Isolation and Characterization of a Novel Conus Peptide with Apparent Antinociceptive Activity*

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J. Michael McIntosh†‡§, Gloria O. Corpuz§, Richard T. Layer†, James E. Garrett†, John D. Wagstaff, Grzegorz Bulaj§, Alexandra Vyazovkina§, Doju Yoshikami§, Lourdes J. Cruz‡** and Baldomero M. Olivera§

From the Departments of Psychiatry and Biology, University of Utah, Salt Lake City, Utah 84112, ‡Cognetix, Inc., Salt Lake City, Utah, 84108, and **Marine Science Institute, University of the Philippines, Diliman, Quezon City 1101, Philippines

Cone snails are tropical marine mollusks that envenomate prey with a complex mixture of neuropharmacologically active compounds. We report the discovery and biochemical characterization of a structurally unique peptide isolated from the venom of Conus marmoreus. The new peptide, mr10a, potently increased withdrawal latency in a hot plate assay (a test of analgesia) at intrathecal doses that do not produce motor impairment as measured by rotarod test. The sequence of mr10a is NGVCCGYKLCCHO, where O is 4-trans-hydroxyproline. This sequence is highly divergent from all other known conotoxins. Analysis of a cDNA clone encoding the toxin, however, indicates that it is a member of the recently described T-superfamily. Total chemical synthesis of the three possible disulfide arrangements of mr10a was achieved, and elution studies indicate that the native form has a disulfide connectivity of Cys1-Cys4 and Cys2-Cys3. This disulfide linkage is unprecedented among conotoxins and defines a new family of Conus peptides.

Conus is a genus of predatory marine gastropods that envenomate their prey. Prey capture is accomplished through a sophisticated arsenal of peptides that target specific ion channel and receptor subtypes. Each Conus venom appears to contain a unique set of 50–200 peptides. The structure and function of only a small minority of these peptides have been determined to date. For peptides where function has been determined, three classes of targets have been elucidated: voltage-gated ion channels, ligand-gated ion channels, and G-protein-linked receptors.

Conus peptides that target voltage-gated ion channels include those that delay the inactivation of sodium channels as well as blockers specific for sodium channels, calcium channels, and potassium channels. Peptides that target ligand-gated ion channels include antagonists of N-methyl-D-aspartate and serotonin receptors as well as competitive and non-competitive nicotinic receptor antagonists. Peptides that act on G protein receptors include neuropeptide Y and galanin receptor antagonists. The unprecedented targeting selectivity of conotoxins derives from specific disulfide bond frameworks combined with hypervariable amino acids within disulfide loops (see Ref. 1 for review). Due to the high potency and exquisite selectivity of the conopeptides, several are in various stages of clinical development for treatment of human disorders (2).

In this report we describe the isolation of a new peptide from the venom of the marble cone, Conus marmoreus. C. marmoreus is found in the Indo-Pacific, from India to the Marshall Islands and Fiji. It preys upon various gastropods including other cone snails (3). We previously reported the isolation and characterization of a peptide from this venom that potently inhibits voltage-gated sodium channels (4). In this report, we describe the isolation of a novel peptide that appears antinociceptive and likely represents a defining member of a new family of Conus peptides.

EXPERIMENTAL PROCEDURES

Materials and HPLC1 Conditions

The venom of C. marmoreus was obtained from snails collected in the Philippines. The venom was lyophilized and stored at −70 °C until use. Crude venom was extracted using previously described methods (5). Reverse phase HPLC purification was accomplished with an analytical (4.6 mm internal diameter × 25 cm) Vydac C18 column. Column pore size was 300 Å. Additional conditions are described in Fig. 1.

Reduction, Alkylation, and Peptide Sequencing

The peptide was reduced, and cysteines were carboxymethylated as described previously (6). The alkylated peptide was purified with a Vydac C18 analytical column using a linear gradient of 0.1% trifluoroacetic acid and 0.092% trifluoroacetic acid in 60% acetonitrile. Alkylated peptide was sequenced by Edman degradation at the Peptide Core Facility at the University of Utah.

Mass Spectrometry

Electrospray ionization mass spectra were measured using a Micromass QII Triple Quadrupole Mass Spectrometer with Micromass MassLynx operating system. The samples (~100 pmol) were resuspended in 0.1 ml of 50% acetonitrile with 0.05% trifluoroacetic acid and automatically infused with a flow rate of 0.05 ml/min in the same solvent system. The instrument was scanned over the m/z range 50–2,000 with a capillary voltage of 2.95 kV and a cone voltage of 64 V. The resulting data were analyzed using MassLynx software.

Chemical Synthesis

Peptides were synthesized, 0.45 mmol/l, on a RINK amide resin (Fmoc-Cys(Trityl)-Wang, Novabiochem (04-12-2050)) using Fmoc (N-(9-
fluorenyl/methoxy carbonyl) chemistry and standard side chain protection except on cysteine residues. Cys residues were protected in pairs with either S-trityl or S-acetamidomethyl groups. Amino acid derivatives were from Advanced Chemtech (Louisville, KY). All three possible disulfide forms of the peptide were synthesized. The peptides were removed from the resin and precipitated, and a two-step oxidation protocol was used to selectively fold the peptides as described previously (7).

**mr10a Precursor cDNA Cloning**

The sequence of the mr10a peptide was used to design degenerate oligonucleotide primers for use in 5' and 3' RACE amplification of the mr10a precursor cDNA. A 3' RACE forward primer (mr10a-F, CAG-GATCC AA/TCC GGT CTC/TGG TCC/TGG/CTT) was based on the peptide sequence GYKLCHP. A 5' RACE reverse primer (mr10a-R, CTGGATCG AAG/AGC GCA/GAC/AGA/T CTT/CTT/GTA/TA IC) was based on the peptide sequence GYKLCCHP. *C. marmoreus* venom duct RNA was prepared, and cDNA appended with 3' and 5' adapter sequences was synthesized by standard methods (8). To facilitate cloning of the RACE amplification products, the mr10a primers incorporated BamHI sites, and the 3' and 5' adapter primers contained XhoI sites. RACE amplifications were performed using a “touchdown” cycling protocol consisting of an initial denaturation of 95 °C for 30 s followed by 30 cycles of 95 °C for 10 s, 65 °C for 15 s, decreasing 0.5 °C each cycle, 72 °C for 10 s, then 15 cycles of 95 °C for 10 s, 50 °C for 10 s, and 72 °C for 10 s. Polymerase chain reaction amplifications were performed using Toy polymerase (PE Applied Biosystems, Foster City, CA), and reaction conditions were optimized for expression of glyceraldehyde 3-phosphate dehydrogenase by electrophoresis of 30% of the gel (Qiagen II DNA purification resin; Qiagen, Inc., Santa Clarita, CA), digested with BamHI and XhoI, and cloned into the plasmid vector pBluescript II SK+ (Stratagene, La Jolla, CA). Plasmids containing inserts of the appropriate size were selected and sequenced on an ABI Prism 373 Fluorescent DNA Sequencer.

**Experimental Animals**

Adult male CF-1 mice (25–35 g) were used for all experiments. Mice were housed five per cage, maintained on a 12-h light/dark cycle, and allowed free access to food and water.

**Hot Plate Test**

Analgesic activity was assessed by placing mice in a plexiglass cylinder (10.2-cm-diameter × 30.5 cm high) on a hot plate (Miral model HP72935, Barnstead/Thermolyne, Dubuque, IA) maintained at 55 °C. Thirty minutes before the hot plate test, animals were treated with a dose of 30 mg/kg of 3-allyl-5,6,7,8-tetrahydro-2(1H)-quinolinone (9) in a volume of 5 μl. The time from being placed on the plate until each mouse either licked its hind paws or jumped was recorded with a stopwatch by a trained observer unaware of the treatments. An arbitrary cut-off time of 60 s was adopted to minimize tissue injury. Hot plate test data were analyzed by analysis of variance followed by Dunnett’s test for multiple comparison, with p < 0.05 considered significant.

**Electrophysiological Assays**

*Rana pipiens*, 2.5 to 3 inches long, were used. The dissected preparation consisted of the 9th and 10th spinal nerves and their continuation down the sciatic nerve to the posterior crural nerve, freed from the skin that it innervates (see e.g. Ref. 10). Each spinal nerve was severed from the spine several millimeters proximal to its sympathetic ramus such that it could be electrically stimulated without activating sympathetic axons (see e.g. Ref. 11).

For extracellular stimulation and recording, the preparation was placed in a chamber fabricated from the silicone elastomer Sylgard (Dow Corning). The chamber was essentially a series of wells, each separated from its closest neighbor by about 1 mm. The proximal ends of the 9th and 10th nerves were placed in separate wells; the sciatic nerve was separated from its closest neighbor by about 1 mm. The proximal ends of the 9th and 10th nerves were placed in separate wells; the posterior crural nerve, freed from the skin that it innervates (see Ref. 10). Each spinal nerve was severed from the spine several millimeters proximal to its sympathetic ramus such that it could be electrically stimulated without activating sympathetic axons (see e.g. Ref. 11).
were incubated with 5.0 nM [3H]GABA (70–90 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 0–4 °C for 60 min. GABA (1.0 μM) was used to define nonspecific binding (24, 25).

**Glutamate, N-Methyl-t-aspartate Agonist Site Binding Assay—**Rat forebrain membranes were incubated with 2.0 nM [3H]NMDA (39,565 ± 1,500 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 0–4 °C for 60 min. N-Methyl-t-aspartate (1.0 μM) was used to define nonspecific binding (26, 27).

**Glycine, Striyncholine-sensitive Binding Assay—**Rat spinal cord membranes were incubated with 16.0 nM [3H]strychnine (15–40 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 25 °C for 90 min. Striyncholine nitrate (1.0 μM) was used to define nonspecific binding (28, 29).

**Histamine H1 Binding Assay—**Bovine cerebellar membranes were incubated with 2.0 nM [3H]pyrilamine (15–25 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.5) at 25 °C for 60 min. Triprolidine (10 μM) was used to define nonspecific binding (30–32).

**Muscarnic Central Binding Assay—**Rat cortical membranes were incubated with 0.15 nM [3H]quinuclidinylbenzilate (30–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 0–4 °C for 60 min. Atropine (0.1 μM) was used to define nonspecific binding (33–35).

**Neurotensin Binding Assay—**Rat forebrain membranes were incubated with 2.0 nM [3H]neurotensin (70–120 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 300 mM NaCl at 0–4 °C for 60 min. Neurotensin (1.0 μM) was used to define nonspecific binding (36, 37).

**Opiate 1 Binding Assay—**Rat forebrain membranes were incubated with 1.0 nM [3H]deltorphin II (30–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 25 °C for 90 min. [D-Pen2, D-Pen5]-enkephalin (1.0 μM) was used to define nonspecific binding (38, 39).

**Opiate 1 Binding Assay—**Rat forebrain membranes were incubated with 0.7 nM [3H]Tyr-D-Ala-Gly-ol (DAMGO) (30–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 25 °C for 90 min. Naloxone (0.1 μM) was used to define nonspecific binding (40–42).

**Opiate 2 Binding Assay—**Rat forebrain membranes were incubated with 1.0 nM [3H]Tyr-n-Ala-Gly-N-methyl-Phe-Gly-ol (DAGO) (30–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 5 mM KCl at 0–4 °C for 4 h. Desipramine (1.0 μM) was used to define nonspecific binding (43, 44).

**Norepinephrine Transporter Binding Assay—**Rat forebrain membranes were incubated with 1.0 nM [3H]nisoxetine (60–85 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 5 mM KCl at 0–4 °C for 4 h. Desipramine (1.0 μM) was used to define nonspecific binding (45, 46).

**Sertotonin Transporter Binding Assay—**Rat forebrain membranes were incubated with 0.7 nM [3H]citalopram (70–87 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl and 5 mM KCl at 25 °C for 60 min. Imipramine (10 μM) was used to define nonspecific binding (50, 51).

**Dopamine Transporter Binding Assay—**Guinea pig striatal membranes were incubated with 12.0 μM [3H]WIN35,428 (60–87 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl at 0–4 °C for 2 h. GBR-12909 (0.1 μM) was used to define nonspecific binding (52, 53).

**RESULTS**

**Purification and Sequencing of mr10a—**Extracted crude venom from *C. marmoreus* was initially size-fractionated using a Sephadex G-25 (dry bed diameter 20–80 μm) packed with Sephadex G-25 (dry bed diameter 20–80 μm). Elution buffer was 1.1% acetic acid at 4 °C. Flow rate was ~18.3 ml/h. B, fractions from the indicated area in panel A (bracket) were combined, lyophilized, and resuspended in 0.1% trifluoroacetic acid and purified on a Vydac C-18 column (see "Experimental Procedures") using a linear 1% buffer B/min gradient where buffer A is 0.1% trifluoroacetic acid and buffer B is 0.092% trifluoroacetic acid, 60% acetonitrile. The gradient began at 10% buffer B. C, the material indicated in panel B (arrow) was lyophilized and resuspended in 0.05% heptfluorobutyric acid. It was then purified using a Vydac C-18 column using a linear 1% buffer B/min gradient where buffer A is 0.05% HFBA and buffer B is 0.05% HFBA, 60% acetonitrile. The gradient began at 30% buffer B. D, the material purified in panel C (arrow) was lyophilized and dissolved in 10 mM NaH2PO4, 50% CH3CN (pH 2.5) (buffer A). The material was then purified using a Vydac protein-SCX (strong cation exchange) column (0.75 × 5 cm) using a linear 1% B/min gradient where buffer B is the same as buffer A but with the addition of 250 mM NaCl. The flow rate was 1 ml/min in panels B, C, and D. Absorbance was monitored at 233, 214, 214, and 220 nm in panels A, B, C, and D, respectively.

**Peptide Synthesis—**The sequence of the peptide was independently confirmed by preparation of synthetic peptide as described under "Experimental Procedures." The mr10a peptide has four Cys residues and, therefore, three possible disulfide linkages. All three disulfide bond linkages were synthesized to unequivocally identify the native configuration. Peptides were initially synthesized in linear form using pairwise protection of Cys residues (see "Experimental Procedures"). Acid cleavage from resin removed trityl-protecting using strong cation exchange chromatography. The fraction was then desalted using reverse phase HPLC. Final purified product is shown in Fig. 2, panel B.

The disulfide bonds of the purified peptide were reduced, and Cys residues were carboxymethylated. The alkylated peptide was then chemically sequenced and yielded NGVC-CGYKLCHOC, where O is 4-(trans)-hydroxyproline. Mass spectrometry of the peptide verified the sequence and indicated that Cys residues are present as disulfides and the C terminus is the free carboxyl (monoisotopic MH+ (Da): calculated, 1408.5; observed, 1408.5).

Peptide Synthesis—The sequence of the peptide was independently confirmed by preparation of synthetic peptide as described under "Experimental Procedures." The mr10a peptide has four Cys residues and, therefore, three possible disulfide linkages. All three disulfide bond linkages were synthesized to unequivocally identify the native configuration. Peptides were initially synthesized in linear form using pairwise protection of Cys residues (see "Experimental Procedures").
groups, and ferricyanide oxidation was used to close the first disulfide bridge. Iodine oxidation was subsequently used to remove S-acetamidomethyl protection groups and close the second bridge. Using this method, each possible disulfide arrangement was synthesized, i.e., Cys1-Cys2, Cys3-Cys4; Cys1-Cys3, Cys2-Cys4, and Cys1-Cys4, Cys2-Cys3. Final purified yields of each peptide were 12.5, 5.2, and 12.4%, respectively. Synthesis of each isomer was confirmed with mass spectrometry (calculated monoisotopic MH$^+$, 1408.5; observed, 1408.6, 1408.7, and 1408.6, respectively). The three forms of the peptide were distinguishable using reverse phase HPLC based on elution time. In addition, they were distinguishable by peak width, with the (Cys1-Cys4, Cys2-Cys3) form having the narrowest peak width (Fig. 2). Both the elution time and peak shape of the (Cys1-Cys4, Cys2-Cys3) disulfide form match that of the native peptide. Additionally, co-injection of each synthetic form indicates that native mr10a co-elutes only with synthetic peptide of the (Cys1-Cys4, Cys2-Cys3) configuration, providing unambiguous evidence for this disulfide linkage being native (Fig. 2).

**mr10a Precursor Structure**—The mr10a peptide sequence was used to design degenerate polymerase chain reaction primers for 5’ and 3’ RACE (rapid amplification of cDNA ends) amplification of the complete mr10a precursor cDNA. The polymerase chain reaction primers were designed to yield overlapping 3’ and 5’ RACE products, allowing the complete cDNA sequence to be assembled from the two sequences. Amplification of *C. marmoreus* cDNA gave specific products of 610 base pairs in the 3’ RACE and 300 base pairs in the 5’ RACE reactions. These polymerase chain reaction products were cloned, and multiple clones of both the 5’ and 3’ RACE products were isolated and sequenced. For both the 5’ and 3’ RACE products, the multiple clones all represented the same sequence, and the appropriate segments of the mr10a peptide sequence were represented by the cloned sequence. The 5’ and 3’ RACE product sequences were assembled to give the complete mr10a prepropeptide precursor cDNA sequence (Fig. 3).

The first ATG start codon encountered from the 5’ end of the cDNA initiates an open reading frame of 61 amino acids, encoding a protein with a structure typical of a conotoxin prepropeptide. The first 24 N-terminal amino acids compose a hydrophobic signal sequence region. The mature mr10a peptide sequence is located at the C-terminal end of the precursor sequence, immediately preceded by a basic amino acid (Arg) signaling proteolytic peptide processing. The stop codon is immediately downstream of the last cysteine residue of the mr10a peptide. A 3’-untranslated region of ~500 base pairs is terminated by a typical poly(A)$^+$ addition signal (AATAAA) and a poly(A) tail.

The mr10a precursor exhibits significant sequence homology to the previously identified family of conotoxin genes, the T-superfamily, although the mature mr10a peptide is distinct from any of the previously isolated T-superfamily conotoxins (Fig. 4). Previously isolated T-superfamily conotoxins all share the cysteine framework CC–CC (7). The cysteine framework of the mr10a conotoxin is similar to that of the α-conotoxins, a large family of nicotinic receptor antagonists, yet the sequence alignment of the prepeptides clearly indicates that mr10a...
and \( \alpha \)-conotoxins are derived from completely unrelated precursors (Fig. 4). The occurrence of the mr10a conotoxin within the T-superfamily provides a demonstration of the ability of \( \textit{Conus} \) species to evolve novel toxin peptide frameworks within the same conotoxin superfamily.

**Biological Activity**—The biological activity of many \( \textit{Conus} \) peptides can be grouped into three major categories: those that produce excitotoxic shock, those that produce paralysis, and those that inhibit sensory circuits. Intrapерitoneal injection of 30 nmol of mr10a into three mice produced neither neuromuscular excitation nor muscle paralysis. We next tested the peptide for analgesic activity using mice in a hot plate assay. Native peptide (2 nmol) injected intrathecally into three mice produced a latency to first hind paw lick (a nociceptive response) of 39.5 ± 13.5 s, suggestive of potent analgesic activity. Due to a limited quantity of native material, a complete dose-response study could not be performed, and all further tests were performed with synthetic peptide.

Intrathecal administration of synthetic mr10a produced a dose-dependent (0.1-10 nmol) increase in the latency to first hind paw lick (a nociceptive response) of 39.5 ± 13.5 s, suggestive of potent analgesic activity. Due to a limited quantity of native material, a complete dose-response study could not be performed, and all further tests were performed with synthetic peptide.

Motor impairment was assessed in all injected mice by means of a rotarod test (see “Experimental Procedures”). Motor impairment was not seen in any mouse injected either intrathecally or intraperitoneally. Injection of high doses of mr10a (25 nmol) by intracerebral ventricular administration resulted in akinesia and seizures in two mice tested. Further testing by intracerebral ventricular administration was not performed.

**Molecular Target**—mr10a was tested in a number of electrophysiological and binding assays (see “Experimental Procedures”) in an effort to define its molecular target. To test effects on Na\(^+\) channels, we used a frog peripheral nerve preparation containing sensory fibers. Mr10a (100 \( \mu \)M) produced no appreciable effects on CAPs recorded from the posterior crural nerve, which innervates the skin (Fig. 6). Clearly evident in the traces are fast-conducting A\(\alpha\)- and A\(\beta\)-CAPs as well as much slower conducting C-CAPs. The A-CAPs and C-CAPs in the posterior crural nerve are tetrodotoxin-sensitive and tetrodotoxin-resistant, respectively,\(^2\) just like those seen in the frog sciatic nerve (cf. Refs. 55 and 56). It is notable that many, but not all, of the CAPs in the posterior crural nerve are sensitive to other conotoxins known to affect action potentials,\(^3\) for example \( \delta \)-conotoxin PVIIA, which targets Na\(^+\) channels (57), and \( \alpha\)-conotoxin SIVA, which targets K\(^+\) channels (58). Mr10a also was without effect on nicotinic acetylcholine receptors, the site of action of \( \alpha \)-conotoxins. Ten \( \mu \)M mr10a does not activate \( \alpha 4 \beta 2 \) nicotinic acetylcholine receptors expressed in Xenopus oocytes, nor does it compete for \( ^{3} \text{H} \)-cytisine binding to putative \( \alpha 4 \beta 2 \) receptors in rat brain membranes.

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\(^1\) S. D. Abbaszadeh and D. Yoshikami, unpublished information.

\(^2\) D. Yoshikami, unpublished information.

\(^3\) S. D. Abbaszadeh and D. Yoshikami, unpublished information.
TABLE I
Conus peptides and disulfide bridge patterns

| Toxin | Sequence                  | Disulfide Linkages |
|-------|---------------------------|--------------------|
| mr10a | NGVCQGYKLEHC             | CC-CCC             |
| T-superfamily toxins |                           |                    |
| ts5a  | γCCyDGH6CCT*AAO           |                    |
| p5a   | GCCKQMRCCTL*              | CC-CC              |
| au5a  | FCPPFIRYCCW               |                    |
| au5b  | FCPPVIRYCCW               |                    |
| α-Conotoxins |                     |                    |
| Mf    | GRCCPHACGKNYSC*           |                    |
| MiI   | GCCSNNVCHLESNHLC*         | CC-CCC             |
| ImI   | GCDSDDPCAR2RC*            |                    |
| AstB  | GCCSYPCCFAATNPDC*         |                    |

DISCUSSION

We describe the characterization of a novel peptide with apparent potent antinociceptive activity isolated from the venom of the mollusk-hunting species, *C. marmoreus*. Like many Conus peptides, mr10a is rich in disulfides, with 4 of 13 residues being Cys residues. Two other groups of Conus peptides were previously shown to have four Cys residues, the α-conotoxins (59) and T-superfamily conotoxins (7). All α-conotoxins and T-superfamily conotoxins characterized to date (1, 7, 60) have Cys1-Cys3, Cys2-Cys4 connectivity. In contrast, mr10a has Cys1-Cys4, Cys2-Cys3 connectivity, a pattern unprecedented among Conus peptides. In addition to the novel disulfide bond connectivity, mr10a bears little if any sequence similarity to the α-conotoxins or other T-superfamily peptides, and clearly represents a new class of Conus peptide (Table I).

Conus peptides are initially translated from mRNA as prepropeptide precursors that are subsequently processed into the small mature neuroactive toxins. Conopeptides can be grouped into superfamilies based on the signal sequences of the precursors and on the disulfide framework of the mature toxin. Thus, in the O-superfamily for example there are ω-conotoxins (Ca2+ channel antagonists), μ-Conotoxins (Na+ channel blockers), δ-conotoxins (peptides that delay inactivation of Na+ channels), and κ-conotoxins (K+ channel blockers). Peptides in these four families share a highly conserved signal sequence as well as the same disulfide framework. Thus, the polypeptides belonging to the same superfamily can be processed to mature conotoxins that are biochemically and pharmacologically diverse.

The mr10a peptide described in this report provides a new twist to this paradigm. Analysis of a cDNA clone of mr10a clearly indicated that this peptide is a member of the T-superfamily. In members of the O-superfamily, although there is hypermutation of toxin sequences, the disulfide connectivity is conserved. In contrast, the previously identified T-superfamily conotoxins versus mr10a have both a divergent arrangement of Cys residues and, most surprisingly, a different disulfide bond linkage. This is the first known example of such a divergent disulfide connectivity within members of a Conus peptide superfamily. Thus, the mr10a peptide defines a distinct branch of the T-conopeptide superfamily clearly different from T-superfamily peptides previously characterized. It seems likely that more than one pharmacological family of Conus peptides will compose each branch.

Although the purpose for which *C. marmoreus* employs mr10a is unknown, it is possible that this is an example of a peptide that inhibits specific neuronal circuits in prey. We demonstrate in this report that mr10a produces inhibition of withdrawal response when tested in a mouse hot plate assay at intrathecal doses that do not produce gross motor impairment or impair performance on the rotarod test. We have postulated elsewhere that a "nirvana cabal" of peptides is used to inhibit sensory circuitry of the fish prey of piscivorous Conus species that use a net strategy (61); the discovery of the mr10a peptide raises the possibility that the mollusk-hunting *C. marmoreus* uses a similar nirvana cabal strategy.

Due to the potency and selectivity of Conus peptides, several are now in various stages of clinical trials. Two Conus peptides are being developed for the treatment of pain. The most advanced is ω-conotoxin MVIIA (ziconotide), an N-type calcium channel blocker (62). ω-Conotoxin MVIIA, isolated from *Conus magus*, is approximately 1000 times more potent than morphine, yet does not produce the tolerance or addictive properties of opiates. ω-Conotoxin MVIIA has completed Phase III clinical trials in humans and now awaits FDA approval as a new therapeutic agent. ω-Conotoxin MVIIA is introduced into human patients by means of an implantable, programmable pump with a catheter threaded into the intrathecal space. Preclinical testing is being carried out on another Conus peptide, contulakin-G, isolated from *Conus geographus* (63); contulakin-G is an agonist of neuropeptide Y receptors but, interestingly, appears significantly more potent than neuropeptide Y in inhibiting pain in vivo assays. Contulakin-G is being investigated for use in post-surgical pain. For a review of conotoxins and therapeutic applications, see Jones and Bulaj (2) and Adams et al. (65). The mechanism of action of mr10a is unknown, but results of the present study indicate that its mechanism is distinct from α-, ω-, δ-, or κ-conotoxins as well as contulakin-G. In addition, it fails to act at numerous other receptor sites associated with analgesia.

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