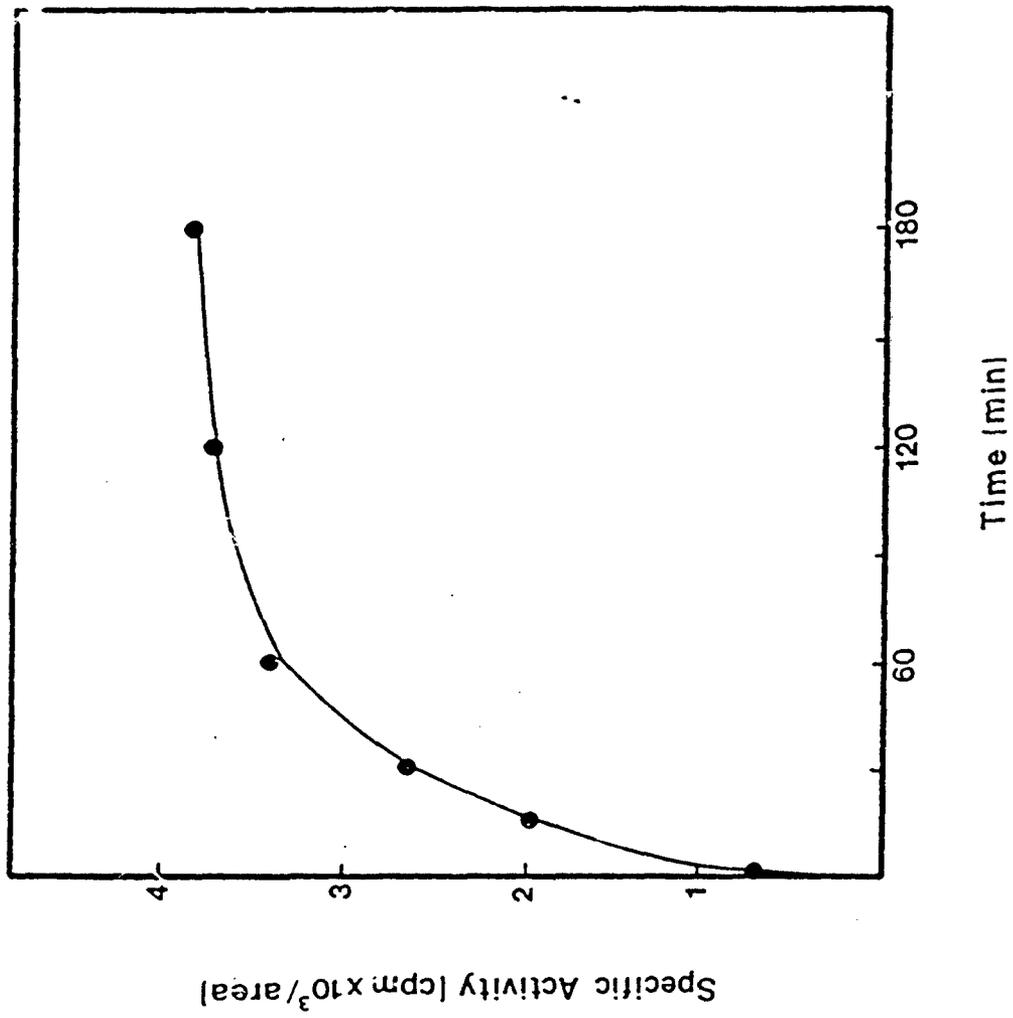


FIGURE 1

Absorbance at 214 nm

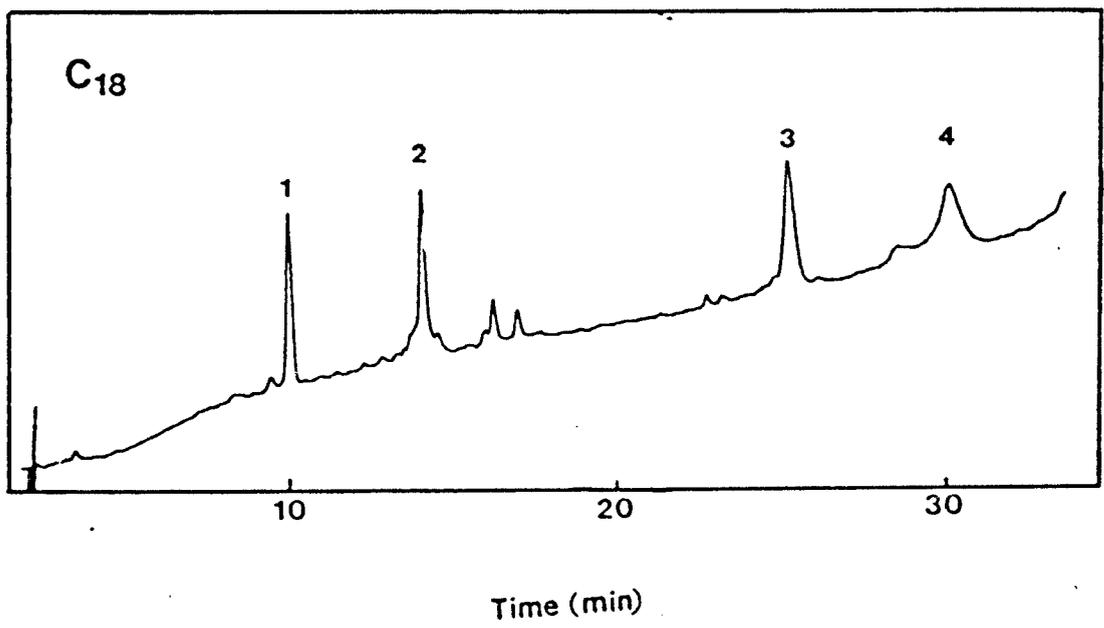


FIGURE 2

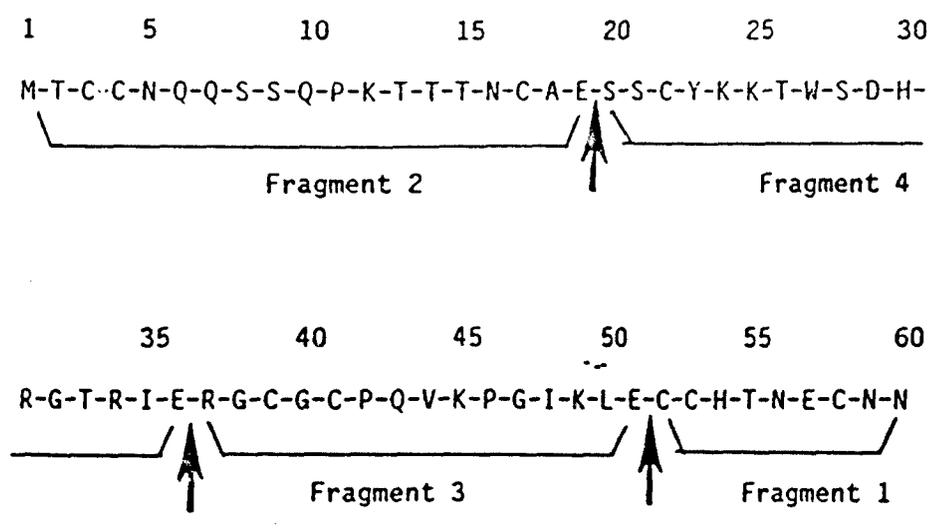


FIGURE 3

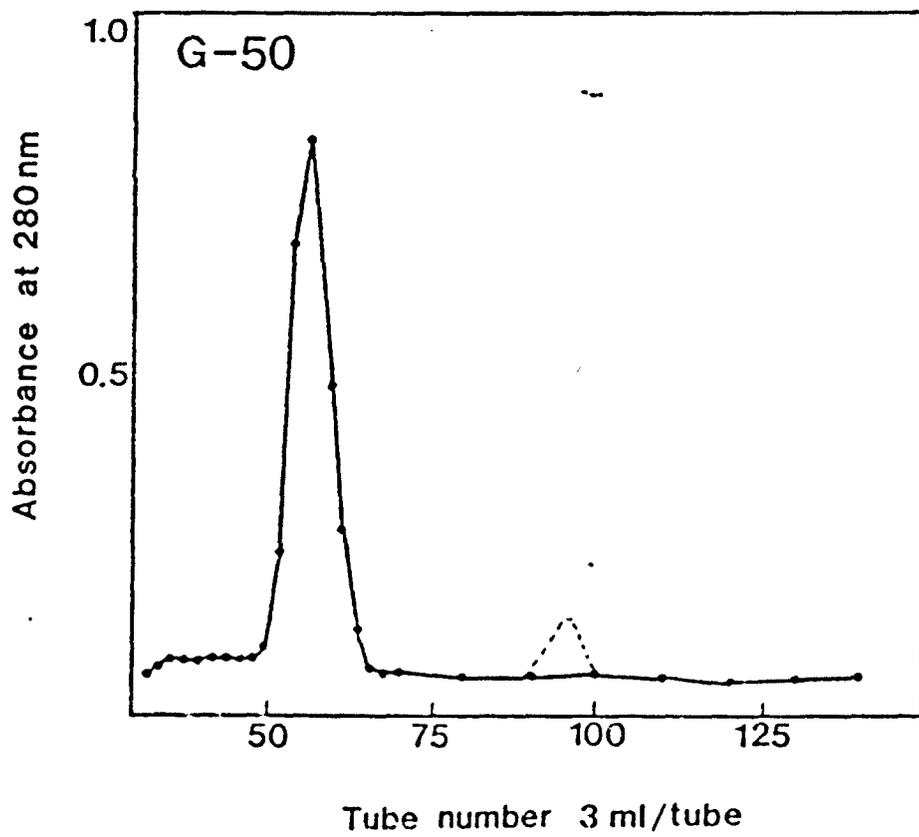


FIGURE 4

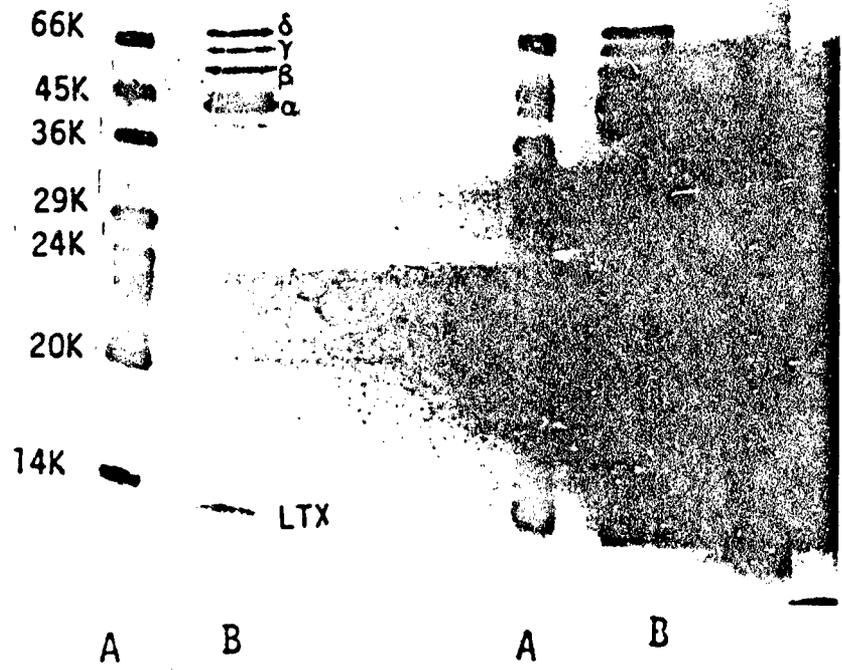


Figure 5

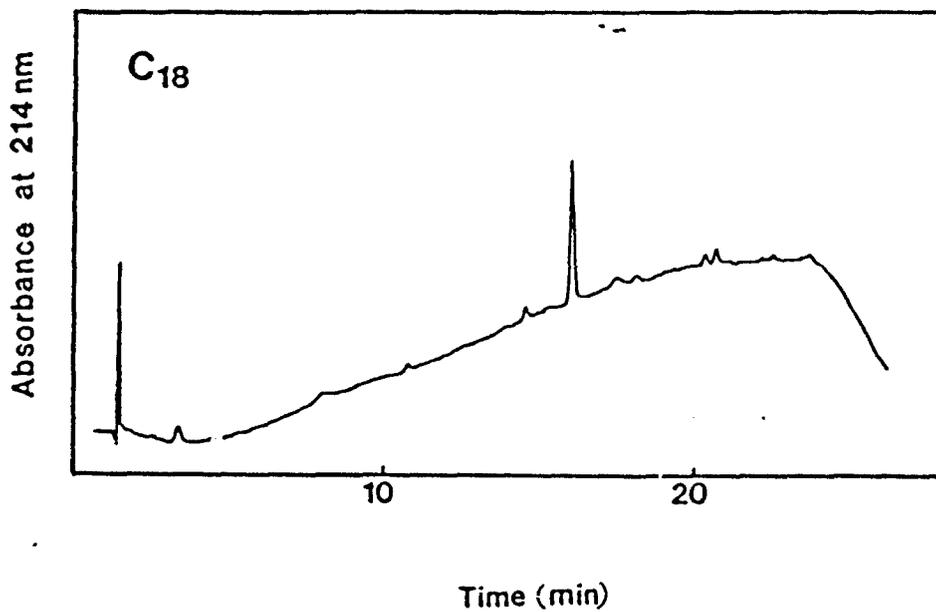


FIGURE 6

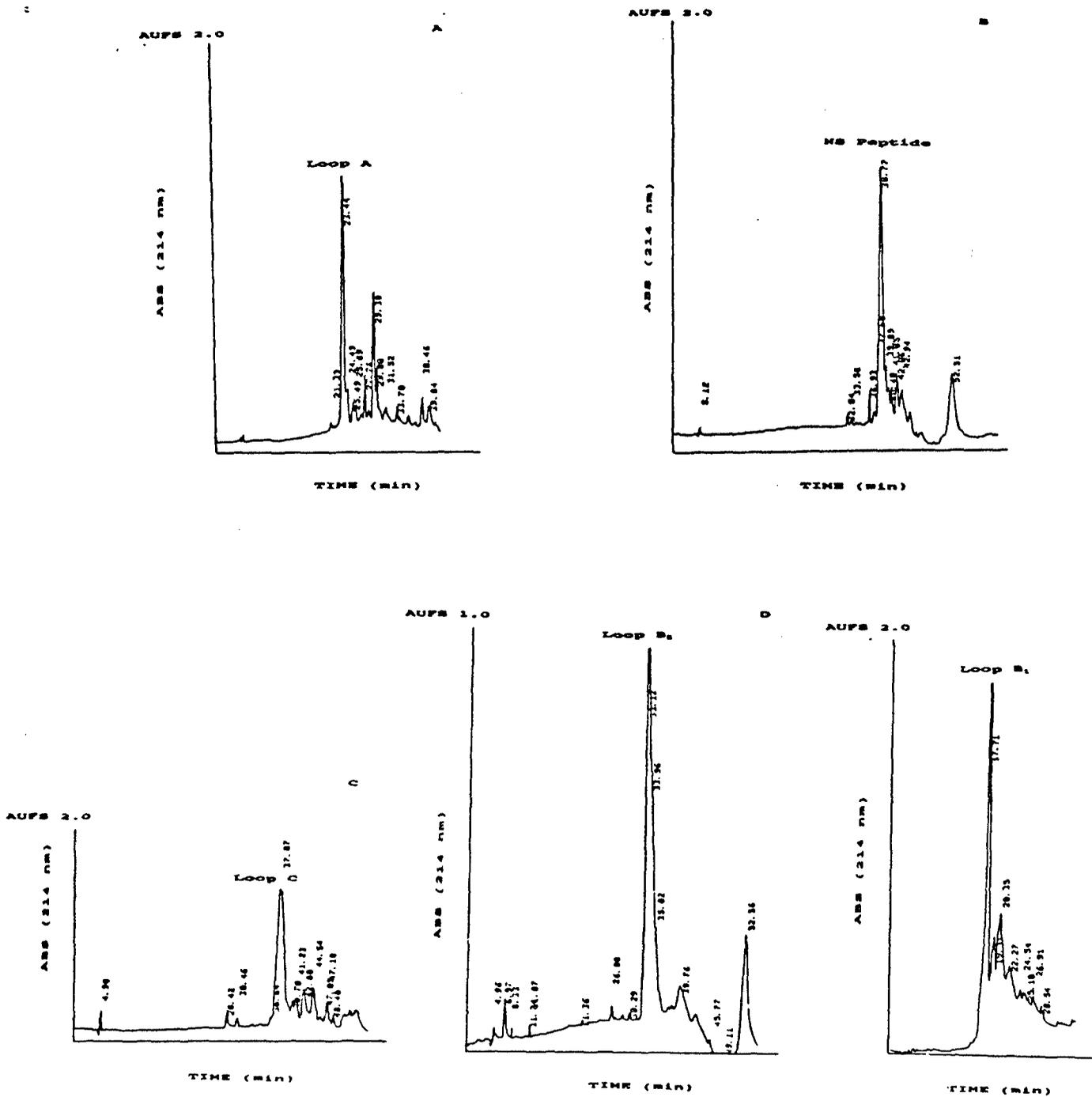
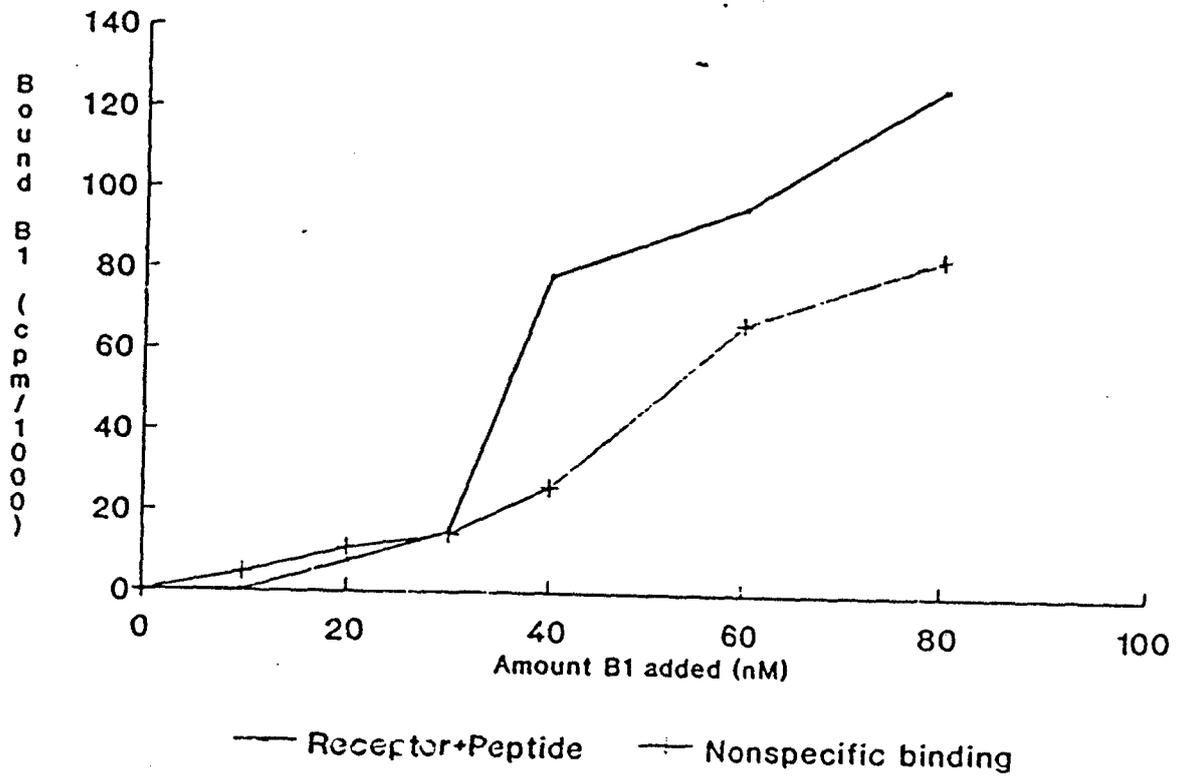


Figure 7

AChR Binding of B1 Peptide Synthetic Peptide B1 (18 mer)



AChR amount constant (5 nM)

FIGURE 8

Table I: Incorporation of Phenyl[2-¹⁴C]glyoxal into Free and AChR-Bound Lapemis Toxin

sample	experiment 1 (cpm)	experiment 2 (cpm)
unbound (free) <u>Lapemis</u> toxin	1688	1501
receptor - <u>Lapemis</u> toxin complex	3756	3793
<u>Lapemis</u> toxin detached from the complex	176	185

Table II: Amino Acid Composition Arginine-Modified Lapamis Toxin

amino acid	residues per mole of protein			Published* data	difference
	Present Experiment				
	actual	integer			
Asp	5.6	6	6		
Glu	8.4	8	8		
Ser	4.6	5	5		
Gly	4.3	4	4		
Thr	7.3	7	7		
His	1.9	2	2		
Ala	1.3	1	1		
Pro	3.3	3	3		
Arg	1.2	1	3	-2	
Tyr	1.0	1	1		
Met	0.5	1	1		
Val	1.3	1	1		
Half-Cys	8.5	9	9		
Ile	2.3	2	2		
Leu	1.2	1	1		
Lys	5.2	5	5		

*Fox, J. W., Elzinga, M., & Tu, A. T. (1977) FEBS Lett. 80, 217.

Table III: Amino Acid Composition of the Endoproteinase Glu-C
 Digested Fragments from Free Lapemis Toxin

amino acid	Fragment			
	1	2	3	4
Asp	2.8 (3)	2.3 (2)		0.6 (1)
Glu	0.7 (1)	2.6 (3)	2.3 (2)	0.8 (1)
Ser		2.1 (2)		3.1 (3)
Gly			3.0 (3)	1.2 (1)
Thr	1.2 (1)	4.1 (4)		1.8 (2)
His	1.0 (1)			1.1 (1)
Ala		1.3 (1)		
Pro		1.3 (1)	2.3 (2)	
Arg			1.0 (1)	0.3 (0)
Tyr				1.0 (1)
Met		0.6 (1)		
Val			0.75 (1)	
Half-Cys	3.1 (3)	2.8 (3)	2.0 (2)	1.1 (1)
Ile			1.1 (1)	0.8 (1)
Leu			1.2 (1)	
Lys		0.6 (1)	2.4 (2)	1.7 (2)

Table IV Lapemis toxin, Fmoc Synthetic Sequences
of Completed Peptide Loops Based on *Lapemis hardwickii*
Short Chain Postsynaptic Neurotoxin

Lapemis toxin: (1-60) MW = 6,680

10 20 30 40 50 60
| | | | | |
MTCCNQSSQPKTTTNC AESSCYKKTWSDHRGTRIERGCGCFQVKPGIKLECCHTNECNN

Loop A: (3-17) (Fmoc method) (15 mer)

3 10 17
| | |
CCNQSSQPKTTTNC

Loop B: (22-39) (Fmoc method) (18 mer)

22 30 39
| | |
CYKKTWSDHRGTRIERGC

Loop B: (23-38) (Fmoc method) (16 mer)

23 30 38
| | |
YKKTWSDHRGTRIERG

Loop C: (41-52) (Fmoc method) (12 mer)

41 50 52
| | |
CPQVKPGIKLEC

Negative Control Nonsense Peptide (Fmoc method) (20 mer)

10 20
| |
EACDFGHIKLMNPQRSTVWY

Single Letter Designation Code of the Amino Acids

A - Alanine	G - Glycine	M - Methionine	S - Serine
C - Cysteine	H - Histidine	N - Asparagine	T - Threonine
D - Aspartic acid	I - Isoleucine	P - Proline	V - Valine
E - Glutamic acid	K - Lysine	Q - Glutamine	W - Tryptophan
F - Phenylalanine	L - Leucine	R - Arginine	Y - Tyrosine