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A Nicotinic Acetylcholine Receptor Ligand of Unique Specificity, α-Conotoxin ImI*

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We report the isolation, characterization, and total synthesis of a small peptide ligand for nicotinic acetylcholine receptors (nAChRs). It is highly active against the neuromuscular receptor in frog but not in mice. In contrast, it induces seizures when injected centrally in mice and rats, suggesting that it may target neuronal nAChRs in mammals. Although such receptors may be important in both normal cognition and the pathophysiology of several neuropsychiatric disorders, there are few ligands to discriminate between the multiple receptor subtypes.

The new peptide is a highly divergent α-conotoxin from the snail *Conus imperialis*, which preys on polychaete worms. In this article, the purification, structural analysis, synthesis, and preliminary physiological characterization of α-conotoxin ImI (α-CTx-ImI) are reported. The sequence of the peptide is: Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2. The peptide shows striking sequence differences from all α-conotoxins of fish-hunting *Conus*, but its disulfide-bridging is similar: [2–8; 3–12]. We suggest that cone venoms may provide an array of ligands with selectivity for various neuronal nAChR subtypes.

Mammalian nicotinic acetylcholine receptors (nAChRs)[1] can be divided into two groups: 1) those found in skeletal muscle, which have the subunit composition of $(\alpha)_2\beta\gamma\delta$ in developing muscle and $(\alpha)_2\beta\epsilon\delta$ in mature muscle; and 2) those found in neurons, which are much more diverse and believed to be made up of combinations of α (α_2-α_7) and β (β_2-β_4) subunits (1). Compared to muscle nAChRs, little is known about the functional aspects of neuronal nAChRs. This is in part due to the paucity of ligands that target specific neuronal subtypes.

Although a number of antagonists for nAChRs have been described, most of these, such as neosurugatoxin and lophotoxin, are not subtype-specific and appear to inhibit all neuronal nAChR subtypes tested (2). The only subtype-specific inhibitors of nAChRs that have been characterized so far come from the venom of the Taiwanese banded krait, *Bungarus multicinctus*. There is now good evidence that the major cholinergic toxin in this venom, α-bungarotoxin, specifically inhibits nicotinic receptors that contain an α_7-subunit (3).

There is also a minor component of this venom known by convention as neuronal bungarotoxin (also known as κ-bungarotoxin, toxin F, or Bgt 3.1), which acts at $\alpha_3\beta_2$- and to a lesser extent $\alpha_4\beta_2$-containing receptors. This toxin is only available by isolation from natural sources and has been difficult to obtain in significant quantities with high purity. Indeed, at the present time it is commercially unavailable.

Conus is a large genus of predatory snails that feed on fish, snails, and marine worms. Their venoms are complex, often containing hundreds of different peptidic and non-peptidic components. The peptides are notable for their small size, which allows for straightforward chemical synthesis, potency, and receptor subtype selectivity (for general reviews, see Refs. 4–7). For instance, ω-conotoxins are now the defining ligand for N-type (neuronal) calcium channels (8–11) and have become standard reagents for inhibiting neurotransmitter release. Similarly, μ-conotoxins selectively antagonize muscle sodium channels (12, 13), and conantokins specifically target the *N*-methyl-D-aspartate-subtype of glutamate receptors (14–16) and appear to distinguish between subtypes of *N*-methyl-D-aspartate receptors (17). These toxins are available in synthetic form and are routinely used by numerous investigators due to their specificity and ready availability. Nevertheless, only a small fraction of the hundreds of cone venoms have been analyzed.

Another series of structurally and functionally related peptides from *Conus* venoms are the α-conotoxins. These are small, disulfide-rich peptides that target to nAChRs of vertebrates. Like the α-neurotoxins from snake venoms, previously isolated α-conotoxins block the nAChR at the mammalian neuromuscular junction, inducing paralysis. Compared to the snake toxins however (approximately 80 amino acids in length), the α-conotoxins are much smaller (typically 12–15 amino acids in length) (5, 7, 18), and readily synthesized.

Seven α-conotoxins have been characterized to date from the venoms of three fish-eating cone snails (*C. geographus*, *C. magus*, and *C. striatus*). The great majority of the 500 species of cone snails, however, do not use their venom to paralyze fish, but rather to paralyze polychaete worms (>100 species), other molluscs (50–100 species), and various invertebrates such as echiuroid worms and hemichordates. Each cone species is typically very specialized, sometimes eating only one prey species. Since the venoms are essential for paralyzing prey, and many of the known prey of *Conus* use cholinergic transmission at their neuromuscular junctions, it seems likely that the venoms of each cone snail species will have at least one nAChR antagonist. Correspondingly, because of the wide diversity of different prey, and presumably of their respective nAChRs, the cholinergic peptides found in *Conus* venoms are likely to include ligands for the ontogenetically diverse array of receptors within

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[1] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; BTX, bungarotoxin; TCEP, tris-(2-carboxyethyl)phosphine; HPLC, high performance liquid chromatography; CCh, carbamylcholine; MEPP, miniature endplate potential; TBTU, 2-(1H-benzotriole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

the mammalian central nervous system.

As part of our broader effort to identify toxins that might serve as ligands for neuronal nAChRs in the mammalian brain, we are analyzing a venom from a non-fish-hunting *Conus* species to explore whether it might have significantly different α-conotoxins. In this report, we describe the purification and characterization of the first α-conotoxin isolated from a vermivorous cone snail, *Conus imperialis*, and demonstrate that it shows strikingly different biochemical and pharmacological properties from previously characterized α-conotoxins.

EXPERIMENTAL PROCEDURES

Materials

Crude venom from dissected ducts of *Conus imperialis* was collected in the Philippines, lyophilized, and stored at −70 °C until use. β-Mercaptoethanol was from Pierce Chemical Co.; dithiothreitol was from Boehringer Mannheim; trifluoroacetic acid (sequencing grade) was from Aldrich; acetonitrile (UV grade) was from Baxter (Muskegon, MI). Iodoacetamide and α-bungarotoxin (α-BTX) were from Sigma. α-Conotoxin GI (α-CTx-GI) was synthesized as described previously (19). Tris-(2-carboxyethyl)phosphine (TCEP) was synthesized by the method of Burns *et al.* (20).

Peptide Isolation and Sequencing

Peptide Enrichment—Crude *C. imperialis* venom (100 mg) was extracted with 0.1% trifluoroacetic acid at 4 °C as previously described (21). The supernatant from this extraction was transferred to a Centricon 3 Microconcentrator (Amicon, Beverly, MA) which has a 3,000 M_r cut-off. The Centricon Microconcentrator was centrifuged at 7,500 rpm in a Sorvall SS-34 rotor overnight at 4 °C, and the filtrate was used for further purification.

HPLC Purification—HPLC consisted of HPXL pumps and a Dynamax model UV1 detector (Rainin, Woburn, MA). For isolation of peptide from venom, buffer A consisted of 0.1% trifluoroacetic acid and buffer B was 0.1% trifluoroacetic acid, 90% acetonitrile. For purification and analysis of synthetic peptide, buffer A consisted of 0.1% trifluoroacetic acid and buffer B was 0.1% trifluoroacetic acid, 60% acetonitrile. Peptides were fractionated on C_{18} Vydac or Microsorb columns (4.6 mm × 25 cm, 5-μm particle size, from Rainin, Woburn, MA) (see Fig. 2).

Pyridylethylation and Purification of Modified Peptide—Peptide from the final purification was stored in the 0.1% trifluoroacetic acid/acetonitrile elution buffer. One hundred fifty μl of this purified peptide solution was combined with 30 μl of 0.1% trifluoroacetic acid, and to this was added 60 μl of reducing buffer (0.25 M Na_2HPO_4, 100 mM β-mercaptoethanol, 10 mM EDTA, and 10 mM dithiothreitol). The reducing buffer was sufficient to raise the pH to a value between 7 and 8 as measured by pH paper. The reaction vessel was flushed with argon and the reaction incubated at 65 °C for 15 min. The solution was allowed to cool; 2 μl of 4-vinyl pyridine was added, and the solution was reacted for an additional 25 min at room temperature in the dark. The solution was then diluted 3-fold with 0.1% trifluoroacetic acid, and the alkylated peptide was purified on the Microsorb column with gradient program 2 as described in Fig. 2.

Sequence Analysis—Sequencing was performed with Edman chemistry on an Applied Biosystems model 477A Protein Sequencer at the Protein/DNA Core Facility at the University of Utah Cancer Center.

Peptide Synthesis and Disulfide Analysis

Linear Peptide—Linear peptide was built by the manual solid phase method following a butoxycarbonyl/benzyl strategy, and employing a *p*-methylbenzhydrylamine resin to obtain the peptide amide. All amino acids were purchased from Bachem (Torrance, CA). Side-chain protection used was: *S*-methylbenzyl-L-cysteine, β-*O*-benzyl-L-serine, L-aspartic acid β-cyclohexyl ester, and N^g-toluenesulfonyl-L-arginine. Coupling reactions were carried out using TBTU (22). Prior to HF cleavage, the α-amino function of the last amino acid was deprotected by treatment of the resin with trifluoroacetic acid/ethanedithiol/dichloromethane (50/5/45) for 30 min. For cleavage, 3 g of peptidyl-resin was treated with liquid HF at 0 °C in the presence of anisole (10%) and dimethylsulfide (3%) for 75 min. This procedure simultaneously cleaved peptide from the resin and deprotected all side chains.

Peptide Cyclization—After removal of HF under vacuum, the linear peptide and resin were washed with ether and the mixture (peptide + resin) was introduced into 3500 ml of isopropanol/H_2O (50/50) and gently stirred. The pH (checked by pH paper) was maintained at 8–9 by adding diisopropylethylamine during the air oxidation reaction. Progress of the oxidation was monitored by HPLC and the Ellman test (23) and was judged to be complete after 5 days. The solution was filtered to remove the resin, the pH was adjusted to 5 by the addition of acetic acid, and isopropanol was evaporated. After dilution with water, the peptide was purified by preparative reversed-phase HPLC on a Vydac C_{18} column, using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Its identity with natural peptide was verified by co-elution experiments on HPLC and capillary zone electrophoresis (CZE), by mass spectrometry, and by disulfide "fingerprinting" (below).

Disulfide Analysis—The partial reduction strategy of Gray (24) was used. Twenty nanomoles of synthetic peptide, as eluted from HPLC, was incubated at room temperature with 10 mM TCEP in 0.1 M citrate, pH 3. After 2–5 min the reaction mixture was diluted with 0.1% trifluoroacetic acid to decrease acetonitrile concentration and immediately injected onto the HPLC column. Peptide fractions, as identified by UV absorbance were collected manually into 1.5-ml polypropylene centrifuge tubes. Monocyclic intermediates were alkylated with 100 mg of iodoacetamide using the rapid alkylation method (24). Labeled peptides were submitted to sequence analysis to determine the locations of *S*-carboxamidomethyl-L-cysteine residues, and hence the disulfide connectivity.

Disulfide Fingerprinting—Approximately 200 pmol of peptide in 50 μl of HPLC eluent were mixed with 50 μl TCEP (20 mM in 0.2 M citrate, pH 3). After brief incubation at room temperature, the mixtures were analyzed by HPLC, to compare product distributions of natural and synthetic materials.

Electrophysiology

Intracellular Recording from R. pipiens Muscle Fibers—Freshly dissected cutaneus pectoris muscle was pinned on a thinly Sylgard-coated (<0.5 mm) 25-mm diameter #1 coverslip and placed in an ~1.2-ml chamber which was perfused with Ringer's solution consisting of 10 mM Na HEPES, pH 7.2, 111 mM NaCl, 1.8 mM $CaCl_2$, and 2 mM KCl. Intracellular recording and iontophoretic application of carbamylcholine were performed under visual control as described previously (25).

Iontophoretic Application of Carbamylcholine (CCh) and Focal Toxin Application—The tip of the iontophoretic CCh pipette (containing 1 M CCh) was placed near a neuromuscular junction and CCh was ejected with current pulses (~50 nA, 5-ms duration) applied at a frequency of 0.1 Hz. The response to each pulse was recorded, and the peaks of the response amplitudes were plotted (see Fig. 5). A puffer-pipette was used to apply peptide toxin as previously described (26). Specifically here, a glass micropipette (tip inner diameter, 6 μm) was filled with 50 μM α-CTx-ImI in Ringer's. Its tip was placed about 50 μm away from the tip of the CCh pipette at the neuromuscular synapse, and peptide was ejected by application of puffs of pressure (2 p.s.i., 50-ms duration) to the pipette. The puffer pipette was placed at a fairly large distance (50 μm) to insure that 1) the toxin diffused to a postsynaptic area which encompassed the area affected by iontophoresed CCh, and 2) the puff of toxin did not mechanically disturb the position of the tip of the CCh pipette relative to the postsynaptic membrane. Data were acquired on a Macintosh computer with an A/D board (Lab NB, National Instruments, Austin Texas) and our own virtual instruments (constructed with the graphical programming language LabVIEW also from National Instruments).

Toxin Effect on MEPPs—Spontaneous miniature endplate potentials (MEPPs) were recorded intracellularly as above. To facilitate recording of MEPPs, their amplitudes and durations were increased by irreversibly blocking acetylcholinesterase in the preparation by exposing the muscle to 1 mM methanesulfonyl fluoride (Eastman Chemicals, Rochester, NY) in Ringer's for 1 h (*cf.* Ref. 27). Spontaneous MEPPs were acquired on a Macintosh as described above with a virtual instrument which captured all transients with amplitudes exceeding 0.4 mV. The recordings of individual transients were visually inspected off-line, and all those which did not have profiles characteristic of MEPPs were culled out.

Extracellular Recording in R. pipiens—Extracellular recording of postsynaptic responses to motor nerve stimulation in a cutaneus pectoris muscle from *R. pipiens* was performed essentially as described previously (28). Specifically, the muscle was placed in a Sylgard recording chamber (15 mm long × 1 mm wide × 2.5 mm deep); the motor nerve was passed through a petroleum jelly (Vaseline®) gap into an adjacent well containing embedded Pt stimulating electrodes. The muscle was bathed with frog Ringer's solution supplemented with 0.25 mg/ml lysozyme to minimize possible nonspecific absorption of the conotoxins. α-Conotoxins were dissolved in normal frog Ringer's containing 0.25 mg/ml lysozyme. Toxins were applied to the preparation and a plastic coverslip was placed over the recording chamber to minimize buffer evaporation. The motor nerve was electrically stimulated about once

every 30–60 s with a supramaximal 0.1-ms pulse. The resulting action potential in the muscle was recorded extracellularly by means of Pt electrodes (embedded in the Sylgard chamber) connected to a Grass amplifier (model P15B). Data was captured by computer as described above.

Extracellular Recording from Electrocytes in Eigenmannia—Fish were anesthetized by chilling in ice-cold water and decapitated, and the distal approximately 4 cm of the tail was removed and further prepared as follows: starting at the anterior end of the piece of tail, the entire skin surrounding the tail was peeled away from the immediately underlying tissue (consisting of muscle/electrocytes). The tissue surrounding the anterior end of the spinal column was removed over a 2–4-mm length approximately 14 mm from the distal end of the tail to yield a "spinal stump." The posterior tip (~14 mm) of the tail was severed away, and the preparation was placed in a recording chamber constructed of Sylgard (15 mm long × 2 mm wide × 2 mm deep) filled with Hickman's solution (110 mM NaCl, 2 mM KCl, 3 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM $NaHCO_3$, 1 mM $Na_2H_2PO_4$, pH 7.2) plus 0.25 mg/ml lysozyme. The spinal stump was placed across a petroleum jelly gap and electrically stimulated (via Pt electrodes on either side of the gap) every 30–60 s with a supramaximal 0.1-ms pulse. The resulting action potential from the electrocytes or electric organ discharge (EOD) was recorded extracellularly with Pt electrodes embedded in the chamber. Recording electronics were as described for the *R. pipiens* preparation above. Toxins were dissolved in the Hickman's solution plus lysozyme buffer and applied directly to the bath. Recordings were taken for 20–25 min.

Bioassay—Biological activity of peptide fractions was tested by intraperitoneal or intracranial injection into young mice as described previously (29). Twenty-μl volumes were injected intracerebrally, and 50-μl volumes were injected intraperitoneally, using insulin syringes with 29-gauge needles. Intracerebral-ventricular injections were made in Sprague-Dawley rats. Rats (450–550 g) were deeply anesthetized with pentobarbital and placed in a Stoelting stereotaxic apparatus. The scalp was incised, and a 1-mm hole was drilled in the skull at the stereotaxic coordinates 0.92 mm posterior to bregma and 1.4 mm lateral to midline. A 25-gauge stainless steel cannula (outer diameter, 0.02, inner diameter, 0.01, preset in a 1/8 nylon set screw, was inserted to a depth of 3.5 mm. A "copper crown" made of 0.5 in diameter × 1/4 in length copper pipe was placed around the cannula to prevent mechanical dislodgment. Cannula and crown were secured with dental cement. Animals were allowed to recover for at least 3 days prior to use. Three-μl injections were made with a 10-μl Hamilton syringe and 33-gauge blunt tip needle. In some cases, position of the cannula in the lateral ventricle was confirmed histologically.

RESULTS

Isolation of α-Conotoxin ImI (α-CTx-ImI)—Our long term goal is to identify α-conotoxin-type peptides from non-fish-hunting *Conus* venoms, with an emphasis on those which might act on subtypes of mammalian neuronal nicotinic acetylcholine receptors. The initial venom examined, and the subject of this report, was that of the imperial cone, *Conus imperialis*, obtained by dissecting venom ducts from specimens mostly collected off Cape Bolinao, Luzon Island, Philippines. This species hunts the polychaete worm, *Eurythoe complanata*. Photographs of *C. imperialis* and other cone shells are shown in Fig. 1.

Several guidelines were used to identify candidate peptides with the desired characteristics. Since these were likely to be related structurally to the α-conotoxins, which are the smallest of the major toxins in fish-hunting cone venoms, we first examined the smaller peptides found in *C. imperialis* venom. The first step in purification involved filtration through a membrane with a cut-off of 3000 daltons (the Centricon 3 microconcentrator).

The filtrate was then purified using a C_{18} reverse phase column (see Fig. 2*A*). The UV absorbance pattern is relatively simple, since larger peptides and polypeptides were retained by the filtration procedure. Peptides targeted for further purification were those that were biologically active upon intracranial injection into mice. This has been a reliable method for isolating central nervous system-targeted ligands. The fraction indicated by an *arrow* in Fig. 2*A* induced seizures upon intracranial injection.

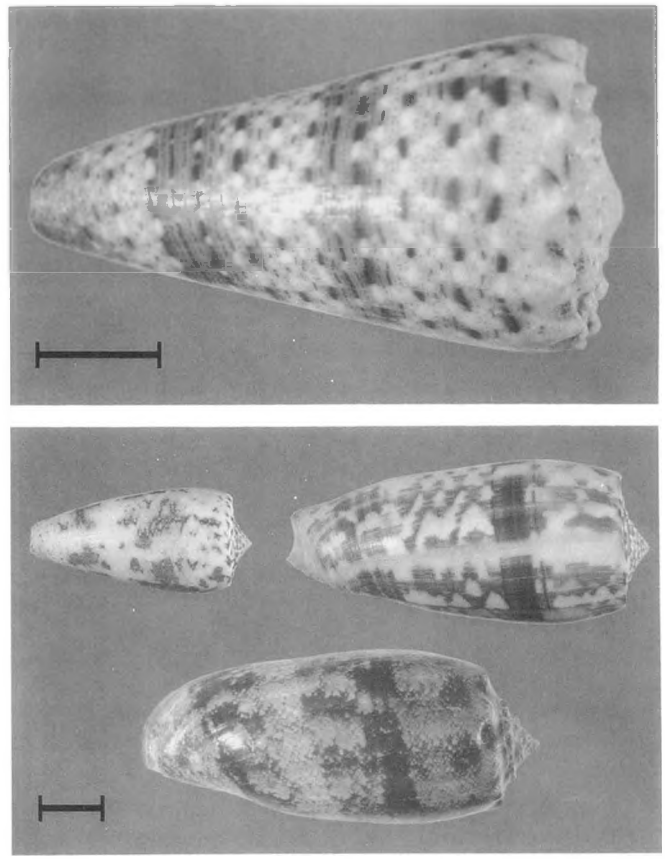

FIG. 1. **Cone snails from which α-conotoxins have been isolated.** *Top panel*, the imperial cone snail, *Conus imperialis*. *Bottom panel*, three fish-eating cone snails: *C. magus* (*top left*), *C. striatus* (*top right*), and *C. geographus* (*bottom*). *Bar* = 1 cm in both panels.

This fraction was rechromatographed with a much shallower gradient of acetonitrile (Fig. 2*B*). The earlier-eluting peptide elicited scratching upon intracranial injection, and proved to be Lys-conopressin G (30). The major component was further purified on a Microsorb column (Fig. 2*C*) and biochemically characterized.

Biochemical Characterization of α-Conotoxin ImI—The peptide purified as described above was reduced, alkylated and sequenced as described under "Experimental Procedures." The results of the sequence analysis are shown in Table I; these were verified with a second independent analysis, which gave similar results. The peptide has only 12 amino acids, including 4 Cys residues. Fast atom bombardment mass spectrometry indicated that Cys residues are present as disulfides and that the C terminus is amidated (monoisotopic MH^+: calculated, 1351.48; found, 1351.5).

Synthetic peptide prepared as described in Methods co-eluted with natural peptide on HPLC and CZE (Fig. 3) and had the correct molecular mass (monoisotopic MH^+: calculated, 1351.48; found, 1351.4). Partial reduction by TCEP of 200 pmol of natural and synthetic peptides gave essentially identical product profiles on HPLC (Fig. 4). This is a sensitive test of identity of disulfide bridging. The two monocyclic peptides R1A and R1B were isolated from a larger scale reduction of synthetic material and alkylated with iodoacetamide. Sequence analysis showed that R1A was alkylated exclusively at Cys-2 and Cys-8, while R1B was alkylated exclusively at Cys-3 and Cys-12. Thus the disulfide bridging pattern is [2–8; 3–12], similar to that in α-conotoxin GI: [2–7; 3–13], where brackets enclose pairs of numbers denoting the partners in a disulfide.

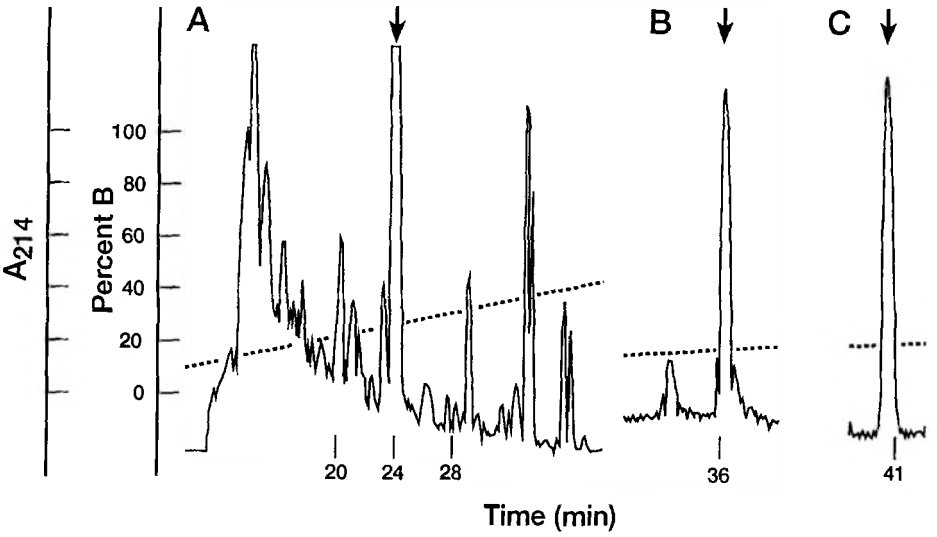

FIG. 2. **Purification of α-CTx-ImI from *C. imperialis* venom.** Buffer A = 0.1% trifluoroacetic acid; buffer B = 0.1% trifluoroacetic acid, 90% acetonitrile. *Panel A*, venom fraction from ultrafiltration (see text) was applied to a C_{18} Vydac column. Gradient program 1 was 0–45% B/40 min, followed by 45–100% B/15 min. *Panel B*, material eluting at 23.9 min (*arrow* in *panel A*) was rerun on the same column with gradient program 2 (2–10% B/3 min; 10–25% B/57 min). *Panel C*, material eluting at 36.4 min (*arrow* in *panel B*) was rerun on the Microsorb column with gradient program 2 to obtain the final purified product. The *solid line* is the absorbance at 214 nm, and the *dashed line* is the gradient of acetonitrile.

TABLE I
Sequence analysis of α-conotoxin ImI from C. imperialis
PTH, phenylthiohydantoin-derivative.

Step	Assigned residue	Yield
		pmol of PTH
1	Gly	188.2
2	Cys	240.4
3	Cys	253.5
4	Ser	64.0
5	Asp	87.1
6	Pro	92.3
7	Arg	76.2
8	Cys	114.3
9	Ala	88.6
10	Trp	17.8
11	Arg	50.3
12	Cys	30.0

Thus, although most of the sequence is quite divergent, and the number of amino acids within the disulfide loops is different from those in previously isolated peptides, the disulfide arrangement clearly places the peptide in the α-conotoxin class. As will be described below, the peptide does indeed target to nicotinic acetylcholine receptors. We have, therefore, named this peptide α-conotoxin ImI (α-CTx-ImI), using the nomenclature for conotoxins previously proposed.

Biological Activity in Vivo of α-Conotoxin ImI—An assessment of the biological activity of α-CTx-ImI by intracranial injection in young mice (6–10 g weight) showed natural and synthetic peptides to be indistinguishable. At low doses (0.5–2 nmol), the symptoms included flattening of ears, back arching, slight spreading of hindlegs, rapid walking, and climbing the sides of the cage, as well as hypersensitivity to tactile stimuli. When a puff of air was blown onto the fur, the test mice would jump much higher than saline-injected controls. At intermediate doses (3–5 nmol) symptoms were more pronounced, and a coarse tremor and complex generalized seizures were also observed. At 6–10 nmol, the wide-based gait was so pronounced that the legs were sometimes completely outstretched. In addition, most mice died following the complex seizures. In contrast, intraperitoneal injection of up to 10 nmol of α-conotoxin ImI did not elicit any notable symptoms. At 20 nmol, some suggestion of ear flattening and hypersensitivity to tactile stimuli was observed, which may be due to a small fraction of peptide crossing the blood-brain barrier and entering the central nervous system (ω-conotoxin-GVIA is known to cross the blood-brain barrier in small amounts).

The effect of intracerebral-ventricular injections of synthetic α-conotoxins into Sprague-Dawley rats was also assessed. Following injection of 5–10 nmol of α-CTx-ImI, crouching, a coarse tremor and wide-based gait were observed, while following 20-nmol injections complex general seizures were induced lasting from 10–30 s, followed by 2–4-min interictal periods. After several seizures, one of two animals died. In contrast, intracerebral-ventricular injection of 40 nmol of the previously characterized α-conotoxin, GI (α-CTx-GI), elicited no effects in two rats tested. The two rats that showed no effect from α-CTx-GI were injected intracerebral-ventricularly the next day with 15 nmol of α-CTx-ImI, which produced the expected symptoms. Intracerebral-ventricular injection of 12 nmol of α-bungarotoxin (α-BTX) produced tremor and a single seizure, while injection of 20 nmol induced repetitive seizures and death.

As shown in Table II, the effects of α-conotoxins in mammalian systems are more restricted than those of α-BTX. In mammals, α-BTX has both peripheral and central nervous system effects, α-CTx-GI has peripheral effects and is inactive upon intracerebral-ventricular injection, while the new peptide, α-CTx-ImI, shows the reciprocal profile with only central effects and no paralytic symptoms upon intraperitoneal injection at the doses tested.

Activity of α-Conotoxin ImI at Neuromuscular Junctions—The cysteine framework and biological activity of α-CTx-ImI strongly suggested that it is a ligand for an nAChR. To test this hypothesis electrophysiologically, experiments were performed on the frog neuromuscular junction. Application of toxin from a pipette containing 50 μM toxin blocked depolarizations induced by iontophoretically applied carbamylcholine (Fig. 5). Fig. 6 shows that bath-applied peptide (0.2 μM) blocks MEPPs. At this concentration of toxin, the mean MEPP amplitude was reduced to 29% of the control value. There was no effect on MEPP frequency indicating that the peptide has no apparent presynaptic effects. When higher concentrations of toxin were used (*e.g.* 50 μM applied through a puffer pipette), complete but reversible block of MEPPs occurred (results not shown). These results indicate that the toxin blocks the postsynaptic nAChR.

Since α-CTx-ImI was isolated from a worm-hunting cone, it was of interest to compare its phylogenetic activity profile to that of α-CTx-GI (isolated from a fish-hunter). Extracellular recording of synaptically evoked action potentials at the frog neuromuscular junction and electrocytes from the weakly electric fish *Eigenmannia* was used for this purpose.

As shown in Table III, the IC_{50} of α-CTx-ImI was 250–500 nM in frog. Under the same conditions, α-CTx-GI caused inhibition of the action potential with an IC_{50} of 2–4 μM. Effects of both

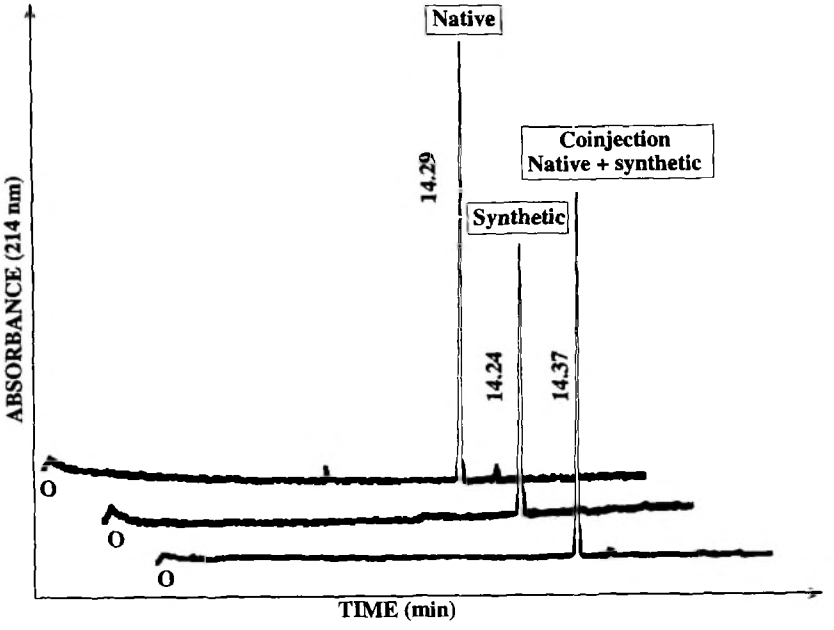

FIG. 3. **Comparison of natural and synthetic α-conotoxin ImI by capillary zone electrophoresis (CZE).** Analysis was performed at 30 °C on a Beckman P/ACE System 2050, monitored at 214 nm, with SpectraPhysics ChromJet SP4400 integrator. Peptides were applied by pressure injection onto fused silica capillaries from Beckman (50 cm × 75 μm). Buffer was 100 mM H_3PO_4 adjusted to pH 2.5 by addition of 2 M NaOH. Analysis was carried out at 15 kV, 80 mA.

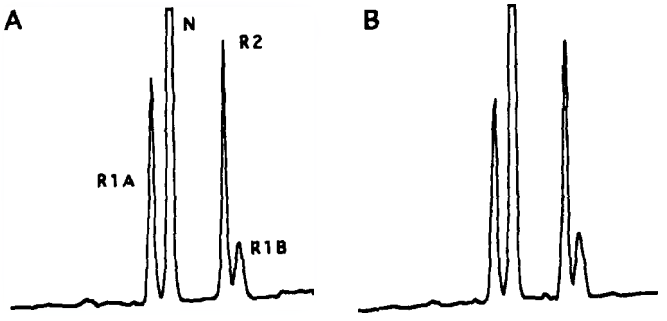

FIG. 4. **Disulfide fingerprinting of synthetic (*panel A*) and natural (*panel B*) α-conotoxin ImI.** Partial reduction was carried out by incubating 200 pmol of peptide with 10 μM TCEP in 100 ml of 0.1 M sodium citrate, pH 3, for 4 min at 25 °C. Samples were analyzed by reverse-phase HPLC as described under "Experimental Procedures." Gradient was 15–35% B in 40 min; segment depicted in each case is from 27–35% B. Peptides corresponding to the absorbance peaks in *panel A* were identified as follows: *N*, unreduced; *R2*, fully reduced; *R1A* and *R1B*, monocyclic intermediates.

TABLE II
Comparison of biological effects in mammals

Intraperitoneal injections of toxins were done in mice and intracerebral-ventricular injections were made stereotactically in rats. α-CTx-ImI produces complex behavioral effects including seizures when injected centrally but does not cause paralysis when injected peripherally. α-CTx-GI produces the opposite result: no effect on intracerebral-ventricular injection and paralysis and death with peripheral injection. α-BTX produces seizures when injected centrally and paralysis when injected peripherally.

	IP	IC
α-CTx-ImI	–	+
α-CTx-GI	+	–
α-BTX	+	+

toxins were reversible with 30 min of washing (results not shown). Thus, α-CTx-ImI is significantly more potent than α-CTx-GI at the frog neuromuscular junction.

However, the reverse order of potency was found when these peptides were tested on electrocytes from *Eigenmannia*. α-CTx-ImI had an IC_{50} of 25–50 μM, while α-CTx-GI had an IC_{50} of

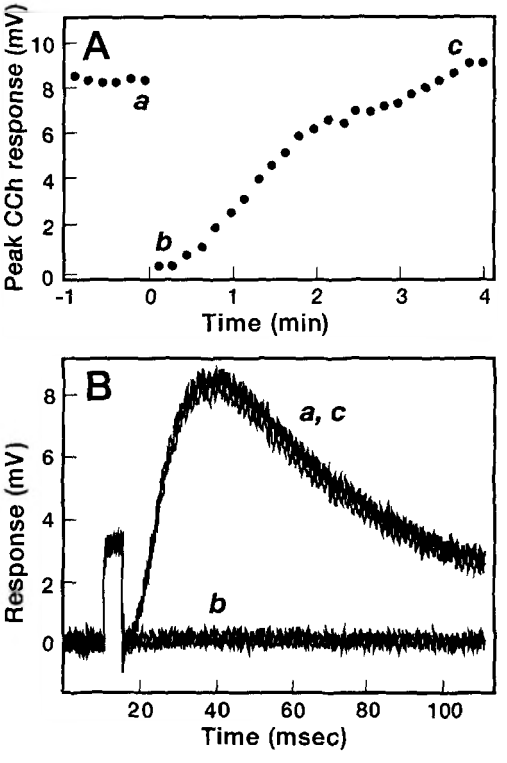

FIG. 5. *Panel A*, time course of block by α-CTx-ImI. CCh was iontophoretically applied every 10 s throughout the experiment. At time zero, a puff of α-CTx-ImI was delivered from a pipette filled with 50 μM peptide. The response to CCh immediately declined, then gradually recovered. *a*, just before toxin puff; *b*, immediately after toxin puff; *c*, after recovery. *Panel B*, sample responses to CCh before, during, and after toxin application. Two responses at each time point are shown (times correspond to points indicated by *a*, *b*, and *c* in *panel A*). The fixed, 5-ms iontophoretic pulse of CCh was applied 10 ms into the trace (note rectangular artifact with fixed amplitude and duration from ms 10 to 15). The application of toxin totally blocked the CCh response (*b*). The response recovered completely when toxin had diffused away. Note that *traces a* (before toxin) and *c* (after recovery) are virtually superimposable. The resting potential remained essentially constant throughout this period, indicating that the toxin had no untoward effects on the muscle membrane *per se*.

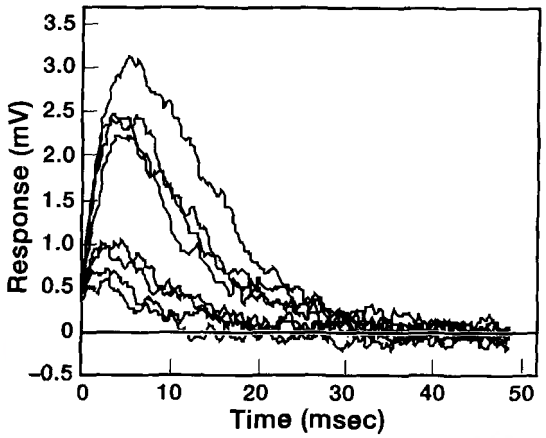

FIG. 6. **α-CTx-ImI reduces MEPP amplitude but not frequency.** Shown are sample traces of MEPPs before (largest four responses) and during (smallest four responses) exposure to 200 nM toxin. Mean amplitude ± S.D. of MEPPs was 2.37 ± 0.97 mV (control, n = 59) and 0.69 ± 0.26 (toxin present, n = 67). The frequency of MEPPs was essentially unaffected by toxin; MEPP frequency was 6.8/min in control Ringer's and 6.3/min when toxin was in the bath. This slightly lower apparent MEPP frequency in the presence of toxin is thought to result from an increased frequency of unrecorded MEPPs, whose amplitudes were too low to trigger capture by the computer.

TABLE III
Relative potencies of α-conotoxin ImI and α-conotoxin GI

The potency of α-CTx-ImI and α-CTx-GI was compared electrophysiologically in fish and frog. The concentration (in μM) of toxin required to inhibit the synaptically mediated action potential by at least 50% was determined. α-CTx-ImI is 100-fold more potent in frog than in fish. In contrast, α-CTx-GI is 40-fold less potent in frog than in fish.

	Fish	Frog
	μM	μM
α-CTx-ImI	25–50	0.25–0.5
α-CTx-GI	0.05–0.10	2.0–4.0

50–100 nM. Thus, α-CTx-GI is approximately 500 times more potent than α-CTx-ImI in blocking a teleost neuromuscular preparation (see Table III), while in the amphibian system, α-CTx-ImI is 8-fold more potent than α-CTx-GI.

DISCUSSION

The venom of *Conus imperialis* is a complex mixture of biologically active agents. We previously reported the isolation of a vasopressin analog (30) and serotonin (21) from this venom. This report describes the first paralytic peptide characterized from *C. imperialis*, the novel nAChR antagonist, α-conotoxin ImI. Numerous other peptides are present which remain to be fully characterized. Since ACh is the neurotransmitter at annelid neuromuscular junctions, it is reasonable to suppose that a peptide targeted to the nAChR would be a major component of the venom of a vermivorous species such as *C. imperialis*. On the other hand, since the invertebrate prey of *C. imperialis* are quite distinct phylogenetically from the fish prey of previously characterized cones (*e.g. C. geographus*), it is not surprising that their respective α-conotoxins would differ both structurally and functionally.

Structural Relationships—Previously characterized α-conotoxins can be divided into two structural classes. These two classes have distinctive sequence features, particularly in the second disulfide loop (see Table IV) where the presence of a proline residue in α-CTx-SI and α-CTx-SII is a diagnostic feature. In other respects however, all previously characterized α-conotoxins have a core sequence CC(N/H)PACG*XX*(Y/F)*X*C in common.

TABLE IV
α-Conotoxin sequence comparison

α-Conotoxins from several species of *Conus* are shown. *C. geographus*, *C. magus*, and *C. striatus* are fish-eaters. The sequence of α-CTx-Im-I from the worm-eating *C. imperialis* differs from the others not only in the number of amino acids between cysteines (4,3) but also in overall amino acid composition. Note that disulfide bond pattern has not been definitively determined for GII, SIA, or SII but is assumed to be conserved. A single or double asterisk indicates that the α-carboxyl group is known to be either amidated or the free acid, respectively.

αCTX	Sequence	Source	Ref.
ImI	GCCSDPRCAWRC*	*C. imperialis*	a
GI	ECCNPACGRHYSC*	*C. geographus*	b
GIA	ECCNPACGRHYSCGK*	*C. geographus*	b
GII	ECCHPACGKHFSC*	*C. geographus*	b
MI	GRCCHPACGKNYSC*	*C. magus*	c,d
SIA	YCCHPACGKNFDC*	*C. striatus*	e
SI	ICCNPACGPKYSC*	*C. striatus*	f
SII	GCCCNPACGPNYGCGTSCS**	*C. striatus*	g

[a] This paper.
[b] Gray et al., 1981 (31).
[c] McIntosh *et al.*, 1982 (18).
[d] Gray *et al.*, 1983 (32).
[e] Myers *et al.*, 1991 (33).
[f] Zafaralla *et al.*, 1988 (34).
[g] Ramilo *et al.*, 1992 (35).

Clearly, α-CTx-ImI does not fit the consensus sequence above, and is much more divergent than the two previously characterized groups of α-conotoxins are from each other. The first disulfide loop contains four amino acids rather than three, and lacks the characteristic (N/H)PA sequence of all other α-conotoxins. Also, in contrast to the 5 amino acids in the second loop of other α-conotoxins, ImI has only 3. Despite the divergence in sequence and size of the disulfide loops, the linkage of the disulfides [2–8; 3–12] is exactly analogous to that in α-conotoxin GI [2–7; 3–13]. Further studies on disulfide isomers and folding of ImI will be reported elsewhere.[2] It will be of great interest to compare the three dimensional structure of the different groups of α-conotoxins. The solution structures of α-CTx-GI and α-CTx-SI have been determined by NMR spectroscopy (36).[3] NMR studies of α-CTx-ImI are currently in progress.

Phylogenetic Specificity for Muscle nAChRs—In view of the structural divergence, it is not surprising that α-CTx-ImI has a unique functional profile. Based on their activity in phylogenetically distinct organisms, the two previously characterized groups of α-conotoxins have distinctive specificities. The first group (typified by α-CTx-GI and α-CTx-MI) are active in all vertebrates tested including mammals, while the second group (typified by α-CTx-SI and α-CTx-SII), are highly potent in teleosts, but have little activity in mammals.

We have used the cutaneus pectoris preparation from *R. pipiens* to demonstrate that α-CTx-ImI is a potent inhibitor of

[2] W. R. Gray, E. Mahe, J. M. McIntosh, B. M. Olivera, and J. E. Rivier, manuscript in preparation.
[3] D. J. Christensen, C. D. Poulter, R. A. Myers, and B. M. Olivera, manuscript in preparation.

the nAChR in frog neuromuscular junction. In contrast, however, α-CTx-ImI is two orders of magnitude less potent in blocking the synaptically mediated action potential in electrocytes from weakly electric fish and is inactive peripherally when injected intraperitoneally into mice. Thus, in accord with its very distinct amino acid sequence, the phylogenetic activity profile of α-CTx-ImI places this peptide into a novel third category.

Mammalian nAChR Specificity—Another unique feature of α-conotoxin ImI is that the peptide is active in mammalian central nervous system, but not at the neuromuscular junction. In this regard, it is instructive to compare and contrast the activities of three cholinergic polypeptide ligands, α-BTX, α-CTx-GI, and α-CTx-ImI. All three toxins block cholinergic transmission at the frog neuromuscular junction. In mammalian systems (*e.g.* rodent), however, the three ligands have different activity profiles at the doses tested. α-BTX causes seizures upon intracerebral-ventricular injection and paralysis upon intraperitoneal injection. α-CTx-GI induces no symptoms upon intracerebral-ventricular injection and causes paralysis upon intraperitoneal injection. α-CTx-ImI injected intracerebral-ventricularly causes seizures at doses comparable to α-BTX, but upon intraperitoneal injection shows no paralytic activity even at doses 100 × (2 nmol/g mouse) greater than the LD_{50} (18.75 pmol/g mouse) for α-BTX.

α-BTX has at least two different mammalian nAChR targets, the peripheral neuromuscular receptor, and a central nervous system nAChR subtype(s), which recent work suggests includes α_7-containing nAChR complexes (2). α-CTx-GI appears to selectively block the neuromuscular receptor (37, 38), and has no activity in the central nervous system. In contrast, α-CTx-ImI does not inhibit the mammalian neuromuscular nAChR, but is centrally active. Whether the neuronal receptor subtypes affected by α-CTx-ImI correspond precisely to the central nervous system targets of α-BTX remains to be investigated. Although α-CTx-ImI blocks the nAChR at frog neuromuscular junction, it is conceivable that the central nervous system target in mammals is not a neuronal nAChR but some other receptor. However, preliminary evidence from *Xenopus* oocyte expression studies indicates that α-CTx-ImI targets a specific neuronal nAChR.[4]

Neuronal nAChRs appear important in human cognition (39) and nicotinic cholinergic transmission has been implicated in neuropsychiatric disorders including schizophrenia (40), movement disorders (41, 42) and Parkinson's disease (43, 44). Despite their importance, there are relatively few ligands to distinguish between the multiple neuronal nAChRs. *C. imperialis* represents only one of many worm-eating cone snails. A preliminary genetic analysis of two additional non-fish-hunting species has revealed the presence of several α-conotoxin-like sequences.[5] These sequences are as different from α-CTx-ImI as α-CTx-ImI is from the peptides produced by fish-hunting cones. Thus, because of the variability of α-conotoxins in non-piscivorous cone snail venoms, such venoms constitute a resource of great potential for the isolation of novel neuronal nAChR-targeted peptides.

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[4] D. S. Johnson and J. M. McIntosh, unpublished data.
[5] A. D. Santos, D. R. Hillyard, and B. M. Olivera, unpublished data.

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