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

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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 NGVCCGYKLCHOC, 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 neurotensin and vasopressin receptor agonists. 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 HPLC[1] 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 C_{18} 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 C_{18} 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 Quattro II 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/g, on a RINK amide resin (Fmoc-Cys(Trityl)-Wang, Novabiochem (04-12-2050)) using Fmoc (*N*-(9-

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends; CAP, compound action potential; HFBA, heptafluorobutyric acid; GABA, γ-aminobutyric acid.

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fluorenyl)methoxycarboxyl) 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(T/C) GGI GT(C/T/G) TG(T/C) TG(T/C) GG) was based on the peptide sequence NGVCCG. A 5′ RACE reverse primer (mr10a-R, CTGGATCC GG (A/G)TG (A/G)CA (C/A/G)A(G/A) (T/C)TT (G/A)TA ICC) was based on the peptide sequence GYKLCHP. *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 *Bam*HI sites, and the 3′ and 5′ adapter primers contained *Xho*I 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 *Taq* polymerase (PE Applied Biosystems, Foster City, CA), and amplification reactions were analyzed by electrophoresis on 2% agarose gels. RACE amplification products were isolated from the gel (Qiaex II DNA purification resin; Qiagen, Inc., Santa Clarita, CA), digested with *Bam*HI and *Xho*I, 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 (Mirak model HP72935, Barnstead/Thermolyne, Dubuque, IA) maintained at 55 °C. Thirty minutes before the hot plate test, animals were treated with a dose of mr10a or vehicle (0.9% saline) by free-hand intrathecal injection (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. Statistical analysis was performed with GraphPad Prism software (San Diego, CA). Shortly after the hot plate test, mice were placed on a 3-cm-diameter rotarod turning at 6 rpm (model 7650, Ugo Basile, Comerio, Italy). Mice were considered impaired if they fell three times in 1 min.

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 portion lay in a third well, whereas the attached lateral crural nerve spanned two additional wells, each 4 mm in diameter. All wells were filled with frog Ringer's solution (111 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM Na-HEPES (pH 7.2)). All portions of the nerve that draped over the partitions between wells and otherwise exposed to air were covered with Vaseline. One of a pair of stainless steel wire stimulating electrodes was placed in the well on either side of the Vaseline gap covering a spinal nerve. One of a pair of stainless steel wire recording electrodes was placed in the well on either side of the Vaseline gap covering the lateral crural nerve. Thus for both stimulation and recording, one electrode was in the same well that containing a cut end of the nerve, whereas its counterpart was in the well containing the portion of the nerve just distal (stimulation) or proximal (recording) to the cut end. The stimulating electrodes led to a Grass SIU5 stimulus isolation unit connected to a Grass S-88 stimulator. The recording electrode in the most distal well, containing the cut end of the posterior crural nerve, led to the positive input of a Grass P-55 differential preamplifier, whereas its counterpart, led to the negative input. Recordings were made with a preamplifier gain of 1000, RC-filtered (1 Hz alternating current and 1 kHz low pass) and digitized at a sampling rate of 4 kHz with a National Instruments Lab-NB board in a Macintosh Quadra 840 computer. Homemade virtual instruments (National Instruments LabVIEW) were used for data acquisition and analysis.

Supramaximal stimuli (1 ms, ~30 V) were applied to a spinal nerve once a minute while recording propagated compound action potentials (CAPs) reaching the other end of the preparation. Conopeptide was introduced into the well just proximal to that containing the distal cut end of the posterior crural nerve.

Oocyte Assays

Xenopus oocytes were injected with cRNA encoding human nAChR subunits as described previously (12, 13). Oocytes injected with $\alpha_4\beta_2$ subunits were placed in a recording chamber (~30 μl), voltage-clamped, and assessed for expression of receptor using a 1-s pulse of 300 μM acetylcholine (see Lu *et al.* (14) for details). Peptide was then added to the bath to achieve a final concentration of 10 μM.

Receptor Binding Assays

ω-Conotoxin GVIA Binding—ω-Conotoxin GVIA was iodinated by previously described methods (15). The binding protocol was a modification of that described in Hillyard *et al.* (16). Crude brain membranes from Harlan Sprague-Dawley rats were prepared as described by Catterall (17), with modifications in buffer components as described in Cruz and Olivera (15). The binding of ^{125}I-labeled ω-conotoxin GVIA to rat brain membrane was measured in a 200-μl assay mix that contained approximately 10 μg of membrane protein, 100,000 cpm of carrier-free ω-[^{125}I]conotoxin GVIA (approximately 150 pm), 0.2 mg/ml lysozyme, 0.32 M sucrose, 100 mM NaCl, and 5 mM HEPES/Tris (pH 7.4). Nonspecific binding was measured by preincubating the membrane preparation with 1 μM unlabeled ω-conotoxin GVIA for 30 min on ice before the addition of ω-[^{125}I]conotoxin GVIA. mr10a was assessed for activity by preincubating the toxin for 30 min on ice. The final assay mix was then incubated at room temperature for 30 min and diluted with 1.5 ml of wash buffer containing 160 mM NaCl, 1.5 mM $CaCl_2$, 2 mg/ml bovine serum albumin, 5 mM HEPES/Tris (pH 7.4). Membranes were collected on glass fiber filters (Whatman GF/C soaked in 0.1% polyethyleneamine) using a Brandell apparatus model M-24 and washed with 1.5 ml of wash buffer four times. The amount of radioactivity in the filters was then measured.

$\alpha_4\beta_2$ Nicotinic Acetylcholine Receptor Binding—The procedure of Pabreza *et al.* (18) was used. [^{3}H]Cytisine (15–40 Ci/mmol) was from PerkinElmer Life Sciences. Rat forebrain membrane was incubated for 75 min at 4 °C in 50 mM Tris-HCl (pH 7.0 at room temperature) containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$. Nonspecific binding was defined with 10 μM nicotine.

The remaining assays were carried out under contract with Novascreen (Hanover, Maryland). The essentials of the procedures used are summarized below.

Adrenergic α1 Binding Assay—Rat forebrain membranes were incubated with 0.3 nM [^{3}H]prazosin (70–87 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.7) at 25 °C for 60 min. Prazosin (1.0 μM) was used to define nonspecific binding (19, 20).

Adrenergic α2 Binding Assay—Rat cortical membranes were incubated with 1.0 nM [^{3}H]RX821002 (40–67 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 25 °C for 75 min. RX821002 (0.1 μM) was used to define nonspecific binding (20, 21).

Adrenergic β1 Binding Assay—Rat cortical membranes were incubated with 0.2 nM (−)[^{125}I]iodopindolol (2200 Ci/mmol) and 120 nM ICI-118,551 (to block adrenergic β2 receptors). Reactions were carried out in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 2.5 mM $MgCl_2$, and 0.5 mM ascorbate at 37 °C for 60 min. Alprenolol HCl (10 μM) was used to define nonspecific binding (22, 23).

$GABA_A$ Agonist Site Binding Assay—Bovine cerebellar membranes

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were incubated with 5.0 nM [^{3}H]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-*D-aspartate Agonist Site Binding Assay—Rat forebrain membranes were incubated with 2.0 nM [^{3}H[CGP 39653 (25–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 0–4 °C for 60 min. *N*-Methyl-D-aspartate (1.0 mM) was used to define nonspecific binding (26, 27).

Glycine, Strychnine-sensitive Binding Assay—Rat spinal cord membranes were incubated with 16.0 nM [^{3}H]strychnine (15–40 Ci/mmol). Reactions were carried out in 50 mM Na_2HPO_4 and 50 mM KH_2PO_4 (pH 7.1) containing 200 mM NaCl at 0–4 °C for 60 min. Strychnine nitrate (1.0 mM) was used to define nonspecific binding (28, 29).

Histamine H_1 Binding Assay—Bovine cerebellar membranes were incubated with 2.0 nM [^{3}H]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).

Muscarinic Central Binding Assay—Rat cortical membranes were incubated with 0.15 nM [^{3}H]quinuclidinylbenzilate (30–60 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) at 25 °C for 75 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 [^{3}H]neurotensin (70–120 Ci/mmol). Reactions were carried out in 50 mM Tris-HCl (pH 7.4) containing 0.04% bacitracin, 0.1% bovine serum albumin, and 1 mM Na_2EDTA at 25 °C for 60 min. Neurotensin (1.0 μM) was used to define nonspecific binding (36, 37).

Opiate δ1 Binding Assay—Rat forebrain membranes were incubated with 1.0 nM [^{3}H]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-Pen^2, D-Pen^5]-enkephalin (1.0 μM) was used to define nonspecific binding (38, 39).

Opiate κ1 Binding Assay—Guinea pig cerebellar membranes were incubated with 0.75 nM [^{3}H]U-69593 (40–60 Ci/mmol). Reactions were carried out in 50 mM HEPES (pH 7.4) at 30 °C for 120 min. U-69593 (1.0 μM) was used to define nonspecific binding (40–42).

Opiate μ Binding Assay—Rat forebrain membranes were incubated with 1.0 nM [^{3}H]Tyr-D-Ala-Gly-*N*-methyl-Phe-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 (1.0 μM) was used to define nonspecific binding (43, 44).

Norepinephrine Transporter Binding Assay—Rat forebrain membranes were incubated with 1.0 nM [^{3}H]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).

Serotonin 5HT$_3$ Binding Assay—N1E-115 cells were incubated with 0.35 nM [^{3}H]GR65630 (30–70 Ci/mmol). Reactions were carried out in 20 mM HEPES (pH 7.4) containing 150 mM NaCl at 25 °C for 60 min. MDL-72222 (1.0 μM) was used to define nonspecific binding (47–49).

Serotonin Transporter Binding Assay—Rat forebrain membranes were incubated with 0.7 nM [^{3}H]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 [^{3}H]WIN,35,428 (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 column (see Fig. 1). Column fractions eluting in a range corresponding to small peptides were further purified using reverse phase HPLC utilizing conditions described under "Methods and Methods" and in Fig. 1. Throughout subsequent purification, HPLC fractions were assayed by means of intracerebral ventricular injection into mice (54). Intracerebral ventricular injection of fractions containing mr10a produced hypokinetic "sluggish" symptoms. The venom fraction was initially purified using a trifluoroacetic/acetonitrile gradient system. To obtain further purification, the new fractions were lyophilized and resuspended in 0.05% heptafluorobutyric acid (HFBA) and run on a reverse phase C_{18} column using a 0.05% HFBA, acetonitrile gradient. Further purification was obtained

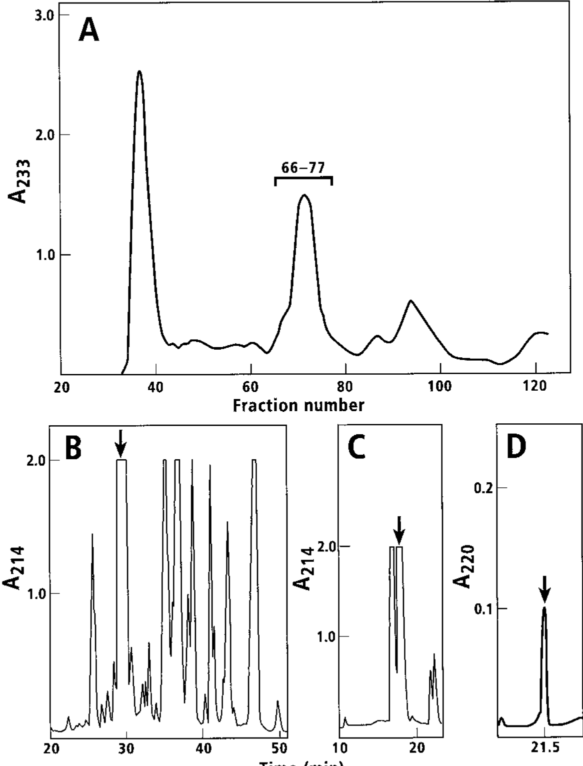

FIG. 1. **Purification of mr10a.** *A*, crude venom extract was size-fractionated using a column (2.54-cm diameter × 188-cm length) packed with Sephadex G-25 (dry bead diameter 20–80 μm). Elution buffer was 1.1% acetic acid at 4 °C. Flow rate was ~18.3 ml/h. *B*, fractions from the indicated area in *panel A* (*bracket*) were combined, lyophilized, and resuspended in 0.1% trifluoroacetic acid and purified on a Vydak 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% heptafluorobutyric acid. It was then purified on 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 indicated in *panel C* (*arrow*) was lyophilized and dissolved in 10 mM NaH_2PO_4, 50% CH_3CN (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.

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 NGVCCGYKLCHOC, 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"). Acid cleavage from resin removed trityl-protecting

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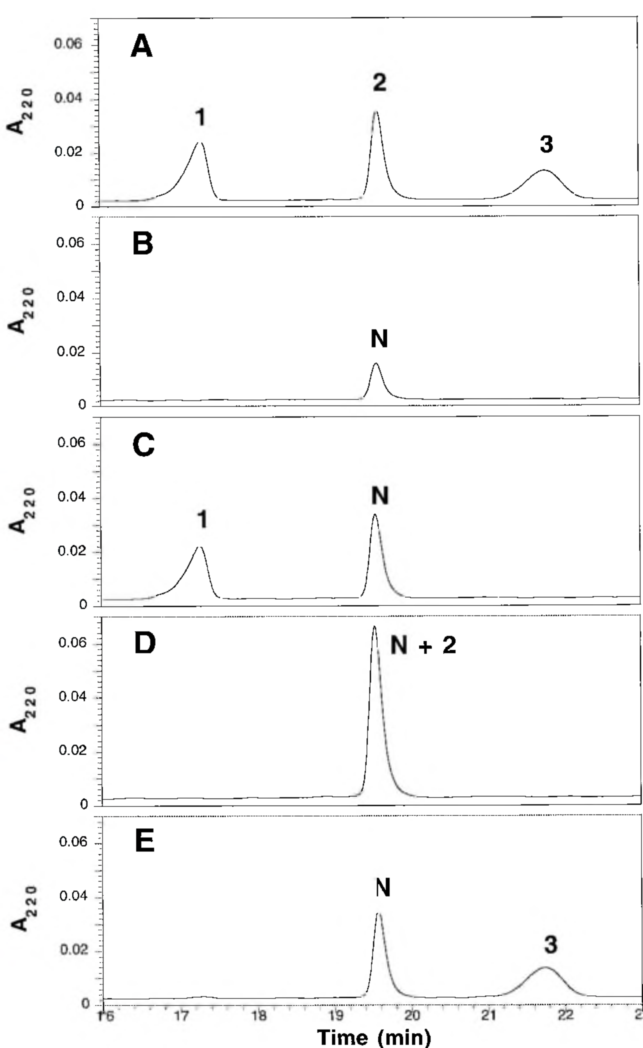

FIG. 2. **Elution studies of mr10a.** *A*, ~200 pmol each of the three possible disulfide forms of synthetic mr10a were chromatographed using reverse phase HPLC. The disulfide connectivities are: *1*, Cys1-Cys3, Cys2-Cys4; *2*, Cys1-Cys4, Cys2-Cys3; *3*, Cys1-Cys2, Cys3-Cys4. *B*, 100 pmol of native mr10a. *C*, ~200 pmol each of synthetic Cys1-Cys3, Cys2-Cys4 and native mr10a. *D*, ~200 pmol each of synthetic Cys1-Cys4, Cys2-Cys3 and native mr10a. Note that the two materials co-elute. *E*, ~200 pmol each of synthetic Cys1-Cys2, Cys3-Cys4 and native mr10a. In all HPLC runs, buffer A = 0.1% trifluoroacetic acid, and buffer B = 0.092% trifluoroacetic acid, 60% acetonitrile. The gradient began at 15% B and increased 1% B/min. The column was an analytical C-18 (see "Experimental Procedures"). The flow rate was 1 ml/min. Absorbance was monitored at 220 nm.

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

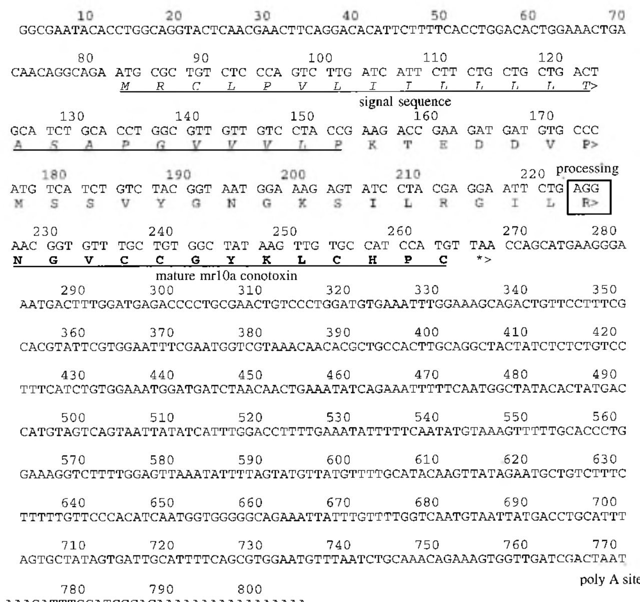

FIG. 3. **The Mr10.1 cDNA and encoded protein are shown.** The signal sequence and mature toxin regions are *underlined*. The putative proteolytic processing site (*R*) is indicated.

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 3′ and 5′ 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 a 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 prepropeptides clearly indicates that mr10a

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Mr10.1 Alignment

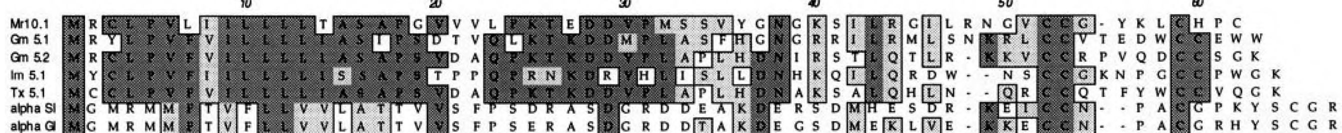

FIG. 4. **The mr10a prepropeptide sequence is shown aligned with representative members of the T-superfamily of conotoxins (Gm5.1, Gm5.2, Im5.1, Tx5.1), along with two examples of α-conotoxin precursors (α-SI, α-GI).** The mr10a precursor has substantial sequence homology with T-superfamily precursor sequences, although the mature peptide has a distinct cysteine pattern, and there is little amino acid similarity. Although the mr10a conotoxin has a cysteine pattern similar to that of α-conotoxins, the mr10a precursor is unrelated to the α-conotoxin prepropeptide, and the mature toxins have no amino acid similarities other than the cysteine residues.

and α-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 *Conus* species to evolve novel toxin peptide frameworks within the same conotoxin superfamily.

Biological Activity—The biological activity of many *Conus* peptides can be grouped into three major categories: those that produce excitotoxic shock, those that produce paralysis, and those that inhibit sensory circuits. Intraperitoneal 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 ($F(3,15) = 7.5$, $p < 0.01$) in the hot plate test. At doses of 1 and 10 nmol, mr10a significantly increased the latency to lick the hind paw in this test (Fig. 5).

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 μM) 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 Aα- and Aβ-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 δ-conotoxin PVIA, which targets Na^+ channels (57), and κA-conotoxin SIVA, which targets K^+ channels (58). mr10a also was without effect on nicotinic acetylcholine receptors, the site of action of α-conotoxins. Ten μM mr10a does not activate α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes, nor does it compete for [^{3}H]cytisine binding to putative α4β2 receptors in rat brain membranes.

[2] D. Yoshikami, unpublished information.

[3] S. D. Abbaszadeh and D. Yoshikami, unpublished information.

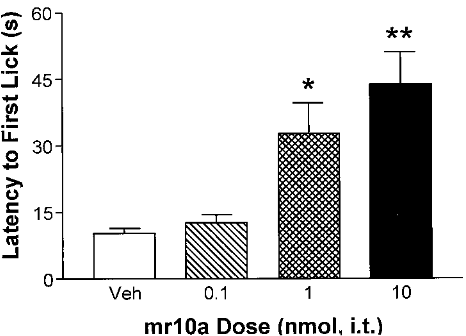

FIG. 5. **mr10a produces dose-dependent analgesia in the hot plate test.** Male CF-1 mice were injected intrathecally (*i.t.*) with either vehicle (0.9% saline) or mr10a. Thirty minutes later mice were placed on the hot plate and latency to first hind paw lick was recorded. Each *bar* shows the mean ± S.E. of 3–6 mice per group; *, $p < 0.05$, and **, $p < 0.01$ *versus* vehicle-treated control group.

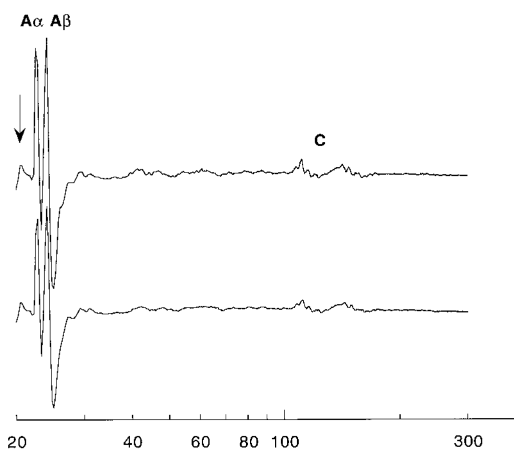

FIG. 6. **mr10a does not affect CAPs in the posterior crural nerve innervating skin.** Control response (*lower trace*) and that following 17-min exposure to 100 μM mr10a (*upper trace*) are very similar, and small differences in amplitudes of the CAPs in these two traces are likely due to fluid evaporation at the Vaseline gap across which the CAPs were recorded. Resolution of fast Aα- and Aβ-CAPs (first two major peaks) in the same sweep that displays C-CAPs (multi-peaked waveform at $t > 100$ ms) is achieved by use of a logarithmic time base (*cf.* Ref. 66). Time-axis units are ms, and traces are displaced from each other by 1.5 mV. Stimulus (duration 1 ms) was applied at $t = 20$ ms, resulting in the artifact seen as the initial small deflection (see *arrow*) preceding the large twin A-CAP peaks.

mr10 also failed to compete for binding to many other receptor targets associated with analgesia including *N*-type calcium channels (the binding site of ω-conotoxins GVIA and MVIIA), neurotensin receptors (the molecular target of contulakin-G), α1, α2, or β1 adrenergic receptors, $GABA_A$, glycine or histamine 1, central muscarine, δ1-, κ1-, or μ-opiate receptors, or $5HT_3$ receptors and did not compete for binding to norepinephrine, serotonin, or dopamine transporters.

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TABLE I
Conus peptides and disulfide bridge patterns
γ,γ-carboxyglutamate; W+, bromotryptophan; T§, O-glycosylated threonine; *, C-terminal amidation.

Toxin	Sequence	Disulfide Linkages
mr10a	NGVCCGYKLCHOC	CC----C--C (Cys1-Cys4, Cys2-Cys3)
T-superfamily toxins		
tx5a	γCCγDGW+CCT§AAO	
p5a	GCCPKQMRCCTL*	CC----CC (Cys1-Cys3, Cys2-Cys4)
au5a	FCCPFIRYCCW	
au5b	FCCPVIRYCCW	
α-Conotoxins		
MI	GRCCHPACGKNYSC*	
MII	GCCSNPVCHLEHSNLC*	CC----C--C (Cys1-Cys3, Cys2-Cys4)
ImI	GCCSDPRCAWRC*	
AuIB	GCCSYPPCFATNPDC*	

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 (Ca^{2+} channel antagonists), μO-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 is an agonist of neurotensin receptors but, interestingly, appears significantly more potent than neurotensin in inhibiting pain in *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 κA-conotoxins as well as contulakin-G. In addition, it fails to act at numerous other receptor sites associated with analgesia.

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