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A New Family of Conotoxins That Blocks Voltage-gated Sodium Channels*

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***Conus* peptides, including ω-conotoxins and α-conotoxins (targeting calcium channels and nicotinic acetylcholine receptors, respectively) have been useful ligands in neuroscience. In this report, we describe a new family of sodium channel ligands, the μO-conotoxins. The two peptides characterized, μO-conotoxins MrVIA and MrVIB from *Conus marmoreus* potently block the sodium conductance in *Aplysia* neurons. This is in marked contrast to standard sodium channel blockers that are relatively ineffective in this system. The sequences of the peptides are as follows.**

μO-conotoxin MrVIA: ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV

μO-conotoxin MrVIB: ACSKKWEYCIVPILGFVYCCPGLICGPFVCV

μO-conotoxin MrVIA was chemically synthesized and proved indistinguishable from the natural product. Surprisingly, the μO-conotoxins show no sequence similarity to the μ-conotoxins. However, ananalysis of cDNA clones encoding the μO-conotoxin MrVIB demonstrated striking sequence similarity to ω- and δ-conotoxin precursors. Together, the ω-, δ-, and μO-conotoxins define the O-superfamily of *Conus* peptides. The probable biological role and evolutionary affinities of these peptides are discussed.

Recent progress in molecular neuroscience has demonstrated that all signaling components in the nervous system are represented by multiple molecular forms ("subtypes"). The identification of different receptor and ion channel subtypes by molecular cloning proceeds at an ever-accelerating rate. In contrast, the structural and functional definition of each subtype has lagged far behind. A major approach to the functional biology of subtypes is to obtain highly subtype-specific ligands that can be used to discriminate between closely related molecular forms.

In this respect the conotoxins, peptides found in the venoms of the predatory cone snails, constitute a unique resource. For incidental biological reasons, it appears that these peptides are under strong selection to be unusually subtype-specific. Several *Conus* peptide families which our laboratories have developed are already being used to discriminate between closely related subtypes. For example, in the calcium channel field, the ω-conotoxins have provided the means for discriminating between various channel subtypes that play a role in neurotransmitter release (see Ref. 1 for review).

Conus peptides that target voltage-sensitive sodium channels have also been described. The best characterized of these are the μ-conotoxins, which selectively target vertebrate skeletal muscle subtypes of voltage-sensitive sodium channels (2–4). A review of the present state of knowledge regarding the functional role of the various sodium channel subtypes (5) makes it clear that it is desirable to develop other highly specific ligands for this group of channel proteins.

In this paper, we describe the isolation, characterization, and synthesis of novel peptides, μO-conotoxins MrVIA and MrVIB, from the venom of the snail-hunting species *Conus marmoreus* (Fig. 1). Although these peptides produce a biological effect which is similar to that of the μ-conotoxins, they are unrelated structurally. Thus, the μO-conotoxins define a new family of peptides which block Na^+ currents of voltage-sensitive sodium channels. The discovery of this novel family provides a new opportunity to obtain a large armamentarium of subtype-specific sodium channel-targeted *Conus* peptides.

EXPERIMENTAL PROCEDURES

Peptide Purification

Crude venom from dissected ducts of *C. marmoreus* was collected in the Philippines, lyophilized, and stored at −70 °C until use. Lyophilized crude venom (500 mg) was extracted (6), placed in a Centriprep 30 microconcentrator, and centrifuged at 1500 × *g* for 8 h at 4 °C. Filtrate was purified on a C18 Vydac column (22 × 250 mm) using a gradient system. Buffer A = 0.1% trifluoroacetic acid and buffer B = 0.1% trifluoroacetic acid, 60% acetonitrile. The gradient was 0–15% buffer B/15 min, then 15–39% buffer B/72 min, then 39–65% buffer B/15 min, then 65–100% buffer B/5 min and held at 100% buffer B for 10 min. Flow rate was 10 ml/min. For subsequent purification steps Vydac C8 columns (10 × 250 mm, or 4.6 × 250 mm, 5 μm particle size) were used with buffer A as above and buffer B = 0.1% trifluoroacetic acid, 90% acetonitrile. The gradient was 5–55% buffer B/15 min, followed by 55–70% buffer B/45 min. Flow rate was 5 ml/min for the semipreparative column and 1 ml/min for the analytical column. Absorbance was monitored at 220 and 280 nm.

Sequence Analysis

Peptides from the final purification were reduced and pyridylethylated by previously described methods (7). The alkylated peptides were then purified by reversed phase high performance liquid chromatography. 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. Mass spectra were measured with a Jeol JMS-HX110 double focusing spectrometer fitted with a Cs^+ gun.

Chemical Synthesis

Synthesis was carried out by the strategy developed for ω-conotoxin MVIID (8). Briefly, standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)

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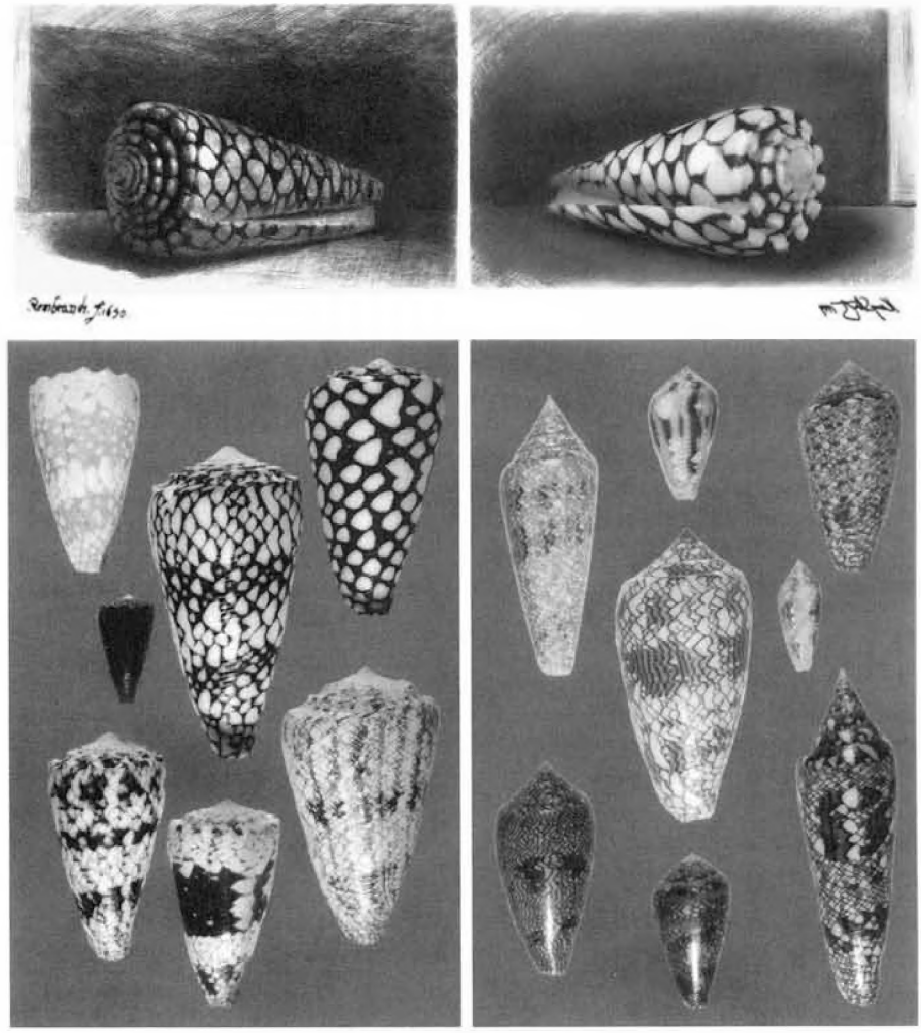

FIG. 1. **The marble cone *C. marmoreus* and other snail-hunting Conus species.** *Top left*, an etching of *C. marmoreus* by Rembrandt. Note that Rembrandt neglected to reverse the picture, so that when printed, the helical shell comes out sinistral, instead of dextral. He did, however, remember to render his signature as a mirror image so the correct signature impression is made. *Top right*, a photograph of *Conus marmoreus*. The photographer has signed as Rembrandt did. In a mirror, the photograph has the handedness of the etching and *vice versa*. *Lower panels* show members of two major clades of snail-hunting *Conus*. In the *C. marmoreus* clade, *left*, the following species and forms are included: *top row, left to right*, *Conus vidua* and *C. marmoreus* (both Philippines). *Middle row*, a dwarf, melanistic *C. marmoreus* specimen (for nigrescens) (Samoa) and *Conus bandanus* (Hawaii). *Bottom row*, *Conus nicobaricus* (Sulu Sea), *Conus nocturnus* (Palawan Island), and *Conus araneosus* (India). In the *lower right panel* is the *C. textile* clade of snail-hunting species. *Top row, left to right*, *Conus gloriamaris* (Philippines), *Conus retifer* (Okinawa), and *Conus natalis* (South Africa). *Middle row*, *C. textile* and *Conus legatus* (both Philippines). *Bottom row*, *Conus eutrios* (Mozambique), *Conus dalli* (Mexico), and *Conus bengalensis* (Bay of Bengal). Photographs by Kerry Matz.

chemistry was used, with single couplings using dicyclohexylcarbodiimide and hydroxybenzotriazole. All amino acid derivatives were purchased from Bachem (Torrance, CA) and were loaded into cartridges for use in an ABI model 430A peptide synthesizer. Side chain protection used was *t*-butoxycarbonyl (Lys), *t*-butyl (Glu, Tyr, Ser), and pentamethylchromansulfonyl (Arg). Cysteine was protected as *S*-trityl (residues 2, 19, 20, 30) and S-acetamidomethyl (residues 9 and 25). Cleavage from the resin and subsequent workup followed essentially the same procedures as for conotoxin GmVIA (9).

Molecular Cloning

A cDNA clone encoding μO-conotoxin MrVIB was identified from a size-fractionated cDNA library constructed from *C. marmoreus* venom duct mRNA as described previously (10). The clones were identified by colony hybridization screening the *C. marmoreus* cDNA library using an oligonucleotide probe specific for the signal sequence of *Conus* peptides belonging to the O-superfamily. The oligonucleotide probe with the sequence 5′ ATG AAA CTG ACG TGC ATG ATG 3′ was radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Hybridization was carried out using duplicate nylon filters (Hybond-N, Amersham Corp.) to which colonies of the *C. marmoreus* cDNA library were bound for 16 h at 42 °C using standard hybridization conditions (5 × Denhardt's solution, 6 × NET (100 mM NaCl, 1 mM EDTA, 10 mM Tris, (pH 8.0), 0.1% SDS, 100 μg/ml salmon sperm DNA). The filters were washed at 51 °C in 0.1 × SSC, 0.1% SDS, and positive colonies were identified by autoradiography. A second round of screening was carried out on 50 putative clones that were radiolabeled in the first round of colony hybridization. Clones which were positive in both screening rounds were sequenced. Plasmid DNA (5 μg) was prepared for sequencing by adding 0.1 volume of 2 M NaOH, 2 mM EDTA at 37 °C for 30 min (thereby denaturing the DNA), and the mixture was then neutralized with 0.1 volume of 3 M sodium acetate (pH 4.5). DNA was precipitated with 2 volumes of ethanol, pelleted, washed with 70% ethanol, dried, and redissolved in distilled H_2O (7 μl). Sequenase reaction buffer (2 μl) and the oligonucleotide indicated above (1 μl) were added, the mixture heated to 65 °C for 5 min and cooled to 30 °C to allow annealing of the oligonucleotide. One DNA strand from each clone was then sequenced using the Sequenase version 2.0 DNA sequencing kit and ^{35}S-dATP (Sequenase version 2.0 seventh edition protocol). The MrVIB sequence was found in four independent clones.

Procedures for Electrophysiology

Neuronal Culture—RB neurons from the abdominal ganglion of the sea hare *Aplysia oculifera* collected in the Gulf of Eilat or *Aplysia californica* shipped from the University of Miami (*Aplysia* Resource Facility) were isolated and grown in culture (11–13). The neurons were cultured at very low densities of three to five cells per culture dish, to prevent synaptic interactions between them. Experiments were performed on neurons 1–4 days in culture.

Electrophysiology—Analysis of the toxin action was carried out using both intracellular current clamp recording and stimulation as well as whole-cell patch clamp techniques (14). For the intracellular current clamp experiments a micro-electrode was inserted into the cell body. This electrode was used for both current injection and voltage recordings. An EPC-9 computerized amplifier (Heka Electronics, Lembracht, Germany) was used to record from neurons in the whole cell patch configuration. Patch pipettes had a typical resistance of 1–1.5 megaohms. The series resistance never exceeded 2.5 megaohms and was stable throughout the experiments. The series resistance was compensated by 70–90%. Experiments in which voltage-current relationship was monitored were carried out only when the series resistance was less than 2.5 megaohms. Thus, the maximal voltage error in these experiments was smaller than 3 mV. Leak and capacitance currents were subtracted. To achieve adequate space clamp, the large axon of the neuron was trimmed off about 30 min prior to the experiment (11, 15). The experiments were carried out at room temperature ranging be-

tween 20–24 °C.

20–100 μl of toxin solution were applied to the experimental dish (0.25–0.75 ml) using a Gilson tip pipette placed a few millimeters away from the neuron under study. The indicated toxin concentrations correspond to the final concentration in the experimental dish.

Solutions—Control experiments were carried out in artificial sea water composed of: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES. The pH was adjusted to 7.6.

To minimize potassium currents we used an external potassium channel-blocking solution, in which KCl was substitute for by CsCl. In addition, the solution contained 50 mM tetraethylammonium chloride and 0.1 mM 3,4-diaminopyridine. The osmolarity of the solution was adjusted by reducing the NaCl concentration to 410 mM.

To monitor sodium current, a calcium-free potassium channel-blocking solution was used. In this solution, Ca^{2+} was substituted for by Mg^{2+}, and the solution was supplemented by 8 mM Co^{2+} to prevent any Ca^{2+} influx. For these experiments the patch pipette contained 440 mM CsCl, 40 mM CsOH, 20 mM NaCl, 6.8 mM $MgCl_2$, 10 mM EGTA, 100 mM HEPES, and 3 mM adenosine 5′-triphosphate (ATP). The pH was adjusted to 7.3.

To monitor calcium currents the external sodium ions were replaced by tetraethylammonium chloride. The patch pipette contained 440 mM CsCl, 40 mM CsOH, 2 mM CaCl, 6.8 mM $MgCl_2$, 100 mM HEPES, 10 mM Cs_4BAPTA[1] (Molecular Probes, Eugene, OR), and 5.6 mM glucose. In order to prevent run-down of calcium channels (16, 17), the internal solution was supplemented with 0.5 mM guanosine 5′-triphosphate (GTP) and "ATP-regenerating system," 10 mM ATP, 20 mM creatine phosphate (Sigma) and 50 units/ml of phosphocreatine kinase (Sigma). In those conditions, run-down of calcium current was less than 10% per hour. The pH was adjusted to 7.3.

To simultaneously monitor calcium and sodium currents, the experiments were carried out in potassium channel-blocking solution, and the patch pipette contained the same internal solution used for calcium current monitoring supplemented by 20 mM NaCl.

Potassium-containing external and internal solutions were used when outward currents were under study. Sodium currents were eliminated by replacing the sodium ions with Trizma 7.4 (Sigma). In these experiments the patch pipette contained 480 mM KCl, 20 mM NaCl, 6.8 mM $MgCl_2$, 10 mM EGTA, and 100 mM HEPES. The pH was adjusted to 7.3.

Here, and throughout the manuscript the "±" stands for standard error of the mean.

RESULTS

Purification and Characterization of μO-conotoxin MrVIA—We screened venom fractions from *C. marmoreus* for potential high affinity blockers of the tetrodotoxin-insensitive inward sodium current which underlies the generation of propagating action potentials in cultured *Aplysia* neurons. Two strongly hydrophobic fractions of this venom potently abolished the sodium component of the action potential. The active fractions were purified to homogeneity as is shown in Fig. 2.

The purified components were analyzed by amino acid sequencing as described under "Experimental Procedures," revealing two novel peptides, μO-conotoxins MrVIA and MrVIB. Their sequences are as follows.

μO-conotoxin MrVIA: ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV

μO-conotoxin MrVIB: ACSKKWEYCIVPILGFVYCCPGLICGPFVCV

Liquid secondary ion mass spectrometry indicated that Cys residues are present as disulfides and that the COOH-terminal α-carboxyl group is the free acid for both peptides (monoisotopic MH^+: MrVIA calculated 3487.66, observed 3487.8; MrVIB calculated 3404.58, observed 3404.8). The sequence was further verified by chemical synthesis and cDNA cloning (see below).

Surprisingly, the new conotoxins show no detectable amino acid sequence homology to the well characterized sodium channel blocking peptides from *Conus* venoms, the μ-conotoxins from fish-hunting *Conus* snails. Instead, the cysteine pattern resembles that of the ω-conotoxins, the large family of *Conus* peptides which are specifically targeted to calcium channels and of the δ-conotoxins that slow down the inactivation rate of sodium channels (1, 4, 9, 18–21).

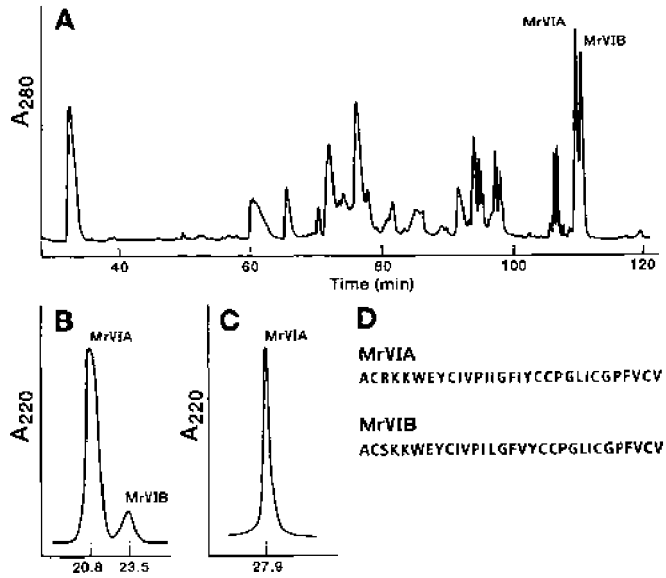

FIG. 2. **Isolation and characterization of μO-conotoxins MrVIA and MrVIA from *C. marmoreus* venom.** *A*, RPLC of crude venom filtrate. The very hydrophobic MrVIA and MrVIB elute as the last two major peaks at 109.4 and 110.3 min. *B*, RPLC of the fraction eluting at 109.4 min separates MrVIA from MrVIB. An analogous RPLC was performed to isolate MrVIB (not shown). *C*, rechromatography of the MrVIA (*arrow*) and MrVIB (not shown) containing fractions shows homogeneous appearing peaks. *D*, the complete sequences are shown using single-letter amino acid code.

Chemical Synthesis—Solid-phase chemical synthesis of μO-conotoxins MrVIA and MrVIB was undertaken by the two-stage strategy of Monje *et al.* (8), as modified for the hydrophobic δ-conotoxins TxVIA and GmVIA (9). On the assumption that the disulfide bridging would be conserved, Cys^9 and Cys^{25} were protected as the stable Cys(*S*-acetamidomethyl), whereas the other four Cys residues were protected as the acid-labile Cys(*S*-trityl). After cleavage from the resin, the linear peptide was purified by RPLC, and then oxidized to a mixture of three bicyclic peptides. Trial experiments indicated which of these corresponded to the correct isomer, and the main batch of peptide was subjected to further oxidation by iodine in 10% trifluoroacetic acid. After destroying excess iodine with ascorbic acid, the tricyclic peptide was purified by RPLC (0.1% trifluoroacetic acid with an acetonitrile gradient of 32–68% in 40 min). Synthetic peptide was shown to co-elute with the natural material and was equipotent in biological assays.

Precursor Sequence—In order to determine the precursor sequence of these peptides, a cDNA library was prepared from venom ducts of *Conus marmoreus* and clones related to the new peptides were identified. Two cDNA clones for μO-conotoxin MrVIB were characterized. The nucleotide sequence and predicted amino acid sequence of the encoded precursor is shown in Fig. 3. It is seen that the last 31 residues exactly match the experimentally determined sequence of μO-conotoxin MrVIB. The putative 82-amino acid precursor sequence is presumably cleaved after Arg-51 to yield the mature 31-amino acid peptide. The cDNA sequence also verifies the biochemical assignment for a free carboxyl terminus for MrVIB.

Electrophysiology—Action potentials generated by intracellular stimulation of cultured *Aplysia* neurons were rapidly

[1] The abbreviations used are: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; RPLC, reversed phase liquid chromatography.

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                                                    10
met lys leu thr cys met met ile val ala val leu phe leu thr ala
ATG AAA CTG ACG TGC ATG ATG ATC GTT GCT GTG CTG TTC TTG ACA GCC

        20                                                  30
trp thr leu val met ala asp asp ser asn asn gly leu ala asn his
TGG ACG CTC GTC ATG GCT GAT GAC TCC AAC AAT GGA CTG GCG AAT CAT

                        40
phe leu lys ser arg asp glu met glu asp pro glu ala ser lys leu
TTT TTG AAA TCA CGT GAC GAA ATG GAG GAC CCC GAA GCT TCT AAA TTG

    50                                                  60
glu lys arg ala cys ser lys lys trp glu tyr cys ile val pro ile
GAG AAA AGG GCG TGC AGC AAA AAA TGG GAA TAT TGT ATA GTA CCG ATC

                70                                          80
leu gly phe val tyr cys cys pro gly leu ile cys gly pro phe val
CTT GGA TTC GTA TAT TGC TGC CCT GGC TTA ATC TGT GGT CCT TTC GTC

cys val OPA AMB OPA
TGC GTT TGA TAG TGA
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FIG. 3. **The precursor sequence of μO-conotoxin MrVIB.** Nucleic acid sequence and inferred amino acid sequence of MrVIB-encoding cDNA clone. A *C. marmoreus* cDNA library was prepared as described by Colledge *et al.* (10). Fifteen clones were identified in two rounds of colony hybridization of this library using a probe specific for ω-conotoxin signal sequence. The 15 clones were then sequenced with this same oligonucleotide. Of these, four encoded μo-conotoxin MrVIB.

(30 s) blocked by bath application of 350 nM conotoxin MrVIA (Fig. 4, *A* and *B*). The action potential blockade was not associated with changes in the transmembrane potential or input resistance. An increase in the intracellular stimulus intensity failed to evoke a full-blown action potential, but induced a small local response (not shown). The local response was most likely generated by unblocked voltage-gated calcium channels, as it could be abolished by the addition of 8 mM cobalt ions to the bath solution (see also Fig. 5*A*).

To identify the mechanisms underlying the action potential blockage by μO-conotoxins MrVIA/B, we used experimental protocols that permitted us to examine the isolated macroscopic currents of either K^+, Ca^{2+}, or Na^+. The toxins had no effects on the voltage-gated potassium currents at concentrations of up to 10 μM.

To examine the effects of μO-conotoxins MrVIA/B on the sodium current we carried out a series of experiments ($n = 6$ for MrVIA and $n = 3$ for MrVIB) in which the potassium and calcium currents were eliminated (see "Experimental Procedures"). The voltage clamp records of Figs. 4*B* and 5*A* show that 150–250 nM MrVIA completely blocks the inward sodium current within 30 s after toxin application. The blockade of I_{Na^+} was not associated with changes in the rise time or decay time kinetics of the macroscopic current as evidenced by the fact that the normalized control I_{Na^+} and partially toxin-blocked I_{Na^+} superimpose on each other with no deviation. The blockade of I_{Na^+} is not associated with a change in the current-voltage relations (Fig. 4*C*), nor by a change in the steady state inactivation or activation curves (data not shown).

Complete blockade of I_{Na^+} occurs at MrVIA concentration of 250 nM. At this concentration the toxin had no detectable effects on the calcium currents. A preliminary report of a MrVIA homolog which blocked *Lymnaea* calcium currents at high concentrations (22) prompted us to test high concentrations of MrVIA in *Aplysia*. At these toxin concentrations (>2 μM) MrVIA reduced the inward calcium current. This is illustrated by the experiment of Fig. 5, in which the potassium current was blocked (see "Experimental Procedures") and the neuron was depolarized from a holding potential of −50 mV to 20 mV. Under these conditions the inward current is composed of two peaks. The first is the inward sodium current with fast activation and inactivation kinetics. The second is the calcium current. Its peak is somewhat delayed and is characterized by slow

TABLE I
Bioassay μO-conotoxin MrVIA

Toxin was injected intracranially (i.c.) or intraperitoneally (i.p.) into young (5–8 g) mice (29). NE, no effect; NT, not tested.

Dose	i.c.	i.p.
nmol		
0.1	Ataxia/light coma	NE
0.5	Deep coma	NE
2.0	NT	NE
10.0	NT	NE

inactivation kinetics (20). Application of 150 nM MrVIA induced a rapid block of the early sodium current (Fig. 5, *A*, and *D*, *diamonds*) but had no effect on the calcium current (Fig. 5, *A* and *D*). Following the application of 3.5 μM MrVIA to the bathing solution the amplitude of $I_{Ca^{2+}}$ was gradually reduced (Fig. 5, *B* and *D*) reaching a 60% blockade of $I_{Ca^{2+}}$ approximately 4 min after toxin application. Complete blockade of $I_{Ca^{2+}}$ was reached at MrVIA concentrations of approximately 100 μM (data not shown). Unlike the blockade of I_{Na^+} that did not recover even after prolonged washing (45 min) (Fig. 5*D*), the blockade of the calcium current recovered to the control level within a few seconds of wash (Fig. 5, *C* and *D*). The high MrVIA concentration necessary to induce the blockade of $I_{Ca^{2+}}$, the slow blocking rate, and the rapid washout of the blockade suggest that this blockade of calcium conductance by MrVIA is unlikely to play a major role in prey immobilization by *C. marmoreus*. Since 250 nM MrVIA is sufficient to completely block I_{Na^+}, a concentration that does not affect $I_{Ca^{2+}}$, μO-conotoxin MrVIA can be used effectively as a sodium channel blocking agent in neurobiological studies of the *Aplysia* nervous system.

μO-conotoxin MrVIA Is Active in Vertebrate Systems—The results above demonstrate that μO-conotoxins purified from the venom of a mollusc-hunting *Conus* potently block voltage-sensitive sodium channels in molluscan systems. The activity of μO-conotoxin MrVIA was also examined using injections into mice; results are shown in Table I. The data reveal that the peptide is extremely potent when injected into the rodent central nervous system. Dramatic symptomatology is observed (ataxia, coma), even upon injection of doses of MrVIA as low as 0.1 nmol. In contrast, no effects are observed at doses up to 100 × greater when the toxin is injected intraperitoneally.

DISCUSSION

The results described above establish that two peptides from *C. marmoreus* venom, μO-conotoxins MrVIA and MrVIB potently block the inward Na^+ current of cultured *Aplysia* neurons. As will be discussed below, the μO-conotoxins show great potential as general pharmacological tools for discriminating among sodium channel subtypes, and together with the ω- and δ-conotoxins, define a "superfamily" of *Conus* peptides. We also comment on the probable biological role of these peptides.

The central nervous system of *Aplysia* is extensively used for the study of various neurobiological questions, yet no efficient sodium channel blocking agents are presently available. The classical sodium channel blockers, tetrodotoxin and saxitoxin, are inefficient in *Aplysia* neurons. For example, tetrodotoxin blocks *Aplysia* sodium channels at concentrations of 100–200 μM, 3–4 orders of magnitude higher concentration than those required to block sodium channels in vertebrate brain, in vertebrate muscles and even the less sensitive channels in cardiac and denervated muscles (5, 23–26). In contrast, the new μO-conotoxins MrVIA and MrVIB block sodium current in *Aplysia* channels at submicromolar concentrations. They should thus provide useful tools for *Aplysia* neurobiological studies and probes for molecular biological analysis of molluscan sodium

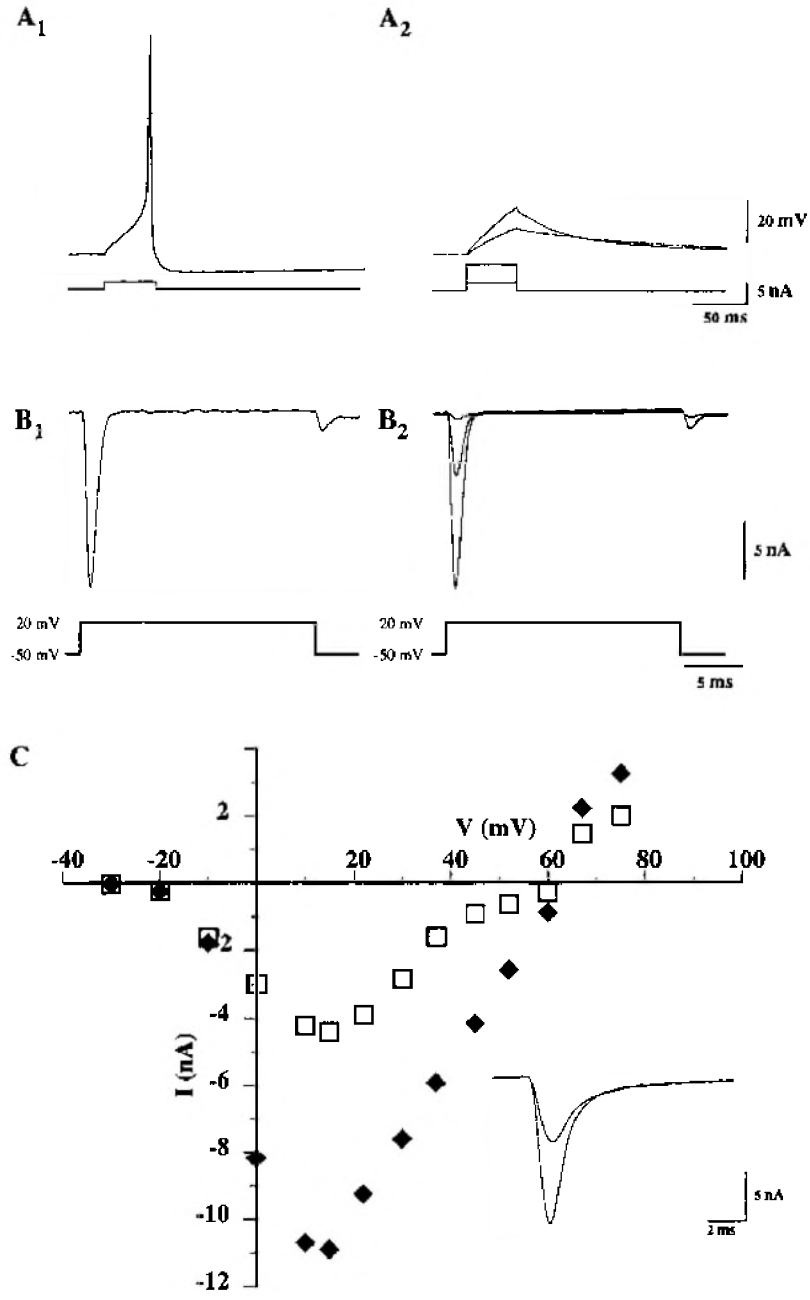


FIG. 4. **Blockade of action potential and inward sodium current by μO-conotoxin MrVIA as revealed by current and voltage clamp experiments.** A_1 (control), the action potential was generated by an intracellular rectangular depolarizing pulse. A_2, 10 s after toxin application to reach a final bath concentration of 350 nM, the action potential was blocked. An increase in the stimulus intensity after the blockade failed to elicit a regenerative response. The inward I_{Na^+} evoked by depolarizing the neuron from a holding potential of −50 to 20 mV (B_1) was completely blocked 30 s following the application of 250 nM MrVIA (B_2). In the record of B_2 the control I_{Na^+} and two consecutive traces are shown. *C*, partial blockade of I_{Na^+} by 40 nM toxin revealed that the block is not associated with a change in the current-voltage relationships. The *inset* shows the control current trace and the partially blocked I_{Na^+}.

channels in general.

Superfamilies of Conus Peptides—Surprisingly, although the new conotoxins clearly block Na^+ currents elicited through voltage-sensitive sodium channels, they show no detectable amino acid sequence identity to the μ-conotoxins from fish-hunting *Conus*, the other group of *Conus* peptides that block sodium currents. Instead, the MrVIA/B peptides have a cysteine pattern similar to that of the ω-conotoxins from fish-hunting snails and the δ-conotoxins from mollusc-hunting snails. An examination of the precursor sequence of the MrVIB peptide reveals considerable homology not only to the ω-conotoxin family, but to the δ-conotoxins which slow down the inactivation rate of sodium channels (9, 19–21). In fact there is greater similarity between the new conotoxin and the δ-conotoxins (35 out of 51 residues identical in the prepro region) than there is to the ω-conotoxins (24 out of 51 residues identical). A comparison of the predicted precursor sequences of these peptides, deduced from cDNA clones, is shown in Fig. 6. The *boxes* indicate sequence identities.

Thus, three distinctive pharmacological groups of *Conus* peptides appear to belong to the same structural group, which we will define as the "O-superfamily" of *Conus* peptides: the ω-conotoxins which inhibit calcium channels, the δ-conotoxins which slow down the inactivation rate of voltage-sensitive sodium channels, and the μO-conotoxins which block voltage-sensitive sodium currents. By contrast, the μ-conotoxins from fish-hunting *Conus*, which show the same general physiological mechanism as the μO-conotoxins (*i.e.* blockage of voltage-sensitive sodium currents), have no detectable homology to any of the peptides in Fig. 6. They clearly belong to a different *Conus* peptide superfamily. By definition, the O-superfamily comprises those peptides with strong similarity to the omega-conotoxins in their precursor sequences; a curious feature is that the signal sequences region is the most highly conserved.

Thus, the work described above suggests that in this single genus there has been functional *convergence* of peptides from two different superfamilies to produce toxins that inhibit sodium channel function, but functional *divergence* within a sin-

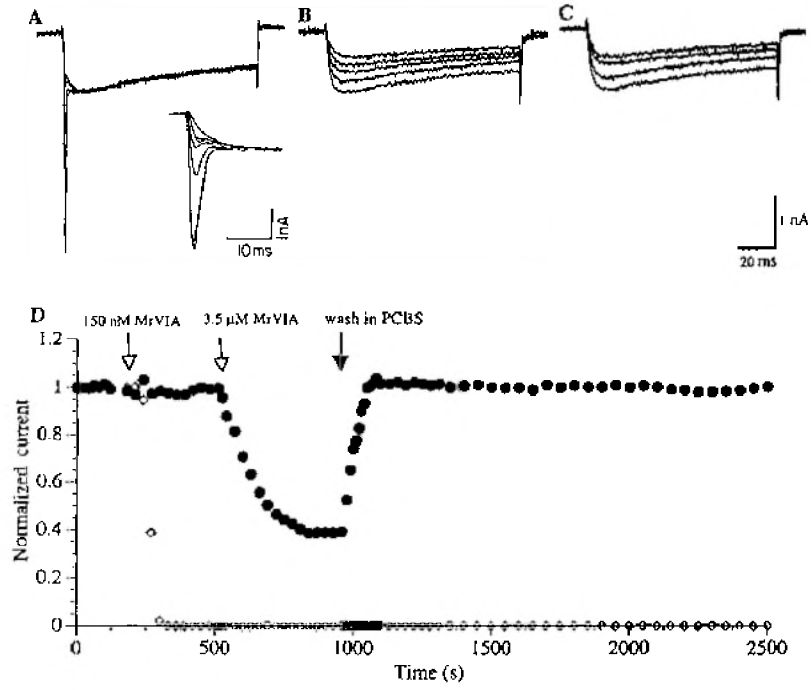


FIG. 5. **Effect of MrVIA on the sodium and calcium current.** For the experiment the potassium currents were blocked. The neuron was depolarized from a holding potential of −50 to 20 mV. In the control (*A*), the depolarization induced an early rapidly inactivating sodium current and a plateau of calcium current. Application of 150 nM MrVIA rapidly blocked the early sodium current (second trace in *A* and see *inset*). The rate at which I_{Na^+} was blocked is shown by the *open diamonds* in *D*. Note that the calcium current was not affected by 150 nM MrVIA (*A* and *black circles* in *D*). Following the application of 3.5 μM MrVIA, $I_{Ca^{2+}}$ was gradually reduced (*B* and *black circles* in *D*). $I_{Ca^{2+}}$ rapidly recovered following wash by artificial sea water, whereas the sodium current remained blocked.

gle superfamily to produce functionally distinct toxin families. Such an extreme interspecific diversification of venom peptides may account in part for the unusual proliferation of species in the genus *Conus*, which is presently believed to be the largest living genus of marine invertebrates (approximately 500 living species) (27).

Biological Role—Our results suggest that snail-hunting *Conus* have evolved at least two solutions to the same biological problem of feeding on snails. When harpooned, the prey would naturally retract into their shells to escape from a predatory *Conus* snail. Our observations of feeding in aquaria suggest that two snail-hunting *Conus* groups have generated very different ways to prevent prey from becoming paralyzed inside the shell. *Conus textile*, after envenomating a snail (such as the *Kelletia* species), induces convulsive contractures, with the victim moving into and out of the shell. In contrast, envenomation by *C. marmoreus* elicits a slow relaxation. Purified δ-conotoxin TxVIA induces the convulsive contractures in snails observed with whole *C. textile* venom, whereas μO-conotoxin MrVIA causes the flaccid relaxation characteristic of crude *C. marmoreus* venom. In either case, access to the prey is ensured by the cone snail after immobilization of the victim, albeit by very different means.

The Potential of Conus Peptides for Generating a Subtype-specific Sodium Channel Pharmacology—μO-conotoxins MrVIA and MrVIB are harbingers of a potentially much wider range of peptides active against sodium channels. The availability of the cloned precursor sequence has allowed us to identify potential homologs from venoms of other *Conus* species.[2] The sequences available suggest that there is considerable sequence hypervariability in this family of *Conus* peptides, making it likely that different members may have differing sodium channel subtype specificity profiles. Similarly, although the structurally unrelated μ-conotoxins have only been characterized from a single fish-hunting *Conus* species, *Conus geographus*, a molecular genetic analysis has revealed that a number of other fish-hunting *Conus* species also have μ-conotoxins; the predicted peptides are presently being synthesized and will be further characterized.

In vivo results already suggest that the first peptides characterized from these two conotoxin families (μ and μO) may have distinctly different sodium channel subtype specificity in mammalian systems. Thus, relatively high doses of μO-conotoxin MrVIA (>10 nmol/10 g) are inactive peripherally in rodents, whereas μ-conotoxin GIIIB is a potent paralytic when injected intraperitoneal (28); this is consistent with the well established specificity of μ-conotoxins for voltage-sensitive sodium channels from mammalian skeletal muscle. In contrast, low doses of μO-conotoxin MrVIA cause ataxia and/or reversible coma in a few minutes when injected intracranially, whereas no detectable symptoms are observed after several hours of a similar central injection of μ-conotoxin GIIIB. These results suggest that MrVIA is a potent ligand in the mammalian central nervous system and may target central sodium channels. In contrast, GIIIB targets the skeletal muscle subtype with high affinity, but not neuronal sodium channels. We cannot exclude the possibility, however, that the differing effects between these toxins are due to differences in state-dependent channel block, rates of absorption, or susceptibility to proteolysis. Preliminary experiments with cloned sodium channels expressed in *Xenopus* oocytes are, however, consistent with the *in vivo* central effects of μO-conotoxins.[3] Many more members of the μ- and μ0-conotoxin families can be identified by molecular genetic techniques and the predicted peptides chemically synthesized. By screening for those peptides that induce different *in vivo* symptoms, an expanding set of promising ligands for establishing a subtype-specific pharmacology for sodium channels should be established.

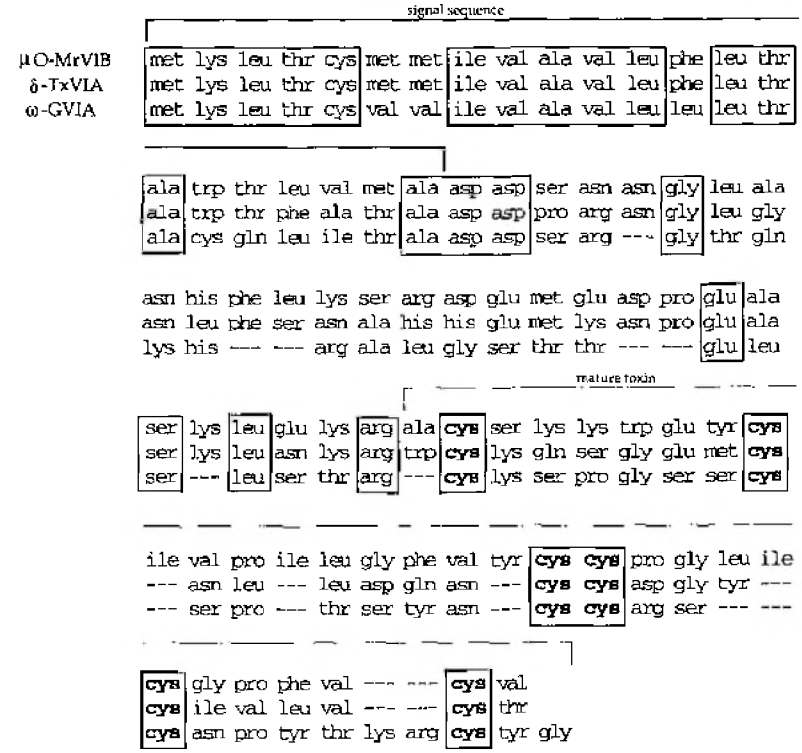
General Perspectives—Different ion channel subtypes might be expected to play distinctive functional roles in nervous systems. For voltage-sensitive calcium channels, this functional differentiation of subtypes has become increasingly well documented. This has been made possible in large part by the availability of a family of *Conus* peptides, the ω-conotoxins: thus, α_{1B}- and α_{1A}-containing calcium channel complexes are differentially targeted by different ω-conotoxins (1).

The functional differentiation of sodium channel subtypes is much more poorly understood; the paucity of sodium channel

[2] M. Marsh and D. Hillyard, unpublished results.

[3] H. Terlau, B. M. Olivera, and W. Stühmer, unpublished results.

A Comparison of O-superfamily precursor sequences



B Summary of Identical Amino Acid Residues

	Signal sequence	"Pro" region	Mature Toxin Cys residues	Mature Toxin Non-Cys Residues
Total residues in μO-MrVIB	22	29	6	25
# Identical to TxVIA	19	19	6	4
# Identical in all three sequences	14	7	6	0

FIG. 6. **Comparison of O-superfamily precursor sequences.** The inferred sequence of the μO-conotoxin MrVIB precursor sequence (see Fig. 3) is aligned with the prepropeptide sequences of δ-conotoxin TxVIA (formerly called the King-Kong peptide) and ω-conotoxin GVIA. The signal sequence and mature toxin regions are indicated, with an intervening proregion. The conserved amino acids are *boxed*. Note the almost total conservation of signal sequences *versus* the striking divergence of the mature toxins.

subtype-specific ligands is a major reason for this situation. This report describes a new family of *Conus* peptides, the μO-conotoxins, which potently block voltage-sensitive sodium currents. The μO-conotoxins are potentially as important to sodium channel subtype differentiation as the ω-conotoxins are to calcium channels.

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