Biochemistry

 Volume 37, Number 46 November 17, 1998

## Accelerated Publications

# An O-Glycosylated Neuroexcitatory *Conus* Peptide†

A. Grey Craig,‡ Glenn Zafaralla,§ Lourdes J. Cruz,§,|| Ameurfina D. Santos,|| David R. Hillyard,⊥ John Dykert,‡ Jean E. Rivier,‡ William R. Gray,|| Julita Imperial,|| Richard G. DelaCruz,|| Annett Sporning,# Heinrich Terlau,# Peter J. West,|| Doju Yoshikami,|| and Baldomero M. Olivera*,||

*The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, P.O. Box 85800, San Diego, California 92186-5800, Marine Science Institute, University of the Philippines, Diliman, Quezon City, 1101, Philippines, Max Planck Institute for Experimental Medicine, Göttingen, Germany, and Departments of Biology and Pathology, University of Utah, 257 South 1400 East, Salt Lake City, Utah 84112*

*Received July 14, 1998; Revised Manuscript Received September 9, 1998*

ABSTRACT: We purified and characterized a novel peptide from the venom of the fish-hunting cone snail *Conus striatus* that inhibits voltage-gated $K^+$ channels. The peptide, κA-conotoxin SIVA, causes characteristic spastic paralytic symptoms when injected into fish, and in frog nerve-muscle preparations exposed to the toxin, repetitive action potentials are seen in response to a single stimulus applied to the motor nerve. Other electrophysiological tests on diverse preparations provide evidence that is consistent with the peptide blocking $K^+$ channels. The peptide has three disulfide bonds; the locations of Cys residues indicate that the spastic peptide may be the first and defining member of a new family of *Conus* peptides, the κA-conotoxins, which are structurally related to, but pharmacologically distinct from, the αA-conotoxins. This 30 AA tricyclic toxin has several characteristics not previously observed in *Conus* peptides. In addition to the distinctive biological and physiological activity, a novel biochemical feature is the unusually long linear N-terminal tail (11 residues) which contains one O-glycosylated serine at position 7. This is the first evidence for O-glycosylation as a posttranslational modification in a biologically active *Conus* peptide.

The fish-hunting cone snails (*Conus*) are improbable predators; a priori, it would seem unlikely for a slow-moving snail to specialize successfully in capturing such agile prey. However, these snails have evolved a sophisticated strategy comprising venoms which contain highly potent neurotoxic peptides (*1*, *2*) as well as a remarkable delivery system consisting of barbed, hollow, harpoon-like teeth which serve as disposable hypodermic needles and also tether the fish prey. Several *Conus* toxins which are sodium channel antagonists have been described (*3*, *4*). However, until the recent characterization of κ-conotoxin PVIIA (*5*, *6*), a conotoxin which targets Shaker $K^+$ channels, no potassium channel antagonists had been identified from *Conus* venoms. In contrast, several potent potassium channel antagonists have been identified in scorpion venom. Scorpion toxins that inhibit ion conduction through potassium channels are

† This work was supported by NIH Grant GM48677. The mass spectrometry was supported by NSF Major Research Instrumentation program DDBI-972450 and with support from the Foundation for Medical Research (A.G.C.).

* To whom correspondence should be addressed. Tel: (801) 581-8370. Fax: (801) 585-5010. E-mail: oliveralab@bioscience.utah.edu.
‡ The Salk Institute.
§ University of the Philippines.
|| Department of Biology.
⊥ Department of Pathology.
# Max Planck Institute for Experimental Medicine.

10.1021/bi981690a CCC: $15.00 
Published on Web 10/26/1998

typically small peptides (35−40 amino acids) and contain three disulfide bridges (*7*, *8*), features shared by many conotoxins. The scorpion toxins bind to the extracellular vestibule of the $K^+$ channel pore (*7*−*9*).

Recently, we identified in the venom of the Eastern Pacific fish-hunting *Conus* species, *Conus purpurascens*, two groups of toxins with different physiological end points (*5*). One set of peptides produced an initial, very rapid immobilization of prey through an excitotoxic shock mechanism. This set of peptides causes excitatory symptomatology when injected into fish or the central nervous system of a mouse. Another set of peptides blocked neuromuscular transmission, producing an irreversible flaccid paralysis of fish. Thus, *C. purpurascens* venom incapacitates prey with a one-two punch, or "double-whammy"; first, rapid stunning by the neuroexcitatory peptides, which provides time for the neuromuscular blockers to get to their targets and cause irreversible paralysis (*5*).

There is increasing evidence for several distinct clades of fish-hunting *Conus* species (*10*). *C. purpurascens* is the only Eastern Pacific cone snail known to hunt fish, and it appears to be closely related to the fish-hunting Atlantic species, *Conus ermineus*. In contrast, there are numerous fish-hunting *Conus* species in the Indo-Pacific marine province (*11*), which are probably long diverged from their Atlantic/eastern Pacific congeneric brethren. Like *C. purpurascens*, most of the Indo-Pacific fish-hunting *Conus* use a hook-and-line strategy; injection of their venom into fish also elicits excitotoxic shock, followed by neuromuscular block (*10*). When observed in aquaria, the venom from these cone snails produces the same immediate rigid immobilization of prey seen for *C. purpurascens*. These observations have been made with Indo-Pacific species such as *Conus striolatus*, *C. stercusmuscarum*, *C. magus*, and *C. circumcisus*. The largest fish-hunting Indo-Pacific species, and one of the most widely distributed geographically, is the striated cone *Conus striatus*. The pioneering work of Alan Kohn on *C. striatus* first established that cone snails could envenomate fish (*12*).

Although fish-hunting Indo-Pacific *Conus* have venom peptides that block neuromuscular transmission, a striking divergence is observed when these peptides are compared with those in *C. purpurascens* venom. For example, the major competitive nicotinic antagonists in *C. purpurascens* and *C. ermineus* are the αA-conotoxins (*13*, *14*). In addition, *C. purpurascens* has $\psi$-conotoxin, a noncompetitive nicotinic antagonist (*15*). These peptides appear to be absent from the venoms of typical Indo-Pacific fish-hunters such as *C. magus* or *C. striatus*. Conversely, *C. magus* and *striatus* have $\omega$-conotoxins, targeted to the $\alpha_{1B}$ subunit of calcium channels (*16*), which do not appear to be present in *C. purpurascens* venom (J. Imperial and R. Jacobsen, unpublished results).

Thus, at the molecular level there may be significant differences in the types of peptides involved in excitotoxic shock between *C. purpurascens* and the Indo-Pacific fish-hunting *Conus* species. As a first step in the investigation of the mechanism underlying the excitotoxic shock syndrome elicited in fish prey by Indo-Pacific fish-hunting cone snails, we are systematically characterizing neuroexcitatory components of *C. striatus* venom. We report the first characterized excitotoxic component from this venom, which by itself produces spastic paralysis in fish. This peptide appears unrelated to any of the excitotoxins in *C. purpurascens* venom and exhibits a number of distinct biochemical and physiological features, suggesting that it may be the first biochemically and functionally characterized member of a new *Conus* peptide family.

## MATERIALS AND METHODS

*Venom Collection, Bioassay*. Specimens of *C. striatus* were collected in the Philippines. The molluscs were buried in ice for 30 min, the venom apparatus dissected and venom scraped from the duct. Animals for bioassay included mice (Japanese sDDy or Swiss Webster) and fish.

*Purification*. Several different batches of the peptide were purified from crude *C. striatus* venom using two different methods. Most studies were originally done on material purified by purification II; however, we have employed purification I as our routine method for obtaining more recent batches of the peptide.

*Purification I*: *Venom Extraction*. Crude venom from dissected ducts of *C. striatus* was pooled and stored at −70 °C. Venom (50 mg) was placed in an Eppendorf tube, and 0.5% trifluoroacetic acid in distilled, deionized water was added (1.5 mL, 0 °C). The tube was placed in ice for 20 min. It was vortexed for 5 min and then centrifuged at 20 000 rpm using a SM-24 rotor in a Sorvall RC2-B centrifuge for 30 min at 4 °C. The supernatant was collected, and 0.5% trifluoroacetic acid (1.5 mL) was added to the remaining pellet; the procedure was repeated again for a second extraction. The two supernatants were then combined.

*HPLC*[1] *Purification*. Crude venom extract (0.5 mL) was run on an analytical Vydac $C_{18}$ column with a guard cartridge; the active fractions from six runs were pooled. The peptide was further purified by running it again on the analytical Vydac $C_{18}$ column without the guard column. For all HPLC chromatography, a gradient from 0.1% trifluoroacetic acid to 0.09% trifluoroacetic acid and 60% acetonitrile was used with a linear increase for acetonitrile of 0.6%/min. Trifluoroacetic acid (sequencing grade) and acetonitrile (HPLC grade) were obtained from Fisher.

*Purification II*. Lyophilized venom (∼0.5 g) was suspended in 1.1% acetic acid (2.0 mL) and stirred, then placed on ice for 30 min and centrifuged at 10 000 rpm (Sorvall SS-34) for 10 min. The supernatant was collected; the pellet was redissolved in the same solvent, sonically disrupted five times for 10 s with 10 s intervals (60−70 W setting, Sonifier Cell Disruptor model W185 equipped with a microtip). Centrifugation followed, and the above procedure was repeated on the pellet. All three supernatants were combined and lyophilized to provide the crude venom extract which was lyophilized and dissolved in 10 mL of 1.1% acetic acid, applied to a Sephadex G-25 column (110 × 2.5 cm), and eluted with 1.1% acetic acid inside the LKB Min Cold Lab set at 5 °C. Blue dextran and bacitracin ($M_r = 2 \times 10^6$ and 1400, respectively) were used as standards. Fractions (10 mL) were collected at a flow rate of 0.27 mL/min.

[1] Abbreviations: AA, amino acid; ESI-MS electrospray ionization mass spectrometry; Hex, hexose; HexNAc, *N*-acetyl hexose; HPLC, high-performance liquid chromatography; LSI-MS, liquid secondary ionization mass spectrometry; MS/MS, tandem mass spectrometry; NSS, normal saline solution; TTX, tetrodotoxin.

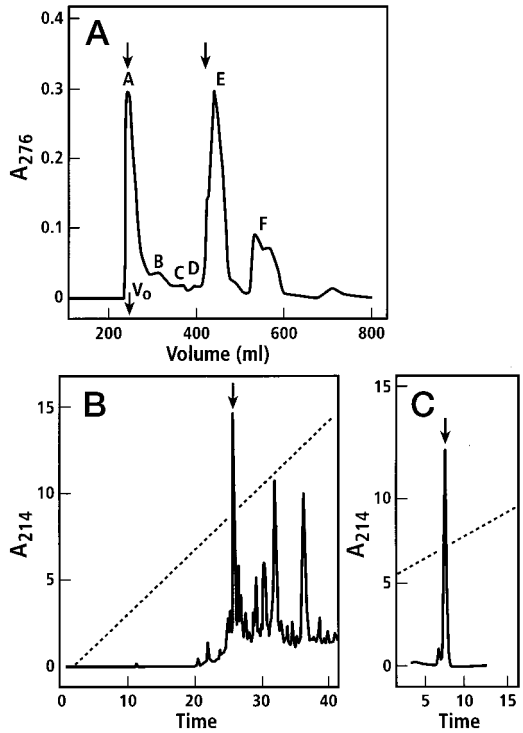



FIGURE 1: Purification of the spastic peptide. (A) Elution profile of crude venom extract on Sephadex G-25. The extract was chromatographed in 1.1% acetic acid. The two arrows indicate the excluded and included volumes. (B) HPLC profile of peak B from Sephadex G-25. Peak B was chromatographed on an Ultropac TSK ODS-120T $C_{18}$ semipreparative column in trifluoroacetic acid with an acetonitrile gradient. The tallest peak, B1, is indicated by the arrow. (C) HPLC profile of B1. B1 was chromatographed on a second Ultropac column in hexafluorobutyric acid with an acetonitrile gradient. Sephadex and HPLC chromatography was performed as described in Purification II in the Materials and Methods.

Fractions from Sephadex G-25 chromatography exhibiting biological activity were pooled, lyophilized, and refractionated by reversed-phase HPLC using an Ultropac TSK ODS-120T semipreparative column (7.8 × 300 mm, 10 $\mu$m particle size, fully capped). Peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (solvent A, 0.1% trifluoroacetic acid, and solvent B, 0.1% trifluoroacetic acid in 60% acetonitrile) at a flow rate of 2 mL/min. Bioactive fractions were rechromatographed two to three times, as needed, on an analytical reversed-phase $C_{18}$ column to remove contaminants. Elution was done with a gradient of acetonitrile in 0.05% heptafluorobutyric acid (solvent A, 0.05% heptafluorobutyric acid, and solvent B, 0.05% heptafluorobutyric acid in 60% acetonitrile) at a flow rate of 1 mL/min. Absorbance of the effluent was monitored at 214 nm and fractions were collected manually.

*Bioassay*. Lyophilized venom extracts and column fractions were resuspended in NSS. For mice (10 g), the fractions were injected intraperitoneally (i.p.) and intracranially (i.c.), and the animals were monitored for peculiar movements and neurological manifestations. Fish (1−2.5 g) were injected i.p. with toxin solution (5 $\mu$L) using a 10 $\mu$L Hamilton syringe in the ventral area between the anal fin and the pelvic fins.

*Other Methods*. The protein content of the venom samples and fractions was determined according to the method of Lowry et al. (*17*), with bovine serum albumin serving as a standard.

Proteolysis was carried out using purified toxin (2.55 $\mu$g of protein) dissolved in 12 $\mu$L of 0.05 M *N*-ethyl morpholine acetate, pH 8.9, 0.5 nM $CaCl_2$ containing 0.2 mg/mL trypsin or α-chymotrypsin. Control toxin solutions containing no proteolytic enzymes were also prepared. Samples were incubated at 37 °C for 4 h then diluted 2-fold with distilled water. Aliquots were assayed for toxicity in fish. At least three fish were injected for each sample and kept under observation for 8 h.

The toxin was reduced by using purified toxin (2.39 $\mu$g of protein) dissolved in 20 $\mu$L of $\beta$-mercaptoethanol (30 $\mu$L of $\beta$-mercaptoethanol in 200 $\mu$L of distilled water). Control toxin solutions containing no reducing agent were also prepared. Samples were incubated at room temperature under nitrogen for 4 h. Aliquots were assayed for toxicity on fish. At least three fish were tested at each dose.

*Amino Acid Analysis and Sequencing*. Amino acid analyses and sequencing were carried out at both the University of Utah Biology Department and the Salk Institute to yield a single consistent sequence.

Amino acid analysis was carried out using the Waters PICO.TAG amino acid analysis system. The peptide samples were first hydrolyzed with 6 N HCl and then derivatized with phenylisothiocyanate to produce phenylthiocarbamyl amino acids which were separated by HPLC. Molar ratios were compared based on amino acid analysis assuming that the amino acids with the lowest percentage are represented once in the polypeptide. Sequence analysis of peptides was carried out by sequential Edman degradation in a Beckman 890D spinning cup sequencer, using the 0.1 M Quadrol Program. Peptide fragments were analyzed by a manual method. Phenylthiohydantoin-amino acids were analyzed by HPLC.

*Mass Spectrometry*. Liquid secondary ionization (*18*) mass spectra (LSI-MS) were measured using a JEOL HX110 (JEOL, Tokyo, Japan) double focusing mass spectrometer operated at 10 kV accelerating voltage. The sample (in 0.1% aqueous trifluoroacetic acid and 25% acetonitrile) was mixed in a glycerol, 3-nitrobenzyl alcohol matrix (1:1). The LSI-MS spectra were measured with electric field scans at a nominal resolution of 1000. Electrospray mass spectra (ESI-MS) were measured using either an Esquire-LC (Bruker Daltonics, Billerica, MA) or an LCQ (Finnigan MAT, San Jose CA) ion trap mass spectrometer. The peptide (0.1% aqueous trifluoroacetic acid diluted with 1% acetic acid in methanol) was analyzed by direct infusion. The mass range of the MS/MS spectrum in Figure 2 was limited to 380−1850 Da.

*Electrophysiology*. Synaptically evoked responses from the cutaneus pectoris muscle of frog were performed as previously described (*6*, *19*). Briefly, a pair of extracellular electrodes were used to stimulate the nerve. A wire electrode placed near the end plate of the muscle and reference electrode placed at the myotendenous end were connected to a differential amplifier to record extracellular responses from the muscle.

Intracellular recording of antidromic action potentials from neurons in intact sympathetic ganglia of the frog was performed as described by others (*20*). Briefly, an intracellular glass microelectrode (∼20 MΩ) measured the membrane potential from the soma of a neuron while the postganglionic

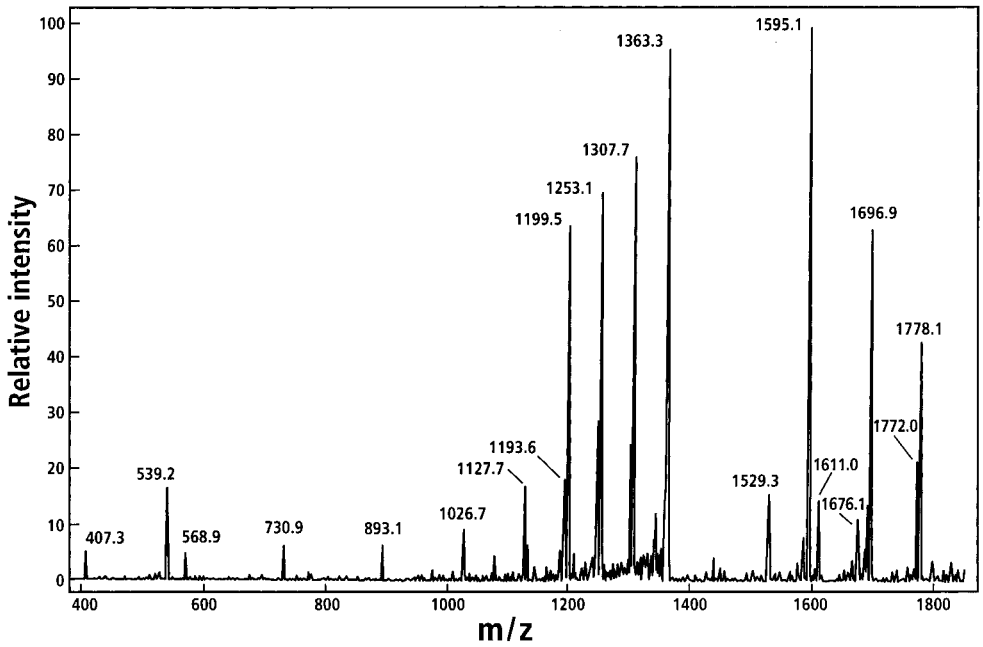



FIGURE 2: The electrospray MS/MS spectrum of the $[M + 3H]^{3+}$ (*m*/*z* 1361) precursor between 380 and 1850 Da indicates the spastic peptide is glycosylated on the basis of singly charged fragment ions with masses which correspond with $HexHAc_2$ (*m*/*z* 407.3), $HexHexNAc_2$ (*m*/*z* 568.9), $Hex_2HexNAc_2$ (*m*/*z* 730.9), $Hex_3HexNAc_2$ (*m*/*z* 893.1); doubly charged fragment ions corresponding with loss of $Hex_2HexNAc$ (*m*/*z* 1778.1), $Hex_3HexNAc$ (*m*/*z* 1696.9), and $Hex_3HexNAc_2$ (*m*/*z* 1595.1) from the intact $[M + 3H]^{3+}$ ion and triply charged fragment ions (*m*/*z* 1307.7, 1253.1 and 1199.5) which correspond with the loss of one, two, and three hexose residues from the intact $[M + 3H]^{3+}$ ion.

nerve was stimulated with a suction electrode. On the other hand, to measure voltage-gated currents, dissociated ganglionic neurons were prepared and whole-cell clamped with patch electrodes.

Whole-cell voltage clamp of *Xenopus* injected with cRNA was performed as previously described (see ref *6* and legend to Figure 3C).

## RESULTS

*Purification of the "Spastic Peptide"*. A fraction of *Conus striatus* venom which induced a spastic paralysis in fish was further resolved by reversed-phase HPLC. This activity was purified to homogeneity—the purification scheme is detailed in Figure 1. We provisionally called the purified activity the "spastic peptide" because of the symptomatology observed in fish.

Results of bioactivity assays of the spastic peptide are shown in Table 1. When injected i.p. in fish, the peptide induced a period of rapid swimming followed by a spastic paralysis with stiff fibrillating fins. At sufficiently high doses, the peptide was lethal to both fish (i.p. > 50 pmol/g) and mice (i.c. > 400 pmol/g).

*Biochemical Characterization of the Spastic Peptide*. The purified spastic peptide was analyzed by liquid secondary ionization-mass spectrometry (LSI-MS); two intact species at *m*/*z* 4084.2 and 4100.5 were observed. Observation of species separated by 16 Da is often indicative of the sample containing a mixture of peptides with methionine and methionine sulfoxide generated upon standing. The sample was subjected to Edman degradation, but no sequence could be determined, suggesting that the peptide was blocked at the N-terminus. When treated with pyroglutamate aminopeptidase to unblock the peptide, sequence analysis gave the partial sequence KSLVP_VITT__GYDOGTM_OO_R_TN, where the level of signal-to-noise after cycle 27 did not allow unambiguous determination of the PTH amino acid in the remaining cycles. After reduction and pyridylethylation, five of the six blank cycles were resolved to give the partial sequence KSLVP_VITTCCGYDOGTMCOOCRC. Microheterogeneity was observed in position 2 of the des-pyroglutamyl peptide, depending on the batch of venom used, with either a Ser (as above) or Glu residue present at this position.

Treatment of the reduced and alkylated peptide with protease Asp-N yielded three major fragments, two hydrophilic and one hydrophobic. The C-terminus of the peptide was determined by chemical sequencing and LSI-MS analysis of the two hydrophilic fragments. For both fragments, the sequence DOGTMCOOCRCTNSC was obtained. The observed masses (*m*/*z* 2053.8 and 2069.2) indicated that the peptide was C-terminally amidated. On the basis of presence of the methionine residue, the two species in this fragment were assigned as methionine- and methionine-sulfoxide-containing analogues (cf. calculated $[M+H]^+$ average masses of 2053.5 and 2069.4 Da). The hydrophobic fragment was identified as the N-terminal fragment based upon the shift in retention time observed after pyroglutamate aminopeptidase treatment. Chemical sequencing of the N-terminal fragment also gave a blank cycle at the seventh position from the N-terminus suggesting the presence of a nonstandard amino acid. While three serine residues were detected in the peptide by amino acid analysis, only two serine residues were found using Edman degradation, suggesting the presence of an additional serine residue (modified) at position 7 and the following sequence:

$$<\text{EKSLVP}\overset{*}{\text{S}}\text{VITTCCGYDOGTMCOOCRCTNSC-NH}_2$$

(where $\overset{*}{\text{S}}$ is a modified Ser, <E is pyroglutamate and O is 4-transhydroxyproline)

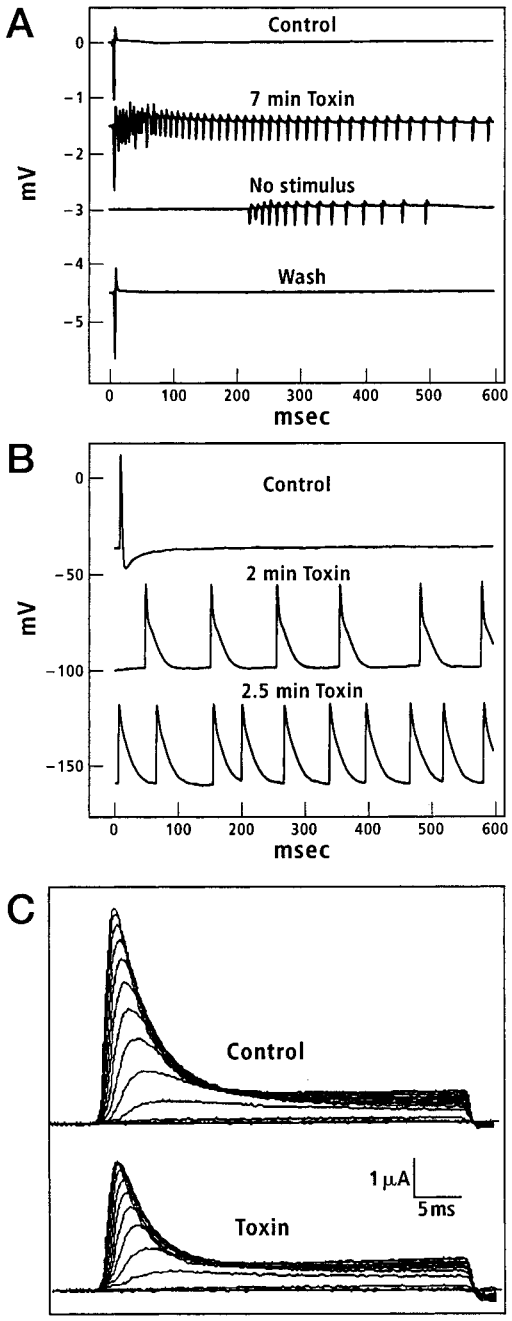



FIGURE 3: (A) Extracellularly recorded responses from the frog cutaneus pectoris muscle reveal that the spastic peptide induces repetitive activity. In the top, second and bottom traces, the nerve innervating the muscle was stimulated once at $t = 5$ ms. After a latency of about a msec, a single biphasic response was recorded (top trace, control). The initial negative deflection is due to a combination of synaptic and action currents originating at the endplate region near the middle of the muscle fibers where the extracellular recording electrode, connected to the positive input of the differential recording amplifier, was located. The later positive phase is due to the action potential which has propagated to the (myotendenous) end of the muscle where the other extracellular recording electrode was located. When the same nerve stimulus was applied after the preparation had been exposed to the spastic peptide (100 nM) for 7 min, repetitive biphasic responses were observed (second trace). Spontaneous responses were also observed within minutes of toxin exposure and persisted as long as toxin was present (third trace, 17 min following addition of toxin). After prolonged washing (1 h), responses similar to controls were observed (bottom trace). Note that each successive trace has been offset from the previous by 1.5 mV. At higher peptide concentration (10 $\mu$M), the repetitive activity remained even after 2 h following washout (not shown).

Table 1: Bioassay of Spastic Peptide

| dose (pmol/g) | observations |
|---|---|
| | (A) Goldfish (i.p.) |
| 5 | no visible effect |
| 10−15 | hyperactivity ∼10 min; darting, tilted swimming ∼20 min; partial paralysis, fins fibrillating ∼180 min |
| 50−55 | darting, mouth open wider ∼4 min; paralysis; fins fibrillating ∼10 min; death ∼100 min |
| >500 | fins spread out, mouth open wider ∼2 min; paralysis, fins fibrillating ∼5 min; death 15 min |
| | (B) Mice (i.c.v.) |
| 50−100 | no visible effect |
| >400 | weak ∼20 min; cannot stand upright ∼25 min; labored breathing ∼30 min; death ∼40 min |

This assignment was verified by sequencing a cDNA clone encoding the peptide (results not shown); the nucleic acid sequence specified a serine codon at position 7.

*MS Evidence for Glycosylation.* The significant difference ($\delta = 893.5$ Da) between the mass of the intact peptide ($m/z$ 4084.2) determined by LSI-MS and that predicted by the proposed sequence (3190.7 Da) suggested that the serine residue was posttranslationally modified. Inspection of the ESI-MS/MS spectrum of the $[M + 3H]^{3+}$ parent ion (Figure 2) reveals several features which indicate that the spastic peptide is glycosylated. The $m/z$ 407.3, 568.9, 730.9, and 893.1 species are singly charged fragment ions with masses which correspond with $HexNAc_2$ (406.8 Da), $HexHexNAc_2$ (568.8 Da), $Hex_2HexNAc_2$ (730.8 Da), and $Hex_3HexNAc_2$ (892.8 Da). The triply charged fragment ions observed at $m/z$ 1307.7, 1253.1, and 1199.5 are consistent with the loss of hexose residues from the intact ion while the doubly charged ions observed at $m/z$ 1778.1, 1696.9, and 1595.1 correspond with loss of $Hex_2HexNAc$, $Hex_3HexNAc$, and $Hex_3HexNAc_2$. Fragment ions involving peptide chain cleavage were also observed in Figure 2 at $m/z$ 539.2, 1026.7, 1127.7, 1529.3, 1611.0, 1676.1, and 1772.0. An extended mass range MS/MS scan ($m/z > 1850$) verified the general trends observed in Figure 2 and revealed that a $m/z$ 1859 doubly charged fragment ion is due to loss of hexose from the $[M + 3H]^{3+}$ ion. These results are consistent with an O-glycosylated serine residue present in position 7. The composition and sequence of the glycan are presently being determined but the mass increment and fragmentation are consistent with $Hex_3HexNAc_2$ (892.817 Da).

*Electrophysiological Studies.* The spastic peptide was tested on the frog neuromuscular preparation. A single

(B) The spastic peptide induces repetitive action potentials in principle neurons of the frog sympathetic ganglion. Shown here are intracellularly recorded action potentials, all from the same neuron, before (top trace, control) and during exposure to 100 nM spastic peptide. Antidromic stimulation was used to elicit the control action potential (top trace). Following exposure to spastic peptide spontaneous action potentials were observed whose frequency increased with duration of exposure. Note that each successive trace has been offset from the previous by 60 mV. The resting potential for all traces was about the same (−38 mV). (C) The spastic peptide blocks conductance of the Shaker $K^+$ channel. An oocyte expressing the Shaker $K^+$ channel was voltage-clamped as previously described (*6*). Traces show outward $K^+$ currents in response to depolarizing steps from −80 to +50 mV in steps of 10 mV before (control) and during exposure to 2.5 $\mu$M spastic peptide. The holding potential was −100 mV.

stimulus to the nerve invariably elicited only a single muscle action potential from the muscle (Figure 3A, top trace). However, when the spastic peptide (100 nM) was present, a train of action potentials was elicited instead (Figure 3A, second trace). Exposure to spastic peptide also produced spontaneous activity (Figure 3A, third trace). Intracellularly recorded action potentials were also examined in intact frog sympathetic ganglia. Action potentials under control conditions were obtained by antidromic stimulation of the postganglionic nerve (Figure 3B, top). Exposure to 100 nM peptide produced spontaneous action potentials; compared to controls, these had a wider overshooting depolarizing phase and no undershoot (Figure 3B, middle and bottom). All these characteristics are consistent with blocking of potassium channels. Furthermore, in preliminary voltage-clamp experiments with TTX-treated dissociated neurons from the ganglion, outward currents elicited by step depolarizations (to −30 mV or more from a −70 mV holding potential) were attenuated by 3 $\mu$M toxin.

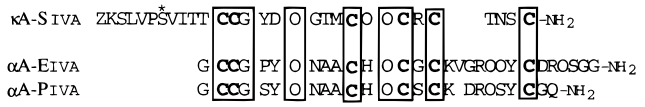
The spastic peptide is an antagonist of cloned *Shaker* $K^+$ channels as illustrated in Figure 3B. The block of $K^+$ currents produced by the peptide was only slowly reversible. Together, the data strongly indicate that the spastic peptide is a potassium channel blocker. However, the molecular identity of the true vertebrate high-affinity $K^+$ channel target of the peptide has not yet been identified. We have designated the spastic peptide as the first member of a new family of *Conus* peptides; the peptide described here is designated $\kappa$A-conotoxin SIVA, consistent with the nomenclature previously used in the *Conus* peptide system.

## DISCUSSION

In this report, we detail the purification and characterization of a novel *Conus* peptide $\kappa$A-conotoxin SIVA which elicits a spastic paralysis when injected into fish. A distinguishing electrophysiological hallmark of the peptide is its ability to elicit repetitive action potentials in the frog nerve-muscle preparation. Although the detailed mechanism of action of $\kappa$A-conotoxin requires further investigation, the neuroexcitatory activity of the peptide appears to be due to blockage of voltage-gated potassium channels, and the peptide appears to be a candidate for contributing to the excitotoxic shock symptomatology observed when *Conus striatus* stings a fish. It is the single most potent (~10 pmol/g) excitotoxic peptide thus far observed when administered i.p. in fish.

At the biochemical level there are striking differences between this peptide and other previously characterized *Conus* peptides. Two unique features are the relatively long N-terminal region (11 AA) preceding the first disulfide linkage and the presence of an O-glycosylated serine residue at position 7. This posttranslational modification has not previously been observed in *Conus* peptides. The blocked amino terminus, the presence of three disulfide bridges, a methionine residue, and the N-terminal extension present in SIVA are all features which are observed in charbydotoxin-type (α-KTx1) scorpion toxins, where five amino acids separate the pyroglutamic acid residue from the N-terminal cysteine residue. However, the absence of charged residues in the SIVA cysteine-rich domain structure stands in contrast with both $\kappa$-conotoxin PVIIA (*6*) and the scorpion $K^+$ channel toxins (*8*).

Like most biologically active peptides in *Conus* venoms, $\kappa$A conotoxin SIVA has multiple disulfide bonds. As shown below, the arrangement and spacing of all but one of the six Cys residues is similar to that of the αA-conotoxins EIVA and PIVA (*13*, *14*).




*****
**S = O-glycosylated Ser residue; Z = pyroglutamic acid; O = transhydroxyproline; X-NH$_2$ = amidated C-terminal residue. Conserved residues are boxed.**

A conserved motif of $CCX_7CX_2CXCX_nC$ (where *n* ranges 3−7) is observed in all three peptides; in addition, two hydroxyproline and one glycine residue are conserved. We note that, like those of the αA-conotoxins, all proline residues between disulfide linkages are hydroxylated; however, in $\kappa$A-conotoxin, the proline residue in the N-terminal tail region remains unmodified. In addition, although the αA-conotoxins are competitive nicotinic receptor antagonists, $\kappa$A-conotoxin is a $K^+$ channel antagonist.

As will be reported elsewhere, we have used peptide purification as well as a molecular biological approach to examine whether similar peptides are present in other Indo-Pacific fish-hunting *Conus* species (A.D.S., D.R.H., M. Watkins, J. M. McIntosh and B.M.O., unpublished results). We found homologues of $\kappa$A-conotoxin SIVA in *C. magus*, *C. stercusmuscarum*, *C. circumcisus* and *C. striolatus*, suggesting a *Conus* peptide family widely distributed in hook-and-line piscivorous *Conus* from the Indo-Pacific. The $\kappa$A-conotoxin is a further example of a peptide which illustrates the distinction between the molecular pharmacology of prey capture in Indo-Pacific and non-Indo-Pacific fish-hunting *Conus* species. We have attempted to identify a spastic peptide homologue in *C. purpurascens* venom without success. It appears to be absent, both from an analysis of the venom and of a cDNA library of this eastern Pacific species. Thus, $\kappa$A-conotoxin SIVA is the first biochemically characterized member of a family of *Conus* peptides, which is widely distributed in hook-and-line fish-hunting Indo-Pacific *Conus* species.

A question which remains to be addressed is the function of the glycan in $\kappa$A-conotoxin SVIA. Like $\kappa$A-SIVA, vespulakinin I and II which are glycopeptides isolated from yellow jacket wasps (*Vespula maculifrons*) (*21*) are polypeptide constituents of venoms. The sites of glycosylation for vespulakinin and $\kappa$A-conotoxin are consistent with the very general motifs for O-linked glycosylation found previously for glycophorin (*22*). The nine C-terminal amino acids of the vespulakinins code for the neuropeptide bradykinin. Comparison of synthetic glycosylated and nonglycosylated vespulakinin analogues indicate that the glycosylated analogue is more active in stimulating guinea pig rectum contraction than the nonglycosylated analogue (*23*). Similarly, preliminary results with synthetic nonglycosylated $\kappa$A-conotoxin analogues indicate that these are far less potent when injected into animals than are the native glycosylated $\kappa$A-conotoxins.

We suggest that $\kappa$A-conotoxin SIVA plays a role analogous to that of $\kappa$-conotoxin PVIIA for *C. purpurascens*, i.e.,

it is one of the major venom components involved in the physiological strategy of the cone snail for eliciting excitotoxic shock in its fish prey that results in immediate immobilization. As was hypothesized elsewhere (*5*), major elements of this excitotoxic immobilization strategy seem to include a $K^+$ channel inhibitor (such as $\kappa$-PVIIA or $\kappa$A-SIVA) along with a peptide that delays sodium channel inactivation (in the case of *C. purpurascens* venom, $\delta$-conotoxin PVIA). In support of this hypothesis, we searched for and found a peptide in *C. striatus* venom which delays sodium channel inactivation (B. Kurz and B.M.O., unpublished results). Thus, our working hypothesis is that $\kappa$A-SIVA and this $\delta$-conotoxin comprise the major venom components that cause the very rapid excitotoxic immobilization observed when *C. striatus* stings its prey. In addition to the peptides which cause the excitotoxic effects on fish, *C. striatus* venom ducts express at least five paralytic peptides which interfere with neuromuscular transmission (*24*−*26*).

According to this hypothesis, in vivo $\kappa$A-conotoxin must be able to incapacitate the appropriate target $K^+$ channels extremely rapidly. Thus, a plausible role for the glycosylation is either increasing the on-time and/or affinity of the peptide for its target $K^+$ channel or increasing the speed of access of the peptide to its target $K^+$ channels. It should be possible in principle to distinguish between these alternatives; given the considerable potency of glycosylated $\kappa$A-conotoxin SIVA, the peptide seems an attractive model system for structure/function studies, not only on the amino acid portion of the glycosylated peptide, but on the glycan residues as well.

## REFERENCES


1. Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V., and Cruz, L. J. (1985) *Science 230*, 1338−1343.
2. Olivera, B. M., Hillyard, D. R., Rivier, J., Woodward, S., Gray, W. R., Corpuz, G., and Cruz, L. J. (1990) in *Marine Toxins*: *Origin, Structure and Molecular Pharmacology* (Hall, S., and Strichartz, G., Ed.) pp 256−278, American Chemical Society, Washington, DC.
3. Cruz, L. J., Gray, W. R., Yoshikami, D., and Olivera, B. M. (1985) *J. Toxicol., Toxin Rev. 4*, 107−132.
4. McIntosh, J. M., Hasson, A., Spira, M. E., Li, W., Marsh, M., Hillyard, D. R., and Olivera, B. M. (1995) *J. Biol. Chem. 270*, 16796−16802.
5. Terlau, H., Shon, K., Grilley, M., Stocker, M., Stühmer, W., and Olivera, B. M. (1996) *Nature 381*, 148−151.
6. Shon, K., Olivera, B. M., Watkins, M., Jacobsen, R. B., Gray, W. R., Floresca, C. Z., Cruz, L. J., Hillyard, D. R., Brink, A., Terlau, H., and Yoshikami, D. (1998) *J. Neurosci. 18*, 4473−4481.
7. Garcia, M. L., Galvez, A., Garcia-Calvo, M., King, V. F., Vazquez, J., and Kaczorowski, G. J. (1991) *J. Bioenerg. Biomembr. 23*, 615−646.
8. Miller, C. (1995) *Neuron 15*, 5−10.
9. MacKinnon, R. (1995) *Neuron 14*, 889−892.
10. Olivera, B. M. (1997) *Mol. Biol. Cell 8*, 2101−2109.
11. Röckel, D., Korn, W., and Kohn, A. J. (1995) *Book Manual of the Living Conidae*, Verlag Christa Hemmen, Wiesbaden, Germany.
12. Kohn, A. J. (1956) *Proc. Natl. Acad. Sci. 42*, 168−171.
13. Hopkins, C., Grilley, M., Miller, C., Shon, K., Cruz, L. J., Gray, W. R., Dykert, J., Rivier, J., Yoshikami, D., and Olivera, B. M. (1995) *J. Biol. Chem. 270*, 22361−22367.
14. Jacobsen, R., Yoshikami, D., Ellison, M., Martinez, J., Gray, W. R., Cartier, G. E., Shon, K., Groebe, D. R., Abramson, S. N., Olivera, B. M., and McIntosh, J. M. (1997) *J. Biol. Chem. 36*, 22531−22537.
15. Shon, K., Grilley, M., Jacobsen, R., Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torres, J., Yoshikami, D., and Olivera, B. M. (1997) *Biochemistry 31*, 9581−9587.
16. 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., and Rivier, J. (1987) *Biochemistry 26*, 2086−2090.
17. Lowry, O., Rosenbrough, N., Farr, A., and Randall, R. (1951) *J. Biol. Chem. 193*, 265.
18. Barber, M., Bordoli, R. S., Elliott, G. J., Sedgwick, R. D., and Tyler, A. N. (1982) *Anal. Chem. 54*, 645A-657A.
19. Yoshikami, D., Bagabaldo, Z., and Olivera, B. M. (1989) *Ann. N. Y. Acad. Sci. 560*, 230−248.
20. Dodd, J., and Horn, J. P. (1983) *J. Physiol. 334*, 255−268.
21. Yoshida, H., Geller, R. G., and Pisano, J. J. (1976) *Biochemistry 15*, 61−64.
22. Piscano, A., Redmond, J. W., Williams, K. L., and Gooley, A. A. (1993) *Glycobiology 3*, 429−435.
23. Gobbo, M., Biondi, L., Filira, F., Scolaro, B., Rocchi, R., and Piek, T. (1992) *Int. J. Pept. Protein Res. 40*, 54−51.
24. Zafaralla, G. C., Ramilo, C., Gray, W. R., Karlstrom, R., Olivera, B. M., and Cruz, L. J. (1988) *Biochemistry 27*, 7102−7105.
25. Myers, R. A., Zafaralla, G. C., Gray, W. R., Abbott, J., Cruz, L. J., and Olivera, B. M. (1991) *Biochemistry 30*, 9370−9377.
26. Ramilo, C. A., Zafaralla, G. C., Nadasdi, L., Hammerland, L. G., Yoshikami, D., Gray, W. R., Kristipati, R., Ramachandran, J., Miljanich, G., Olivera, B. M., and Cruz, L. J. (1992) *Biochemistry 31*, 9919−9926.


BI981690A