Novel α- and ω-Conotoxins from *Conus striatus* Venom†

Cecilia A. Ramilo,‡,§ Glenn C. Zafaralla,‡ Laszlo Nadasdi,‖ Lance G. Hammerland,§ Doju Yoshikami,§ William R. Gray,§ Ramasharma Kristipati,‖ J. Ramachandran,‖ George Miljanich,‖ Baldomero M. Olivera,*,§ and Lourdes J. Cruz‡,§

Marine Science Institute, University of Philippines, Diliman, Quezon City 1101, Philippines, Neurex Corporation, 3760 Haven Avenue, Menlo Park, California 94025-1057, and Department of Biology, University of Utah, Salt Lake City, Utah 84112

Received January 30, 1992; Revised Manuscript Received June 17, 1992

ABSTRACT: Three neurotoxic peptides from the venom of *Conus striatus* have been purified, biochemically characterized, and chemically synthesized. One of these, an acetylcholine receptor blocker designated α-conotoxin SII, has the sequence GCCCNPACGPNYGCGTSCS. In contrast to all other α–conotoxins, SII has three disulfide bonds (instead of two), has no net positive charge, and has a free C-terminus. The other two paralytic peptides are Ca channel-targeted ω-conotoxins, SVIA and SVIB. ω-SVIA is the smallest natural ω-conotoxin so far characterized and has the sequence CRSSGSPCGVTSICCGRCYRGKCT-NH_2. Although ω-conotoxin SVIA is a potent paralytic toxic in lower vertebrate species, it was much less effective in mammals. The third toxin, ω-conotoxin SVIB, has the sequence CKLKGQSCRKTSYD-CCSGSCGRSGKC-NH_2. This peptide has a different pharmacological specificity from other ω-conotoxins previously purified from *Conus* venoms; only ω-conotoxin SVIB has proven to be lethal to mice upon ic injection. Binding competition experiments with rat brain synaptosomal membranes indicate that the high-affinity binding site for ω-conotoxin SVIB is distinct from the high-affinity ω-conotoxin GVIA or MVIIA site.

The venoms of the ca. 500 predatory marine snails belonging to the genus *Conus* are used to paralyze prey. Approximately 50 species hunt fish exclusively and employ potent toxins to immobilize their faster moving prey (Kohn et al., 1960). It has previously been shown that the paralytic components in *Conus* venoms are small peptides, typically 10–30 amino acids long [see Olivera et al. (1990, 1991)].

One unexpected feature of the *Conus* peptide system is the extreme sequence hypervariability, even among homologous toxins from different *Conus* species (Olivera et al., 1991). Thus, diverse amino acid sequences can be found within a peptide family wherein all peptides have the same target in the fish prey (i.e., inactivating a specific receptor or ion channel important in neuromuscular transmission). This hypervariability is potentially useful in neuroscience. An enormous proliferation of different receptor subtypes has apparently taken place in the evolution of the mammalian central nervous system. Families of *Conus* toxins are proving to be useful for studying and distinguishing closely related receptor subtypes. When these sequence-divergent but homologous conotoxins are tested in a complex nervous system such as mammalian brain, any two homologous *Conus* peptides often exhibit somewhat different (but usually overlapping) binding specificities. The multiplicity of receptor subtypes being identified by recombinant DNA technology creates a need to enlarge the collection of complementary pharmacological probes; the homologous, but diverse, *Conus* peptides are helping to fulfill this need.

In this work, we describe the purification, characterization, and synthesis of three new paralytic peptide toxins from *Conus striatus* venom. The striated cone, *C. striatus* (Figure 1), is one of the largest fish-hunting *Conus* snails, a highly successful species found over the entire Indo-Pacific region from the Hawaiian Islands to the east African coast [see Walls (1979)]. The biology of *C. striatus* differs from that of the other large fish-hunting species which we have extensively characterized, the geography cone, *Conus geographus*. *C. geographus* is a reef species which hunts fish using a net strategy; it opens its mouth to a remarkable extent and engulfs sleeping fish before stinging them. In contrast, *C. striatus* prefers sandy areas where it lies generally buried. This predator uses a harpoon-and-line strategy; when it senses a fish, it extends its proboscis and harpoons the fish with the hollow radular tooth which is also used to inject venom. Instances of humans being stung have been described for both species (Cruz et al., 1985b); although a high proportion of *C. geographus* stings are lethal, no fatalities have been reported for *C. striatus* envenomations.

We previously characterized two paralytic toxins from *C. striatus* venom, α-conotoxin SI (Zafaralla et al., 1988) and α-conotoxin SIA (Myers et al., 1991). Both of these peptides have two disulfide linkages and clearly belong to the nicotinic acetylcholine receptor targeted family of peptides, the α-conotoxins (Gray et al., 1981; Myers et al., 1991). In this work, we describe three more paralytic toxins from *C. striatus* venom. One is a unique α-conotoxin; in contrast to all other α-conotoxins described so far, the new peptide has three disulfide bonds instead of two. The other two paralytic toxins belong to the ω-conotoxin family and target to voltage-sensitive Ca channels (Olivera et al., 1985). One of these, ω-conotoxin SVIB, has a different pharmacological specificity from other ω-conotoxins previously characterized.

EXPERIMENTAL PROCEDURES

Purification of Conotoxins from C. striatus Venom. Purification of the peptides from *C. striatus* venom involved initial size fractionation of its components by chromatography on Sephadex G-25 as previously described (Zafaralla et al.,

† This work was supported by Grants GM 22737 and GM 34913 from the National Institute of General Medical Sciences.

* To whom correspondence should be addressed at the Department of Biology, University of Utah, Salt Lake City, UT 84112.

‡ University of Philippines.

§ University of Utah.

‖ Neurex Corporation.

0006-2960/92/0431-9919$03.00/0

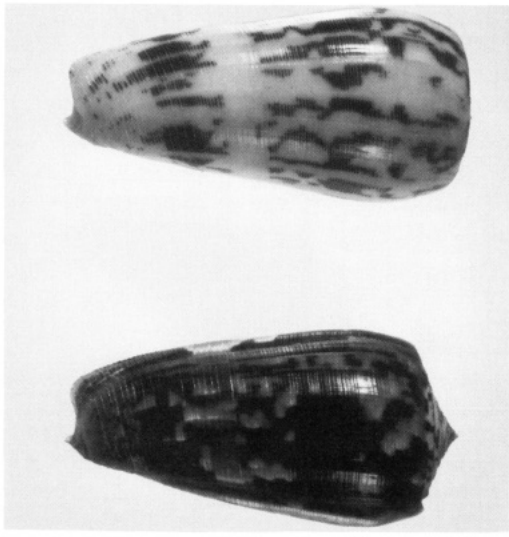

FIGURE 1: Shells of the striated cone, *C. striatus*. This species is found in the entire Indo-Pacific region, from Hawaii to East Africa. The specimens shown are approximately 7 cm long. The two specimens represent extremes both in geographical range and in shell pattern variation. The lighter specimen is from Oahu, HI, and the dark shell is a Reunion Island specimen from the Indian Ocean. Living specimens of *C. striatus* completely bury themselves in the sand, but in the presence of fish they emerge to hunt prey.

1988), followed by HPLC[1] on semipreparative reverse-phase C_{18} columns (see Figure 2). Isolation of peptides from selected peaks usually required one or two more runs on analytical reverse-phase C_{18} columns. Gradients of acetonitrile in 0.1% TFA were used to elute the peptides.

Sequence Analysis of Conotoxins. Two of the peptides (SII and SVIA) were reduced and carboxymethylated and then analyzed in a spinning-cup sequencer as described previously (Edman & Begg, 1967). SVIB was sequenced using an Applied Biosystems 475A liquid pulsed sequenator by Dr. G. Hathaway, of the University of California, Riverside.

Synthesis of Conotoxin SII. Synthesis was carried out by the solid-phase procedure of Merrifield (1963), following the general protocol of Gray et al. (1983). Two grams of *t*-Boc-L-serine resin (Vega Biotechnologies Inc.; 0.60 mmol substitution/g) was used as the starting support, and Boc amino acids were purchased from Bachem. All Boc amino acids, except Boc-Gly, were of the L configuration. Side chains were protected as Cys(Mob), Ser(Bzl), Thr(Bzl). With one exception, couplings were carried out using dichloromethane as solvent and diisopropylcarbodiimide as the coupling agent. Asparagine was coupled without side-chain protection, using dimethylformamide as solvent, and 2 equiv of hydroxybenzotriazole was added to minimize side reactions. Coupling was repeated when Pro residues made the ninhydrin test (Kaiser et al., 1970) an unreliable guide for completeness of reaction.

The peptide was deprotected and removed from the resin (2.0 g of peptide-resin) as the Ser C-terminal, using the low–high HF procedure of Tam et al. (1983).

Extraction and Purification of Reduced SII. After completely distilling off the HF and most of the scavengers, the mixture was extracted with ~200 mL of ether/1% β-mercaptoethanol (βME). The peptide was extracted with ~200 mL of 5% HOAc/1% βME and then filtered through a 0.5-μm Millipore membrane filter to remove the precipitate that formed.

The filtrate was concentrated to ~40 mL by lyophilization and loaded onto an LH-20 column using 30% acetonitrile in 0.1% TFA as eluting buffer at a flow rate of 2.5 mL/min. Fractions were collected every 3 min for a total of 80 tubes, and those fractions that showed positive ninhydrin test were pooled and lyophilized.

Oxidation and Purification of SII. The lyophilized peptide (0.611 g) was treated with 10 mL of 6 M guanidine hydrochloride/10 mM DTT, pH 9.0, and incubated at 55 °C for 1 h. After cooling, the clear, yellow solution was then added dropwise to 4 L of 0.05 M NH_4CO_3, pH 8.08 (previously bubbled with N_2 for 1.5 h). The reaction flask was loosely covered with paper tissue, and the mixture was allowed to air-oxidize (with magnetic stirring) at room temperature until it gave a negative Ellman's test for thiol.

The oxidized mixture was adjusted to pH 5.5 with glacial acetic acid and then filtered on a Buchner funnel (Whatman No. 1 filter paper). The filtrate was chromatographed using a C_{18} RP Waters Custom Prep Pak HPLC column (Delta Pak C_{18}, 300-Å pore size, 15 μm) and eluted with a linear gradient of the TEAP system, pH 6.0 (Rivier et al., 1984), at a flow rate of 40 mL/min for 50 min. Fractions monitored at 280 nm were collected manually every 30 s. The correctly-folded peptide was identified by analytical HPLC using 10–20 μL of each fraction from the peaks and comparing the elution time in TFA/acetonitrile with that of the natural SII peptide. Further purification of the peptide was carried out using a semipreparative column and TFA/acetonitrile buffer system.

Synthesis of Conotoxin SVIA and SVIB. These peptides were synthesized on a replumbed ABI Model 430A peptide synthesizer, using standard *t*-Boc chemistry with some modifications (Yamashiro & Li, 1988). The syntheses were started from 0.4 mmol of MBHA resin (0.61–0.66 mequiv of NH_2/g; Advanced Chem. Tech.), single coupling the first nine amino acids (except Arg and Asn, which are always double coupled as active esters formed from HOBt with DCC) and double coupling the remainder (the first couplings were in DCM, the second couplings in DMFA solution). Amino acid side chain protections were Arg(Tos), Asp(OBzl), Cys(4-MeBzl), Hyp(Bzl), Lys(ClZ), Ser(Bzl), Thr(Bzl), and Tyr-(BrZ). In order to obtain 95+% overall efficiency of synthesis, the coupling steps were monitored with the ninhydrin test and were repeated to achieve 99.5+% amino acid incorporation yield in each cycle.

Peptides were cleaved from the resin (1 g) in liquid HF (15 mL, –10 °C to 0 °C) containing 10% *p*-cresol. The cleavage time varied between 80 and 95 min. The HF was removed with a strong stream of nitrogen, the oily residue was washed with cold AcOEt (3 × 20 mL, –30 °C) and filtered, and the peptide was extracted by washing the residue with 1 × 15 mL of water, 3 × 15 mL of 50% AcOH, and 1 × 15 mL of water. The combined aqueous extracts were lyophilized, and the dried material was redissolved and oxidized by stirring while exposed to the atmosphere at +4 °C. The progress of the oxidation was monitored with HPLC, and completion was confirmed with the Ellman test (at the end, the free SH content was usually less than 5% of the starting value). Upon completion of the disulfide formation, the solution was acidified to pH ~3.5 with acetic acid, concentrated under vacuum to ca. 15–

[1] Abbreviations: ACN, acetonitrile; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DMFA, *N,N*-dimethylformamide; DTT, dithiothreitol; HOBt, 1-hydroxybenzotriazole; HPLC, reverse-phase high-performance liquid chromatography; MBHA, methylbenzhydrylamine resin; TFA, trifluoroacetic acid; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor. ω-Conotoxins SVIA and SVIB are also known as SNX-157 and SNX-183, respectively.

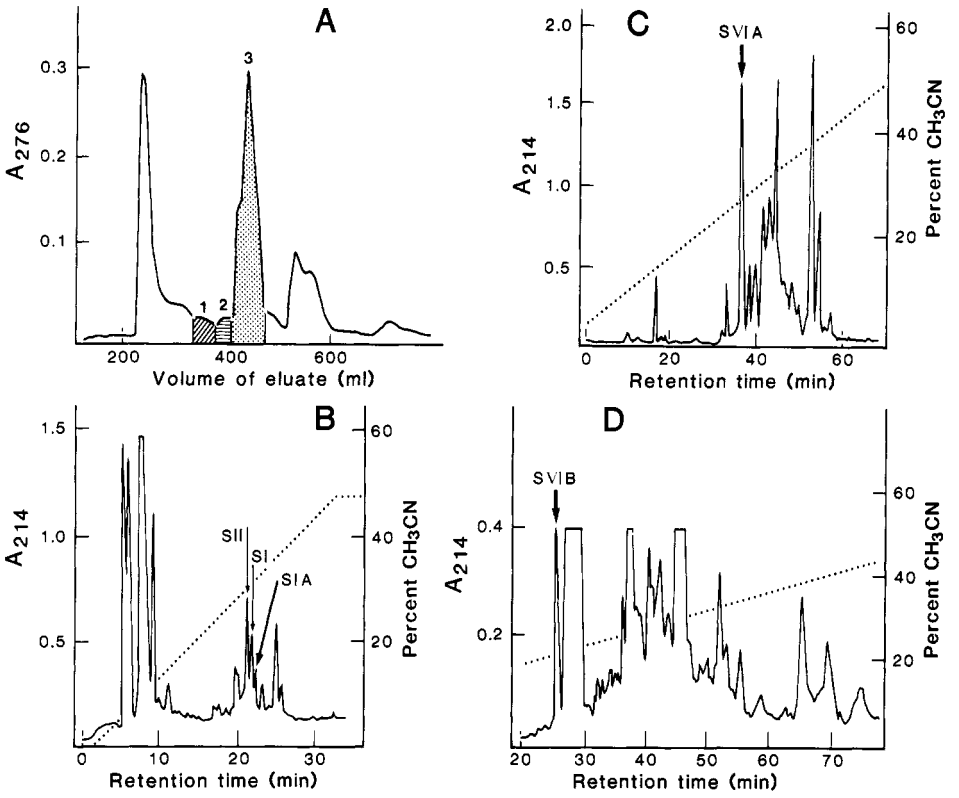

FIGURE 2: Purification of conotoxins SII, SVIA, and SVIB. Panel A shows the Sephadex G-25 chromatogram of *C. striatus* venom. Marked peaks were further fractionated by HPLC on semipreparative C_{18} columns (Ultropac TSK ODS-120T, 7.8 × 300 mm, 10 μm; or VYDAC C_{18}, 10 × 250 mm, 5 μm). Panel B shows the HPLC profile of the dotted peak (3) from which α-conotoxin SII was isolated. Panel C shows the chromatographic profile of the fraction marked by horizontal lines (peak 2) in panel A from which ω-conotoxin SVIA was isolated. Panel D shows the HPLC profile of the fraction marked by diagonal lines (peak 1) in panel A, which contained ω-conotoxin SVIB. The peaks from which the conotoxins described in this paper were isolated are marked by arrows. α-Conotoxins SI and SIA have been described previously (Zafaralla et al., 1988; Myers et al., 1991). The dotted lines indicate gradients of acetonitrile in 0.1% TFA.

20 mL, and gel-filtered on a Sephadex G-25 column (2.5 × 60 cm) eluted with 0.5 M AcOH. The pooled peptide fractions were further purified on a preparative HPLC column (Rainin Dynamax system, 4.14 × 30 cm, C_{18} reverse-phase packing material, 300-Å pore size, 12-μm particle size) using 0.1% TFA in water/0.1% TFA in acetonitrile gradient elution solvent system (40 mL/min pumping rate). The pure fractions were pooled and lyophilized. The yield of purified peptide was usually 10–16% based on the loading capacity of the MBHA-resin.

Amino acid analyses of the final synthetic SVIA and SVIB products were performed. The results of these analyses indicated as mole ratios were as follows: (SVIA) Thr 1.86 (2), Ser 3.54 (4), Hyp 1 (1), Gly 3.76 (4), Val 1.09 (1), Cys 4.79 (6), Ile 0.93 (1), Tyr 0.92 (1), Lys 1.07 (1), Arg 3.13 (3); (SVIB) Asp 0.97 (1), Thr 0.94 (1), Ser 4.64 (5), Gln 1.05 (1), Cys 4.92 (6), Gly 4.04 (4), Leu 1.00 (1), Tyr 0.95 (1), Lys 4.11 (4), Arg 1.97 (2).

Biological Assays. Injection of fish and mice was performed as previously described (Zafaralla et al., 1988).

Synaptic responses were extracellularly recorded from cutaneus pectoris nerve–muscle preparations from *Rana pipiens* as previously described (Yoshikami et al., 1989). Briefly, the lateral third of the muscle was cut away and pinned in a rectangular Sylgard trough. Synaptic responses were recorded with Pt-wire electrodes from the preparations treated with 0.2 μM α-bungarotoxin to block end-plate potentials to levels below the threshold necessary for action potential generation. Conotoxins were bath-applied by replacing the bath solution with toxin-containing solution.

Spontaneous miniature end-plate potentials (mepps) are recorded intracellularly from cutaneus pectoris muscle pinned to Sylgard-coated glass cover slips and then placed in a chamber which was secured to the stage of a fluorescence microscope. Toxin was focally applied in a solution containing a tetramethylrhodamine–lysozyme conjugate (5 μM). The fluorescence of the solution allowed its location to be monitored to be sure that end-plate regions were contacted by the toxin expelled from the puffer pipet. Toxin was washed away from the end plate following withdrawal of the puffer pipet by perfusing the bath.

Electrophysiological data were acquired with virtual instrument software (LabVIEW National Inst.) on Macintosh computers fitted with A/D converter hardward either from National Instruments (Lab NB) or GW Instruments (MacADIOS adio).

RESULTS

Purification and Characterization of α-Conotoxin SII. In the course of purification of α-conotoxins SI and SIA, additional fractions which cause paralysis in fish were identified. One of these activities eluted closely to SI and SIA. The details of the purification of this new activity are shown in Figure 2. The paralytic fraction was purified to >90% homogeneity, and its amino acid composition was determined (Table I). An amino acid sequencing run (Table II) gave results consistent with a 19 amino acid peptide with the sequence indicated at the bottom of Table II. The sequence assignments in Table II match the amino acid composition of Table I. Further confirmation of the sequence assignment was provided by fast atom bombardment (FAB) mass spectrometry. The FAB mass spectrometry determination yielded a MW (MH^+ 1790.56) which is consistent with the

Table I: Amino Acid Analysis of α-Conotoxin SII (Natural)

amino acid	nmol	mole ratio[c]	amino acid	nmol	mole ratio[c]
Asp	4.08	1.89 (2)[d]	Ala	2.16	1.00 (1)
Ser	3.81	1.76 (2)	Pro[a]	7.32	3.39 (2)
Gly	8.62	3.99 (4)	Tyr	1.94	0.90 (1)
Thr	2.02	0.94 (1)	Cys[b]	14.55	6.72 (6)

[a] The Pro anlaysis is subject to interference from a reagent peak. [b] Cysteine was analyzed as the carboxymethyl derivative. [c] The sample contained the following molar ratios of minor constituents: Glu, 0.25; Ile, 0.12; Leu, 0.42; and Lys, 0.19. [d] Values in parentheses indicate the number of residues found by sequence analysis.

Table II: Sequence Analysis of α-Conotoxin SII

step	assigned residue	yield (nmol of PTH-amino acid)	step	assigned residue	yield (nmol of PTH-amino acid)
1	Gly	1.02	11	Asn	0.51
2	Cys[a]	1.32	12	Tyr	0.41
3	Cys	0.88	13	Gly	0.25
4	Cys	0.78	14	Cys	0.21
5	Asn	0.97	15	Gly	0.26
6	Pro	0.52	16	Thr	0.29
7	Ala	1.04	17	Ser	0.19
8	Cys	0.49	18	Cys	0.07
9	Gly	0.59	19	Ser	0.06
10	Pro	0.20			

sequence: GCCCNPACGPNYGCGTSCS

[a] Cys residues were analyzed as the carboxymethyl adduct.

predicted 19 amino acid peptide with a free carboxyl terminus. Although this is larger than any other α-conotoxin so far characterized, its striking homology to α-conotoxin SI makes it virtually certain that the peptide is an α-conotoxin; we are therefore designating this peptide α-conotoxin SII. To confirm the sequence assignment of the natural peptide, and to make more peptide available for further characterization, we synthesized α-conotoxin SII.

α-Conotoxin SII is generally found at lower levels in *C. striatus* venom than α-conotoxin SI, or the major ω-conotoxin, SVIA (see below). However, there is considerable variation in the levels of the toxins from one venom sample to the next; in the venom sample in Figure 2, the levels of SI and SII are approximately equal. More typically, α-SII is found to be ca. 20% of α-conotoxin SI and ca. 10% of α-conotoxin SVIA (C. Hopkins, unpublished results).

Chemical Synthesis of α-Conotoxin SII. The peptide was synthesized by the standard methods detailed in the Experimental Procedures section. Starting with 2 g of Boc-Ser resin, the peptide was synthesized manually by the Merrifield procedure and was released from the resin using HF; approximately 0.6 g of reduced peptide was obtained. The peptide was oxidized by slow air oxidation at 4 °C in a large volume (4 L). After 5 days, the oxidized peptide was purified by HPLC.

The synthetic and natural peptides have identical behavior on HPLC (see Figure 3), and they showed similar effects on fish. Comparable paralysis and death times were observed at the three doses tested (0.5, 1.0, and 5.0 nmol/fish). Thus, by all criteria, the native and synthetic peptides are identical to each other, a direct confirmation of the sequence assignment in Table II. It is notable that although the peptide is a potent paralytic toxin to fish, no obvious effects are seen in 2-week-old mice even at 20 nmol/mouse.

Purification and Characterization of ω-Conotoxins SVIA and SVIB. Two fractions which were paralytic to fish displayed an elution pattern (Figure 2C,D) quite distinctive from those of α-conotoxins SI, SIA, and SII, and these activities

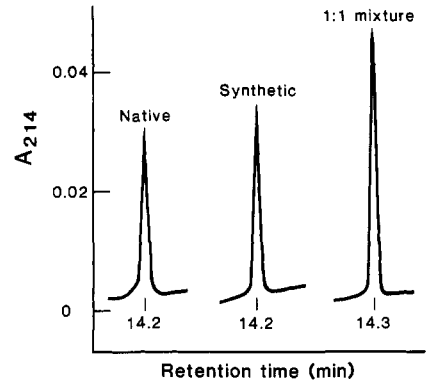

FIGURE 3: HPLC comparison of natural and synthetic α-conotoxin SII. The natural purified peptide, the synthetic peptide, and a mixture of natural and synthetic peptides were analyzed by HPLC. The TFA/acetonitrile system (solvent A, 0.1% TFA; solvent B, 0.1% TFA in 60% acetonitrile) was used to elute the peptide from an analytical Vydac C_{18} column. Peptides were eluted with a linear gradient of 20–50% solvent B in 20 min.

Table III: Amino Acid Analysis of ω-Conotoxins from *C. striatus* Venom

	ω-conotoxin SVIA		ω-conotoxin SVIB	
amino acid	pmol	mole ratio[a]	nmol	mole ratio[a]
Asp			2.06	1.23 (1)[b]
Glu			2.12	1.27 (1)
Hyp	120	0.96 (1)[b]		
Ser	497	3.98 (4)	7.86	4.71 (5)
Gly	526	4.21 (4)	6.88	4.12 (4)
Arg	354	3.84 (3)	2.93	1.75 (2)
Thr	224	1.79 (2)	2.03	1.22 (1)
Tyr	98	0.78 (1)	1.07	0.64 (1)
Val	114	0.91 (1)		
Cys	505	4.04 (6)	7.15	4.28 (6)
Ile	112	0.90 (1)		
Leu			2.48	1.48 (1)
Lys	140	1.12 (1)	5.96	3.57 (4)

[a] The analysis showed the following molar ratios of minor constituents: (SVIA) Asp, 0.18; Glu, 0.30; Ala, 0.23; Leu, 0.24; and (SVIB) Ala, 0.31; Pro, 0.32; Val, 0.13; Met, 0.11. [b] Numbers in parentheses indicate the number of residues found by sequence analysis.

were further purified to greater than 90% homogeneity. The material was analyzed by amino acid analysis (Table III). The purified peptides were sequenced, and the results of the amino acid sequencing runs are shown in Table IV; the amino acid assignments are unequivocal, the main contaminant amino acid seen being a variable tail-over from previous Edman steps. The resultant sequence assignments are given at the bottom of the table.

The peptides belong to the ω-conotoxin family by several criteria. The pattern of Cys residues is related to that of other ω-conotoxins, and the peptides are paralytic to fish. Most strikingly, both peptides induce a shaking syndrome in mice, which is highly characteristic of ω-conotoxins. Finally, both peptides compete for binding with ^{125}I-ω-conotoxin GVIA (Miljanich et al., 1992). A variety of other physiological and pharmacological experiments have been performed, most of which will be detailed elsewhere (Miljanich et al., 1992); the effects on the frog neuromuscular junction are described below. All of the results are consistent with the assignment of these toxins to the ω-conotoxin family, peptides that inhibit voltage-sensitive Ca channels.

On the basis of this tentative assignment, we will refer to the two activities as ω-conotoxins SVIA and SVIB. In order to confirm the primary structure assignments and to determine the status of the C-terminus, the peptides were chemically synthesized.

Table IV: Sequence Analysis of ω-Conotoxins from *C. striatus* Venom

ω-conotoxin SVIA			ω-conotoxin SVIB		
step	assigned residue	yield (nmol of PTH-amino acid)	step	assigned residue	yield (pmol of PTH-amino acid)
1	Cys[a]	4.28	1	Cys	207
2	Arg	0.59	2	Lys	291
3	Ser	1.08	3	Leu	266
4	Ser	1.14	4	Lys	174
5	Gly	0.89	5	Gly	151
6	Ser	0.92	6	Gln	202
7	Hyp	0.83	7	Ser	66
8	Cys	0.50	8	Cys	196
9	Gly	0.49	9	Arg	105
10	Val	0.85	10	Lys	163
11	Thr	0.33	11	Thr	65
12	Ser	0.51	12	Ser	39
13	Ile	0.75	13	Tyr	116
14	Cys	0.22	14	Asp	30
15	Cys	0.42	15	Cys	103
16	Gly	0.35	16	Cys	70
17	Arg	0.10	17	Ser	15
18	Cys	0.43	18	Gly	55
19	Tyr	0.40	19	Ser	17
20	Arg	0.07	20	Cys	63
21	Gly	0.32	21	Gly	47
22	Lys	0.25	22	Arg	30
23	Cys	0.14	23	Ser	14
24	Thr	Nq	24	Gly	30
			25	Lys	37
			26	Cys	11

sequence:[b]
SVIA: CRSSGSPCGVTSICCGRCYRGKCT
SVIB: CKLKGQSCRKTSYDCCSGSCGRSGKC

[a] Cys residues were analyzed as the carboxymethyl adducts. [b] The standard one-letter code is used for amino acids, except P which is hydroxyproline.

Synthesis of ω-Conotoxins SVIA and SVIB. The two peptides were synthesized by standard solid-phase methods and the peptides folded as described under Experimental Procedures. Both peptides were made with amidated C-termini since all previously characterized ω-conotoxins were so modified. Both by chemical methods and by 2D NMR analysis to be described elsewhere, the disulfide bonding of the synthetic peptides has been shown to be the pattern reported by Nishiuchi et al. (1986) for ω-conotoxin GVIA; i.e., the first cysteine is linked to the fourth, the second to the fifth, and the third to the sixth (D. Chung, S. Gaur, J. Bell, L. Nadasdi, and J. Ramachandran, manuscript in preparation; V. Basus, unpublished results).

The native and synthetic toxins appear to be identical by all criteria tested. Since relatively small amounts of native toxins were available, the number of biological experiments done on native material was rather limited. However, as shown in Figure 4, the chromatographic profiles of native and synthetic toxins are identical. In a mixing experiment with native and synthetic toxin, there was no broadening of peaks and a single homogeneous peak was obtained. The biological activities exhibited by native and synthetic toxins were indistinguishable in the range that was tested (see section below). These results confirm the sequences in Table IV and indicate that the native peptides have an amidated C-terminus.

Biological Activity of ω-Conotoxins SVIA and SVIB. In fish, the new ω-conotoxins (SVIA and SVIB) from *C. striatus* venom are comparable to ω-conotoxin MVIIA of *Conus magus* (they cause paralysis and death on intramuscular injection at similar doses, approximately 20 pmol/g).

Compared to ω-conotoxin MVIIA, about 20 times more SVIA must be injected intracranially in mice to produce the shaking characteristic of all previously isolated ω-conotoxins. Although ω-conotoxin SVIB induces shaking in mice at a slightly higher dose than for MVIIA, SVIB causes respiratory distress at around 70 pmol/g mouse and it is lethal at around 300 pmol/g mouse. In contrast, MVIIA and SVIA do not kill mice even at extremely high doses. ω-Conotoxin GVIA is also not lethal in mice.

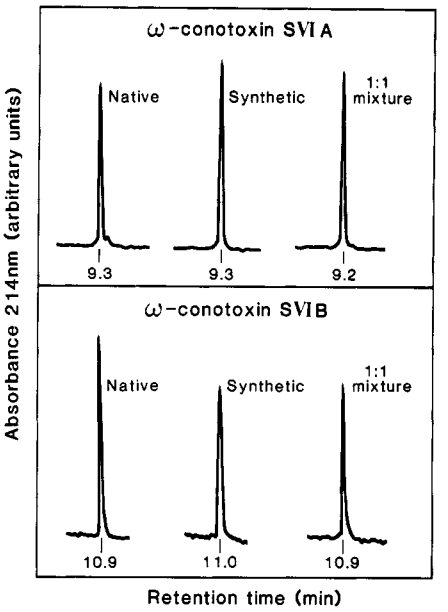

FIGURE 4: HPLC comparison of natural and synthetic ω-conotoxins from *C. striatus*. For each toxin, natural purified peptide, the synthetic peptide, and a mixture of natural and synthetic peptides were analyzed by HPLC on analytical Vydac C_{18} columns. The TFA/acetonitrile solvent system described in Figure 2 was used. ω-Conotoxin SVIA (upper panel) was eluted with the linear gradient 20(0)/20(2)/30(12) and ω-conotoxin SVIB (lower panel) with the gradient 15(0)/15(2)/25(12), expressed as percent solvent B achieved at times given in parentheses (min).

It is clear that although the ω-conotoxins behave similarly in fish, they have different activities and specificities in mammalian systems.

Binding Studies with ω-Conotoxin SVIB. The in vivo effects of ω-conotoxin SVIB on mice suggest that this peptide may have somewhat different receptor target specificity from ω-conotoxins GVIA and MVIIA. In order to examine the high-affinity receptor site of ω-SVIB, radiolabeled toxin was prepared as described previously (Cruz & Olivera, 1986). The ability of different ω-conotoxins to compete for the ω-SVIB site was assessed by the binding competition experiments shown in Figure 5.

It is clear that, of the ω-conotoxins tested, ω-SVIB showed the highest apparent affinity for radiolabeled ω-SVIB binding site ($IC_{50} \cong 4$ nM). Both ω-conotoxins GVIA and MVIIA showed significantly lower affinity ($IC_{50} \cong 100$ nM), and ω-SVIA had the weakest affinity for this site ($IC_{50} > 50$ μM).

It is notable that the IC_{50} values for GVIA and MVIIA in this binding competition assay are orders of magnitude higher than for their own high-affinity binding site. Under similar binding competition conditions with ^{125}I-ω-conotoxin MVIIA, ω-GVIA and ω-MVIIA exhibit IC_{50}'s 3–4 orders of magnitude lower [for MVIIA, 7 pM; for GVIA, 130 pM (Miljanich et al., 1992)]. These results suggest that the high-affinity ω-SVIB binding site is distinct from the high-affinity ω-GVIA binding site. A much more extensive biochemical characterization of the different binding sites will be presented elsewhere (Miljanich et al., 1992).

Effects of SII, SVIA, and SVIB on Frog Neuromuscular Junction Preparations. α-Conotoxin SII reversibly blocked

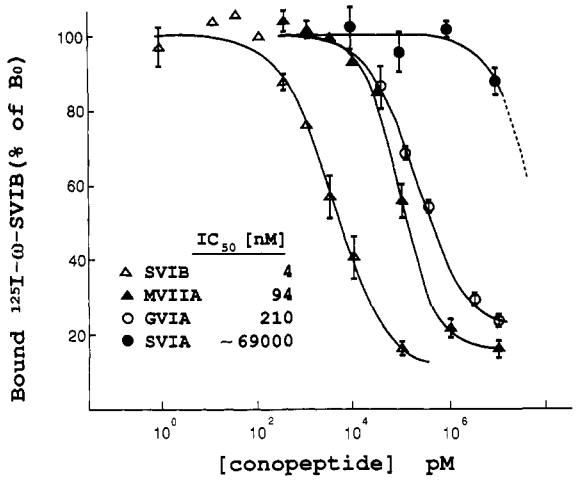

FIGURE 5: Competition binding of several ω-conotoxins against ^{125}I-ω-conotoxin SVIB. The inhibitory constants (IC_{50}'s) for binding to rat brain synaptosomal membranes were determined by competitive filter binding assays as previously described (Cruz et al., 1986). IC_{50}'s were derived from the curves of best fit shown in the figure. B_0 is the amount of radioiodinated SVIB bound in the absence of competing peptide. Error bars represent standard deviations from the means of three determinations for each data point.

electrically evoked postsynaptic responses of the frog neuromuscular junction preparation (result not shown). To test whether SII affected acetylcholine receptors, the toxin's effects on spontaneous miniature end-plate potentials (mepps) were measured (see Experimental Procedures). SII (20 μM) applied from a puffer pipet reduced mepp amplitudes 45%, which recovered following washout of the toxin ($n > 20$). This result indicates that acetylcholine receptors were reversibly blocked by the toxin.

Both SVIA and SVIB also reversibly blocked evoked postsynaptic responses of the frog neuromuscular preparation. SVIB, at a concentration of 10 μM, blocked most, but not all, of the synaptic response, whereas SVIA completely blocked synaptic responses at a concentration of 1 μM (Figure 6). To test whether the acetylcholine receptor was the target of these toxins, their effects on spontaneous mepps were examined. Neither SVIA nor SVIB (50–100 μM applied from a puffer pipet) had an effect on spontaneous mepp amplitudes. This indicates that the toxins have a presynaptic target and they do not affect acetylcholine receptors.

Binding Competition between ^{125}I-Labeled α-Bungarotoxin and α-Conotoxin SII. The results with the frog neuromuscular junction, i.e., the blockade of mini-end-plate potentials (mepps), described above, as well as the homology revealed by sequence analysis of α-conotoxin SII, are strongly suggestive (but not conclusive evidence) that this peptide binds to the acetylcholine receptor at the vertebrate neuromuscular junction. In order to confirm this receptor assignment, we therefore investigated whether α-conotoxin SII bound to the ligand binding site of the nicotinic acetylcholine receptor by assaying for the ability of this peptide to compete with radiolabeled α-bungarotoxin to this site.

The results of such an experiment are shown in Figure 7; it is clear that α-conotoxin SII will completely displace the binding of ^{125}I-α-bungarotoxin to the well-characterized nicotinic acetylcholine receptor found in the *Torpedo* electric organ. Under the binding conditions used, the apparent IC_{50} for α-conotoxin SII was 8 μM; a similar analysis for the previously characterized α-conotoxin SI yielded IC_{50} value of 1 μM under these conditions (data not shown). These data therefore directly demonstrate that α-conotoxin SII interacts

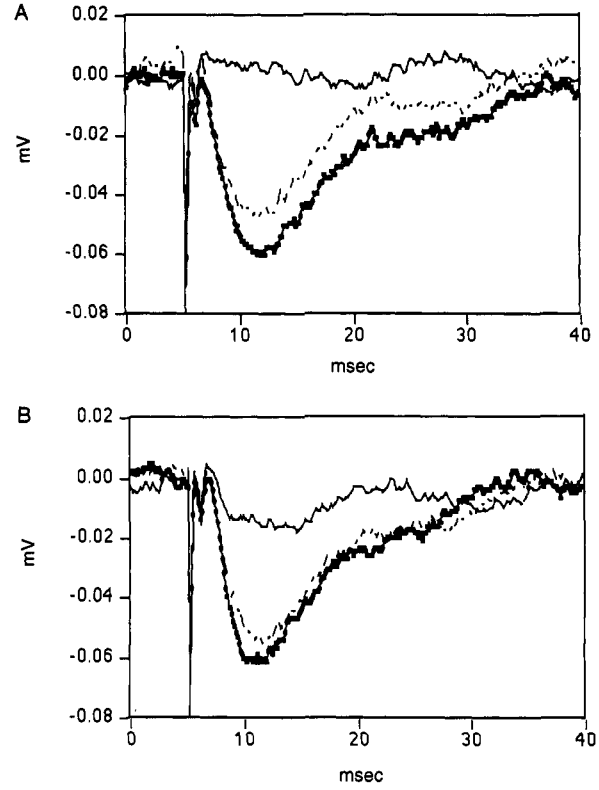

FIGURE 6: Conotoxins SVIB and SVIA reversibly block evoked synaptic responses recorded extracellularly at the frog neuromuscular junction. (A) 1 μM SVIA abolished synaptic responses. (B) 10 μM SVIB blocked synaptic responses by nearly 80%. Vertical axis is peak amplitude of synaptic response in mV, horizontal is time in ms. In both (A) and (B), bold solid line is control, plain solid line is with toxin present, and dotted line is recovery following toxin washout.

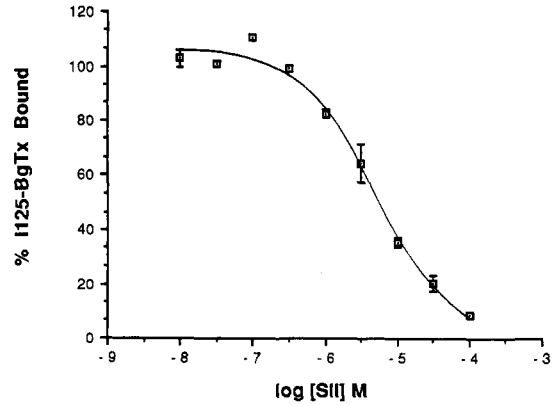

FIGURE 7: Competition binding of SII versus ^{125}I-α-bungarotoxin. Binding experiments were done using a filtration assay of a postsynaptic membrane fraction isolated from electroplax of *Torpedo californica*. Data points are the mean (±SEM) from three different determinations at each concentration. The ^{125}I-α-bungarotoxin (2000 Ci/mmol) was used at ca. 10^5 cpm per assay; 100% binding under the conditions used was 20 000 cpm. Nonspecific binding, determined by preincubation with 1 μM unlabeled α-bungarotoxin, was subtracted out.

with the nicotinic acetylcholine receptor at the ligand binding site. Thus, both the physiological data and the binding data are consistent with α-conotoxin SII belonging to the α-conotoxin class which inhibits acetylcholine binding to the acetylcholine receptor at the neuromuscular junction.

DISCUSSION

In the work above, three new paralytic toxins from *C. striatus* venom have been characterized. The peptides were purified

Table V: α-Conotoxin Amino Acid Sequences

toxin	sequence	charge	ref
GI	ECCNPACGRHYSC-NH_2	+1.5	a
GIA	ECCNPACGRHYSCGK-NH_2	+2.5	a
GII	ECCHPACGKHFSC-NH_2	+2	a
MI	GRCCHPACGKNYSC-NH_2	+3.5	b
SI	ICCNPACGPKYSC-NH_2	+2	c
SIA	YCCHPACGKNFDC-NH_2	+1.5	d
SII	GCCCNPACGPNYGCGTSCS-OH	0	this work

[a] From *C. geographus*: cf. Gray et al. (1988). [b] From *C. magus*: cf. McIntosh et al. (1982). [c] From *C. striatus*: cf. Zafaralla et al. (1988). [d] From *C. striatus*: Myers et al. (1991).

Table VI: ω-Conotoxins

toxin	sequence	charge	ref
GVIA	CKSPGSSCSPTSYNCCR-SCNPYTKRCY-NH_2	+5	a
GVIIA	CKSPGTPCSRGMRDCCT-SCLLYSNKCRRY-NH_2	+6	b
MVIIA	CKGKGAKCSRLMYDCCTGSCR--SGKC-NH_2	+6	c
MVIIB	CKGKGASCHRTSYDCCTGSCN--RGKC-NH_2	+5.5	c
SVIA	CRSSGSPCGVTSI-CC-GRC--YRGKCT-NH_2	+5	This work
SVIB	CKLKGQSCRKTSYDCCSGSC-GRSGKC-NH_2	+6	This work

[a] From *C. geographus*: cf. Olivera et al. (1984). [b] From *C. geographus*: cf. Olivera et al. (1990). [c] From *C. magus*: Olivera et al. (1987). P is hydroxyproline.

from the venom, their amino acid sequences deduced, and their structures confirmed by chemical synthesis. The peptides fall into two pharmacological classes, an α-conotoxin which blocks the acetylcholine receptor and two ω-conotoxins which block presynaptic Ca channels. The electrophysiological data on the frog neuromuscular junction preparation, as well as the ability of the peptide to displace specific ^{125}I-α-bungarotoxin binding to *Torpedo* electric organ membrane (Figure 7), directly demonstrate that α-conotoxin SII is an inhibitor of the nicotinic acetylcholine receptor. The experiments with conotoxins SVIA and SVIB showed that these peptides blocked electrically-evoked postsynaptic responses without affecting spontaneous mepp amplitudes. Thus, SVIA and SVIB do not affect acetylcholine receptors, but do block evoked synaptic responses just as the well-characterized ω-conotoxin GVIA does (Kerr & Yoshikami, 1984). SVIA and SVIB share significant sequence homology with GVIA and other ω-conotoxins which block presynaptic calcium channels. Furthermore, the striking syndrome induced by the peptides in mice in vivo is similar to the ω-GVIA symptomatology. We therefore presume that SVIA and SVIB are ω-conotoxins that target presynaptic Ca channels.

Previously, six α-conotoxins were described, two from *C. striatus* venom, one from *C. magus* venom, and three from *C. geographus* venom. All of these peptides had two disulfide bonds. A summary of these structures is shown in Table V. The novelty of α-conotoxin SII is immediately apparent: it is the only α-conotoxin with three disulfide bonds, a free carboxyl terminus, and no net positive charge.

The two internal loops of α-conotoxin SII are closely related in sequence to α-conotoxin SI. Both peptides are paralytic to fish and relatively inactive in mammalian systems [see Zafaralla et al. (1988)]. In contrast, the third α-conotoxin found in *C. striatus* venom, SIA, is relatively more active in mammalian systems and shows less homology to α-conotoxins SI and SII.

The sequences of the two new ω-conotoxins which we have characterized, ω-conotoxin SVIA and SVIB, are compared to all previously characterized ω-conotoxins in Table VI. ω-Conotoxin SVIA, which contains 24 amino acids, is the smallest natural ω-conotoxin known. Although ω-conotoxin SVIA is clearly paralytic to fish and frogs, compared to other previously characterized toxins in this family (ω-conotoxin GVIA and MVIIA, for example), ω-conotoxin SVIA has a relatively poor activity on most Ca channel targets in mammalian systems. Thus, three of the major toxins in *C. striatus* venom, SI, SII, and SVIA, show much less activity on mammalian systems than their corresponding homologs in *C. geographus*. This may explain in part why stings of *C. striatus* are not fatal to man, while *C. geographus* has caused many human deaths.

The two ω-conotoxins in *C. striatus* venom, SVIA and SVIB, have very different sequences; they have different lengths, and even if gaps are created to allow maximum sequence alignment, less than 40% of the non-cysteine amino acids in SVIA are identical to corresponding residues in SVIB. ω-Conotoxin SVIB has novel pharmacological characteristics. Although ω-conotoxin SVIB might not be as potent as other conotoxins (e.g., MVIIA) in inducing the typical shaker syndrome in mice, it clearly has different biological effects in vivo from other ω-conotoxins: at high concentrations, it is lethal. Even at the highest concentrations injected, neither ω-conotoxins MVIIA nor GVIA are lethal in mice. Rather, the shaker syndrome is intensified and much more rapid in onset than that elicited at low doses. These results indicate that ω-conotoxins SVIB may affect a subset of high-affinity Ca channels that are not targets of ω-conotoxins MVIIA and GVIA. Direct evidence for this is provided by the binding competition experiment shown above; this result strongly indicates that the high-affinity binding site for ^{125}I-SVIB is distinct from the high-affinity GVIA and MVIIA sites (see Figure 5). Finally, 2D NMR characterization of SVIB (V. Basus, unpublished results) suggests that, despite the conserved disulfide framework, it has a conformation that is significantly different from ω-conotoxins MVIIA and GVIA. Thus, although several other α-conotoxins and ω-conotoxins have been previously characterized, the newly characterized peptides from *C. striatus* have a number of unique and potentially useful properties.

REFERENCES

Cruz, L. J., & Olivera, B. M. (1986) *J. Biol. Chem. 261*, 6230–6233.

Cruz, L. J., Gray, W. R., Olivera, B. M. Zeikus, R. D., Kerr, L., Yoshikami, D., & Moczydlowski, E. (1985a) *J. Biol. Chem. 260*, 9280–9288.

Cruz, L. J., Gray, W. R., Yoshikami, D., & Olivera, B. M. (1985b) *J. Toxicol., Toxin Rev. 4*, 107–132.

Edman, P., & Begg, C. (1967) *Eur. J. Biochem. 1*, 80–91.

Ellman, G. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., & Cruz, L. J. (1981) *J. Biol. Chem. 256*, 4734–4740.

Gray, W. R., Rivier, J. E., Galyean, R., Cruz, L. J., & Olivera, B. M. (1983) *J. Biol. Chem. 258*, 12247–12251.

Gray, W. R., Olivera, B. M., & Cruz, L. J. (1988) *Annu. Rev. Biochem. 57*, 665–700.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.

Kohn, A. J., Saunders, P. R., & Weiner, S. (1960) *Ann. N.Y. Acad. Sci. 90*, 706–725.

McIntosh, M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R., & Olivera, B. M. (1982) *Arch. Biochem. Biophys. 218*, 329–334.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149–2154.

Miljanich, G., Nadashi, L., Gohil, K., Woppman, A., Kristipati, R., Rivnay, B., Tarczy-Hornoch, K., Gaur, S., Adrianensseno, P., Yamashiro, D., Bell, J., Hillyard, D., Monje, V. D., Zafaralla, G. C., Cruz, L. J., Olivera, B. M., & Ramachandran, J. (1992) *Neuron* (submitted for publication).

Myers, R. A., Zafaralla, G. C., Gray, W. R., Abbott, J., Cruz, L. J., & Olivera, B. M. (1991) *Biochemistry 30*, 9370–9377.

Nishiuchi, Y., Lumagaye, K., Noda, Y., Watanabe, T. X., & Sakakibara, S. (1986) *Biopolymers 25*, S61–S68.

Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., & Gray, W. R. (1984) *Biochemistry 23*, 5087–5090.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V., & Cruz, L. J. (1985) *Science 230*, 1338–1343.

Olivera, B. M., Cruz, L. J., de Santos, V., LeCheminant, G., Griffin, D., Zeikus, R., McIntosh, J. M., Galyean, R., Varga, J., Gray, W. R., & Rivier, J. (1987) *Biochemistry 26*, 2086–2090.

Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., & Cruz, L. J. (1990) *Science 249*, 257–263.

Olivera, B. M., Rivier, J., Scott, J. K., Hillyard, D. R., & Cruz, L. J. (1991) *J. Biol. Chem. 266*, 22067–22070.

Rivier, J., McClintock, R., Galyean, R., & Anderson, H. (1984) *J. Chromatogr. 288*, 303–328.

Saunders, P. R., & Wiener, S. (1960) *Ann. N.Y. Acad. Sci. 90*, 706.

Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442–6445.

Walls, J. G. (1979) *Cone Shells: A Synopsis of the Living Conidae* T. H. F. Publications, Hong Kong.

Yamashiro, D., & Li, C. H. (1988) *Int. J. Peptide Protein Res. 31*, 322–334.

Yoshikami, D., Bagabaldo, Z., & Olivera, B. M. (1989) *Ann. N.Y. Acad. Sci. 560*, 230–248.

Zafaralla, G. C., Ramilo, C., Gray, W. R., Karlstrom, R., Olivera, B. M., & Cruz, L. J. (1988) *Biochemistry 27*, 7102–7105.

Registry No. SVIA, 137706-81-1; SVIB, 143306-19-8; SII, 143294-31-9.