A Novel Hydrophobic ω-Conotoxin Blocks Molluscan Dihydropyridine-Sensitive Calcium Channels†

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ABSTRACT: A novel calcium channel blocking peptide designated ω-conotoxin-TxVII has been characterized from the venom of the molluscivorous snail *Conus textile*. The amino acid sequence (CKQADEPCD-VFSLDCCTGICLGVCMW) reveals the characteristic cysteine framework of ω-conotoxins, but it is extremely hydrophobic for this pharmacological class of peptides and further unusual in its net negative charge (−3). It is further striking that the sequence of TxVII, a calcium current blocker, is 58% identical to that of δ-conotoxin-TxVIA, which targets sodium channels. TxVII effects were examined in the caudodorsal cell (CDC) neurons from the mollusc *Lymnaea stagnalis*. The toxin has no significant effect on sodium or potassium currents in these cells, but it clearly blocks the calcium currents. TxVII most prominently blocks the slowly inactivating, dihydropyridine- (DHP-) sensitive current in CDCs, while blockade of the rapidly inactivating current is less efficient. This novel ω-conotoxin is apparently targeted to DHP-sensitive calcium channels and thereby provides a lead for future design of selective conopeptide probes for L-type channels.

Natural toxins are powerful pharmacological tools that can be used as discriminative probes for closely related ionic channels, such as high-voltage activated (HVA)[1] calcium channels. Thus the well-characterized peptide toxins ω-conotoxin-GVIA and ω-agatoxin-IVA are the definitive tools used in characterizing N-type and P-type calcium channels, respectively [reviewed by Olivera et al. (1994)]. L-type calcium channels are defined pharmacologically on the basis of sensitivity to small organic compounds, primarily of the dihydropyridine (DHP) family. All L-type calcium channels are affected by DHPs, although the class is extremely heterogeneous at the molecular level [see for reviews Miller (1992) and Hofmann et al. (1994)]. The only peptide toxins affecting L-type channels characterized to date are calciseptine from snake venom (De Weille et al., 1991) and ωAgaIIIA from spider venom (Mintz et al., 1991), both of which affect a wide range of L-type channels, and in the case of ωAgaIIIA also other subtypes of HVA calcium channels (Olivera et al., 1994). Calciseptine has a broad range of efficacies for different L-type channels, mostly in the low micromolar range (De Weille et al., 1991).

The marine *Conus* snails have been a valuable source of selective peptide neurotoxins (Myers et al., 1993; Olivera et al., 1994). Conidae include a group of approximately 50 mollusc-hunting species. Since in some molluscan systems the calcium channels involved in transmitter or hormone release appear to be DHP sensitive (Edmonds et al., 1990, Dreijer & Kits, 1995, Dreijer et al., 1995), we searched for toxins targeted to L-type calcium channels in venoms from mollusc-hunting *Conus* species. We used the caudodorsal cell (CDC) neurons of *Lymnaea stagnalis*, a homogeneous population of peptidergic neurons expressing two subtypes of HVA calcium current, which are thought to be important in regulation of hormone release (Kits & Bos, 1982; Dreijer & Kits, 1995; Dreijer et al., 1995). In this study we describe a novel hydrophobic, negatively charged ω-conotoxin which blocks mainly the sustained DHP-sensitive calcium current in the CDC. This toxin provides a new pharmacological tool for analysis of HVA calcium currents, and its sequence provides a lead for the design of small peptide probes for L-type channels.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. *Conus textile* venom was obtained from specimens collected in the Northern Red Sea. All other toxins and chemicals were of analytical grade from commercial suppliers.

Column Chromatography. *Conus textile* venom was extracted in 30% acetonitrile/0.1% trifluoroacetic acid (TFA), pelleted by microcentrifugation, and the soluble peptides were fractionated by high-performance gel-permeation chromatography on Beckman SW2000 and SW3000 linked columns according to Li et al. (1993). Subsequent fractionation was by reverse-phase HPLC on Poros 20 R1 (PerSeptive Biosystems), using a gradient of acetonitrile in

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[1] Abbreviations: CDC, caudodorsal cell; DHP, dihydropyridine; ESI-MS, electrospray ionization mass spectrometry; HBS, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid- (HEPES-) buffered saline; HPLC, high pressure liquid chromatography; HVA, high-voltage activated; TFA, trifluoroacetic acid.

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0.1% aqueous TFA; followed by analytical RP-HPLC on C8 (Alltech, 25 × 0.46 cm, 5-μm particle size), in a gradient system of acetonitrile/2-propanol (1:1) in 0.1% aqueous TFA.

Amino Acid Sequence Determination. Purified peptide was reduced in 0.1 M NH_4HCO_3 (pH 8) containing 6 M guanidine hydrochloride, 10 mM EDTA, and dithiothreitol at 37 °C for 2 h under argon. Reduced peptide was then alkylated by addition of fresh distilled 4-vinylpyridine and incubation for another 2 h at 37 °C under argon. The alkylated peptide was purified by RP-HPLC on narrow-bore C18 immediately after the reaction. Alkylated purified peptide was applied to glass fiber filters and sequenced by automated Edman degradation on an Applied Biosystems 473A protein sequencing system. Cyanogen bromide cleavage of pure TxVII was performed according to the procedure of Villa et al. (1989) for cysteine-rich peptides; after which the digest was applied to glass fiber for automated sequencing.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) served for initial examination of homogeneity and was performed as previously described (Li et al., 1993). HPLC fractions or purified peptides were brought to 50% acetonitrile, acidified by addition of dilute TFA, and injected into a BioQ triple-quadrupole mass spectrometer (Fisons, U.K.) fitted with an electrospray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile and the flow rate was 5 μL/min. For high-resolution mass measurements of native TxVII, we used a VG Autospec 5000 oa-TOF mass spectrometer (Fisons, U.K.) equipped with an ESI source. The instrument was set to a resolution of 4000 so that charge states could be clearly distiguished. Samples were dissolved in 50% methanol and 1% acetic acid, which was also used as mobile phase. Five microliters of sample was injected and delivered into the mass spectrometer at a flow rate of 5μL/min.

Electrophysiology. Adult snails from a laboratory-bred population of *L. stagnalis* were used. CDC were isolated from the central nervous system by mechanical dissociation after incubation for 30 min at 37 °C in HEPES-buffered saline (HBS) (see below) supplemented with 0.2% trypsin (type III, Sigma). Isolated cells (diameter 30−50 μm) with round-shaped cell bodies and without neurites were used, always within 7 h after isolation. Isolated CDC were plated in 35-mm petri dishes (Costar) and bathed in HBS. To record calcium, sodium, or potassium currents, HBS was washed out and replaced under continuous perfusion by the appropriate saline. HBS composition (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. Calcium current selective saline had TEACl 40, $CaCl_2$ 4, $MgCl_2$ 1, HEPES 10, 4-aminopyridine 2, pH (7.8) set with TEAOH. Sodium current selective saline contained: NaCl 47.5, $CaCl_2$ 4, $MgCl_2$ 1, $CdCl_2$ 0.1, HEPES 10, 4-aminopyridine 1, pH (7.8) set with NaOH. Nonselective pipette saline had the following composition: KCl 29, $CaCl_2$ 2.3, EGTA 11, HEPES 10, ATPMg 2, GTPtris 0.1, pH (7.4) adjusted with KOH; calculated free [Ca^{2+}] was 10 nM. Pipette saline used to record selectively sodium or calcium currents had CsCl 29, $CaCl_2$ 2.3, EGTA 11, HEPES 10, ATPMg 2, GTPtris 0.1, pH (7.4) adjusted with CsOH; calculated free [Ca^{2+}] was 10 nM. Venom fractions and purified toxins were administered by means of a pressure ejection system built in the laboratory, allowing rapid application (~300 ms) of the toxins. Toxins were applied continuously during a series of depolarizing voltage steps.

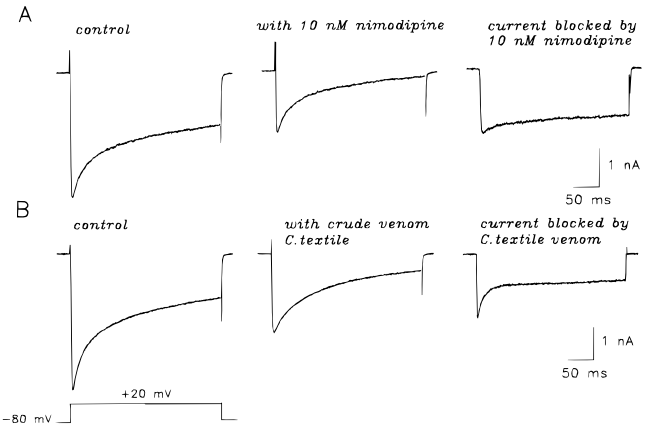

FIGURE 1: Comparison of effects of nimodipine (A) or *Conus textile* venom (B) on CDC HVA Ca^{2+} current. (Left panel) Control traces at +20 mV. (Middle panel) Currents in the presence of 10 nM nimodipine (A) or 5 mg equiv *C. textile* venom extract (B). (Right panel) Traces of the blocked components of the current, obtained by subtraction of the traces in presence of the toxins from the control traces. All responses were measured at voltage steps from −80 to +20 mV, as indicated.

Whole-cell voltage-clamp experiments were performed using the Axoclamp 2A amplifier (Axon Instruments) 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, U.K.) (seal resistance > 1 GΩ). After disruption of the patch membrane series resistance (<7 MΩ) was compensated for ~70%. With current amplitudes of <5 nA, the maximal voltage error is estimated to be ≤10 mV. Cell capacitance (~100 pF) was not compensated. When calcium or sodium currents were recorded, measurements were only started ~20 min after gaining access to the cell in order to equilibrate the intracellular fluid with the pipette solution. Data acquisition was controlled by a CED 1401 AD/DA converter (Cambridge Electronics Design, 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 kHz and sampled at 3 kHz (calcium currents and potassium currents) or filtered at 3 kHz and sampled at 9 kHz (sodium currents) and stored on-line. This system allowed simultaneous application of voltage steps, acquisition of current recordings, and timed application of toxins.

RESULTS

Purification and Chemical Characterization of Conotoxin TxVII. We screened the venoms of a number of molluscivorous *Conus* species for effects on HVA calcium currents in CDC. These cells express two HVA calcium currents, previously characterized as transient and sustained subtypes, as revealed by their inactivation kinetics (Dreijer & Kits, 1995). The sustained current is blocked by nanomolar concentrations of the DHPs nimodipine or nitrendipine (Figure 1A; J.C.L. and K.S.K., unpublished data); thus it is by definition an L-type current. The transient current can also be blocked by dihydropyridines, at concentrations 3 orders of magnitude above those required for the sustained current (data not shown). From all the venoms tested, only that of *Conus textile* was found to mimic the effects of nimodipine in preferentially blocking the sustained calcium current (Figure 1B). Aliquots (50 mg) of venom were

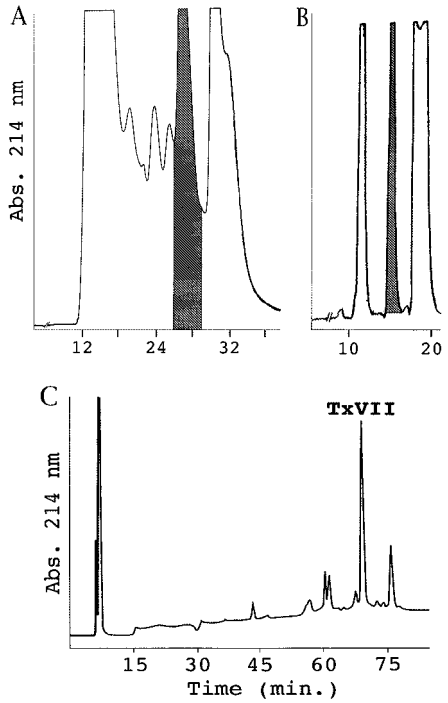

FIGURE 2: Purification of conotoxin-TxVII. (A) Crude *C. textile* venom was extracted in 30% acetonitrile/0.1% TFA, pelleted, and the supernatant was applied to Beckman SW2000−3000 high-performance gel permeation columns, equilibrated and eluted at 1 mL/min in the same solvent. The marked peak, eluting between 25 and 27 min, contained Ca^{2+} current blocking activity. (B) The active fraction from (A) was applied to a Poros 20 R1 column, equilibrated at 4 mL/min in 30% acetonitrile and 0.1% TFA. After a wash in this solvent, the bound peaks were eluted in a linear gradient of 30−40% acetonitrile and 0.1% TFA, from 10 to 20 min. The central peak shaded gray contained Ca^{2+} current blocking activity. (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 with a linear gradient of 0−80% acetonitrile/2-propanol (1:1) in aqueous 0.1% trifluorocetic acid from 0 to 80 min. The main peak indicated is pure TxVII.

extracted in 30% acetonitrile and fractionated on Beckman high-performance gel-permeation chromatography (Figure 2A). The indicated fraction was pooled from four runs (total 200 mg venom equiv) and the total fraction pool was loaded directly into a Poros 20 R1 column. The column was washed with 30% acetonitrile, eluting a pool of relatively hydrophilic components (not shown), and then eluted with a gradient of 30−40% acetonitrile (Figure 2B). The peaks shown in Figure 2B were analyzed by ESI-MS, revealing that the third doublet peak is composed of a mixture of conotoxins TxVIA and TxVIB; the other peaks contain uncharacterized peptides. The indicated central peak in Figure 2B was active in blockade of CDC sustained calcium current and was further purified on an Alltech C8 column, yielding a major component subsequently designated conotoxin-TxVII (Figure 2C).

Homogeneity of purified TxVII was further examined by RP-HPLC in two different systems, with analysis of fractions by ESI-MS (not shown). Both the HPLC and ESI-MS were consistent with the presence of a single peptide. This was further supported by structural analyses of TxVII, including Edman degradation, cyanogen bromide cleavage, and high-resolution mass spectrometry (see below).

The amino acid sequence of TxVII was determined by automated Edman sequencing after reduction and pyridylethylation. An unambiguous sequence of 25 amino acid residues was obtained in two separate runs, including six

Table 1: Initial Edman Degradation Sequencing of TxVII[a]

cycle no.	assigned residue	yield (pmol)	cycle no.	assigned residue	yield (pmol)
1	Cys	24	14	Asp	27
2	Lys	111	15	Cys	17
3	Gln	99	16	Cys	17
4	Ala	125	17	Thr	4
5	Asp	54	18	Gly	17
6	Glu	54	19	Ile	14
7	Pro	62	20	Cys	5
8	Cys	37	21	Leu	12
9	Asp	45	22	Gly	17
10	Val	56	23	Val	11
11	Phe	47	24	Cys	2
12	Ser	5	25	Met	6
13	Leu	29	26		

[a] Yields shown are not corrected for residue-specific differences in efficiency for sequencing. Background peaks of less than 5% of the major signal are not detailed in the table.

Table 2: Amino Acid Sequence of ω-Conotoxin-TxVII Compared with Other Members of the Same Superfamily[a]

Toxin	Sequence	Channel Target
ωMVIIA	**C** KGKGAK **C** SRLMYD **CC** TGS **C** RSGK **C**	Ca^{2+} N-type
ωGVIA	**C** KSOGSS **C** SOTSYN **CC** R S **C** NOYTKR **C** Y	
ωTxVII	**C** KQADEP **C** DVFSLD **CC** TGI **C** LGV **C** MW	Ca^{2+} L-type
δTxVIA	W **C** KQSGEM **C** NVLDQN **CC** DGY **C** IVLV **C** T	Na^+
δNgVIA	SK **C** FSOGTF **C** GIKOGL **CC** SVR **C** FSLF **C** ISFE	

[a] The conserved cysteine framework is in bold type. Spaces inserted to maximize homologies. O, *trans*-4-hydroxyproline.

cysteines in the typical framework for ω-conotoxins (Table 1). The ESI-MS average mass measurement for the native peptide was 2832.5 ± 0.8, approximately 178 mass units higher than that calculated from the Edman sequence in Table 1 (2654.2). This discrepancy suggested the presence of at least one additional residue at the C-terminus, after Met25. Cyanogen bromide cleavage of native TxVII was therefore performed according to methods previously described for cysteine-rich peptides (Villa et al., 1989). ESI-MS of the digest product revealed a product of average mass 2624.5 ± 1.7, consistent with cleavage of 178 mass units and transformation of Met25 to homoserine. Edman analysis of the CNBr reaction products mixture revealed a tryptophan residue in the first cycle; no additional unpredicted amino acids were observed in further cycles. Thus the C-terminal residue of TxVII is Trp26.

The sequence was further confirmed by measuring the accurate mass of the native peptide using high-resolution mass spectrometry. The electrospray mass spectrum showed that the doubly charged monoisotopic peak of the peptide was at m/z 1417.11 while the triply charged one was at *m*/*z* 945.08. The measured monoisotopic mass of the native peptide is thus 2832.23 Da. This is in good accordance with the monoisotopic mass predicted from the sequence, with all cysteines in disulfide bridgings and a free acid C-terminus (predicted 2832.14 Da). The complete sequence of TxVII is shown in Table 2, in comparison with other peptides from the same superfamily.

Electrophysiological Characterization of the Effects of TxVII. Calcium current blocking actions of TxVII were examined on cells voltage clamped at −80 mV, with depolarizing steps every 15 s to voltages ranging from −40

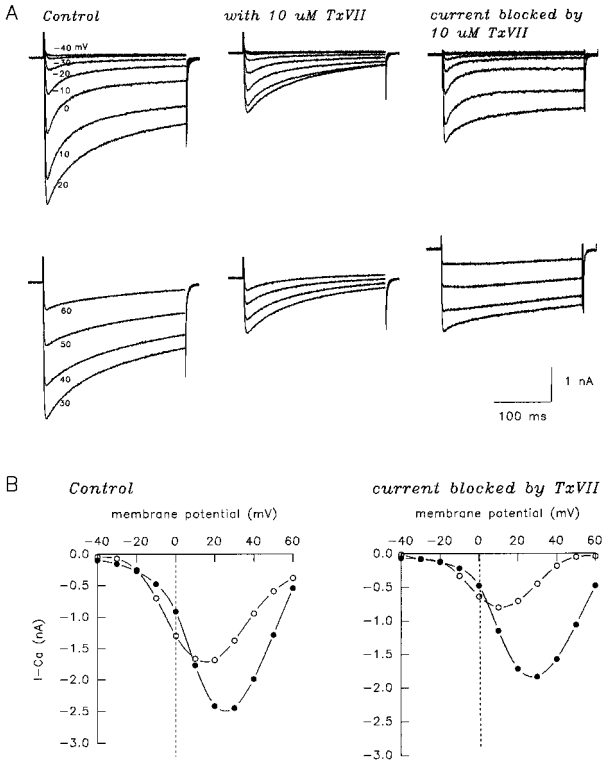

FIGURE 3: Blocking effects of TxVII on the HVA calcium currents in CDC. (A) (Left) Control current traces at −40 to +20 mV (upper panels) and at +30 to +60 mV (lower panel), as indicated. (Middle) Current traces at −40 to +60 mV in the presence of 10 μM TxVII, thus showing the TxVII-resistant current. (Right) Traces at −40 to +60 mV showing the component blocked by TxVII. These traces were derived by subtraction of the traces in the presence of TxVII from the control current traces. (B) I−V relations of the transient (●) and sustained (○) currents under control conditions (left) and the currents blocked by TxVII (right). Note that the predominant effect is on the sustained current. The I−V relations were measured by quantifying the sustained current as calcium current amplitude at 125 ms, at which point the transient current is completely inactivated. The transient current was then determined by subtracting this quantity of sustained current from the early peak of total calcium current.

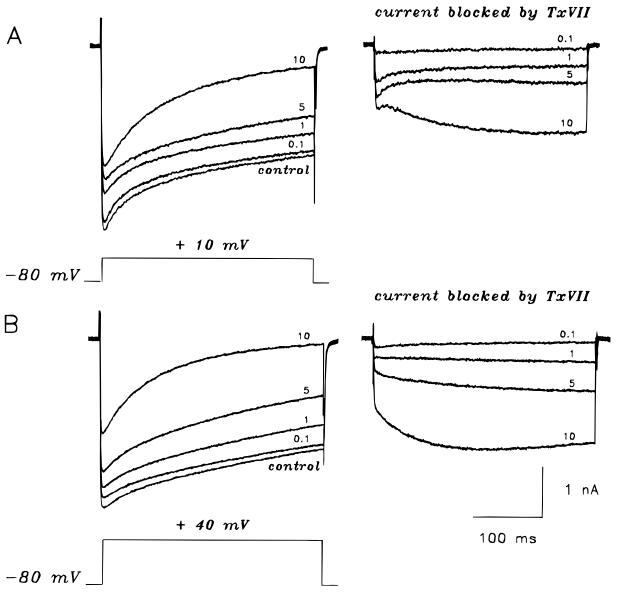

FIGURE 4: Dose dependence of TxVII effects on HVA current in CDC. (A) (Left) Current traces in the absence of TxVII (control) and current responses obtained with 0.1, 1, 5, and 10 μM TxVII (as indicated). (Right) Plots of the current blocked by 0.1, 1, 5, and 10 μM TxVII, as indicated (plots obtained by subtraction). All responses obtained by stepping from −80 to to +10 mV. Note in the plots of the blocked current that, in addition to the sustained current, a minor initial transient component is present. (B) As in (A), but for current responses at +40 mV. Note the slow rise phase, probably arising from open channel block of the sustained current.

to +60 mV. Control currents exhibited fast inactivation kinetics at potentials of <20 mV and slow inactivation kinetics at potentials of >20 mV, reflecting the prevalence of the transient and sustained current in the corresponding voltage ranges (Figure 3A, left panels). TxVII at 10 μM blocks a considerable fraction of the total current (Figure 3A, center panels). Subtraction of the TxVII-resistant current from the total current reveals the identity of the TxVII-sensitive component (Figure3A, right panels). This shows that the sustained, L-type current was the main current that was blocked. TxVII (10 μM) blocked the sustained current by about 85% at +10 mV and by about 95% at +40 mV. The selectivity for the sustained current was further tested in protocols using prepulses to 20 mV to inactivate the transient current. Under these conditions, the isolated sustained currents evoked by test pulses to 10 and 40 mV were strongly affected by TxVII (data not shown). However, while both experimental paradigms show that the stronger effect was on the sustained current, TxVII also causes a partial block of the transient current. The IV plots shown in Figure 3B further demonstrate that the most significant effect of TxVII is on the sustained, DHP-sensitive current.

The dose dependency of TxVII is shown in Figure 4. Threshold concentration for block of the sustained current was 100 nM, while a maximal effect was seen at 10 μM. The blocking action of the toxin develops over a period of 5−10 min after application and is completely reversible within 10 min of washing ($n = 7$). These doses are higher than the effective doses usually expected for classical ω-conotoxins on vertebrate N- and P-type channels (Olivera et al., 1994). However, the rigorous HPLC and ESI-MS analyses of purified TxVII rule out the possibility of the effects described above being due to a contaminant. Furthermore, the activity profiles of the peaks adjacent to TxVII (Figure 2B) do not suggest the presence of a coeluting high-potency calcium channel blocker. The dose range for TxVII effects is quite similar to that previously reported for calciseptine, a snake toxin selective for L-type currents (De Weille et al., 1991).

As also indicated by the data of Figure 4, TxVII appears to block the sustained current through an open channel block mechanism. This is suggested from current traces of the blocked current, which revealed a large sustained component with a very slow rate of rise. This is especially clear at +40 mV, where the sustained current is predominant (Figure 4B). The open channel block implies that the residual sustained current has a transient appearance. Thus, the transient character of the remaining current in the presence of TxVII (Figure 3A, center panels) is explained by unblocked transient current and residual sustained current, making it difficult to quantify the remaining transient current.

To test the selectivity of TxVII for calcium currents, we examined its effects on the isolated sodium current and on the total outward current, which consists mainly of potassium currents. The outward currents were recorded using non-selective media (HBS in the bath and nonselective pipette saline) to which 0.1 mM Cd^{2+} was added to block the calcium currents. No significant effect on voltage-dependent sodium or potassium currents was observed in the presence of 10 μM TxVII ($n = 4$ in each case).

DISCUSSION

In the present study we have identified a novel ω-conotoxin affecting DHP-sensitive calcium channels in molluscan neurons. TxVII provides a selective tool for molluscan calcium channels, but perhaps more interestingly a lead compound for future design of small peptide probes for L-type channels. Its amino acid sequence is extremely different from those of classical ω-conotoxins, while preserving the six cysteine−four loop framework C···C···CC···C···C (Table 2). In this context it is noteworthy that in TxVII the fifth residue is Asp, whereas in all other ω-conotoxins this residue is a conserved Gly, which participates in a type II β turn observed in the NMR solution structures of ωGVIA and ωMVIIC [summarized in Farr-Jones et al. (1995)]. Other toxins belonging to this superfamily include the δ-conotoxins, which inhibit inactivation of sodium channels (Fainzilber et al., 1994), conotoxin-GS, which acts as a sodium channel blocker (Yanagawa et al., 1988), conotoxins MrVIA and MrVIB, which affect both sodium and calcium channels (Fainzilber et al., 1995; McIntosh et al., 1995), and conotoxin-TxVIIA (formerly called TxIIA), which may act as a sodium channel activator (Fainzilber et al., 1991; Nakamura et al., 1996). TxVII, although acting as a calcium channel blocker, actually appears more homologous to the δ-conotoxins in its hydrophobicity in reverse-phase chromatography, short hydrophobic C-terminal loop, and net negative charge. Thus, for example, the sequence homology between TxVII and ωMVIIA is 45% identity and 54% similarity. The sequence homologies to δ-conotoxin-TxVIA (from the same species) include six identical and three similar residues, apart from the cysteine framework (Table 2). This is an identity of 58% and an overall similarity of 68%. Furthermore, the two Asp residues in the second loop of TxVII are replaced by Asn residues in identical positions in TxVIA, a substitution requiring single nucleotide changes in the relevant codons. TxVII and TxVIA may well represent very recently diverged genes; thus the dramatic differences in their activities and channel targets are intriguing. In this context it should be noted that TxVIA has no observable effect on calcium currents in *Lymnaea* CDC (K.S.K. and J.C.L., unpublished data). On the other hand, HPLC fractions containing TxVII had no observable effects on action potential amplitude or duration in *Aplysia* neurons (Fainzilber et al., 1991), which have been the most sensitive system to date for TxVIA effects. Thus the differences between these two closely related peptides define crucial structural elements for their divergent activities.

TxVII is the only ω-conotoxin described to date that may be specific for an L-type channel, although it should be noted that the N-type channel blocker ωGVIA has also been reported to reversibly block avian L-type channels (Aosaki & Kasai, 1989). The pharmacological selectivity of TxVII will require verification by testing on the other calcium channel subtypes (N, P, Q) in the future. Its efficacy for the molluscan L-type channel tested herein is less than that of classical ω-conotoxins for their respective channel targets; however, its potency is similar to that of calciseptine, a snake venom toxin that is the only previously described protein ligand specific for L-type channels (De Weille et al., 1991). Calciseptine affects a wide range of L-type channels, with 50% effective concentrations of up to 3 μM (De Weille et al., 1991).

An important question when assessing the utility of TxVII as a probe for L-type channels is the degree of phyletic selectivity it might exhibit, as well as the degree of cross-phyletic conservation of toxin binding sites on L-type channels. Certain molluscivorous *Conus* toxins exhibit a high degree of phyletic selectivity (e.g., Fainzilber et al., 1991, 1994). Although the dihydropyridine pharmacology of the sustained HVA calcium channel in *Lymnaea* CDC clearly classifies it as L-type, it does not necessarily follow that TxVII will target L-type channels in vertebrates. However, preliminary experiments with TxVII on calcium currents in rat melanotropic pituitary cells show a clear effect on the dihydropyridine sensitive current (K.S.K. and J.C.L., unpublished observations). It should be stressed that these are preliminary observations, but they enable a prediction that TxVII and related or synthetic derivative toxins should provide probes for a diversity of L-type channels.

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