New Sodium Channel-Blocking Conotoxins Also Affect Calcium Currents in *Lymnaea* Neurons†

Michael Fainzilber,* Roel van der Schors, Johannes C. Lodder, Ka Wan Li, Wijnand P. M. Geraerts, and Karel S. Kits

Graduate School Neurosciences Amsterdam, Institute of Neuroscience Vrije Universiteit, Faculty of Biology, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

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ABSTRACT: Two new conotoxins that affect both sodium and calcium currents have been characterized from the venom of *Conus marmoreus*, using direct assays on voltage-gated currents in caudodorsal neurons (CDC) of the freshwater snail *Lymnaea stagnalis*. The designations and amino acid sequences of the new toxins are MrVIA, ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV, and MrVIB, ACSKKWEYCIVPILGFVYCCPGLICGPFVCV. Both toxins block voltage dependent sodium currents in snail neurons with ED_{50}'s of 0.1−0.2 μM. Effects are also observed on the fast-inactivating calcium current subtype in the CDC at ≥1 μM. At concentrations of 1−5 μM, MrVIA acts as a calcium current agonist whereas MrVIB acts as a blocker. At higher doses both toxins block the fast-inactivating calcium current. Almost no effects of MrVIB are seen on the second (sustained kinetics) CDC calcium current subtype, while MrVIA also slightly blocks the sustained current. The calcium current block is rapidly reversible, whereas in contrast recovery of the sodium current requires extensive wash. MrVIA/B have the same cysteine framework as the ω- and δ-conotoxins and a high content of hydrophobic residues, in common with the δ-conotoxins. There is only one localized concentration of charged residues in MrVIA/B, in the first intercysteine loop. These two conotoxins provide unique probes for structure and function studies on voltage-gated sodium and L-type calcium channels. Their unusual cross-channel activity suggests they may represent an "intermediate" variant of conotoxin, in the diversification of one conotoxin structural family that selectively targets either sodium or calcium channels.

Conus snails are selective predators which utilize a sophisticated peptidergic venom system to paralyze their prey. The most well-known components in *Conus* venoms are the conotoxins, small, typically 10−30-residue, disulfide-rich neuroactive peptides (Olivera et al., 1990, 1991). They are utilized as research tools in many fields of neurobiology since they provide high-affinity antagonists for receptor and ion channel subtypes, and being small peptides, they are synthesizable and modifiable, thus increasing their availability and potential utility. Conotoxins in general exhibit a high degree of selectivity in their receptor targets (Olivera et al., 1990, 1991) and in certain cases also distinguish between phyletic variants of ion channels and receptors (Fainzilber et al., 1991, 1994a,b; Zaffarella et al., 1988).

The utility of conotoxins as pharmacological tools have motivated studies on conotoxins selective for invertebrate receptors. Molluscan neuronal systems such as those of *Aplysia* and *Lymnaea* are in wide use as model systems and recent efforts have focused on characterization of conotoxins that may serve as selective tools for these systems. One intriguing outcome of these studies has been the characterization of the δ-conotoxin family that affects sodium channels (Hasson et al., 1993; Fainzilber et al., 1994a) but shares critical structural features with the calcium channel-blocking ω-conotoxins (Hillyard et al., 1989; Fainzilber et al., 1991). This includes the conserved cysteine framework and a glycine residue that are important for correct folding of these peptides (Davis et al., 1993; Pallaghy et al., 1993; Sevilla et al., 1993; Skalicky et al., 1993). Furthermore the prepro regions in the cDNA's encoding these pharmacologically diverse peptides exhibit a striking degree of homology, suggesting they may represent members of a strongly diversified gene family (Woodward et al., 1990; Colledge et al., 1992). The possibility that both sodium and calcium channel specific ligands have evolved from a common origin is reminiscent of the evolution of the targeted channels, in which sodium and calcium channels are thought to form one lineage which diversified through gene duplications from an ancestral calcium channel (Strong et al., 1993).

We have been analyzing molluscivorous *Conus* venoms in an effort to characterize novel conotoxins that target molluscan calcium channels. When testing effects of fractions from *Conus marmoreus* venom on L-type HVA calcium currents in a snail neuroendocrine cell, we identified two toxins that were found to affect both calcium and sodium channels. These new toxins have the same cysteine framework as the δ- and ω-conotoxins, thus suggesting that they may represent structural and pharmacological intermediates in the superfamily of conotoxins that target sodium or calcium channels.

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* Address correspondence to: M. Fainzilber, Molecular Neurobiology, Faculty of Biology, Vrije Universiteit Amsterdam, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands. Fax: 31-20-444 7123. Tel: 31-20-444 7121. e-mail: mike@bio.vu.nl.

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EXPERIMENTAL PROCEDURES

Animals and Cells. Adult snails from a laboratory-bred population of *Lymnaea stagnalis* were used. CDC[1] were isolated from the CNS by mechanical dissociation after a 30 min incubation at 37 °C in HEPES-buffered saline (HBS) (see below) supplemented with 0.2% trypsin (type III; Sigma, St. Louis, MO). The isolated cells used for experiments had round-shaped cell bodies (diameter ~ 50 μm) without neurites. Cells were always used within 7 h after isolation.

Chemicals. C. marmoreus venom was extracted from adult specimens collected on Lizard Island, Great Barrier Reef, Australia. Synthetic conotoxin-MrVIA was a generous gift from Dr. J. M. McIntosh (University of Utah). All other chemicals were analytical grade from commercial suppliers.

Venom Extraction and Fractionation. Lyophilized venom (200 mg) was extracted as previously described (Fainzilber et al., 1991, 1994b) and fractionated on Sephadex G-50 and semipreparative reverse phase C18 columns (Figure 1). The active fractions were purified to homogeneity by reverse phase chromatography on a C8 column as described in Figure 1C. Purified peptides were quantified by amino acid analysis.

Peptide Sequencing. Purified toxins were reduced and alkylated with 4-vinylpyridine as previously described (Fainzilber et al., 1991). Amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems 473 pulse-liquid phase protein sequencing system. The chromatography system was calibrated prior to each analysis with phenylthiohydantoin (PTH) amino acid standards.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) of the purified peptides was performed as previously described (Li et al., 1993), with minor modifications. Peptides were dissolved in 50% acetonitrile, 7.5 mM trifluoroacetic acid and injected via a 10 μL loop into a BioQ triple-quadropole mass spectrometer (Fisons, U.K.) fitted with an electrospray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile and the flow rate 5 μL/min.

Electrophysiology. Isolated CDC were kept in Petri dishes (Costar) and bathed in HBS (in mM: NaCl 30, $NaCH_3SO_4$ 10, $NaHCO_3$ 5, KCl 1.7, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10; pH 7.8 set with NaOH). To record calcium, sodium, or potassium currents, HBS was replaced under continuous perfusion by the appropriate saline. The compositions of extracellular and pipette solutions used to selectively record specific currents were as follows (in mM): extracellular I_{Ca} saline-TEACl 40, $CaCl_2$ 4, HEPES 10, 4-aminopyridine 2, pH 7.8 set with TEAOH; extracellular I_{Na} saline-NaCl 47.5, $CaCl_2$ 4, $MgCl_2$ 1, HEPES 10, $CdCl_2$ 0.1, 4-aminopyridine 1, pH 7.8 set with NaOH; pipette saline (I_{Ca} and I_{Na}) CsCl 29, $CaCl_2$ 2.3, HEPES 10, EGTA 11, ATPMg 2, GTP-Tris

[1] Abbreviations: CDC, caudodorsal neurons; CNS, central nervous system; ED_{50}, effective dose 50%; ESI-MS, electrospray ionization mass spectometry; GIIIA, μ-conotoxin-GIIIA from *Conus geographus*; GVIA, ω-conotoxin-GVIA from *C. geographus*; GS, μ-conotoxin-GS from *C. geographus*; HBS, HEPES-buffered saline; HPLC, high-pressure liquid chromatography; HVA, high-voltage activated; MVIIA, ω-conotoxin-MVIIA from *Conus magus*; MrVIA, conotoxin-MrVIA from *Conus marmoreus*; MrVIB, conotoxin-MrVIB from *Conus marmoreus*; PTH, phenylthiohydantoin; SVIB, ω-conotoxin-SVIB from *Conus striatus*; TFA, trifluoroacetic acid; TxVIA, δ-conotoxin-TxVIA from *Conus textile*.

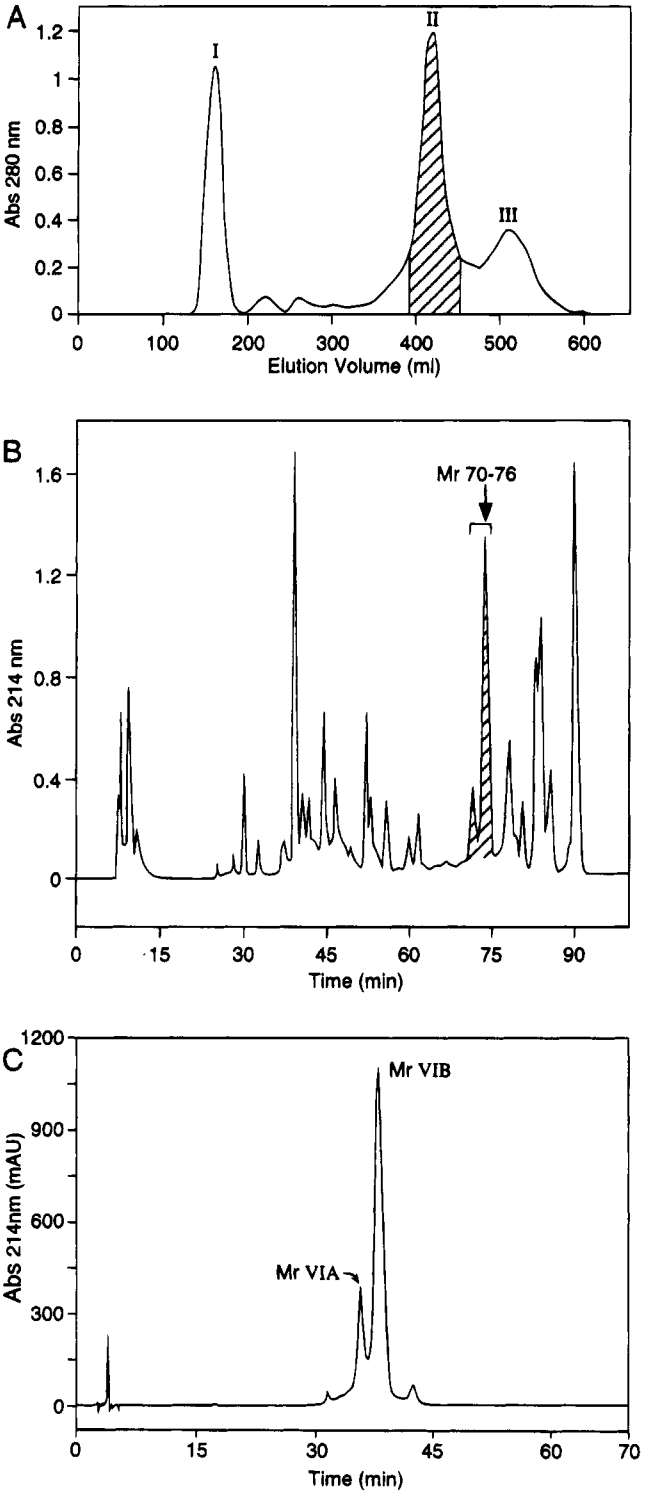

FIGURE 1: Purification of the toxins MrVIA and MrVIB. (A) Lyophilized *C. marmoreus* venom (200 mg) was extracted extensively in ammonium acetate, 0.1 M, pH 8.0, and separated on a Sephadex G-50 column (120 × 2.2 cm), equilibrated and eluted in the same buffer at a flow rate of 40 mL/h in the cold. (B) The marked fraction was further fractionated by reverse phase HPLC on a semipreparative C18 column (25 × 1 cm, 10 μm particle size), eluted at a flow rate of 2 mL/min with a linear gradient from 0 to 80% acetonitrile in 7.5 mM TFA. (C) The active fraction indicated in B was further purified on an Alltech C8 column (25 × 0.46 cm, 5 μm particle size), eluted at 0.5 mL/min. The solvent system was a gradient of acetonitrile/2-propanol (1:1) in 7.5 mM TFA, running from 0 to 40% organic solvent in 10 min and then to 80% organic solvent at 50 min.

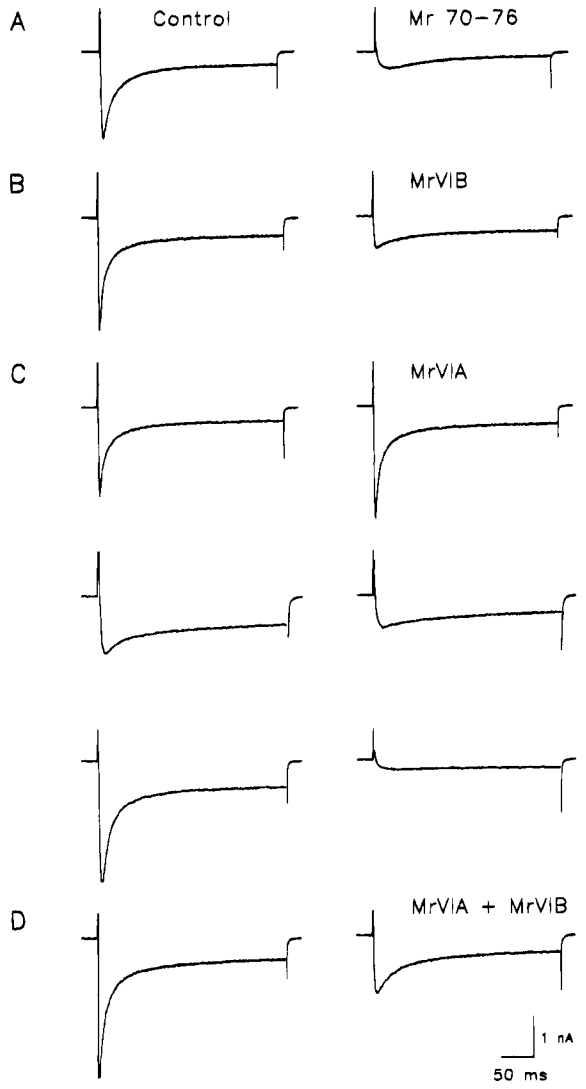

FIGURE 2: Antagonistic and agonistic effects of the MrVI toxins on the transient Ca^{2+} current in CDC. In all panels the left trace is control Ca^{2+} current and the right is in the presence of the fraction or toxin. Currents were evoked by a 300 ms step to 0 mV from a holding potential of −80 mV. (A) Blocking effect of a crude fraction of *C. marmoreus* venom. The fraction Mr70−76 (see Figure 1B) was applied at 5 mg of crude venom equiv to the preparation. Crude venom and subsequent fractions were tested on a total of 27 cells (n = 27). (B) Similar block observed upon application of 10 μM MrVIB (n = 11). (C) Upper trace: Increase of peak Ca^{2+} current upon application of 5 μM MrVIA (n = 9). Middle trace: Slight blocking effect of 10 μM MrVIA. Lower trace: Strong blocking effect of 200 μM MrVIA (for the blocking effects n = 5). Current traces shown are from three different cells, each tested at a single concentration. (D) Combined effect of a mixture of purified MrVIA and B (5 and 10 μM, respectively). Note that at this concentration ratio a small transient Ca^{2+} current can be observed in the presence of the toxins.

0.1, pH 7.4 adjusted with CsOH; pipette saline (nonselective) KCl 29, $CaCl_2$ 2.3, HEPES 10, EGTA 11, ATPMg 2, GTP-Tris 0.1, pH 7.4 adjusted with KOH. Venom fractions and purified toxins were administered by means of a laboratory-built pressure ejection system through a small glass pipette (tip diameter 20 μm) placed at ~100 μm from the recorded cell. This enabled rapid application of toxins, which were applied continuously during a series of depolarizing voltage steps.

Membrane potential measurements were performed using sharp microelectrodes filled with 0.5 M KCl (40 MΩ) using an Axoclamp 2A (Axon Instr., Foster City) amplifier in the bridge balance mode. Whole-cell voltage−clamp experiments were performed using the Axoclamp 2A amplifier in the continuous single-electrode voltage−clamp mode. Pipettes (2−6 MΩ) were pulled on a Flaming/Brown P-87 (Sutter Instruments, CO) horizontal microelectrode puller from Clark GC-150T glass (Clark Electromedical Instruments, England) (seal resistance > 1 GΩ). After disrupture of the patch membrane, series resistance (<10 MΩ) was compensated for ~80%. With current amplitudes of <5 nA, the maximal voltage error is estimated to be ≤10 mV. Cell capacitance (~100 pF) was not compensated. Measurements of calcium or sodium currents were commenced 20 min after access to the cell, in order to allow equilibration with the pipette solution. Data acquisition was controlled by a CED AD/DA converter (Cambridge Electronics Design, Cambridge, U.K.) connected to an Intel 80486-based computer, run with voltage−clamp software developed in our laboratory. The current recordings were filtered at 1−5 kHz, sampled at 1 kHz (calcium currents and K^+ currents) or 3 kHz (Na^+ currents), and stored on-line. This system allowed simultaneous application of voltage steps, acquisition of current recordings, and timed application of toxins.

Table 1: Edman Degradation of MrVIA and MrVIB

	MrVIA		MrVIB	
cycle	assigned residue	yield (pmol)	assigned residue	yield (pmol)
1	Ala	255	Ala	417
2	Cys	209	Cys	292
3	Arg	163	Ser	65
4	Lys	197	Lys	297
5	Lys	232	Lys	311
6	Trp	79	Trp	35
7	Glu	116	Glu	141
8	Tyr	153	Tyr	194
9	Cys	143	Cys	160
10	Ile	141	Ile	199
11	Val	143	Val	203
12	Pro	87	Pro	107
13	Ile	98	Ile	123
14	Ile	133	Leu	104
15	Gly	76	Gly	98
16	Phe	80	Phe	106
17	Ile	74	Val	108
18	Tyr	72	Tyr	105
19	Cys	52	Cys	69
20	Cys	67	Cys	122
21	Pro	28	Pro	59
22	Gly	19	Gly	54
23	Leu	7	Leu	33
24	Ile	7	Ile	31
25	Cys	3	Cys	20
26	Gly	3	Gly	24
27	Pro	2	Pro	9
28	Phe	1	Phe	7
29	Val	1	Val	3
30	Cys	1	Cys	2
31	Val	0.2	Val	0.3

RESULTS

Purification and Chemical Characterization of Conotoxins MrVIA and MrVIB. Venom fractions were assayed by direct screening on calcium currents in voltage-clamped CDC. The voltage dependent calcium currents in these cells have been characterized in detail by Dreijer and Kits (1995) and consist of two subtypes, one with rapid activation and inactivation kinetics (hereby refered to as the transient current) and the second with slower inactivation kinetics (hereby refered to as the sustained current) (see Figure 3). Both current

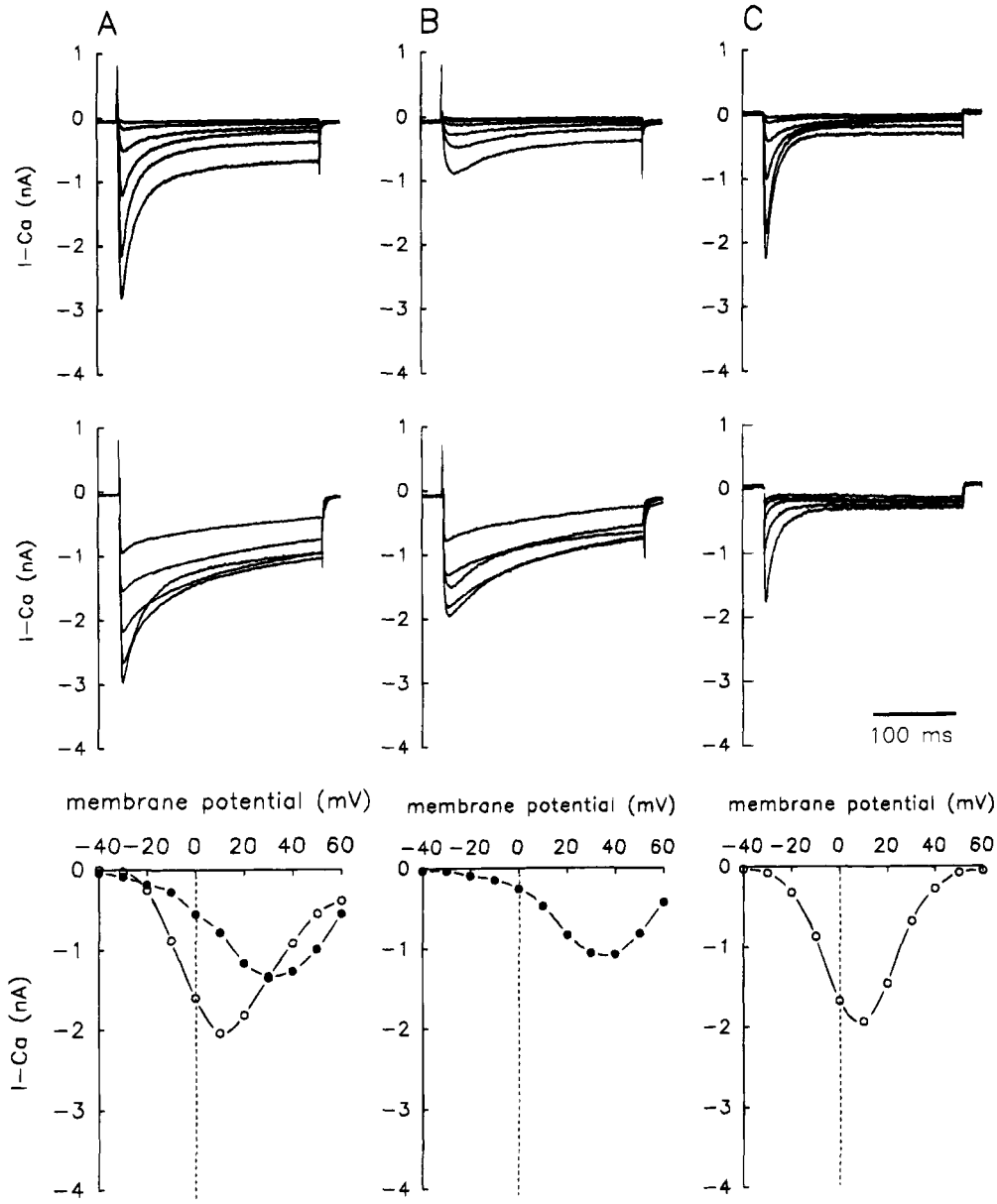

FIGURE 3: Selective block of the transient Ca^{2+} current in CDC's by MrVIB. Currents were elicited by 300 ms steps to the test potential. Holding potential was −80 mV, and pulse intervals were 10 s. Horizontal scale bar is 100 ms. (A) Upper panels: Control current traces from −40 to +20 mV. Middle panels: Control currents from +20 to +60 mV. Lower panels: Current−voltage (I−V) relations of the transient current (open circles) and the sustained current (filled circles). The I−V relation of the sustained current was obtained by measuring the current amplitude at t = 125 ms, i.e., when the inactivation of the transient current is complete (inactivation time constant of the transient current is <25 ms at all potentials where activation takes place). The I−V relation of the transient current was obtained by subtracting the amplitude of the sustained current from the early peak amplitude. (B) Current traces as in A but in the presence of 10 μM MrVIB, thus showing the MrVIB resistant current. The I−V relation of the sustained current, obtained as in A, is plotted, showing that this current is hardly affected. (C) Current traces showing the component blocked by MrVIB. These traces were derived by subtraction of the traces in B from those in A. From these traces, the I−V relation of the transient component was obtained as in A. Comparison of this I−V with that of the transient current in A reveals that this current is completely blocked.

subtypes are sensitive to organic blockers from the 1,4-dihydropyridine group (Dreijer & Kits, 1995) and are therefore classified pharmacologically as L-type calcium channels. *C. marmoreus* venom and its low molecular mass peptide fraction (Figure 1A) caused a clear block of the transient calcium current subtype in the CDC (Figures 2 and 3). The Sephadex peptide fraction was separated by reverse phase HPLC on a semipreparative C18 column. The major fractions affecting the transient calcium current subtype in the CDC were found to elute late in the reverse phase profiles (Figure 1B). These fractions were purified to homogeneity by another step of reverse phase chromatography on a C8 column (Figure 1C). Purity was monitored by ESI-MS for all fractions. The amino acid sequences of the new toxins were then determined by Edman degradation and verified by ESI-MS.

Sequence analyses were performed by automated Edman degradation after reduction and pyridylethylation of the peptides. Single unambiguous sequences of 31 amino acids were obtained for both toxins (Table 1), with six cysteines and a high content of hydrophobic residues. The toxin masses by ESI-MS were in good agreement with those predicted from the Edman data (MrVIA predicted mass 3488.4, observed 3488.1; MrVIB predicted mass 3405.2, observed 3404.9), assuming three disulfide bridges in the peptides and amidated C-termini. ESI-MS of the reduced

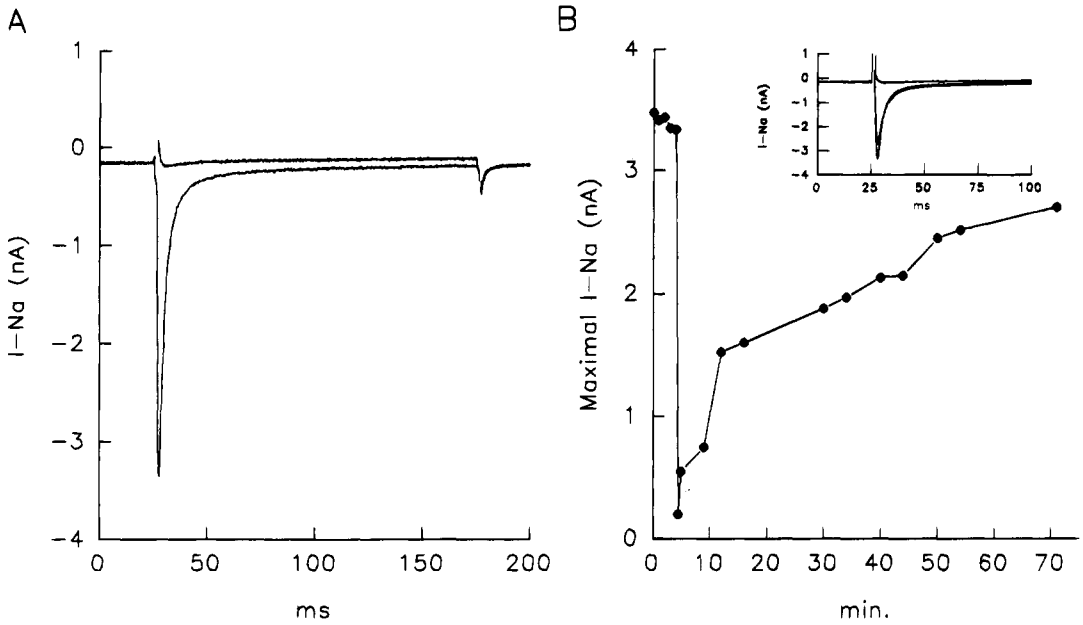

FIGURE 4: MrVIA and MrVIB effects on the voltage dependent Na^+ current (I_{Na+}) in CDC's. (A) Complete block of I_{Na+} by 1 μM MrVIA ($n = 5$). Currents were elicited by 150 ms steps to the test potential. Holding potential was −80 mV, and pulse intervals were 5 s. (B) Very slow reversibility of the blocking effect of 1 μM MRVIB on the voltage dependent Na^+ current ($n = 4$). Even after >1 h, the amplitude of I_{Na+} had not completely recovered to the control value. Inset shows the current traces at 5 min (control), 6 min (maximal block), and 65 min (partial recovery).

and pyridylethylated peptides revealed the predicted mass increments for six cysteine residues, thus further confirming the sequence assignments. As seen from the summarized sequence information (Table 1 and Figure 6), and from their elution profiles in reverse phase HPLC, the conotoxins characterized in this study are extremely hydrophobic peptides. After purification and characterization of these toxins, we became aware of the fact that essentially identical peptides had been characterized in parallel from Philippine *C. marmoreus*, on the basis of a sodium current-blocking assay in *Aplysia* neurons (McIntosh et al., submitted for publication). The agreed designations for these toxins (MrVIA, MrVIB) include an abbreviated species identification (Mr for *C. marmoreus*) and the roman numeral VI to identify the cysteine framework, which is the same as that found in ω- and δ-conotoxins.

Electrophysiological Characterization of the Purified Toxins. Initial experiments on the partially purified fractions suggested that the MrVI toxins affect both sodium and calcium currents in the CDC. Detailed electrophysiological studies on the purified toxins confirmed this initial observation. The current traces in the presence of 10^{-5} M MrVIB or the originating fraction Mr70−76 revealed a complete block of the transient calcium current component (Figure 2A,B). Intriguingly, when MrVIA was applied at lower concentrations (≤ 5 μM), the currents were not reduced but instead a net increase of the peak current could be observed (Figure 2C, upper traces). Higher concentrations of MrVIA, however, yielded a dose dependent block of the transient calcium current and, to a lesser extent, some block of the sustained component (Figure 2C, middle and lower traces). Figure 3 illustrates that MrVIB was selective for the transient subtype of the calcium current, also when high toxin concentrations were applied. This can clearly be observed also in the plots of current/voltage relations (Figure 3C). The effects of both toxins on CDC calcium currents were rapidly reversible, and currents recovered to control levels within 1 min of wash.

MrVIA and MrVIB had no effect on potassium currents when tested at 10^{-5} M (data not shown). In contrast, both toxins proved to be potent blockers of the voltage dependent sodium currents in *Lymnaea* CDC (Figure 4). The effects on Na^+ current are observed from threshold concentrations of 20 nM, and a complete and very slowly reversible block could be seen at doses above 500 nM (Figures 4 and 5). Thus, the concentration of MrVIA or MrVIB required for a complete block of CDC Na^+ current is in the range of the threshold concentrations at which effects are first observed on Ca^{2+} currents (Figure 5). In contrast to the complex effects observed on calcium channels, with very steep dose−response curves (Hill coefficients = 4) and rapid reversibility, the blocking effects of MrVIA and MrVIB on CDC sodium channels are characterized by less steep dose−response relationships (Hill coefficients = 2) and a very slow reversibility, requiring up to a 1 h wash when the toxin was applied at a dose of 1 μM (Figure 4).

It should be noted that the MrVI peptides used for these experiments were extensively repurified, and lack of contaminants was verified by both ESI-MS and repeated N-terminal sequencing. Furthermore, similar effects on both calcium and sodium currents in the CDC were obtained using synthetic conotoxin-MrVIA (data not shown). Thus, the dual effects of the MrVI toxins on both sodium and calcium channels in *Lymnaea* CDC cannot be attributed to any impurities or contaminations in the toxin preparations.

In Vivo Effects of the Toxins on Adult Lymnaea. As *C. marmoreus* presumably produces its toxins for use in paralysis of prey snails, it was of interest to examine the *in vivo* effects of the purified toxins upon injection into *Lymnaea*. Both toxins cause symptoms of paralysis in adult *Lymnaea* (2 g body weight, ±1 mL hemolymph volume), which is observed as an overall flaccidity of the snail, a lack

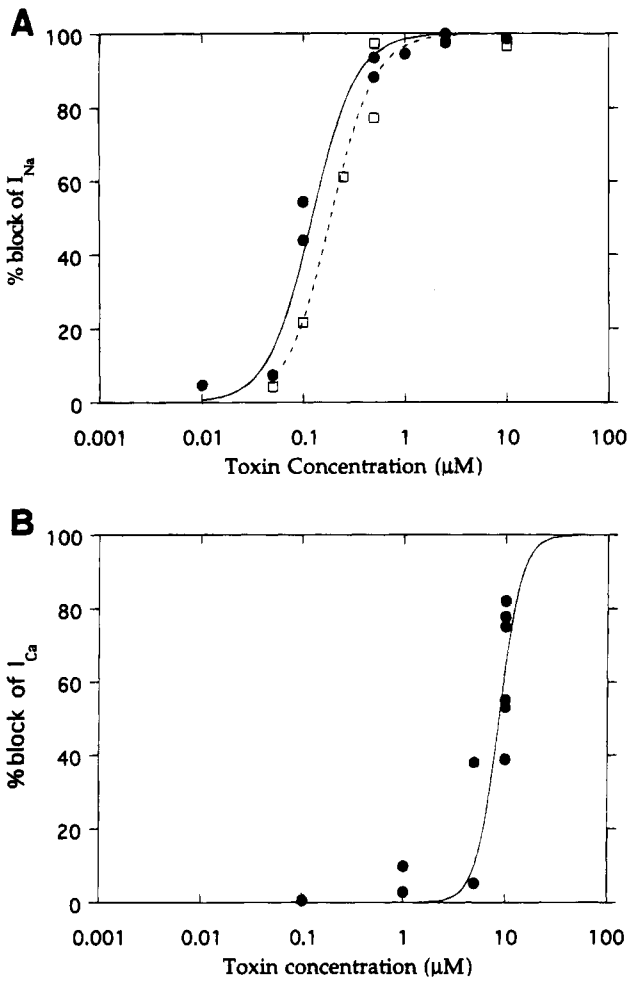

FIGURE 5: Dose–response relations of MrVIA and MrVIB effects on sodium versus calcium currents. (A) Dose–response curves of the blocking action of MrVIA (circles; $n = 3$) and MrVIB (squares; $n = 2$) on the voltage dependent Na^+ current in CDC. Fitted lines are Langmuir adsorption curves, assuming a Hill coefficient of 2. The $K_{0.5}$ for Na^+ current block is 0.12 μM for MrVIA and 0.19 μM for MrVIB. (B) Dose–response curve of the blocking action of MrVIB on the transient HVA Ca^{2+} current in CDC (curve composed from observations on eight cells, most of which were tested at three concentrations). The fitted line indicates a Hill coefficient of 4. The $K_{0.5}$ for Ca^{2+} current block is 8.7 μM.

of response to noxious stimuli, and a loss of the ability to retract into the shell. Interestingly, these effects are observed only in the micromolar dose range, i.e., the same as those required for *in vitro* effects on Ca^{2+} channels. Thus, no effects whatsoever could be seen upon injection of doses below 0.2 nmol of MrVIB/animal (corresponds to ± 0.2 μM hemolymph concentration), a weak and rapidly reversible paralysis was observed at doses of 0.2–0.5 nmol/animal [$\pm(0.2-0.5)$ μM], and a somewhat more sustained paralysis (up to 1 h) was observed at doses above 1 nmol/animal (>1 μM).

DISCUSSION

In the present study we have identified and characterized two new conotoxins that affect both sodium and calcium currents in molluscan neurons. As will be detailed below, these toxins provide intriguing probes for ion channels and may represent structural and pharmacological intermediates in the diversification of a group of related toxin ligands.

The electrophysiological data we have presented (Figures 2–5) clearly demonstrate that MrVIA and MrVIB affect both sodium and calcium currents in *Lymnaea* CDC. Rigorous verification of toxin purity by ESI-MS and replication of the dual effects with synthetic MrVIA clearly rule out any possibility of one or the other activity being due to the presence of an unknown contaminant. The effects of the MrVI toxins on CDC sodium currents are more potent, as they are observed at lower concentrations of toxin (Figure 5), and are only very slowly reversible. Effects on calcium currents become apparent at toxin concentrations in which sodium current effects are clearly saturated (Figure 5). Intriguingly, the nature of the effect of MrVIA was dose dependent, being agonistic at low concentrations and antagonistic at high concentrations (Figure 2C). Although MrVIB has a very similar sequence (Figure 6), it did not show this duality. The data strongly suggest that the effects of the MrVI toxins on sodium versus calcium channels are direct effects in both cases, i.e., the effects on one channel type do not result from an indirect mechanism due to the effect on the other channel. This is supported by the differences in dose dependency of the effects (Figure 5), the dual effects of MrVIA on calcium channels (Figure 2) whereas it acts as a simple blocker on sodium (Figure 4), and the differences in the kinetics and reversibility of the different effects (Figure 4, see also text).

The two *C. marmoreus* toxins described in this study clearly belong to the superfamily of toxins defined by the six cysteine-4 loop framework C··C··CC··C··C (see

Toxin	Sequence																Channel Targeted
TxVIA	W	**C**	KQS**G**EM	**C**		NVLDQN	**CC**	DGY	**C**		IVLV	**C**	T				Sodium[a]
GS	A	**C**	SGR**G**SR	**C**		OOQ	**CC**	MGLR	**C**		GRGNPQK	**C**	IGAH*E*DV				Sodium
MrVIA	A	**C**	RKKWEY	**C**		IVPIIGFIY	**CC**	PGLI	**C**		GPFV	**C**	V				Sodium & Calcium
MrVIB	A	**C**	SKKWEY	**C**		IVPILGFVY	**CC**	PGLI	**C**		GPFV	**C**	V				
GVIA		**C**	KSO**G**SS	**C**		SOTSYN	**CC**	R S	**C**		NOYTKR	**C**	Y				Calcium
MVIIA		**C**	KGK**G**AK	**C**		SRLMYD	**CC**	TGS	**C**		RSGK	**C**					
SVIB		**C**	KLK**G**QS	**C**		RKTSYD	**CC**	SGS	**C**		GRSGK	**C**					

FIGURE 6: Sequences of MrVIA and MrVIB compared to other conotoxins with the same cysteine framework. Identical residues are shown in bold type and boxed. Standard one-letter code for amino acid residues, except O = *trans*-4-hydroxyproline and *E* = γ-carboxyglutamate. Spaces were inserted to allow maximal homology. Note the underlined glycine residue in the first loop, which is conserved in all other toxins in this group except MrVIA and MrVIB. [a]TxVIA is an inhibitor of sodium current inactivation, i.e., its effect is to cause a net *increase* in the current. All the other toxins shown above act as blockers of their respective channel targets.

Figure 6). Other toxins belonging to this superfamily include the ω-conotoxin calcium channel blockers (Olivera et al., 1991), the δ-conotoxins which inhibit inactivation of sodium channels (Fainzilber et al., 1994a), and the conotoxin-GS which acts as a sodium channel blocker (Kobayashi et al., 1989). The MrVI toxins differ from all previously described members of this structural superfamily in the lack of a glycine residue important for folding in the first loop and in the excessive length of their hydrophobic second loop (Figure 6). The high content of hydrophobic residues, and the C-terminal loop region, is somewhat homologous to that found in δ-conotoxins (Figure 6). It is unusual that all the charged residues in MrVIA and MrVIB are concentrated in the first N-terminal loop, forming a complex of adjacent mainly positive charges. Previous studies on both ω- and μ-conotoxins have emphasized the importance of positively charged residues for toxin activity. A number of studies on μGIIIA have shown that Arg_{13} is crucial for sodium channel block, and a number of other positively charged residues are also important (Sato et al., 1991; Wakamatsu et al., 1992; Becker et al., 1992). Lampe et al. (1993) performed site specific acetylation studies on ωGVIA and showed that both the amino terminus and the lysine side chains are important for N-type calcium channel blocking activity. The background of these studies, coupled with the observed localization of positively charged residues in MrVI sequences, suggests that these residues are a priority for analysis in future structure–function studies on these peptides.

In contrast to the effects on sodium currents, the dose dependence of toxin effects on calcium currents is very steep (Figure 5), and the effects are rapidly reversible. It is interesting that these effects appear to be relatively selective for the transient subtype of calcium currents in the CDC (Figure 3). The dose–reponse data may suggest that MrVI toxin interactions with calcium channels are low affinity in comparison with their binding to sodium channels. However, the high degree of cooperativity in the effects on calcium currents (Figure 5B) might suggest a requirement for binding of a number of toxin molecules per channel in order to observe an effect. The dual effects of MrVIA, increasing currents at lower concentrations and blocking at higher (Figure 2), also support this possibility. The most parsimonious hypothesis to explain these data would be to assume that the MrVI toxins bind to calcium channels at sites not directly occluding the channel pore. Thus, partial occupancy of binding sites on the channel might in the case of MrVIA lead to a conformational shift of the channel, increasing the observed current. A complete block of the channel would require occupancy of a number of sites simultaneously. Clearly, ligand-binding studies are required to test this hypothesis.

It is noteworthy that the concentrations of MrVI toxins required for effects on CDC calcium currents are in the same range as the concentrations of calciseptine required to block L-type channels in dorsal root ganglion neurons (De Weille et al., 1991). Calciseptine is the only selective peptide blocker for L-type Ca^{2+} channels, and its efficacy in various systems may differ by over 1 order of magnitude. Also, calciseptine is completely ineffective in vertebrate assay systems with external [Ca^{2+}] above 10 mM (De Weille et al., 1991). If this is also the case for the MrVI toxins, then effects on calcium currents might be overlooked in marine invertebrate preparations, for which the normal saline concentrations include 11 mM Ca^{2+}. Thus in such systems, the MrVI toxins may appear to be highly selective sodium channel blockers. Our *in vivo* injections into adult *Lymnaea* suggest that the MrVI toxins may not affect (or have very low efficacy for) peripheral sodium channels in the snail (see closing paragraph of results). Although these are initial observations which should be extended, it is noteworthy that another structurally unrelated conotoxin family is also selective for sodium channels in snails (Hasson et al., 1995; M. Fainzilber et al., unpublished data).

Characterization of novel conotoxins selective for L-type Ca^{2+} channels reveals sequences rich in negatively charged residues but with hydrophobic stretches somewhat homologous to MrVI sequences (K. S. Kits et al., unpublished data). Together with the above data, this may suggest that the MrVI toxins interact with sodium or calcium channels via different recognition sites on the toxin. If this hypothesis is correct, the MrVI toxins may represent a structural and pharmacological "ω/μ" hybrid or intermediate in this conotoxin superfamily. Another possible interpretation of the data would be that "design" of a high-affinity snail sodium block recognition site on these toxins creates at the same time a low-affinity blocker site for L-type Ca^{2+} channels. This would then suggest conservation of certain structural features between the two channel types. Detailed pharmacological and structural analyses will be required to distinguish between these intriguing possibilities, and we expect future studies on the MrVI toxins will reveal unexpected insights on ion channel structure and functions.

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