Purification, Characterization, Synthesis, and Cloning of the Lockjaw Peptide from *Conus purpurascens* Venom[†]

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ABSTRACT: The major groups of *Conus* peptides previously characterized from fish-hunting cone snail venoms (the α-, μ-, and ω-conotoxins) all blocked neuromuscular transmission. A novel activity, the "lockjaw peptide", from the fish-hunting *Conus purpurascens*, caused a rigid (instead of flaccid) paralysis in fish and increased excitability at the neuromuscular junction (instead of a block). We report the purification, biological activity, biochemical and preliminary physiological characterization, and chemical synthesis of the lockjaw peptide and the sequence of a cDNA clone encoding its precursor. Taken together, the data lead us to conclude that the lockjaw peptide is a vertebrate-specific δ-conotoxin, which targets voltage-sensitive sodium channels. The sequence of the peptide, which we designate δ-conotoxin PVIA, is (O = 4-*trans*-hydroxyproline) EACYAOGTFCGIKOGLCCSEFCLPGVCFG-NH_2. This is the first of a diverse spectrum of *Conus* peptides which are excitotoxins in vertebrate systems.

Fish-hunting cone snails use a diverse set of small, disulfide-rich peptides, the conotoxins, as potent pharmacological agents to paralyze their prey (Olivera et al., 1990; Myers et al., 1993). To date, three major classes of paralytic conotoxins have been characterized from the venoms of fish-hunting cone snails: the α-conotoxins, which inhibiit nicotinic acetylcholine receptors; the μ-conotoxins, which target to skeletal muscle sodium channels at the tetrodotoxin/saxitoxin site; and the ω-conotoxins, which block voltage-sensitive calcium channels at presynaptic termini. These peptides potently block neuromuscular transmission, resulting in flaccid paralysis of the prey.

We observed that, for the fish-hunting *Conus purpurascens*, envenomation is not immediately followed by skeletal muscle flaccidity. Instead, an initial muscle contraction is characteristically observed; often, the prey fish extend their mouths. An illustration of the latter effect is shown in Figure 1. Like other members of the genus, *C. purpurascens* harpoons its fish prey with a disposable harpoon-like tooth, which also serves as hypodermic needle to inject venom. In the figure, a fish was harpooned at the mouth exterior; the harpoon is still visible where the fish was stung. It is apparent that the muscles around the mouth of the fish are extremely contracted, resulting in a remarkable, rigid extension of the fish's mouth (the "fish lockjaw syndrome"). This condition persisted for many hours after the fish was stung. Because of the unusual site of injection, the venom did not cause general systemic effects and was presumably localized around the mouth musculature, causing the bizarre lockjaw effect.

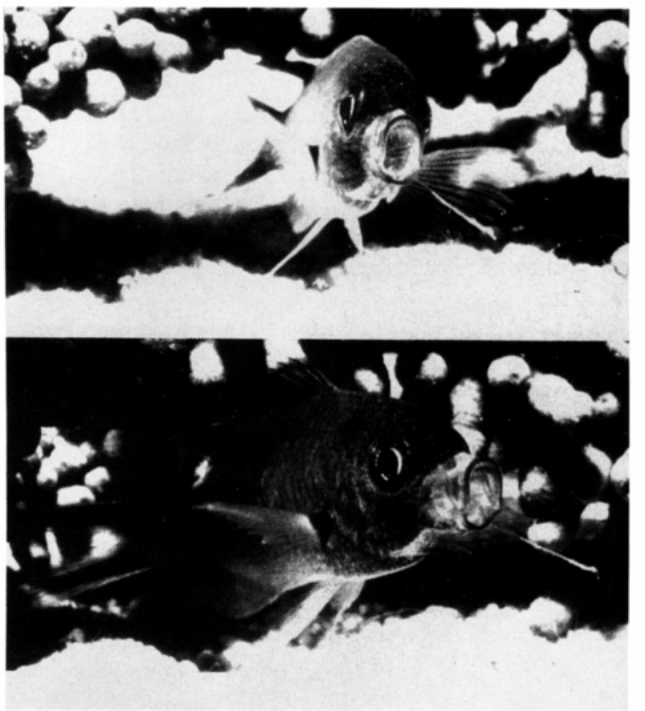

FIGURE 1: Effects of *C. purpurascens* sting. A scissor-tail damsel fish (*Chromis atrilobatus*) from the Gulf of California was harpooned in the mouth by a purple cone (*C. purpurascens*). The cone snail's harpoon is visible still attached to the fish's mouth. The grossly abnormal mouth extension, illustrated in the two panels, persisted for many hours. The venom component which elicited this unusual symptomatology is characterized in this study. (Photograph by Alex Kerstitch.)

In this paper, we describe the purification and biochemical characterization of a novel peptide from *C. purpurascens* venom, which appears to be the major component responsible for the rigid contracture of skeletal muscle, resulting in the "lockjaw" effect. It is unique among known conotoxins

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[1] Abbreviations: acm, acetamidomethyl; boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOBT, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; *t*-Bu-*tert*-butyl; TFA, trifluoroacetic acid; trt, trityl; 4-VP, 4-vinylpyridine.

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analyzed from piscivorous snails so far, in producing contraction rather than relaxation of muscle.

METHODS

Milked Venom Extraction. *C. purpurascens* specimens were collected from the Gulf of California, and venom was collected by a milking procedure to be described in detail elsewhere (C. Hopkins et al., manuscript in preparation). Milked venom (0.5 mL) was pooled from 50 Eppendorf tubes stored in a −70 °C deep freezer. The pooled venom was kept over ice and diluted with 10 mL of 0.1% TFA[1] in water. The solution was spun for a few minutes using a bench-top microfuge, and the supernatant was immediately subjected to purification.

Peptide Purification by HPLC. A preparative scale reversed-phase HPLC was used for first-line purification of the milked venom. The entire 10 mL was applied to a C_{18} Vydac preparative column (22.0 × 250 mm; 20 mL/min) with a guard column (22.0 × 50.0 mm). As a secondary purification, a C_{18} Vydac analytical column (218TP54, 4.6 × 250 mm; 1 mL/min) was used. HPLC buffers were (A) 0.1% TFA in water and (B) 0.085% TFA in 90% acetonitrile. For both preparative and analytical runs, the peptides were eluted with a linear gradient of 1% buffer B increase per minute. The C_{18} analytical column was also used for purifying alkylated peptides for amino acid sequence analysis.

Amino Acid Sequence Analysis. Peptide reduction and alkylation protocols were as described in Shon et al. (1994). Reversed-phase HPLC was used for repurifying alkylated peptide. The eluted peptide was adsorbed onto Biobrene-treated glass fiber filters, and the amino acid sequence was analyzed by automated Edman degradation on an ABI Model 477A instrument.

Solid-Phase Peptide Synthesis. The protected peptide resin was built using standard fmoc chemistry, couplings being carried out with equimolar amounts of amino acid derivative, DCC, and HOBT. All amino acids were purchased from Bachem (Torrance, CA), and the side chains were protected as follows: Hyp (*t*-Bu), Lys (boc), Ser (*t*-Bu), Tyr (*t*-Bu), Glu (*t*-Bu), and Thr (*t*-Bu). Cys residues 3, 17, 18, and 27 were protected by trt, while Cys residues 10 and 22 were protected by acm.

At the completion of synthesis, the terminal fmoc group was removed by standard treatment with piperidine/NMP (20% by volume). Peptide was removed from the resin by treatment for 2 h at 20 °C with TFA/H_2O/ethanedithiol/phenol/thioanisole (90/5/2.5/7.5/5 by volume), and the whole mixture was filtered rapidly into *tert*-butyl methyl ether at −10 °C. The pellet, almost free of ether, was dissolved in 60% acetonitrile containing 0.1% TFA.

A two-step oxidation protocol was used as described in Shon et al. (1994) with a few minor changes. Crude linear peptide cleaved from 100 mg of resin was directly subjected to oxidation in 20 mM FeCN in 0.1 M Tris−acetate buffer (pH = 8.0) containing 60% acetonitrile. The peptide solution (diluted to 200 mL containing 60% acetonitrile) was dripped slowly (at least 30 min) into 200 mL of the FeCN solution in order to minimize any intermolecular disulfide bond formation. On average, 1 h is usually enough for complete oxidation. The oxidation reaction resulted in three bicyclic peptides with disulfide bonds among Cys 3, 17, 18, and 27. The three isomers were purified using a reversed-phase HPLC preparative column with a gradient of acetonitrile (27−50%) in 0.1% TFA and a flow rate of 20 mL/min. One of the three isomers gave native-like material after oxidation with 1 mM I_2 in 10% TFA and acetonitrile (5 min at room temperature, followed by a quench with 30 mM ascorbic acid).

Biological Assays. Goldfish (1.0−1.5 g) were injected into the intraperitoneal cavity, and 10−14-day-old Swiss Webster mice were injected intracranially (Olivera et al., 1984). The garden snail *Helix aspersa* was injected into the head as described (Shon et al., 1994).

Electrophysiology. The cutaneus pectoris muscle of the leopard frog *Rana pipiens* was prepared and placed in a recording chamber as previously described (Yoshikami et al., 1989) except that the muscle was not pretreated with any toxins or drugs unless otherwise indicated. The motor nerve was electrically stimulated every 30 s with a rectangular suprathreshold pulse lasting 0.1 ms, and extracellular recording electrodes were used to monitor the compound action potential from the muscle.

Membrane Preparation. The crude membrane fraction was obtained from the whole brain of 6−8-month-old Sprague-Dawley rats as previously described (Cruz & Olivera, 1986).

Radiolabeling of Conotoxins. Iodination of δ-conotoxin TxVIA was carried out using the water-soluble reagent chloramine T. Two nanomoles of δ-conotoxin TxVIA dissolved in 50% acetonitrile in water was incubated for 10 min at room temperature with 2 nmol of $Na^{125}I$ (1.1 mCi/nmol) and 10 nmol of chloramine T in 200 mM Tris (pH 8.6). The reaction was quenched with 50 μL of 500 mM ascorbic acid and 50 μL of 200 mM methionine, and the solution was gently extracted twice with 500 μL of diethyl ether. Upon application onto an C_{18} analytical column (Vydac), the monoiodinated TxVIA eluted shortly after the unmodified δ-conotoxin TxVIA at approximately 56% acetonitrile on a linear gradient of acetonitrile (36−63%). The label was stored as a HPLC effluent at −20 °C with 57 mM methionine and centrifuged before use in binding assays. ω-Conotoxin GVIA was labeled by resuspending 10 nmol of peptide in 0.1% TFA, adding an equal volume of 0.25 M Tris-HCl, pH 7.0, and incubating with an equivalent amount of chloramine T and 4 nmol of $Na^{125}I$ (2.2 mCi/nmol) for 10 min at room temperature. The [^{125}I]ω-GVIA was purified by HPLC as previously described (Cruz & Olivera, 1986; Cruz et al., 1987).

Competition Binding Assays. Two assay procedures were used. The first was optimized for [^{125}I]δ-conotoxin TxVIA binding (Fainzilber et al., 1994b). The second conditions were standard ω-conotoxin binding assays (Hillyard et al., 1992), modified by adding 130 mM NaCl, 5 mM $CaCl_2$, 1.3 mM KCl, and 0.8 mM $MgCl_2$ to the assay mix.

Molecular Biology. A cDNA clone encoding the lockjaw peptide was purified from a library of *C. purpurascens* ω- and δ-conotoxin cDNAs using the lockjaw-specific oligonucleotide DHOG 538 (5′ GAR GCN TGY TAY GCN CC 3′, Y = C or T, V = A or C or G, R = A or G, N = A or C or G or T) as probe (Colledge et al., 1992). The library was constructed in the pUC vector ptz18u by cloning PCR amplicons generated using reversed-transcribed *C. purpurascens* venom duct RNA (AMV reverse transcriptase, Boehringer Mannheim) and an oligonucleotide A corresponding

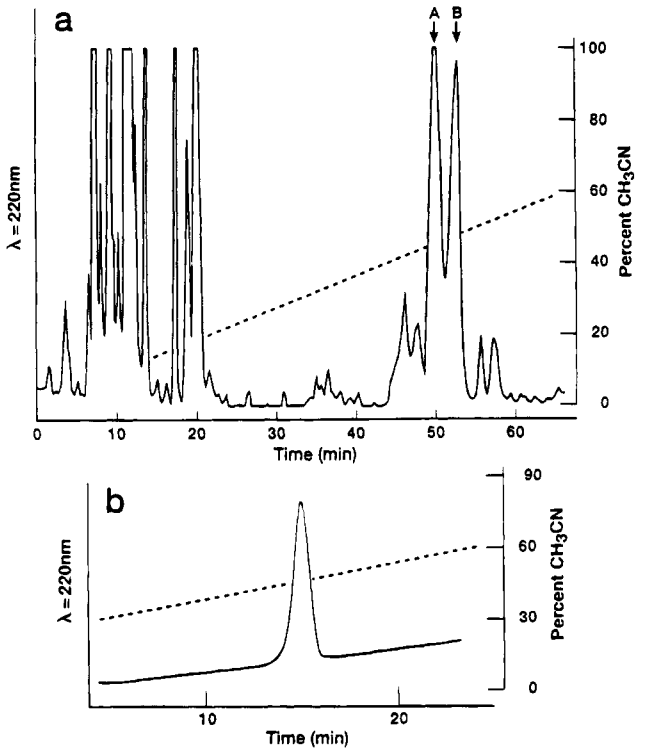

FIGURE 2: (a) Reversed-phase HPLC chromatogram of *C. purpurascens* milked venom. Milked venom (0.5 mL) was diluted and injected onto a C_{18} Vydac preparative column. Peptides were eluted using a linear gradient of 1% buffer B increase per minute with a flow rate of 20 mL/min. Buffer B was 0.085% TFA in 90% acetonitrile. Peaks A and B, indicated with arrows correspond to the active frations which elicited the lockjaw syndrome. (b) Reversed-phase HPLC chromatogram of chemically synthesized lockjaw peptide (free carboxyl form) using an analytical C_{18} Vydac column with a gradient of 1% buffer B increase per minute and a flow rate of 1 mL/min. A single homogeneous peak of characteristic shape eluted at 45% acetonitrile.

to the signal sequence of ω- and δ-conotoxins and oligonucleotide poly(dT) as primers. Putative clones were sequenced using the Sequenase version 2.0 DNA sequencing kit and [^{35}S]dATP (Sequenase version 2.0 seventh edition protocol).

RESULTS

Purification and Sequencing. Crude *C. purpurascens* venom was collected by milking aquarium specimens as described under Methods. The crude venom was fractionated by reversed-phase HPLC as shown in Figure 2 using protocols previously described (Shon et al., 1994). There are several prominent components of the venom; when these were tested for biological activity, several of the major peaks caused flaccid paralysis of fish. Some of these have been shown to inhibit the nicotinic acetylcholine receptor; these studies will be detailed elsewhere (C. Hopkins et al., manuscript in preparation). In a pooled venom preparation, the late-eluting fractions shown in Figure 2 caused paralysis and death in fish accompanied by muscle contracture and the lockjaw symptoms. When injected directly into the central nervous system of mice, these same fractions elicited a variety of symptoms (rapid running, seizures, and convulsions) consistent with increased excitability in the mammalian central nervous system.

The major components which elicit these symptoms were purified to apparent homogeneity from peaks A and B, shown

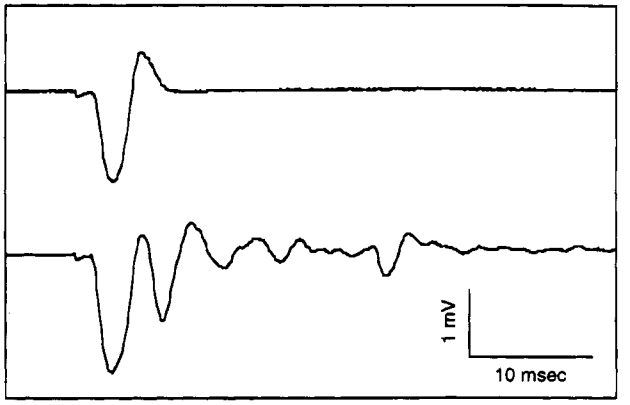

FIGURE 3: The lockjaw peptide elicits repetitive action potentials in frog muscle in response to a single nerve stimulus. Responses of the muscle preparation were recorded extracellularly as described in Methods. The nerve was stimulated with a 0.1-ms pulse (5 ms from start of trace) while the muscle was exposed to control solution (top trace) and solution containing 10 μM lockjaw peptide (bottom trace, obtained 4 min after introduction of toxin into the bath). In the control solution, the muscle produced a single action potential; in contrast, when exposed to toxin the muscle responded with multiple action potentials. The response returned to that of the control when the toxin was washed out (not shown), indicating that the toxin's action was reversible.

with arrows in Figure 2. The amino acid sequence of each homogeneous peptide was determined by standard Edman degradation procedures. The two sequencing runs gave the identical sequence, revealing a hydrophobic 29 amino acid peptide, which we will refer to as the lockjaw peptide. The purified components were analyzed by mass spectrometry, for one component, a mass (monoisotopic MH^+ = 2997.3; theoretical 2997.22) consistent with the predicted sequence from the Edman degradation with an amidated C-terminus. Together, the sequencing data and the mass spectroscopy predict the sequence:

EACYAOGTFCGIKOGLCCSEFCLPGVCFG

where O = 4-*trans*-hydroxyproline. However, the other purified component did not yield a satisfactory mass spectrometric analysis. Since both components had the same sequence, it remained a possibility that these represented forms of the peptide with free carboxyl and amidated C-termini. In order to confirm the sequence assignment, and assess the functional effects of the presence or absence of C-terminal amidation, both the free and amidated forms of the peptide were synthesized and folded as described under Methods. Both synthetic peptides proved to be biologically active; the same *in vivo* symptomatology was induced by the synthetic forms and the native peptide purified from venom (results detailed below). The earlier eluting native lockjaw peptide peak exhibits the same retention time as the synthetic amidated peptide.

Electrophysiology. The effects of the lockjaw peptide on a frog neuromuscular juntion preparation were examined (Figure 3). Clearly, neuromuscular transmission was not blocked by the lockjaw peptide; instead, trains of action potentials were produced in response to a single nerve stimulus when the preparation was exposed to toxin. The effects of the toxin were reversible. The experiment shown in the figure was one of six performed; in every case repetitive action potentials were observed. The number of action potentials produced by stimulation increased with time

of exposure to the toxin. The results provide a plausible explanation for the fish lockjaw syndrome; in effect, repetitive action potentials in the fish jaw musculature elicited by the lockjaw peptide would result in a tetanic paralysis and the rigid extension of the fish mouth parts observed in Figure 1.

Molecular Genetic Analysis of Precursor Structure: Suggestive Evidence That the Peptide Is a δ-Conotoxin. The peptide profile shown in Figure 2 was not reproducibly obtained in all preparations of *C. purpurascens* venom. Although some venom preparations (Figure 2) exhibited a substantial level of the lockjaw peptide, in other samples of *C. purpurascens* venom the lockjaw peptide was missing. To verify that the lockjaw peptide is in fact encoded by *C. purpurascens*, and to confirm the sequence assignment, an analysis of venom duct mRNA was carried out. These studies have confirmed that the lockjaw peptide is encoded by *C. purpurascens* venom duct mRNA. The variation in levels of lockjaw peptide in milked venom preparations may be a result of differential adsorption of the very hydrophobic peptide during venom storage.

A PCR amplification/cloning strategy (described in Methods) was used to identify a lockjaw cDNA clone. The nucleotide sequence of the clone and the inferred amino acid sequence are shown in Table 1. The open reading frame encodes a typical signal sequence, with the prepropeptide organization found in other conotoxins (Olivera et al., 1990). The precursor sequence has a typical conotoxin proteolytic cleavage site (Woodward et al., 1990; Colledge et al., 1992). The predicted mature toxin sequence is identical to the peptide purified from venom, with a C-terminal amide group. The C-terminal −CFGG-OH sequence of the precursor would be processed to a $-CFG-NH_2$ sequence by peptidylglycine α-amidating monooxygenase (Bradbury et al., 1982; Murthy et al., 1987).

The arrangement of cysteine residues in the lockjaw peptide is characteristic of the so-called "four-loop" family of *Conus* peptides. In fish-hunting cones, the major peptide family with the four-loop Cys motif is the ω-conotoxins, which inhibit voltage-sensitive Ca channels (Myers et al., 1993). Indeed, when the sequence of the lockjaw peptide precursor is aligned with the precursor sequence of an ω-conotoxin, considerable identity is observed (Table 1). For this reason, we tested whether the purified lockjaw peptide bound to the ω-conotoxin site. In a competition binding experiment using ^{125}I-radiolabeled GVIA, we found no displacement of specific ω-conotoxin binding (see Table 2), suggesting that the lockjaw peptide is *not* a member of the ω-conotoxin family.

However, the *C. purpurascens* peptide exhibits an even greater similarity to the precursor sequence of a previously characterized peptide from a snail-hunting *Conus* venom, δ-conotoxin TxVIA (Woodward et al., 1990). The δ-conotoxins were previously shown to bind specifically to voltage-sensitive Na channels (Fainzilber et al., 1994a), causing a delay in channel inactivation resulting in an increase in Na conductance (Hasson et al., 1993; Shon et al., 1994). The sequence homology in Table 1 strongly suggested that the lockjaw peptide might be a member of the δ-conotoxin family.

Binding Evidence That the Lockjaw Peptide Is a δ-Conotoxin. In order to confirm whether the *C. purpurascens* peptide was in fact a δ-conotoxin, binding competition was performed using radiolabeled δ-conotoxin TxVIA as the probe for high-affinity sites on rat brain Na channels (Fainzilber et al., 1994b). The results are shown in Table 2. It is clear that the peptide completely displaced specific δ-conotoxin TxVIA binding under assay conditions where there was not detectable displacement of ω-conotoxin GVIA binding. The experiments in Table 2 were carried out under conditions optimal for δ-conotoxin TxVIA binding; even under assay conditions optimal for ω-conotoxin GVIA binding, the lockjaw peptide displaced the δ-conotoxin but not [^{125}I]ω-GVIA (results not shown). These results, together with the precursor sequence homologies, support the conclusion that the *C. purpurascens* peptide targets to the δ-conotoxin binding site on Na channels and is not an ω-conotoxin. The electrophysiological effects in Figure 3 are thus rationalized by the lockjaw peptide increasing voltage-gated Na^+ currents (Fainzilber et al., 1991), thereby making the neuromuscular junction more electrically excitable.

In Vivo Experiments. The molecular genetic and binding data which indicate that the lockjaw peptide is a δ-conotoxin and not an ω-conotoxin are reinforced by the observed *in vivo* biological activity of the peptide on fish and mice. The ω-conotoxins cause a characteristic shaking syndrome when injected intracranially into mice. In contrast, injection of 0.5 nmol of the purified lockjaw peptide caused hyperactivity, rapid running, limb extension, and death. At higher doses (~5 nmol), the peptide was remarkably toxic to mice, causing death in 10 s. Thus, the peptide is a potent excitotoxin in mammals, a result consistent with a Na channel-targeted ligand, which increases conductance, rather than a calcium channel blocker of the ω-conotoxin class.

In fish, the peptide elicited spurts of rapid swimming, with twisted motions, quivering fins, and the lockjaw extended mouth syndrome. Rigid paralysis and death were observed if 0.5−5.0 nmol was injected.

Previously characterized δ-conotoxins were highly potent in all molluscs tested (Hillyard et al., 1989; Fainzilber et al., 1991, 1994b; Shon et al., 1994). The peptide from *C. purpurascens* was injected into a mollusc. In contrast to the results with δ-conotoxins GmVIA and TxVIA which cause typical "King-Kong type" symptomatology (Hillyard et al., 1989) in this snail, the lockjaw peptide elicited no detectable biological effects. Thus, the lockjaw peptide is a potent toxin in vertebrate systems but is inactive in this mollusc.

DISCUSSION

The venom from *C. purpurascens*, a fish-hunting cone snail, contains a novel lockjaw peptide which we have purified and characterized. An initial excitotoxic symptomatology has been observed immediately after injection of venom from several *Conus* species; the lockjaw peptide is the first biochemically characterized toxin shown to underlie such symptoms, as well as to increase excitability at the vertebrate neuromuscular junction. When the peptide was injected into fish intraperitoneally or intramuscularly, a characteristic rapid and very jerky swimming behavior was followed by rigid paralysis, the lockjaw syndrome, and death. Excitotoxic activities were also induced upon intracranial injection of the peptide into mice.

The data above strongly indicate that the lockjaw peptide is a vertebrate-targeted δ-conotoxin. The *C. purpurascens*

Table 1

(A) Nucleic Acid and Predicted Amino Acid Sequence of a cDNA Clone Encoding the Lockjaw Peptide Precursor and a Comparison of the Lockjaw Precursor Peptide to Two Other Related *Conus* Peptide Precursor Sequences[a]

signal sequence

1. met lys leu thr cys val val ile val ala val leu leu leu thr ala cys gln
2. met lys leu thr cys met met ile val ala val leu phe leu thr ala trp thr
3. met lys leu thr cys val met ile val ala val leu phe leu thr ala trp thr

ATG AAA CTG ACG TGC GTG ATG ATC GTT GCT GTG CTG TTC TTG ACT GCC TGG ACA

leu ile thr ala asp asp ser arg --- gly thr gln lys his --- --- arg ala
phe ala thr ala asp asp pro arg asn gly leu gly asn leu phe ser asn ala
phe val thr ala asp asp ser lys asn gly leu glu asn his phe trp lys ala

TTC GTC ACG GCT GAT GAC TCC AAA AAT GGA CTG GAG AAT CAT TTT TGG AAG GCA

leu gly ser thr thr --- --- glu leu ser --- leu ser thr arg --- ---
his his glu met lys asn pro glu ala ser lys leu asn lys arg --- trp
arg asp glu met lys asn arg glu ala ser lys leu asp lys lys glu ala

CGT GAC GAA ATG AAG AAC CGC GAA GCC TCT AAA TTG GAC AAA AAG GAA GCC

mature toxin

cys lys ser pro gly ser ser **cys** ser pro thr ser tyr asn **cys cys**
cys lys gln ser gly glu met **cys** asn leu leu asp gln asn **cys cys**
cys tyr ala pro gly thr phe **cys** gly ile lys pro gly leu **cys cys**

TGC TAT GCG CCT GGT ACT TTT TGT GGC ATA AAG AAG CCC GGG TGC TGC

--- arg ser **cys** asn pro tyr thr lys arg **cys** tyr gly
asp gly tyr **cys** ile val leu val --- --- **cys** thr
ser glu phe **cys** leu pro gly val --- --- **cys** phe gly gly

AGT GAG TTT TGT CTC CCG GGC GTC --- --- TGC TTC GGT GGT

(B) Comparison of Mature δ-Conotoxin Sequences

Lockjaw peptide (PVIA) EA**C**YAOGTF**C**GIKOGL**CC**SEF-**C**LPGV**C**FG

TxVIA WC**K**QSGEM**C**NLLDQN**CC**DGY-**C**IVLV**C**T

GmVIA VKP**C**RKEGQL**C**DPIFQN**CC**RGWN**C**-VLF**C**V

(O = 4-transhydroxyproline)

[a] (a) ω-conotoxin GVIA, (2) δ-conotoxin TxVIA, and (3) lockjaw peptide. Sequences have been aligned to yield maximal identity. The N-terminal 22 amino acids comprise the putative signal sequence, while the mature toxin is at the C-terminus. The ω-conotoxin GVIA and δ-TxVIA sequences were described by Colledge et al. (1992) and Woodward et al. (1990), respectively. Amino acids identical in all three sequences are boxed.

lockjaw peptide was inactive in the molluscan test system at doses 100-fold higher than required to potently affect both fish and mice. In contrast, δ-conotoxin TxVIA which potently potentiates molluscan Na channels showed no biological activity in any assays involving vertebrate systems.

Nevertheless, δ-conotoxin TxVIA and the lockjaw peptide competed for the same binding site in rat brain membranes. δ-Conotoxin TxVIA binds specifically and with high affinity to voltage-sensitive sodium channels in the mammalian central nervous system, even though it has no inhibitory effect (Fainzilber et al., 1994b). Taken together, the primary structure of the lockjaw peptide, the predicted amino acid sequence of the precursor, the electrophysiological results using the frog neuromuscular junction, the binding data, and

Table 2: Binding Competition Experiments[a]

	[^{125}I]δ-TxVIA label (cpm bound)	[^{125}I]ω-GVIA label (cpm bound)
no additions	4890 ± 691	4405 ± 123
δ-conotoxin TxVIA	925 ± 39	4719 ± 40
ω-conotoxin GVIA	4710 ± 450	954 ± 209
lockjaw peptide (amidated)	889 ± 92	4927 ± 253
lockjaw peptide (nonamidated)	848 ± 196	4393 ± 458

[a] Binding conditions were as in Fainzilber et al. (1994b). For each condition, three independent trials were carried out; the standard deviation is indicated. The binding medium contained 130 mM NaCl, 5 mM $CaCl_2$, 1.3 mM KCl, 0.8 mM $MgCl_2$, 50 mM HEPES (pH 7.4), 5.5 mM glucose, and 1 mg/mL BSA and either carrier-free [^{125}I]δ-conotoxin TxVIA (39 nM) or [^{125}I]ω-conotoxin GVIA (52 nM). All unlabeled peptides were added at a final concentration of 1 μM. The wash was the same as the binding medium, but with no glucose, HEPES at 5 mM, and BSA at 5 mg/mL. The rat brain synaptosome membrane protein concentration was 60 μg per 200-μL binding reaction. Preincubation was for 30 min on ice; label was added and incubation was at room temperature for 45 min. After incubation, binding reactions were diluted with 2 mL of ice-cold wash medium and filtered through GF/F (Whatman) filters using a Skatron cell harvester. Filters were washed with an additional 9 × 2 mL wash medium and counted in a δ counter. A similar set of experiments were carried out under assay conditions optimal for ω-conotoxin binding, with qualitatively similar results. The apparent IC_{50} for the amidated lockjaw peptide under these binding conditions was 50 nM.

the *in vivo* symptoms induced by the peptide are consistent with the conclusion that the lockjaw peptide is a vertebrate-targeted δ-conotoxin. A much more mechanistic biophysical characterization of the effects of the peptide on different Na channel subtypes is in progress; the peptide clearly inhibits inactivation of cloned neuronal Na channels (H. Terlau, B. Olivera, K.-J. Shon, and W. Stühmer, unpublished results).

The cloning data demonstrate that the toxin precursor must have an amidated C-terminus. We designate the amidated lockjaw peptide as δ-conotoxin PVIA and the form of the peptide with the free carboxyl terminus as [deamido]-δ-conotoxin PVIA. It should be noted that another fish-hunting *Conus* species, *Conus striatus*, has a toxin which apparently acts by the same physiological mechanism. However, the purified toxin from *C. striatus* (striatotoxin) has been reported to have a much higher molecular mass (~25 kDa) (Kobayashi et al., 1982), which would appear to make it distinct from all of the δ-conotoxins characterized so far. It will be of interest to determine whether striatotoxin exhibits signifiicant sequence similarity to the δ-conotoxins despite its larger size. We are presently attempting to identify clones encoding putative δ-conotoxins of novel specificity from a number of *Conus* species of diverse prey specificity. Different δ-conotoxins would be expected to show considerable variation in their affinities for different types of sodium channels. The discovery of δ-conotoxin PVIA in the venom of the fish-hunting *C. purpurascens* clearly demonstrates that the δ-conotoxin family of peptides is more cosmopolitan in the genus than previously thought. It seems likely that the δ-conotoxins will prove to be one of the major *Conus* toxin families.

In a survey of a dozen *Conus* venoms, we found that a broad spectrum of mammalian excitotoxins is present. Previously characterized conotoxins (α-, μ-, and ω-) all cause a decrease in electrical excitability or an inhibition of neurotransmission (Olivera et al., 1990; Myers et al., 1993). The comprehensive biochemical characterization and successful chemical synthesis reported in this paper make δ-conotoxin PVIA the first vertebrate-targeted excitotoxin from *Conus* venoms generally accessible to the neuroscience research community.

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