Isolation, Characterization, and Amino Acid Sequence of a Polypeptide Neurotoxin Occurring in the Sea Anemone *Stichodactyla helianthus*†

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ABSTRACT: An aqueous exudate collected from frozen and thawed bodies of a Caribbean sea anemone, *Stichodactyla* (formerly *Stoichactis*) *helianthus*, contained a polypeptide neurotoxin (Sh I) selectively toxic to crustaceans. The polypeptide was purified by G-50 Sephadex, phosphocellulose, and sulfopropyl-Sephadex chromatography and shown to have a molecular size of 5200 daltons and a p*I* of 8.3. The amino acid sequence determined by automatic Edman degradations of whole RCM Sh I and of its clostripain, staphylococcal protease, and cyanogen bromide digest peptides is $A^{1}ACKC^{5}DDEGP^{10}DIRTA^{15}PLTGT^{20}VDLGS^{25}$-$CNAGW^{30}EKCAS^{35}YYTII^{40}ADCCR^{45}KKK$. Only 33% of this sequence is identical with the sequence of *Anemonia sulcata* toxin II, a sea anemone toxin isolated from the taxonomic family Actiniidae. The six half-cystines are located in equivalent positions to those of the actiniid toxins and account for nearly half of the residues common to all of the toxins. However, 69% of the Sh I sequence is identical with that of toxin II from *Heteractis paumotensis*, another sea anemone belonging to the family Stichodactylidae. Stichodactylid toxins lack the initial N-terminal residue of actiniid toxins and possess three consecutive acidic residues at positions 6–8, a single tryptophan at position 30, and four consecutive basic residues at positions 45–48 (C-terminus). A rabbit IgG prepared by Sh I immunization bound Sh I with a $K_{0.5}$ of 4.7 nM but failed to bind homologous actiniid (*Anemonia sulcata* II, *Condylactis gigantea* III) or bolocerid (*Bolocera tuedae* II) polypeptide neurotoxins. We conclude that Sh I and other stichodactylid neurotoxins so far isolated represent a second type of sea anemone long-peptide neurotoxin which is structurally and immunologically distinct from the actiniid type 1 polypeptides, although these two types have obviously evolved from a common ancestral gene.

Polypeptide neurotoxins are often highly specific chemical tools for investigating ion channels in excitable cells. Among the polypeptide toxins affecting voltage-dependent sodium channels, scorpion toxins have been most thoroughly investigated (Catterall, 1977; Ray et al., 1977; Jover et al., 1980; Couraud et al., 1982). The initially investigated α-scorpion toxins delay the closing (inactivation) of sodium channels (Koppenhofer & Schmidt, 1968). Polypeptide toxins isolated from the sea anemones *Anemonia sulcata* (Bêress et al., 1975) and *Anthopleura xanthogrammica* (Norton et al., 1976) apparently act similarly upon the same site on the sodium channel (Catterall, 1980). One problem with sea anemone toxin binding experiments has been that the low affinities of the *Anemonia* and *Anthopleura* toxins for neuronal sodium channels render specific binding estimates more difficult. Consequently, there are some discrepancies in the literature regarding the relative numbers of α-scorpion and sea anemone toxin binding sites and the abilities of these toxins to compete for these sites (Catterall & Bêress, 1978; Yarnich et al., 1978; Vincent et al., 1980; Catterall & Beneski, 1980).

In order to investigate the binding of sea anemone toxins to sites on sodium channels in more detail, and determine the relationship of their receptors with receptors for various other polypeptide neurotoxins, we are isolating and characterizing polypeptide neurotoxins from several families of sea anemones. In this paper, we describe the purification, characterization, and amino acid sequence determination of a polypeptide neurotoxin occurring in a Caribbean sea anemone belonging to the Stichodactylidae, a taxonomic family which comprises the giant sea anemones primarily found in the Indopacific region. On the basis of important structural and immunochemical differences between the neurotoxins isolated from actiniid and bolocerid families, on one hand, and the Stichodactylid toxins, on the other, we propose that the former group be called type 1 toxins and the latter type 2.

Materials and Methods

Toxin Purification. Stichodactyla helianthus was collected by SCUBA in the Florida Keys. The anemones were subjected to two freeze–thaw cycles (−20 °C) and then were immersed in 10 volumes of cold deionized water. The mass was stirred intermittently for 1 h at 5 °C, filtered through cheesecloth, and centrifuged at 16000*g* for 10 min to remove insoluble material. The supernatant was fractionated by ammonium sulfate precipitation at 60% saturation. The precipitate was collected by centrifuging at 16000*g* for 10 min, dialyzed (Spectropor 1000 molecular weight cutoff) against H_2O for 6 h at 5 °C, and then freeze-dried. Gel and ion-exchange chromatography using volatile buffers was done as previously described (Kem, 1976; Kem & Dunn, 1988). Neurotoxic fractions were pooled, dialyzed, and again freeze-dried. Edman N-terminal analysis (described below) and analytical isoelectric focusing on 0.5-mm-thick polyacrylamide gels prepared according to the Bio-Rad manual and containing equal amounts of Servalyte 7–9 and 9–11 ampholytes were used to assess the homogeneity of the purified toxin samples. The p*I* of the toxin was determined by calibrating the gels with Serva marker proteins.

Toxicity Measurements. Fiddler crabs (*Uca pugilator*), collected at Cedar Key, FL, were used to assess neurotoxicity

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with a crustacean whole animal injection method previously described (Kem, 1976; Kem & Blumenthal, 1978). Hemolytic (cytolytic) activity was measured with washed rat erythrocytes after incubation at 37 °C for 1 h (Kem & Blumenthal, 1978; Kem & Dunn, 1988).

Since the *Stichodactyla* cytolysins interfere with the crab neurotoxicity assay, we could only measure neurotoxin levels in crude anemone fractions after separating the neurotoxin from the cytolysins. This was accomplished by exploiting lipophilicity differences between these toxins. In brief, samples were dissolved in 1.0 mL of 0.1 M cold trifluoroacetic acid and then loaded on a Waters C_{18} SepPak. Under these conditions, no toxic activity appeared in the initial eluate. The neurotoxin fraction was eluted with 3.0 mL of 40% acetonitrile containing 0.1 M trifluoroacetic acid, while the cytolysins were eluted with 3.0 mL of 80% acetonitrile–0.1 M trifluoroacetic acid. After dialysis, the fractions were freeze-dried and then subjected to crab toxicity and hemolysis bioassays.

Physical and Chemical Characterization of the Neurotoxin. For carboxymethylation, freeze-dried toxin was dissolved in 6.0 M guanidinium chloride containing 1.0 M Tris, pH 8.3, and then reduced 6 h with a 100-fold molar excess of mercaptoethanol, based upon an expected number of six half-cystines per monomer. Then a 90-fold excess of iodoacetic acid containing iodo[^{3}H]acetate (New England Nuclear) was allowed to react with the toxin for 30 min (Crestfield et al., 1963). The carboxymethylated toxin was desalted by exhaustive dialysis in 0.1 M acetic acid. The molecular size of the reduced and carboxymethylated (RCM)[1] toxin was estimated by Bio-Gel A-0.5m column chromatography in 6.0 M guanidinium chloride, as previously described (Fish et al., 1969; Kem, 1976).

Amino acid analyses were, in most cases, carried out with acid (6 N HCl) hydrolysates obtained by heating at 110 °C for 24, 48, or 72 h. The CM-cysteinyl, seryl, and threonyl estimates were obtained by extrapolating from these time points back to zero time. The isoleucyl and valyl residue estimates seemed to show no time dependence, so the mean values for all times were utilized, as with the other amino acids.

Edman Sequencing. A Beckman 890C sequenator was utilized with the Quadrol program. The PTH-amino acids obtained by manual conversion from the anilinothiazolines were identified by C_{18} HPLC using a methanol–acetate gradient, according to Bhown et al. (1978), or by TLC (Inagami & Murakami, 1972) in order to further identify certain amino acids. In the later stages, an Applied Biosystem 470A sequanator was employed to establish the C-terminal sequence.

Peptide Digests. Clostripain was used to selectively cleave arginyl peptide bonds. Enzyme (0.055 mg, Sigma) was incubated with 2.4 mg of RCM Sh I for 5.5 h at 37 °C in 0.1 M ammonium bicarbonate containing 0.2% mercaptoethanol and 0.05 M calcium chloride. The reaction was terminated by boiling for 2 min, and the products were separated on a C_{18} HPLC column utilizing a methanol gradient. Certain peaks were further resolved on G-10 and G-25 Sephadex columns equilibrated with 10% acetic acid.

In order to cleave acidic peptide bonds, 5.0 mg of RCM Sh I was incubated for 24 h at 37 °C with 0.27 mg of staphylococcal protease (Boehringer-Mannheim) dissolved in 0.05 M potassium phosphate, pH 7.8, containing 1 mM NaEDTA. The resulting peptides were resolved by C_{18} HPLC as above and by Sephadex chromatography in 0.1 M ammonium hydroxide.

In order to selectively cleave the tryptophanyl peptide bond of RCM SH I, 10 mg of toxin was dissolved in 2.0 mL of 88% formic acid and 2.0 mL of heptafluorobutyric acid, according to Ozols and Gerard (1977). Then 1400 mg of cyanogen bromide (Pierce) was added and the reaction allowed to proceed at room temperature for 24 h in the dark. The products were resolved on a G-25-SF Sephadex column (0.9 × 60 cm) eluted with 20% acetic acid.

Radioimmunoassay. The IgG fraction of an antiserum raised to Sh I in a male New Zealand white rabbit was used with iodinated Sh I (chloramine T method; Pennington et al., submitted for publication) for radioimmunoassay.

The rabbit was initially injected with 100 μg of toxin in Freund's complete adjuvant at multiple intradermal and subcutaneous sites on the back. Booster injections of 50 μg of toxin in incomplete adjuvant were made at 3-week intervals for up to a year. The sera from different bleeds were compared by Ochterlony immunodiffusion.

The IgG fraction was prepared by affinity chromatography on a protein A column. The protein A column itself was made as follows: staphylococcal protein A (10 mg, Boehringer Mannheim) was reacted at 4 °C with 3.0 mL of preswollen Affi-Gel 15 (Bio-Rad) for 4 h in 150 mM NaCl–100 mM $NaCO_3$, at pH 8.2. The remaining unreacted imidoesters were reacted with ethanolamine (Eastman) overnight at 4 °C. The column contained at least 3.2 mg of protein A/mL of resin, on the basis of changes in the 280-nm absorbance of the protein A coupling solution. After equilibration of the column with 140 mM NaCl and 25 mM Na_2HPO_4–Na_2PO_4, pH 7.8, antiserum (10 mL) to Sh I was then loaded on the column and eluted with this medium until the 280-nm absorbance approached zero; the IgG fraction was then eluted from the column with 140 mM NaCl, containing 0.58% glacial acetic acid. As the IgG fraction eluted from the column, its pH was immediately neutralized by mixing with 140 mM NaCl containing 1.5 M Tris buffer, pH 8.5.

Radioimmunoassays were carried out by using microtiter plates coated with protein A (Chandler et al., 1984). The anti-Sh I IgG fraction was diluted with RIA saline (25 mM Na_2HPO_4–NaH_2PO_4, 140 mM NaCl, 0.05% Tween 20, and 0.3% bovine serum albumin, pH 7.8) to 1/2000 of its serum volume; 20 μL was then added to each well. After allowing 1.5 h at room temperature for attachment, each well was washed 5 times with 100 μL of RIA buffer. To each well was then added 25 μL of a 1/100 dilution of ^{125}I Sh I (approximately 4000 cpm) and 25 μL of the appropriate dilution of unlabeled toxin. After incubation for 1.5 h at room temperature, each well was again washed 5 times with 100 μL of RIA saline, punched out, and then bound ^{125}I Sh I was counted with a Beckman 5500 γ counter.

Results

Toxin Purification. In our earliest purification attempts, we used Sephadex G-50 chromatography as the first step, but the neurotoxic activity was not separated very well from the more abundant cytolytic activity. In fact, the neurotoxic activity only became apparent after the second step when it

[1] Abbreviations: ^{125}I-Aah II, iodinated derivative of *Androctonus australis Hector* toxin II; Af I, *Anthopleura fuscoviridis* toxin I; Af II, *Anthopleura fuscoviridis* toxin II; As I, *Anemonia sulcata* toxin I; As II, *Anemonia sulcata* toxin II; As V, *Anemonia sulcata* toxin V; Ax I, *Anthopleura xanthogrammica* toxin I; Ax II, *Anthopleura xanthogrammica* toxin II; Bol II, *Bolecera tuediae* toxin II; CB, cyanogen bromide; CM, carboxymethylated; Cg III, *Condylactis gigantea* toxin III; CP, clostripain; Hm II, *Heteractis macrodactylis* toxin II; Hp II, *Heteractis paumotensis* toxin II; Hp III, *Heteractis paumotensis* toxin III; HPLC, high-pressure liquid chromatography; SP-Sephadex, sulfopropyl-Sephadex; PTH, phenylthiohydantoin; RCM, reduced and carboxymethylated; ^{125}I-Sh I, iodinated derivative of *Stichodactyla helianthus* neurotoxin I; Sh I, *Stichodactyla helianthus* neurotoxin I.

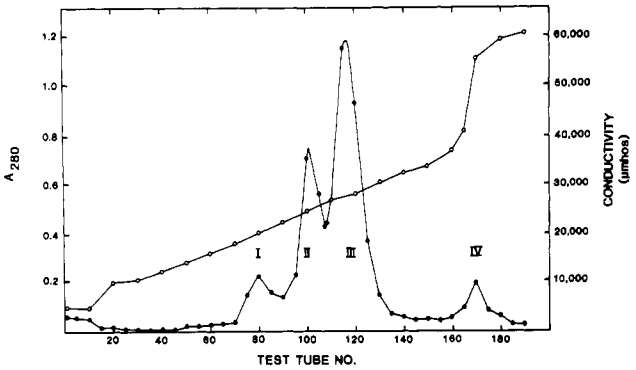

FIGURE 1: Ion-exchange purification of *Stichodactyla helianthus* toxin I by phosphocellulose ion-exchange chromatography (column size, 2.5 cm × 60 cm; eluting gradient, 500 mL of 0.1 M ammonium formate, pH 4.0, 500 mL of 1.0 M ammonium formate, pH 4.0; flow rate, 40 mL/h). A 15-g sample of 60% ammonium sulfate precipitate of the aqueous exudate was initially batch-adsorbed to the column at a conductivity of 4000 $\mu\Omega^{-1}$. Neurotoxic activity was found in peak II.

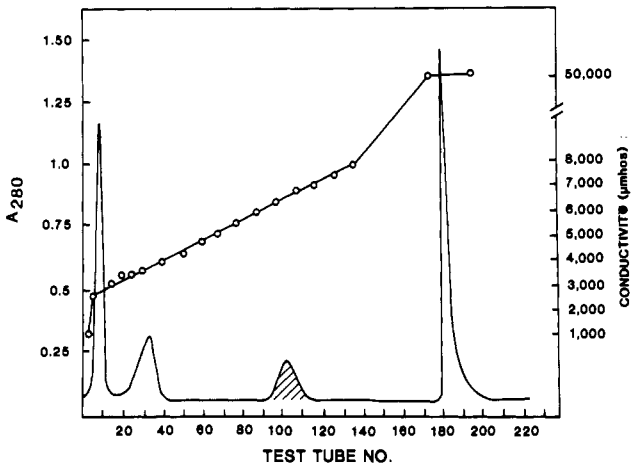

FIGURE 2: Isolation of Sh I by sulfopropyl-Sephadex ion-exchange chromatography at pH 6.5 (column size, 2.5 cm × 60 cm; eluting gradient, 500 mL of 0.01 M ammonium acetate, pH 6.5, and 500 mL of 0.20 M ammonium acetate, pH 6.5; following completion of gradient, column was washed with 1.0 M ammonium acetate, pH 6.5; flow rate, 30 mL/h). A 114-mg sample of phosphocellulose peak II fraction was loaded on the column with buffer possessing an initial conductivity of 650 $\mu\Omega^{-1}$. Neurotoxic activity was found in peak III.

was not retained by the weak cation-exchanger CM-52 cellulose (pH 7.5, 0.05 M ammonium acetate), while the cytolytic activity was. The neurotoxin could then be purified by chromatography on the stronger cation exchangers, phosphocellulose and SP-Sephadex, as outlined below, but this process was needlessly complex.

The best first step with regard to capacity, as well as degree of purification, was to absorb the dialyzed ammonium sulfate precipitate directly onto phosphocellulose. The neurotoxic fraction could then be separated from the more basic cytotoxic fraction by a linearly increasing gradient of ionic strength at pH 4.0 (Figure 1).

Final purification was achieved by a second step of ion-exchange chromatography (SP-Sephadex, Figure 2) and by Sephadex G-50 chromatography (0.05 ammonium acetate, pH 6.8). The resulting neurotoxin was homogeneous by the following criteria: (1) analytical isoelectric focusing; (2) Bio-Gel A-0.5m gel chromatography of the RCM toxin in 6.0 M guanidinium chloride; (3) amino acid composition; (4) N-terminal Edman sequencing.

The yield of toxin obtained during our purification could only be estimated by separating the neurotoxic and cytolytic

Table I: Amino Acid Composition of *Stichodactyla helianthus* Neurotoxin I

amino acid	no. of residues	amino acid	no. of residues
CM-Cys	6.19 (6)[a]	Val	1.18 (1)
Asx	5.96 (6)	Ile	2.63 (3)
Thr	4.30 (4)	Leu	2.19 (2)
Ser	1.66 (2)	Tyr	1.97 (2)
Glx	2.08 (2)	Trp	0.78 (1)
Gly	4.24 (4)	Lys	4.75 (5)
Ala	6.00 (6)[b]	Arg	2.15 (2)
Pro	2.04 (2)	total no.	48.12 (48)[c]

[a] Integral values are in parentheses. [b] All residue estimates were calculated assuming six alanyl residues per molecule. [c] The molecular weight, based upon this analysis, was 5142.

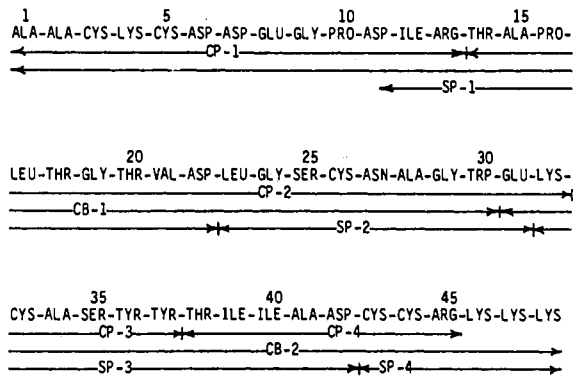

FIGURE 3: Amino acid sequence of *Stichodactyla helianthus* neurotoxin I, determined by automated Edman degradation of the reduced and carboxymethylated toxin and of the peptides generated by clostripain (C), staphylococcal protease (SP), or cyanogen bromide (CB) cleavage.

activities prior to the crab paralysis bioassay. About 120 mg of pure Sh I was obtained from 7.46 kg (115 animals) fresh weight of anemones. Using a Waters SepPak C_{18} cartridge, it was possible to separate these two activities in the crude exudate by varying the eluting acetonitrile concentration. After dialysis and freeze-drying, we detected about 27 mg of Sh I equiv/kg fresh weight of tissue. Thus, we estimate that our purification method yielded about 60% of the neurotoxin present in the aqueous exudate.

Physical and Chemical Characteristics of the Toxin. We determined the size of reduced, carboxymethylated Sh I by Bio-Gel A-0.5m gel chromatography in 6 M guanidinium chloride. The estimated molecular size of 5200 daltons, calculated from a K_d of 0.70 (mean of two measurements with K_d's of 0.68 and 0.71), indicates that the toxin belongs to the most common neurotoxin molecular size group.

The amino acid composition (Table I) of Sh I yielded a minimum monomeric molecular weight of 5142, on the basis of a single valine being present. This is in excellent agreement with a toxin molecular weight of 5142.1, estimated by plasma desorption mass spectrometry (Bennich & Kem, unpublished results). Since native toxin failed to react with Ellman's reagent in 6 M guanidinium chloride, the six half-cystines must contribute three disulfide bonds, the same number found in all other long polypeptide neurotoxins so far reported from sea anemones. The amino acid composition of Sh I is significantly more polar than the actiniid toxins, due to the presence of a larger proportion of ionizable residues and a smaller proportion of aromatic residues. Its composition was more similar to the *Heteractis* neurotoxins investigated by Schweitz et al. (1985).

Amino Acid Sequence of Sh I. The sequence of this toxin is presented in Figure 3. The peptide fragments analyzed are also indicated. Now we describe the strategy we used in obtaining this sequence.

Table II: Amino Acid Composition of the Clostripain (CP) Digest Peptides of *Stichodactyla helianthus* Neurotoxin (Moles of Amino Acid per Mole of Peptide)

peptide:	CP I	CP II	CP III	CP IV		
sequence:	1–13	14–32	33–37	38–45		
yield (%):	53	68	20	18	Σ peptides	whole toxin[d]
Cys	1.92 (2)[a]	0.97 (1)	0.82 (1)	1.85 (2)	6	6
Asp	3.02 (3)	1.94 (2)	0 (0)	1.06 (1)	6	6
Thr	0.14 (0)	2.72 (3)	0.10 (0)	0.89 (1)	4	4
Ser	0[b] (0)	0.96 (1)	0.92 (1)	0.09 (0)	2	2
Glu	1.01 (1)	0.96 (1)	0.05 (0)	0.10 (0)	2	2
Pro	0.78 (1)	1.06 (1)	0.09 (0)	0 (0)	2	2
Gly	1.01 (1)	3.04 (3)	0 (0)	0.15 (0)	4	4
Ala	1.51 (2)	2.22 (2)	0.81 (1)	1.01 (1)	6	6
Val	0 (0)	0.88 (1)	0.09 (0)	0 (0)	1	1
Ile	0.87 (1)	0 (0)	0 (0)	1.40 (2)	3	3
Leu	0 (0)	2.10 (2)	0 (0)	0 (0)	2	2
Tyr	0.23 (0)	0 (0)	2.09 (2)	0 (0)	2	2
Lys	1.01 (1)	0.89 (1)	0.11 (0)	0.18 (0)	2	5
Trp	– (0)	– (1)	– (0)	– (0)	1	1
Arg	0.82 (1)	0 (0)	0 (0)	1.00 (1)	2	2
total	13	19	5	8	45[c]	48

[a] Numbers in parentheses are the values expected from the proposed sequence. [b] A zero-residue estimate indicates less than 0.05 mol of amino acid/mol of peptide. [c] Lysine (0.8 residue per molecule) was released during clostripain proteolysis of the RCM toxin. [d] Composition determined by amino acid analysis.

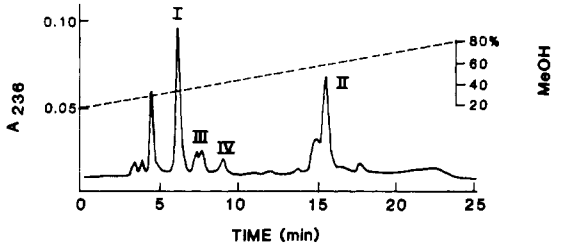

FIGURE 4: Reverse-phase HPLC isolation of the peptide fragments generated by clostripain digestion. Peptides were separated with a Waters C_{18} Nova-pak (0.46 cm × 15 cm) using a gradient of 20–80% methanol into 0.05 M ammonium acetate, pH 4.45, at a flow rate of 1.5 mL/min. The peak number corresponds with the clostripain peptide shown in Figure 3.

The intact RCM toxin was subjected to two automatic Edman degradation runs with a Beckman sequenator. The first run was terminated after 36 steps, since the PTH-amino acid yields at some steps were not sufficient for identification. About 80% of the residues were identified unequivocally. We then proceeded to obtain peptide fragments to elucidate the remaining residues.

We initially utilized clostripain cleavage with the hope of selectively cleaving the two arginyl peptide bonds (Keil, 1982). One Arg residue had been located at position 13. However, HPLC separation of the clostripain digest revealed that more extensive digestion had occurred (Figure 4). After further purification on several Sephadex columns, we obtained five homogeneous peptides, whose compositions and yields are shown in Table II. The cumulative amino acid composition of the clostripain peptides accounted for all of the amino acid residues found in RCM Sh I, except for three lysyl residues. Since 0.8 residue of lysine was liberated per mole of toxin, it was inferred that 1 lysyl residue was located on the amino side of a peptide bond containing another clostripain-sensitive amino acid residue.

Initially, we suspected that one or more of these lysyl residues was interposed between two other clostripain-cleaved peptides in the portion of the polypeptide chain which had not been sequenced using the whole RCM Sh I. That CP III lacked either Arg or Lys residues was unexpected. The basis for this spurious cleavage was not further studied. Although three of the four peptides (C I, II, and IV) were sequenced, the primary contribution of the clostripain digest was verification of the amino acid compositions for each region of sequence.

Table III: Amino Acid Composition of the Staphylococcal Protease (SP) Digest Peptides of *Stichodactyla helianthus* Neurotoxin

peptide:	SP 1	SP II	SP III
sequence:	12–22	23–31	32–42
Cys	0 (0)[a]	0.62 (1)	0.78 (1)
Asp	0.98 (1)	0.92 (1)	1.28 (1)
Thr	2.21 (3)	0.14 (0)	1.08 (1)
Ser	0.14 (0)	0.85 (1)	0.78 (1)
Glu	0.12 (0)	0.99 (1)	0.34 (0)
Pro	1.02 (1)	0.11 (0)	0 (0)
Gly	1.08 (1)	2.00 (2)	0.25 (0)
Ala	1.02 (1)	1.05 (1)	2.00 (2)
Val	1.07 (1)	0.06 (0)	0.28 (0)
Ile	0.78 (1)	0 (0)	1.42 (2)
Leu	1.20 (1)	0.96 (1)	0.22 (0)
Tyr	0 (0)	0 (0)	1.56 (2)
Lys	0 (0)	0.24 (0)	0.62 (1)
Arg	0.77 (1)	0.08 (0)	0.12 (0)
total	11	9	11

[a] Numbers in parentheses are values based upon the proposed toxin sequence.

At this point, we attempted to obtain a series of overlapping peptides by staphylococcal protease cleavage at acidic residue peptide bonds. Due to their limited solubility in acetic or other acids, purification of these peptides was largely accomplished by Sephadex (G-15 or G-25) separations in 0.1 M ammonium hydroxide. The compositions of the three isolated peptides (Table III) were consistent with the sequence shown in Figure 3. Edman degradation of peptide SP III provided the sequence for residues 33–41 for the first time. Unfortunately, the C-terminal peptide could not be isolated for analysis. The staphylococcal protease peptides also confirmed the assignments of Asp-11, Asp-22, Glu-31, and Asp-42, on the basis of this enzyme's specificity for acidic residue peptide bonds.

At this point, the only amino acid residues which had not been assigned in the sequence were three lysyl residues. The C-terminal region had not been as extensively analyzed as other portions of the toxin. We thus attempted to cleave the toxin into two peptides at Trp-30, using cyanogen bromide cleavage in the presence of heptafluorobutyric acid (Ozols, 1977). After desalting with a G-25 Sephadex column, the polypeptide fraction was separated into three peaks on a G-50

Table IV: Amino Acid Composition of the *Stichodactyla helianthus* Neurotoxin Cyanogen Bromide Tryptophan Cleavage Peptides

peptide:	CB I (1–30)	CB II (31–48)
yield (%):	72	76
CMC	1.99 (3)[a]	1.75 (3)
Asp	5.39 (5)	1.26 (1)
Thr	3.04 (3)	1.00 (1)
Ser	1.01 (1)	0.87 (1)
Glu	1.24 (1)	0.97 (1)
Pro	1.45 (2)	0 (0)
Gly	3.70 (4)	0.29 (0)
Ala	4.00 (4)[b]	2.00 (2)[d]
Val	1.08 (1)	0.10 (0)
Ile	1.09 (1)	1.62 (2)
Leu	2.20 (2)	0.12 (0)
Tyr	0 (0)	1.55 (2)
Lys	1.08 (1)	4.32 (4)
Trp	– (1)[c]	– (0)
Arg	1.22 (1)	1.10 (1)
total	30	19

[a] Numbers in parentheses are the values expected from the proposed sequence. [b] Peptide composition was calculated by assuming 4.0 mol of alanine/mol of peptide. [c] Based upon the specificity of the cyanogen bromide–heptafluorobutyric acid cleavage reaction. [d] Peptide composition was calculated by assuming 2.0 mol of alanine/mol of peptide.

Sephadex column. The first peak was uncleaved RCM Sh I, the second peak CB I (residues 1–30), and the third peak CB II (31–48). The CB II peptide (Table IV) was then sequenced on a Beckman 890C sequenator. A definite HPLC peak was not obtained for residue 48. Furthermore, the amino acid composition of this peptide indicated the occurrence of only two lysyl residues, rather than the three expected from the RCM toxin amino acid analysis. Identification of the C-terminal amino acid as Lys-48 was finally obtained when the CB II peptide was sequenced using an ABI-470A gas-phase sequenator. The amount of PTH-Lys at position 48 exceeded the yield for PTH-Lys-47. No other residues were detected after position 48, so assignment of the Lys-46-Lys-47-Lys-48 was complete.

Our attempts to release the carboxyl-terminal amino acids with carboxypeptidases were unsuccessful. Carboxypeptidase A + B or Y digests of the RCM toxin under optimal conditions for cleaving basic amino acids from the C-terminus were consistently unsuccessful, despite the fact we used at least two separate batches of each enzyme preparation and allowed the digests to continue for up to 24 h (data not shown).

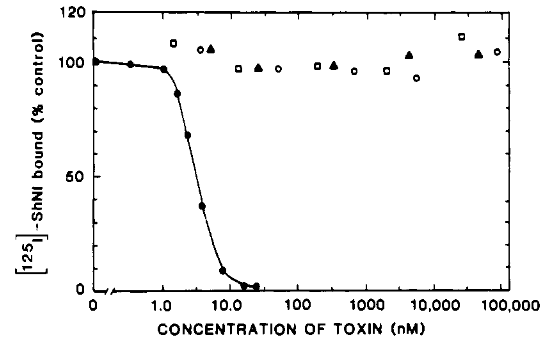

FIGURE 5: Competition between ^{125}I Sh I and different sea anemone toxins for association with Sh I antibodies. The Sh I serum was used at a dilution of 12000. (Ordinate) Ratio of bound Sh I to ^{125}I Sh I obtained by directly counting radioactivity bound to protein A coated wells of microtiter plate. Each point is the mean value obtained from triplicate measurements. (●) Sh I; (○) As II; (▲) Bol II; (□) Cg III.

Immunochemical and Toxic Properties of Sh I. The rabbit polyclonal antibody (IgG fraction) prepared with native Sh I as immunogen bound ^{125}I Sh I with a $K_{0.5}$ of 4.7 nM, as shown in Figure 5. In contrast, 10 000 nM concentrations of *Anemonia sulcata* toxin II, *Condylactis* toxin III, and *Bolocera* toxin II failed to compete with iodinated Sh I for antibody binding. The first two toxins are from sea anemones belonging to the family Actiniidae, and the third presented the family Boloceroididae.

Sh I was highly toxic to crustaceans, but without effect upon mice when injected intraperitoneally (Table VI). Along with toxins purified from the actiniid anemone *Condylactis gigantea*, it seems to be the most crustacean toxic sea anemone neurotoxin so far investigated. Only when injected intracerebroventricularly into the mouse brain at a very high dose (116 μg/kg) was paralysis observed.

Discussion

The method we selected for extracting *Stichodactyla* neurotoxin (and the cytolysins) was milder than the procedures used by other investigators (Bĕress, 1975; Schweitz et al., 1985; Norton et al., 1976). Alcohol–water extraction and subsequent

Table V: Amino Acid Sequences of Sea Anemone Long Polypeptide Toxins Affecting Sodium Channels[a]

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        1       5        10        15        20              25        30        35        40        45
Af I    G V A C L C D S D G P N V R G N T L S G T I W L       A G C P S G W H N C K A H G P T I G W C C K Q
Af II G G V P C L C D S D G P S V R G N T L S G I I W L       A G C P S G W H N C K A H G P T I G W C C K Q
As I    G A P C L C K S D G P N T R G N S M S G T I W V       F G C P S G W N N C E G R A   I I G Y C C K Q
As II   G V P C L C D S D G P S V R G N T L S G I I W L       A G C P S G W H N C K K H G P T I G W C C K Q
As V    G V P C L C D S D G P S V R G N T L S G I L W L       A G C P S G W H N C K K H K P T I G W C C K
Ax I    G V S C L C D S D G P S V R G N T L S G T L W L Y P S G C P S G W H N C K A H G P T I G W C C K Q
Ax II   G V P C L C D S D G P R P R G N T L S G I L W F Y P S G C P S G W H N C K A H G P N I G W C C K K

Hm III    G N C K C D D E G P Y V R T A P L T G Y V D L       G Y C N E G W E K C A S Y Y S P I A E C C R K K K
Hp II     A S C K C D D D G P D V R S A T F T G T V D F       W N C N E G W E K C T A V Y T P V A S C C R K K K
Hp III    G N C K C D D E G P N V R T A P L T G Y V D L       G Y C N E G W E K C A S Y Y S P I A E C C R K K K
Sh I      A A C K C D D E G P D I R T A P L T G T V D L       G S C N A G W E K C A S Y Y T I I A D C C R K K K
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[a] All polypeptides are aligned with respect to As II; the numbering system is also based upon this toxin. References: Af I and Af II, Sunahara et al., 1987; As I, Wunderer & Eulitz, 1978; As II, Wunderer et al., 1976; As V, Scheffler et al., 1982; Ax I (anthopleurin A), Tanaka et al., 1977; Ax II (anthopleurin B), Reimer et al., 1985; Hm III, Zykova et al., 1985; Hp II, Wemmer et al., 1986; Hp III, Metrione et al., 1987; Sh I, this paper.

heating to precipitate the larger polypeptides and proteins could inactivate many other anthozoan toxins and related venom constituents such as phospholipases. We therefore selected the milder freeze–thaw procedure, in order to obtain concurrently any larger toxins which might be present. This procedure efficiently released the *Stichodactyla* cytolysins (Kem & Dunn, 1988) but probably failed to release all of the neurotoxic activity. It has recently been shown that the Sh cytolysins are localized within integumentary gland cells, rather than nematocysts (Kem and Östman, in preparation). Upon examination of freeze–thawed *Stichodactyla* tissues, few discharged nematocysts were observed. If the neurotoxicity of this species is localized within its nematocysts, then it is likely that more drastic extraction procedures (such as alcohol extraction or mechanical disruption) expected to rupture nematocysts would yield greater amounts of neurotoxin, possibly including other isotoxins. We detected trace amounts of another more basic neurotoxin in one purification. Schweitz et al. (1985) previously reported that the related Indopacific species *Stichodactyla gigantea* possesses two polypeptide neurotoxins similar in size and composition to Sh I.

Although polypeptides the size of Sh I have occasionally been fully sequenced in a single continous Edman degradation, this was difficult to achieve with this toxin, due to its C-terminal lysines and the frequent occurrence of seryl, threonyl, and aspartyl residues. These were sometimes difficult to unequivocally assign because of low PTH yields or HPLC retention time overlap. The C-terminal lysines were also assigned with difficulty by others (Zykova et al., 1985; Wemmer et al., 1986; Metrione et al., 1987). Although the tryptophan peptide bond cleavage according to the Ozols and Gerard (1977) procedure was not complete under the conditions employed, we suggest that this chemical cleavage method can be quite useful in future sequence studies of other sea anemone polypeptides, particularly when few methionyl residues are present.

The amino acid sequence of Sh I is quite different from the previously reported actiniid toxins (Table V). Only 16 of the 48 residues in Sh I are the same as for AS II, and 6 of these are half-cystines constituting the 3 disulfide pairs so far found in the medium-sized neurotoxins. However, the toxin sequence is similar to those of other stichodactylid neurotoxins that have been reported. In contrast, 33 residues of Sh I are identical with those occurring in Hp II, isolated from the same family of sea anemones. In general, the stichodactylid toxins all share the following features: (1) the N-terminal amino acid (usually glycine) of actiniid toxins is missing; (2) there are three consecutive carboxylate-containing side chains at positions 6–8; (3) a single tryptophan occurs at position 30; (4) a Tyr-Tyr sequence occurs at positions 36–37; and (5) a highly basic Arg-Lys-Lys-Lys tetrapeptide sequence occurs at the C-terminus.

On the basis of this structural information, it is apparent that there are two distinct families of medium-sized anemone toxins. We propose that the actiniid toxins be called type I toxins since they were first characterized and the stichodactylid toxins be designated as the second type. We are intentionally avoiding the α,β terminology applied to the scorpion polypeptides, as the receptor binding sites for certain sea anemone neurotoxins probably differ from those for both types of scorpion toxin. Since there are many other taxonomic families of sea anemones, future investigations are likely to reveal other toxin types. Analysis of the structure–activity relationships of these various isotoxins should reveal much about the functionality of particular amino acid side chains for affecting different sodium channels.

Table VI: Pharmacological Properties of Purified Sea Anemone Long Polypeptide Toxins Affecting Sodium Channels[a]

	LD_{50} (μg/kg)		rat tissue response (nM)	
toxin	crab	mouse	brain K_D	heart EC_{50}
type 1				
Cg II	0.2	>50000		>1000
Pf I	0.4	>20000		>1000
As I	2	4000	7000	
As II	2	100	150	15
As V	5	19	50	2
Ax-I	11	66	120	3
Ax-II	39	8	35	2
type 2				
Sh I	0.3	>15000		>8000
Sg I	7	>2000	>10000	
Hp III	10	53	300	4000
Hp II	15	4200	>100000	5000
Hp I	36	145	900	3000
Hp IV	90	40	10000	1300
Hm III	820	20		

[a] LD_{50}'s are based on intrahaemocoelic (crab) or intraperitoneal injections; K_D = equilibrium dissociation constant; EC_{50} = median effective concentration increasing sodium fluxes. References: (1) Cg, Pf (Kem, unpublished results); (2) As, Ax, Sg (Schweitz et al., 1981); (3) Hm (Zykova et al., 1985); (4) Hp (Schweitz et al., 1985). Abbreviations: As = *Anemonia sulcata*; Ax = *Anthopleura xanthogrammica*; Cg = *Condylactis gigantea*; Hm = *Heteractis macrodactylus*; Hp = *Heteractis paumotensis*; Pf = *Phyllactis flosculifera*; Sg = *Stichodactyla giganteum*; Sh = *Stichodactyla helianthus*.

We have corroborated the initial observations of Schweitz et al. (1985) concerning the immunological unrelatedness of the stichodactylid toxins with the actiniid toxins. Our radioimmunoassays showed that *Anemonia sulcata* II, *Condylactis gigantea* III, and the *Bolocera* II toxins all failed to react with a polyclonal antibody specific for Sh I, even at 2500-fold higher concentrations than the $K_{0.5}$ for Sh I. Using Ochterlony diffusion assays, we also found that the *Bolocera* antiserum precipitated *Anemonia sulcata* II and that As II antiserum precipitated *Bolocera* toxin II. The sequence of *Bolocera* toxin II (A. Henschen, personal communication) most closely resembles the actiniid (type 1) toxins.

It will be of interest to compare the electrophysiological actions of the type 1 and 2 toxins, in view of the differences in sodium channel receptor binding properties reported by Schweitz et al. (1985). The electrophysiological actions of Sh I are currently being investigated (Salgado and Kem, in preparation). It has been shown that Sh I prolongs the repolarization phase of the action potential in the crayfish median giant axon in a manner similar to the actiniid toxin effects. The sodium channel is the only site of action so far found for both the actiniid and stichodactylid polypeptides (Schweitz et al., 1985; Warshina & Fujita, 1983).

Sh I possesses a high crustacean toxicity but no mammalian toxicity when injected peripherally (Table VI). At the other extreme in the selective toxicity profile is *Heteractis macrodactylus* toxin III, with high vertebrate toxicity but relatively low arthropod toxicity. These two stichodactylid toxins differ only at 10 positions in the sequence, some of which are conservative changes (Val for Ile, Ser for Thr, Glu for Asp); thus, the phylogenetic differences in toxicity seem to depend upon just a few amino acid residues. Further studies of other related sea anemone polypeptides are needed to determine if there is always an inverse relationship between crustacean and vertebrate toxicity in this group of toxins. We have recently succeeded in the chemical synthesis of Sh I (Pennington et al., in preparation). This approach can thus provide numerous Sh I analogues differing by single amino acid substitutions for the pharmacological and biochemical analysis. Investigation

of the selective toxicity of synthetic as well as naturally occurring sea anemone neurotoxin variants, besides permitting insight into phyletic and intraorganismic differences in sodium channels, may eventually provide leads for the design of new highly selective pesticides lacking significant vertebrate toxicity.

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Registry No. Sh I, 119454-65-8; Sh I (reduced), 117860-13-6.

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